

***N*-Methyl-*N*-((1-methyl-5-(3-(1-(2-methylbenzyl)piperidin-4-yl)propoxy)-1*H*-indol-2-yl)methyl)prop-2-yn-1-amine, a New
Cholinesterase and Monoamine Oxidase Dual Inhibitor**

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ABSTRACT: Based on *N*-((5-(3-(1-benzylpiperidin-4-yl)propoxy)-1-methyl-1*H*-indol-2-yl)methyl)-*N*-methylprop-2-yn-1-amine (**II**), and QSAR predictions, in this work we have designed, synthesized, and evaluated a number of new indole derivatives from which we have identified *N*-methyl-*N*-((1-methyl-5-(3-(1-(2-methylbenzyl)piperidin-4-yl)propoxy)-1*H*-indol-2-yl)methyl)prop-2-yn-1-amine (**2**) as a new cholinesterase and monoamine oxidase dual inhibitor.

Key Words: Drug design, QSAR, Synthesis, Biological evaluation, Multipotent Molecules, MAO/ChE Inhibitors, PAMPA-BBB, Molecular modeling, ADMET, Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disorder that accounts for approximately 70% of adult dementia.¹ Worldwide, it is estimated that 40 million people suffer from AD, and the prevalence of AD is expected to rise significantly in the next decades, as the average age of the population increases.² AD is characterized by a progressive memory loss, a decline in language skills and other cognitive impairments.³ Although the etiology of AD is not completely known, common hallmarks, such as amyloid- β ($A\beta$)⁴ deposits, τ -protein aggregation,⁵ and oxidative stress,⁶ are thought to play key roles in the pathophysiology of the disease.⁷ In addition, the selective loss of cholinergic neurons in AD results in a deficit of acetylcholine (ACh) in specific brain regions that mediate learning and memory.⁸ However, alterations in other

neurotransmitter systems, specially serotonergic, and dopaminergic, are also thought to be responsible for the behavioural disturbances observed in AD patients.⁹

Monoamine oxidase (MAO; EC 1.4.3.4), the enzyme that catalyses the oxidative deamination of a variety of biogenic and xenobiotic amines,¹⁰ is also an important target to be considered for the treatment of specific features of AD. MAO exists as two distinct enzymatic isoforms, MAO-A and MAO-B, based on their substrate and inhibitor specificities.¹¹ MAO-A preferentially deaminates serotonin, adrenaline and noradrenaline and is selectively and irreversibly inhibited by clorgyline. In contrast, MAO-B preferentially deaminates β -phenylethylamine and benzylamine and is irreversibly inhibited by *l*-deprenyl.¹² Selective inhibitors for MAO-A are effective antidepressants, whereas MAO-B inhibitors are useful in the treatment of Parkinson's disease (PD)¹³ and might also be valuable for the treatment of AD.¹⁴ In fact, MAO-B inhibitors are also currently in clinical trials for the treatment of AD because an increased level of MAO-B has been detected in the plaque-associated astrocytes of brains from AD patients.¹⁵

At present, there are three FDA-approved drugs (donepezil, galantamine and rivastigmine)^{16,17} that improve AD symptoms by inhibiting acetylcholinesterase (AChE; E.C.1.1.1.7), i.e. the enzyme responsible for the hydrolysis of ACh, and, thereby, raising ACh content in the synapsis. Apart from the beneficial palliative properties of AChE inhibitors in AD,^{18,19} cholinergic drugs have shown little efficacy to prevent the progression of the disease. Consequently, there is no efficient therapy to cure, stop or even slow the progression of the disease; therefore, effective therapeutics are urgently sought and needed.

The failure to find such a drug or treatment is possibly due to the multifactorial nature of AD. Thus, a single drug that acts on a specific target to produce the desired

clinical effects might not be suitable for the complex nature of AD. Accordingly, the multi-target-directed ligand (MTDL) approach²⁰⁻²² has been the subject of increasing attention by many research groups, which have developed a variety of compounds acting on very diverse targets.²³⁻²⁶

We have previously reported *N*-((5-(benzyloxy)-1*H*-indol-2-yl)methyl)prop-2-yn-1-amine (**I**)²⁷ (Figure 1) as a potent and selective propargylamine-containing MAO-B inhibitor (MAOBI) possessing neuroprotective properties that, unlike *l*-deprenyl does not generate amphetamines when metabolised and possesses anti-apoptotic properties.²⁸ Next we used **I** as *hit* compound with therapeutic potential for the treatment of neurodegenerative diseases, and started a project targeted to finding an improved *lead* compound.

As a result of this effort, we found that *N*-((5-(3-(1-benzylpiperidin-4-yl)propoxy)-1-methyl-1*H*-indol-2-yl)methyl)-*N*-methylprop-2-yn-1-amine (**II**, Figure 1) is a multipotent drug able to inhibit human AChE (hAChE) and human butyrylcholinesterase (hBuChE), showing inhibition of human MAO-A and human MAO-B (hMAO-A, hMAO-B).²⁹ The kinetic analysis demonstrated that **compound II** is not only a reversible inhibitor of both hAChE and hBuChE with micromolar affinity, but a highly potent irreversible inhibitor of hMAO-A, similarly to clorgyline.³⁰ The crystal structure of human MAO-B in complex with **compound II** highlighted the covalent adduct formed with the flavin N5 atom which, based on the spectral changes, occurs also with the MAO-A cofactor.³⁰ **Hybrid II** inhibited $A\beta_{1-42}$ self-aggregation more efficiently than that of $A\beta_{1-40}$, limiting the formation of fibrillar and oligomeric species,³¹ and completely blocked the aggregation mediated by AChE of both $A\beta_{1-42}$ and $A\beta_{1-40}$. Interestingly, **compound II** significantly reduced $A\beta_{1-42}$ -mediated toxicity in SH-SY5Y human neuroblastoma cells, and showed a significant ability to capture free-

radical species *in vitro*, as well as a potent effect in preventing the $A\beta_{1-42}$ induced depletion of catalase and SOD-1.^{31,32} Finally, compound **II** is able to cross the blood brain barrier *in vivo*.³³ Overall, these data indicate that hybrid **II** is the desired *lead* compound in our search for neurodegenerative diseases therapy.

