

Cholinesterases Inhibition by Novel *cis*- and *trans*-3-Arylamino-cyclohexyl *N,N*-Dimethylcarbamates: Biological Evaluation and Molecular Modeling

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The present study describes the synthesis, assessment of the anticholinesterase activity and the inhibition type of novel *cis*- and *trans*-3-arylamino-cyclohexyl *N,N*-dimethylcarbamates. *In vitro* inhibition assay by Ellman's method with human blood samples showed that carbamates were selective for butyrylcholinesterase (BuChE) with compound concentration that inhibits 50% of enzyme activity (IC₅₀) between 0.11 and 0.18 mmol L⁻¹. *cis*- and *trans*-3-(4-Methoxyphenylamino) cyclohexyl *N,N*-dimethylcarbamate hydrochloride were the most active for BuChE, showing that the presence of methoxyl group enhanced the anticholinesterase activity. The enzyme kinetics studies indicate a noncompetitive inhibition against acetylcholinesterase (AChE) and mixed type inhibition for BuChE. Molecular modeling studies confirm the ability of carbamates to bind both the active and peripheral sites of the BuChE.

Keywords: carbamate derivatives, butyrylcholinesterase inhibitors, molecular docking, non-covalent interactions

Introduction

Acetylcholinesterase (AChE) is a serine hydrolase that is mainly present in red blood cells, nerve endings and striated muscles.^{1,2} The principal biological role of AChE is regulating the transmission of impulses at cholinergic synapses, catalyzing rapid hydrolysis of the neurotransmitter acetylcholine (ACh).^{3,4} Like AChE, the butyrylcholinesterase (BuChE) has an important role in the nervous system as coregulator of ACh. Studies have shown that BuChE is able to compensate for the lack of AChE, allowing the continued regulation of cholinergic neurotransmission.⁵⁻⁷ Alzheimer's disease (AD), glaucoma and myasthenia gravis are diseases which are related to the cholinergic system and their symptomatic treatment is by administration of drugs that act as cholinesterase inhibitors (ChEI).^{2,8,9} Depending on the AD stage, there is a decline in AChE levels in the brain and a progressive increase of BuChE which becomes responsible for the hydrolysis of acetylcholine.¹⁰⁻¹² Thus, the use of specific BuChE inhibitors may be indicated for the symptomatic treatment of AD.¹³

The attribute of carbamates as cholinesterase inhibitors has been known for decades. Physostigmine, an alkaloid present in Calabar bean, was the first carbamate used clinically as a cholinesterase inhibitor, in the treatment of glaucoma and later in AD. However, due to their high doses and side effects the use of physostigmine has been discontinued.¹⁴ Neostigmine and pyridostigmine are used in the treatment of myasthenia gravis, and neostigmine is also used in the treatment of glaucoma. Rivastigmine has a dual inhibitory action on AChE and BuChE enzymes and is used in the treatment of AD.¹⁵

Since carbamates have been successfully used to treat a variety of diseases involving cholinergic dysfunction, innumerable studies have been performed to find new ChEI that can be used clinically.¹⁶⁻¹⁹

Recently, *in vitro* inhibition tests performed in human blood samples for *cis*- and *trans*-2-arylamino-cyclohexyl *N,N*-dimethylcarbamates showed that carbamates of both series were selective for BuChE.²⁰ Moreover, carbamates tested exhibited a higher activity when compared with 2-*N,N*-dimethylamino-cyclohexyl 1-*N',N'*-dimethylcarbamates analogues,²¹ indicating that the presence of the arylamine group potentiated the BuChE inhibition.

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This work describes the synthesis, biological evaluation and molecular modeling of novel *cis*- and *trans*-3-arylamino-cyclohexyl *N,N*-dimethylcarbamates **5a-5c** and **6a-6c** as potential cholinesterase inhibitors. AChE and BuChE inhibition mode was accessed by kinetics studies, molecular docking and non-covalent interactions analysis.

Results and Discussion

Synthesis

New carbamates derivatives (**5a-5c** and **6a-6c**) were synthesized in three steps starting from the 2-cyclohex-1-enone (**1**) according to route outlined in Scheme 1.