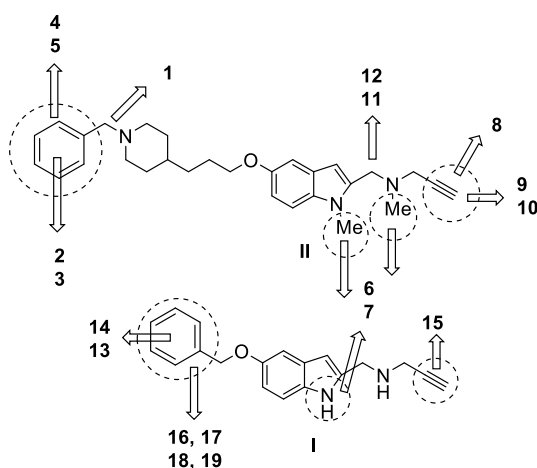


Figure 1. Schematic structural and functional modifications in compounds **I** and **II** leading to the new target molecules **1-19**.

Based on these results, we started a *lead compound optimization program*, whose results are described now in this manuscript. From this study, *N*-methyl-*N*-((1-methyl-5-(3-(1-(2-methylbenzyl)piperidin-4-yl)propoxy)-1*H*-indol-2-yl)methyl)prop-2-yn-1-amine (**2**) has emerged as a permeable, potent, irreversible, and quite selective hMAO-A inhibitor (MAOAI) in the nanomolar range, showing also moderate and almost equipotent hAChE vs hBuChE inhibition power.

RESULTS AND DISCUSSION

Design. In figure 1 we show the modifications in compounds **I** and **II** which have resulted into the new nineteen molecules that we have synthesized. Our selection was

based on QSAR analysis (see below), and simple functional changes on **compound II** (Figure 2) and **I** (Figure 3).

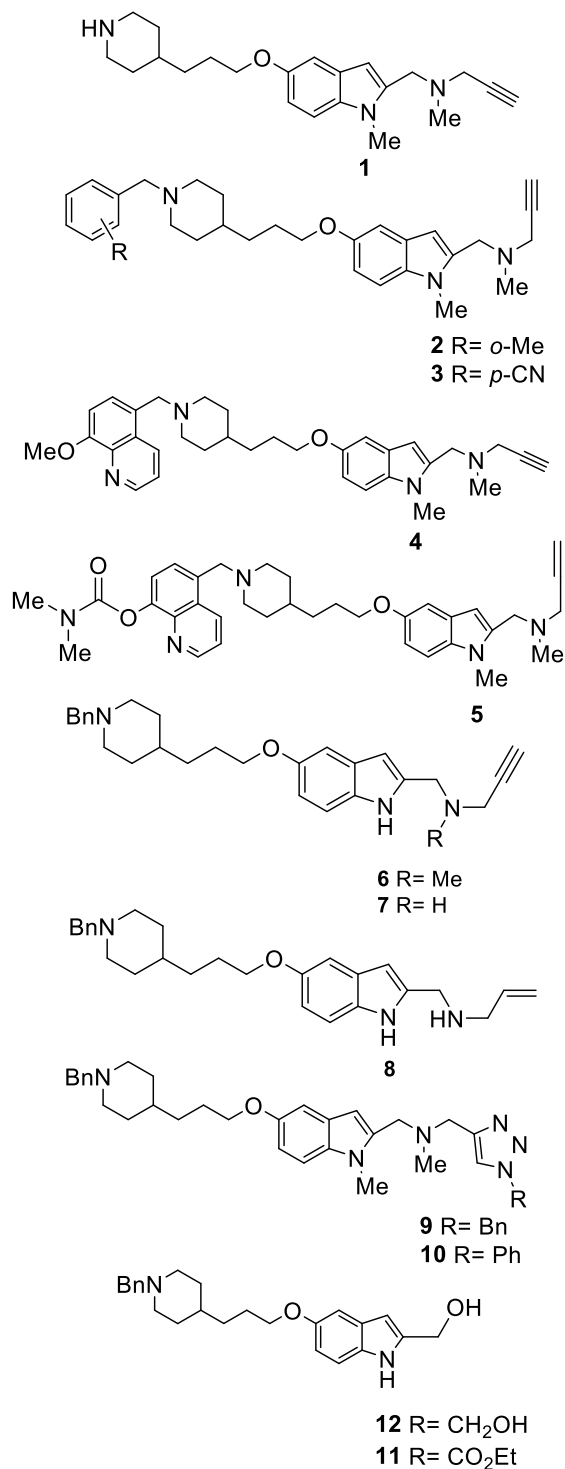


Figure 2. Compounds 1-12 synthesized and designed from **compound II**.

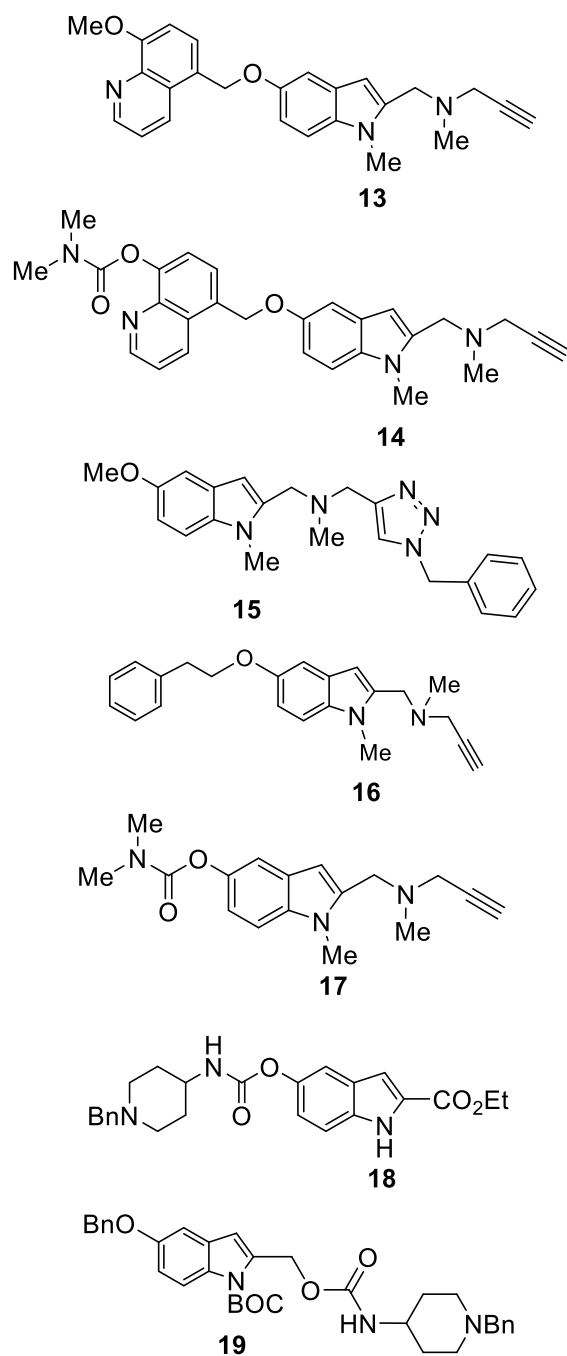


Figure 3. Synthesized compounds **13-19** designed from **compound I**.

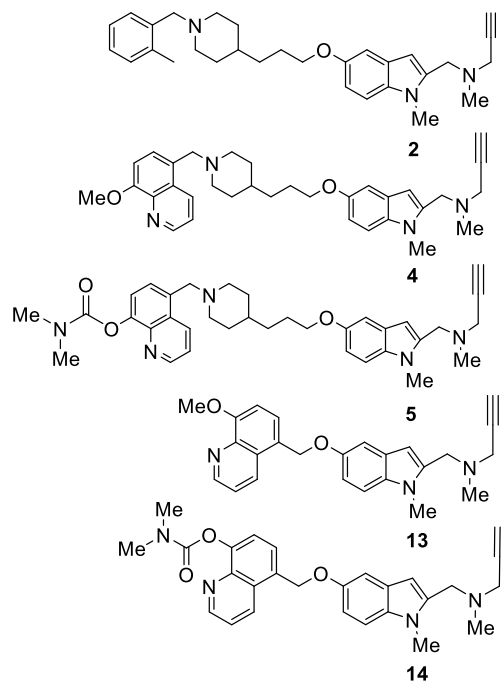
As depicted, the *N*-benzyl motif in **compound II** has been eliminated (**1**), or modified incorporating different substituents in the phenyl ring, such as *o*-MeC₆H₅CH₂ (**2**), *p*-CNC₆H₅CH₂ (**3**), and *N*-[(8-*O*-methyl- or 8-*O*-(*N,N'*-dimethylcarbamoyl)]5-

methylen]quinolone (**4** and **5**) (Figure 2). Taking into account the structure, inhibition power and selectivity of **compound I** for MAO B,²⁷ the *N*(1)Me in **compound II** has been replaced by a *N*1(H) leading to **6**; the *N*(1)Me and (indole)C(2)CH₂N(*Me*)CH₂C≡CH in **hybrid II** has been replaced by a *N*1(H) and (indole)C(2)CH₂N(*H*)CH₂C≡CH in **7** (Figure 2); compound **8** is like **7** (Figure 2), but the acetylene has been substituted by a vinyl group. Compounds **9** and **10** (Figure 2) are the *N*-benzyl- and *N*-phenyl-1,2,3-triazole derivatives,³⁴ respectively, of **compound II**. Similarly, compounds **11** and **12** (Figure 2) result from **hybrid II** by eliminating the C(2)CH₂N(*Me*)CH₂C≡CH side chain by CH₂OH, and CO₂Et,³⁵ respectively, and in both cases we have replaced the *N*(1)Me group by *N*(1)H.

Regarding compound **I**,²⁷ related transformations have afforded compounds **13-16** (Figure 3). Compounds **17-19** (Figure 3) are ladostigil/rasagiline-like carbamates, where we wanted to explore the known ability of compounds bearing this functional motif to inhibit ChEs.³⁶ Results from recent MAO/ChE literature, such as the substitution of an acetylene by a triazole,³⁴ or the use of a multifunctional chelator derived from 8-hydroxyquinoline,³⁷ have been used to design molecules **5, 9, 10, 14**, and **15** (Figures 2 and 3).

QSAR analysis. The 3D-QSAR study has been carried out with main aim to explain and predict the binding on the active sites of human MAO-A, MAO-B, AChE, and BuChE enzymes based on the pharmacological activities^{27,31,35} previously observed for the lead-improving program work with multipotent compound **II** (**Supporting Information**), and the results are shown in table 1.

Table 1. Experimental and QSAR-predicted activities of novel MAO/ChE inhibitors.



CMPD	Experimental IC₅₀ (hMAO-A)^a	Predicted IC₅₀ (MAO-A)
2	6.3 ± 0.4 nM	3.9 nM
4	10.3 ± 0.8 nM	218.8 nM
5	257.6 ± 11.4 nM	306.2 nM
14	310.6 ± 17.1 nM	3.2 μM
13	630.1 ± 16.1 nM	23.7 μM
CMPD	Experimental IC₅₀ (hMAO-B)^a	Predicted IC₅₀ (MAO-B)
2	183.6 ± 7.4 nM	100.7 nM
13	164.7 ± 12.1 nM	662.2 nM
5	196.3 ± 7.8 nM	38.8 μM
14	273.1 ± 8.9 nM	58.5 μM
4	7.9 ± 0.5 nM	73.1 μM
CMPD	Experimental IC₅₀ (hAChE)	Predicted IC₅₀ (AChE)
2	2.8 ± 0.1 μM	849.2 nM
5	8.4 ± 0.9 μM	3.0 μM
14	**	4.0 μM
4	***	10.3 μM
13	**	11.1 μM
CMPD	Experimental IC₅₀ (hBuChE)	Predicted IC₅₀ (BuChE)
2	4.9 ± 0.2 μM	1.3 μM
5	5.9 ± 0.4 μM	4.4 μM
4	**	9.8 μM
14	**	14.8 μM
13	**	202.8 μM

^aAfter 15 min pre-incubation, so not directly comparable with the predicted IC₅₀ values.

** Inactive at 100 μM (highest concentration tested).

*** 100 μM inhibits the corresponding MAO activity by approximately 40-50%. At higher concentration the compounds precipitate.

In the data set of previously characterised indole derivatives,^{27,31,35} the MAO-A pIC₅₀ activity interval spanned 4 log units (3.07–9.10); for pIC₅₀ (MAO-B) it was 5.6 log units (4.00–10.60), for pIC₅₀ (AChE) 4.2 log units (4.00-8.17), and pIC₅₀ (BuChE) 3.4 log units (4.00-7.44). The relatively wide pIC₅₀ interval of the training set provide broad applicability domain for the 3D-QSAR models created. The 3D-QSAR (MAO A) model with two significant components (A= 2), R²: 0.94, leave-one-out cross validation Q²: 0.66, Training Set parameters (R²_{Observed vs. Predicted}: 0.908 and RMSEE: 0.433), and Test Set parameters (R²_{Observed vs. Predicted}: 0.809 and RMSEP: 0.617), was developed. The 3D-QSAR (MAO B) model with two significant components (A=2), R²: 0.97 and leave-one-out cross validation Q²: 0.85, Training Set parameters (R²_{Observed vs. Predicted}: 0.957 and RMSEE: 0.303), and Test Set parameters (R²_{Observed vs. Predicted}: 0.787 and RMSEP: 0.598), was formed. Similarly, the 3D-QSAR (AChE) model with three significant components (A=3), R²: 0.87 and leave-one-out cross validation Q²: 0.61, Training Set parameters (R²_{Observed vs. Predicted}: 0.873 and RMSEE: 0.431), and Test Set parameters (R²_{Observed vs. Predicted}: 0.539 and RMSEP: 0.661), was developed. The 3D-QSAR (BuChE) model with two significant components (A=2), R²: 0.92 and leave-one-out cross validation Q²: 0.72, Training Set parameters (R²_{Observed vs. Predicted}: 0.922 and RMSEE: 0.261), and Test Set parameters (R²_{Observed vs. Predicted}: 0.657 and RMSEP: 0.521), was created.³⁵

The statistical parameters indicated that the 3D-QSAR (MAO-A, MAO-B, AChE, BuChE) models could be used for prediction of MAO-A, MAO-B, AChE, and

BuChE inhibiting activities for novel analogs of compounds **I** and **II**. Reliability of the activities predictions is strongly related to the type and extent of structural changes of the lead compounds.

3D-pharmacophores are derived from 3D-maps of interaction energies between the examined molecule and four chemical probes: DRY (which represent hydrophobic interactions), O (sp^2 carbonyl oxygen, representing H-bond acceptor), N1 (neutral flat NH, like in amide, H-bond donor), and the TIP probe (molecular shape descriptor). The 3D-pharmacophores for MAO-A and MAO-B inhibiting activities of **compound II** contain crucial favorable O-TIP/O-DRY/TIP-TIP/DRY-TIP interactions between the propargylamine moiety and the benzyl group. Therefore, substitution of the benzyl moiety of **hybrid II** with small groups, such as an *o*-Me in **2** (Figure 2), could enhance the MAO-A and MAO-B inhibiting activity of the examined compounds by facilitating the TIP-TIP/DRY-TIP favorable interactions. The 3D-QSAR study confirmed also previous experimental findings that the propargylamine moiety provides an essential positive influence on the MAO-A and MAO-B inhibiting activity.^{35,38,39}

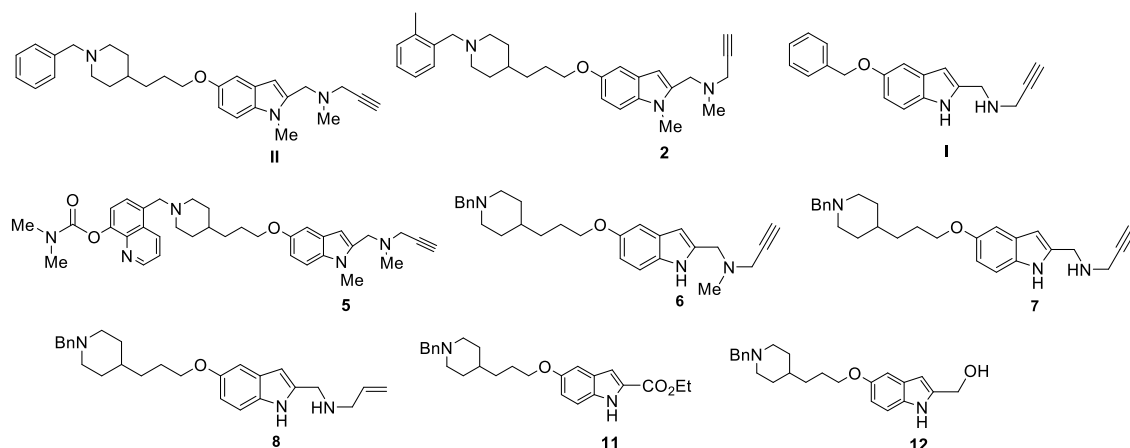
The 3D-pharmacophores for BuChE inhibiting activities of *lead* compound **II** contain specific unfavorable TIP-TIP/DRY-TIP interactions between the indole and the *para* position of the benzyl moieties. Therefore, substitution with bulky substituent at *para* position of the benzyl moiety, such as a cyano group in **3** (Figure 2), could decrease BuChE inhibiting activity of **compound II** derivatives (Table 1). The 3D-pharmacophores for AChE inhibiting activities of **compound II** contain a crucial favourable O-O interaction between the piperidine ring and *ortho* positions of the benzyl group. Thus a small substituent at *ortho* position of the benzyl ring, such as the methyl group in compound **2** (Figure 2) could enhance AChE inhibiting activity of *lead*-compound **II** (Table 1).

In addition to **2**, compounds **4**, **3**, **13** and **14** have also been investigated by QSAR analysis, and their predicted IC₅₀ values are also shown in table 1. The predicted IC₅₀ values clearly suggest that: (a) **compound II**-donepezil type hybrids **2**, **4**, and **5**, in this order, should be the most potent MAO-AI; (b) the **compound I**-like derivative **14** should be a more potent inhibitor of MAO-A, AChE, and BuChE than **13**; and (c) **2** should be the most potent inhibitor against all four enzymes.

Next, the designed **compound II** analogues were synthesized and examined for MAO/ChE inhibitory activity.

Chemistry. The synthesis of the new molecules (Figures 2 and 3) has been achieved by standard or reported methods, in very efficient and short synthetic sequences, in good overall yields (see **Supporting Information**). All new molecules gave satisfactory analytical and spectroscopic data, in good agreement with their structures.

Table 2. IC₅₀ Values for the Inhibitory Effects of Test Compounds on the Enzymatic Activity for human MAO or ChE isoforms and Selectivity Ratios for MAO-B [IC₅₀ (MAO-A)]/[IC₅₀ (MAO-B)] or for AChE ([IC₅₀ (AChE)]/[IC₅₀ (BChE)]^a



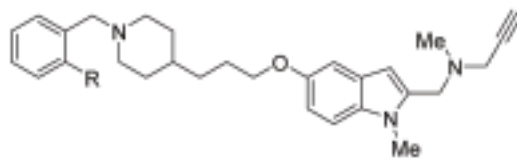
CMPD	hMAO-A	hMAO-B	Ratio	hAChE	hBuChE	Ratio
2 (MBA236)	$6.3 \pm 0.4 \text{ nM}^b$	$183.6 \pm 7.4 \text{ nM}$	0.03	$2.8 \pm 0.1 \mu\text{M}$	$4.9 \pm 0.2 \mu\text{M}$	0.57
5	$257.6 \pm 11.4 \text{ nM}^c$	$196.3 \pm 7.8 \text{ nM}$	1.3	$8.4 \pm 0.9 \mu\text{M}$	$5.9 \pm 0.4 \mu\text{M}$	1.4
6	$9.1 \pm 0.7 \mu\text{M}^c$	$35.1 \pm 2.8 \mu\text{M}$	0.26	$15.4 \pm 0.9 \mu\text{M}$	$7.1 \pm 0.5 \mu\text{M}$	2.2
7	$19.2 \pm 1.3 \mu\text{M}^c$	$33.6 \pm 1.5 \mu\text{M}$	0.57	$4.9 \pm 0.3 \mu\text{M}$	$7.3 \pm 0.8 \mu\text{M}$	0.67
8	$45.3 \pm 1.6 \mu\text{M}^c$	$21.3 \pm 1.9 \mu\text{M}$	2.1	$4.6 \pm 0.3 \mu\text{M}$	$40.6 \pm 2.2 \mu\text{M}$	0.11
11	$876.6 \pm 25.2 \text{ nM}$	$1.0 \pm 0.02 \mu\text{M}$	0.87	$9.6 \pm 0.08 \mu\text{M}$	$9.7 \pm 0.7 \mu\text{M}$	0.99
12	$9.1 \pm 0.3 \mu\text{M}$	$13.5 \pm 1.1 \mu\text{M}$	0.67	$13.6 \pm 1.4 \mu\text{M}$	$31.2 \pm 3.1 \mu\text{M}$	0.43
II	$58.2 \pm 1.2 \text{ nM}^a$	$1.2 \pm 0.1 \mu\text{M}$	0.05	$3.4 \pm 0.2 \mu\text{M}$	$3.3 \pm 0.2 \mu\text{M}$	1.03
I	$287.3 \pm 11.2 \text{ nM}^b$	$4.1 \pm 0.7 \text{ nM}$	70	**	**	
Clorgyline	$4.7 \pm 0.2 \text{ nM}^b$	$65.8 \pm 1.6 \mu\text{M}$	0.0000 71	**	**	
<i>l</i>-Deprenyl	$63.6 \pm 1.3 \mu\text{M}^b$	$18.2 \pm 0.9 \text{ nM}$	3,494	**	**	
Donepezil^d	nd	nd		$0.016 \pm 0.001 \mu\text{M}$	$8.2 \pm 0.2 \mu\text{M}$	

^a All IC_{50} values shown in this Table are the mean \pm SD from five experiments. **hMAO**: IC_{50} values were determined after 15 min preincubation of inhibitor with the enzyme. **hChE**: IC_{50} values were determined after 10 min preincubation of inhibitor with the enzyme. Level of statistical significance: ^b $P < 0.01$ or ^c $P < 0.05$ versus the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett's. ^d Ref. 23. ** Inactive at 100 μM (highest concentration tested). nd: not determined.

Biological evaluation. The biological evaluation, comprising the hMAO-A/ hMAO-B, and hAChE/ hBuChE inhibition, has been carried out according to the protocols described in the **Supporting Information**, following the usual methods.^{40,41} In Table 1S (**Supporting Information**) we have shown the IC_{50} values for the inhibition of the MAOs and ChEs with all the nineteen new compounds, using **compounds I and II**, re-analyzed in this work⁴² as reference compounds. The established MAOI, clorgyline and

l-deprenyl, and the ChEI, donepezil, are also reported for comparative purposes. Note that only seven compounds, **2**, **5-8** **11**, and **12**, were active simultaneously in the four enzymes (Table 2). As shown in table 1S, compound **6** is a moderate hMAO-AI and hBuChEI, in the high micromolar range, but shows very modest hMAO-B and hAChE inhibition power. **12** is almost equipotent with **6** for the hMAO-A and hAChE inhibition, but 2.6-fold more, and 4.4-fold less potent than **6**, for the inhibition of hMAO-B and hBuChE, respectively. **7** is a poor hMAO-AI and hMAO-BI, but a moderate hAChEI and BuChEI, in the micromolar range. **8** is a quite poor hMAO-AI, hMAO-BI and hBuChEI, but a modest hAChEI. Furthermore, **5**, and **11** were non-selective, potent hMAOI in the low micromolar range, but moderate and equipotent ChE inhibitors.

However, among all these products, as predicted by the QSAR analysis (see above), compound **2** (Table 2) was the most potent hMAO-A, hMAO-B AChE, and BuChE multipotent inhibitor found in this work. Compound **2** is a very potent, in the nanomolar range, and selective hMAO-AI (with an apparent mixed inhibitor $K_i = 7.5 \pm 0.9$ nM), showing a potent and slightly selective hAChE inhibition profile ($K_i = 1.3 \pm 0.5$ μ M), in the micromolar range. Compared with the reference compound **II**, the new compound **2** is 9.2-fold and 6.5-fold more potent hMAO-AI and hMAO-BI, respectively, 1.2-fold more potent hAChEI, and only 1.5-fold less potent hBuChEI. Comparing with compound **I** and the standard MAOIs, for the inhibition of MAO-A, compound **2** is 45-fold more potent than compound **I**, 1.3-fold less than clorgyline, and 10095-fold more potent than *l*-deprenyl. Conversely, for the inhibition of MAO-B, compound **2** is 45-fold less, 358-fold more and 10-fold less potent than compound **I**, clorgyline and *l*-deprenyl, respectively.



2 (MBA236) (R= Me)	II (ASS234) (R= H)
$IC_{50}(\text{hMAO-A}): 6.3 \pm 0.4 \text{ nM}$	$58.2 \pm 1.2 \text{ nM}$
$IC_{50}(\text{hMAO-B}): 183.6 \pm 7.4 \text{ nM}$	$1.2 \pm 0.1 \mu\text{M}$
$IC_{50}(\text{hAChE}): 2.8 \pm 0.1 \mu\text{M}$	$3.4 \pm 0.2 \mu\text{M}$
$IC_{50}(\text{hBuChE}): 4.9 \pm 0.2 \mu\text{M}$	$3.3 \pm 0.2 \mu\text{M}$

Figure 4. Structure of compound **2**, and its IC_{50} values for the inhibition of MAO and ChEs, compared with the reference compound **II**.

To sum up, **compound 2** is a multipotent molecule of MAO and ChE, showing a very attractive pharmacological profile, as a selective and very potent MAO-AI, with high MAO-B and potent hAChE/ hBuChE inhibition power. It must be also concluded that all our present and past efforts²⁹ to transform compound **I** into a multipotent MAO/ChE compound, by transforming the benzyl group into the ChE donepezil-like pharmacophore have met with success, but at the cost of losing significant MAO-B inhibition capacity.

Taking into account these findings, we have performed further studies on the inhibition mechanism of action of compound **2**.

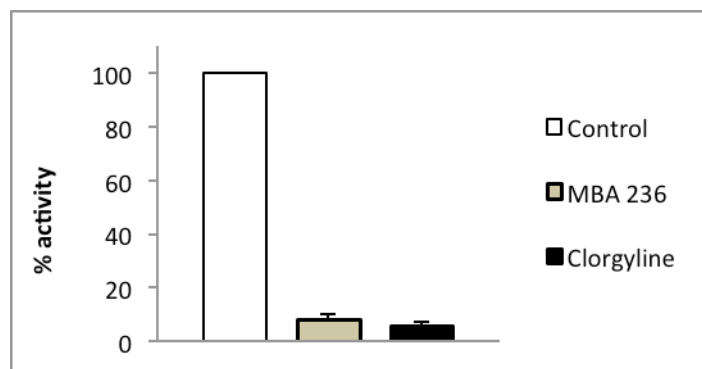


Figure 5. Recovery of hMAO-A activity after dilution following incubation (30 min at room temperature) of the 100x-enzyme concentration with 10-fold IC_{50} concentration of **compound 2** or clorgyline. The control was carried out by pre-incubating in the absence of inhibitor and diluting in the same way. Results are expressed as percentage of control. Represented data are mean \pm SD of **five** independent assays.

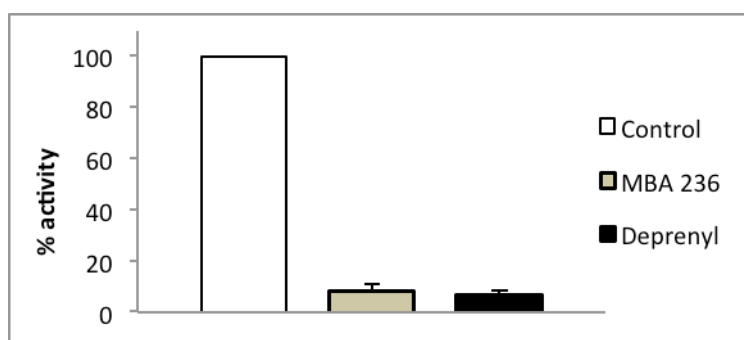


Figure 6. Recovery of hMAO-B activity after dilution following incubation (30 min at room temperature) of the 100x-enzyme concentration with 10-fold IC_{50} concentration of **compound 2** or *l*-deprenyl. The control was carried out by pre-incubating in the absence of inhibitor and diluting in the same way. Results are expressed as percentage of control. Represented data are mean \pm SD of **five** independent assays.

Figures 5 and 6 show the results of the reversibility test⁴³ on hMAO. **Compound 2** binds as an irreversible inhibitor on both enzyme isoforms, maintaining near a 90% inhibition after the dilution of the pre-incubated mixture. The IC_{50} decreased from 14.6 ± 1.2 nM in reversible conditions (no preincubation and substrate concentration $2 \times K_m$) to 6.3 ± 0.4 nM assayed with saturating substrate after a 15 min incubation.

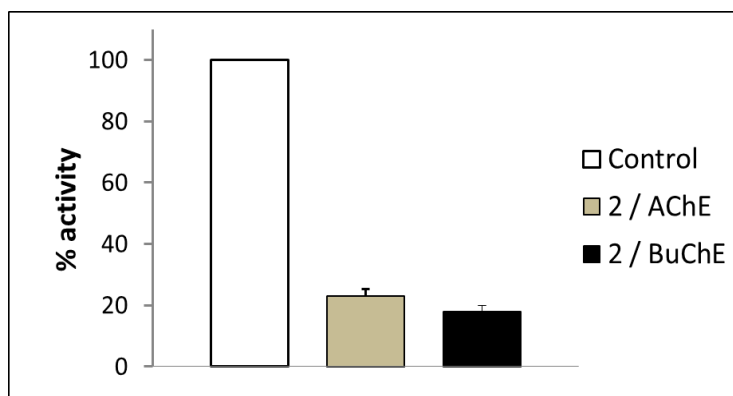


Figure 7. Recovery of hAChE (grey column) and hBuChE (black column) activity after the dilution of the incubation (30 min at room temperature) of the 100X-enzyme concentration and 10-fold IC_{50} of compound **2**. The control was carried out by pre-incubating in the absence of inhibitor and diluting in the same way. Results are expressed as percentage of control. Represented data are mean \pm SD of five independent assays.

Nevertheless, the recovery of ChE activity after compound was significantly greater (around 20%), allowing us to classify it as a slow inhibitor (Figure 7). Kinetic assays performed with this compound have shown ambiguous results, probably due to the low degree of reversibility of inhibition achieved on hAChE and hBuChE. As a result of the low reversibility, the pattern of the Lineweaver-Burk representation appears to indicate a mixed model for both enzymes (results not shown).

With the aim of investigating **compound 2** recognition with respect to either MAO-A, MAO-B, AChE and BuChE enzymes, molecular modeling studies have been carried out. The theoretical investigation has been focused on the ligand enzyme interaction and target structural perturbation produced by **compound 2** comparing these information with respect to the same data coming from the related compound **II** (**Supporting Information**). Only one proposed structure was found for **compound 2** in hMAO-A and two for hMAO-B, with a global minimum population equal to 96.11%.

Compounds **II** and **2** both adopted bent conformations in hMAO-A, but were linear in hMAO-B (Figure S1, **Supporting Information**). Overall, the interactions between compounds **II**, **2** and the MAOs are driven by both steric hindrance and hydrophobic contribution with an additional electrostatic term present in hMAO-A. Using the target RMSd matrix, each MD trajectory was clustered into nine groups. The visual inspection of the ten structures for each inhibitor with each enzyme clearly indicates the conformation stabilizing effect of the *o*-methyl group in hybrid **2** (Figure S4, **Supporting Information**).

In this work we have designed a new and multipotent MAOI. MAOI are well known for showing diverse therapeutic potential as the result of combining selectivity and (ir)reversible modes of binding.¹⁵ MAO-AI have been used mostly in the treatment of depression and anxiety,⁴⁴ and MAO-BI for the treatment of PD.⁴⁵ Recent studies have shown that MAO-A is involved in the regulation of serotonin for ventricular remodeling *via* activation of the serotonin2A receptors.⁴⁶ In the aged heart, the increased MAO-A concentrations results in high levels of oxygen peroxide leading to cardiomyocyte severe damage.⁴⁷ A survey of the current literature on MAOIs shows also that one of the major reasons for designing new and specific inhibitors is to function as cardioprotectants for the age-dependent increase of MAO A in the heart.⁴⁸ Although MAO-AIs enhance extracellular levels of dopamine produced from L-dopa, irreversible, and selective MAO-AI cannot be used in the PD therapy, because of cardiovascular side effects.⁴⁹ Another disadvantage of an irreversible MAO-AI is the hypertensive “cheese effect”,⁵⁰ due to the predominance of MAO-A in the gut wall to metabolize tyramine in fermented foods such as cheese and beer. Hypertensive crises may be prevented by using reversible rather than irreversible MAO-AI, or tissue-specific inhibitors such as the brain-selective ladostigil.⁵¹

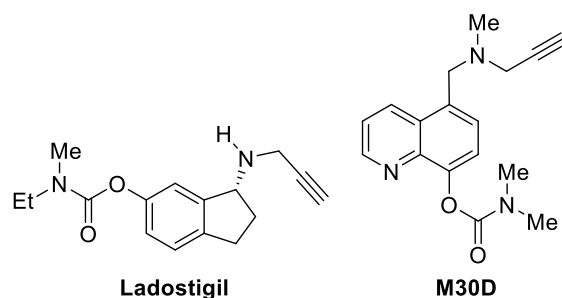


Figure 8. Structures of **ladostigil**, and **M30D**.

The cases of **ladostigil**⁵² and **M30D**⁵³ (Figure 8) are of interest for this project and future possible endeavours, because both are ChE, MAO-A and MAO-B irreversible inhibitors with potential therapeutic applications as antidepressants, antiparkinson and anti-Alzheimer drugs.⁴⁴ **Ladostigil**⁵¹ (Figure 8) was designed from rasagiline, a MAO-BI, bearing a propargylamine motif, responsible for the neuroprotective properties, and a carbamate group, affording ChE inhibitory activity. **M30D**⁵³ (Figure 8) shows an inhibitory profile against ChE [hAChE IC₅₀= 0.52 ± 0.07 μM; hBuChE IC₅₀= 44.90 ± 6.10 μM] and against MAO [ratMAO-A: IC₅₀= 0.0077 ± 0.0007 μM; ratMAO-B: IC₅₀= 7.90 ± 1.34 μM] very similar the one observed for **2** (Table 2), as well as other properties as potential drugs for treating AD.

***In Vitro* Blood–Brain Barrier Permeation Assay.** Finally, in order to evaluate the brain penetration we used the PAMPA-BBB method described by Di,⁵⁴ and subsequently optimised by Rodríguez-Franco et al. for molecules with limited water-solubility.⁵⁵⁻⁵⁷

The *in vitro* permeability (P_e) value of compound **2** through a lipid extract of porcine brain was determined by using PBS: ethanol (70:30). In the same assay, 11 commercial drugs of known CNS penetration were also tested and their experimental

values were compared to reported values, giving a good linear correlation, P_e (exp.) = $1.4442 P_e$ (bibl.) + 6.5263 ($R^2 = 0.95$). From this equation and taking account the limits established by Di for BBB permeation,⁵⁴ we found that compounds with permeability values above $12.3 \cdot 10^{-6} \text{ cm s}^{-1}$ could penetrate into the CNS. Derivative **2** showed $P_e = 14.4 \pm 0.4 \cdot 10^{-6} \text{ cm s}^{-1}$ and thus, it could cross BBB by passive diffusion (**Supporting Information**).

CONCLUSIONS

In this paper we have reported the design, synthesis and biological evaluation of nineteen new donepezil-indolyl hybrids as multifunctional drugs able to bind MAO and ChE enzymes, using the MAO B inhibitor **I** hit, and the multipotent **II** hybrid lead as references for lead-optimization in order to discover a new lead-compound for deeper *in vivo* pre-clinical investigations targeted to neurological disorders. The QSAR-designed compound **II** analogues have been synthesized and examined for MAO/ChE inhibitory activity. As shown in figure 4, the potent MAO/ChE inhibiting activity for the novel ligand **2** confirmed the quality of the optimized 3D-pharmacophores for use in future work to design novel donepezil-indolyl hybrids. We have found significant biological activity on the four enzymes studied in almost half of the synthesized structures. The most promising compound of the series, **2**, acted as an irreversible hMAO-AI, potent in the nanomolar range, **nine** times more potent than the reference compound **II**, and **29**-fold more selective for hMAO-A over hMAO-B. Inhibition of the ChEs by hybrid **2** is in the micromolar range, slightly better than compound **II** for hAChEs although slightly poorer for hBuChE. Although we are confronted with unbalanced, highly preferred MAO vs ChE inhibition, **the potency and desired balance**

between targets in MTDL-based strategies is not known.⁵⁸ Molecular modeling studies⁵⁹ reported similar binding modes of compounds **II** and **2** in all the evaluated biological targets. The *o*-Me group in **compound 2** improves the ligand recognition increasing the ligand-enzyme hydrophobic interaction in hBuChE, and π - π stacking in hMAO-A, hMAO-B and hAChE. Thus, it is clear that simple modification of **compound II**, such as the incorporation of an *o*-methyl instead of a hydrogen in the phenyl ring of the *N*-benzylpiperidine motif, produces significant qualitative and quantitative pharmacological changes in the inhibition of MAO and ChE enzymes. Finally, the ADMET virtual analysis⁵⁹ suggests that inhibitor **2** should not be hepatotoxic, not show hERG liability, and should present good drug-like characteristics similar to **compound II**, with a slightly better brain penetration ability, a prediction experimentally confirmed by the *in vitro* blood-brain barrier permeation assay.

Consequently, we have fulfilled our initial expectations, that a new, easily available, permeable multipotent MAO inhibitor has been discovered. The related pharmacological properties with **ladostigil**, clearly support projected *in vivo* studies for proof of concept targeted to probe the suitability of **compounds 2** and **II** to treat neurodegenerative disorders such AD or PD.

ASSOCIATED CONTENT

Supporting Information

QSAR methods, ADMET analysis and descriptors, the synthesis of the target compounds, the ¹H and ¹³C spectra, biological evaluation, molecular modeling, and *in*

vitro blood–brain barrier permeation assay of compound **2**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

OMBA carried out the synthesis of the target molecules. OMBA, MC, and AS carried out the spectroscopic analyses. KN, SF and DA carried out the QSAR predictions. MY undertook the enzyme inhibition and mechanistic studies. ES did the ADMET-analysis. FO and SA carried out the computational studies. *LdA did the PAMPA-BBB experiment, under MIRF supervision.* RRR evaluated the data and contributed to writing the manuscript. JMC conceived, designed and supervised the project.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ACh, acetylcholine; AChEI, acetylcholinesterase inhibitors; hAChE, human acetylcholinesterase; AD, Alzheimer's disease; ADMET, Absorption, Distribution, Metabolism, Excretion, Toxicity; hBuChE, human butyrylcholinesterase; hERG, human *Ether-à-go-go*-Related Gene; MAO, monoamine oxidase-A/B; MAOI, monoamine oxidase inhibitors; MTDL, Multi-Target-Directed Ligand; PAMPA-BBB, Parallel Artificial Membrane Permeation Assay/Blood-Brain-Barrier; QSAR, Quantitative Structure-Activity Relationships.

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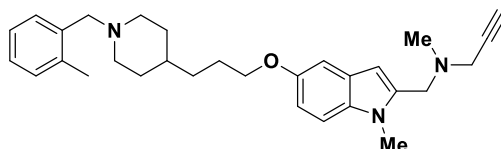
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(59) ADMET, and molecular modeling details and results have been reported in the **Supporting information**.

”Table of Contents graphic”

A New Cholinesterase and Monoamine Oxidase Dual Inhibitor



N-Methyl-*N*-((1-methyl-5-(3-(1-(2-methylbenzyl)piperidin-4-yl)propoxy)-1*H*-indol-2-yl)methyl)prop-2-yn-1-amine (**2**)

$IC_{50}(hMAO-A)$ $IC_{50}(hMAO-B)$ $IC_{50}(hAChE)$ $IC_{50}(hBuChE)$

6.3 ± 0.4 nM **183.6 ± 7.4 nM** **2.8 ± 0.1 μM** **4.9 ± 0.2 μM**