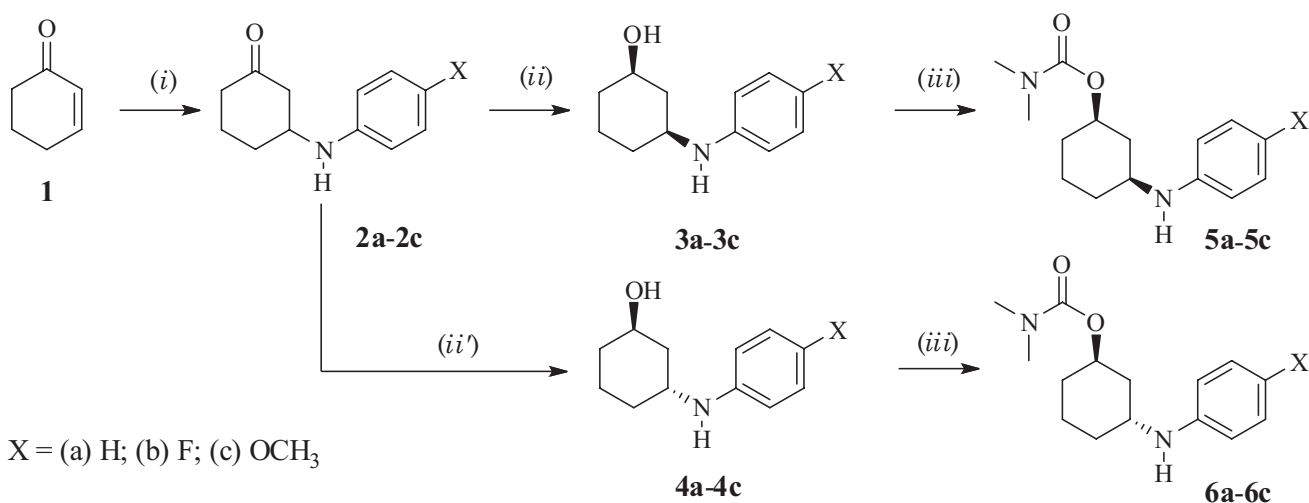
In the first step, we obtained the 3-arylamino-cyclohexanones (**2a-2c**) via aza-Michael addition of arylamines with 2-cyclohex-1-enone (**1**). The more common protocols of aza-Michael reaction use strong bases and acids as catalyst, and milder Lewis acidic catalysts. However, no conventional methodology was effective, since the arylamines exhibit a low nucleophilicity. Ying *et al.*²² have successfully used the basic ionic liquid 1,8-diazabicyclo[5.4.0]-undec-7-en-8-ium acetate ([DBU][Ac]) as promoter for aza-Michael addition under solvent free conditions. The authors propose that the ionic liquid increases the nucleophilicity of the arylamines. We modified the methodology proposed by Ying *et al.*²² using equimolar amounts of the Michael acceptor and arylamine and keeping the reaction time and temperature. The authors purified the compounds by column chromatography; to reduce the use of solvents we purified the products by recrystallization with good yields (75-82%). In the next step, the ketones **2a-2c** were reduced by two ways to

obtain the *cis*- and *trans*-3-arylamino-cyclohexanols (**3a-3c**; **4a-4c**). Using *N*-selectride as reducer²³ at low temperature ($-80\text{ }^{\circ}\text{C}$) the *trans*-3-arylamino-cyclohexanols **4a-4c** were obtained with high diastereoselectivity (ca. 100%) in good yields (72-85%). However, when ketones **3a-3c** were reduced with NaBH_4 ,²⁴ we observed that the product was obtained as the mixture of the isomers *cis/trans* in a ratio of 75/25. The identification of the isomers was conducted by comparative analysis of the chemical shifts of geminal hydrogen to hydroxyl group (H_1). The chemical shifts of the H_1 for the products obtained by reduction with *N*-selectride were 4.10 to 4.20 ppm, while the chemical shifts of the H_1 for the products obtained using NaBH_4 as reducer were 3.60 to 3.81 ppm. According to literature, the hydrogens oriented in the equatorial position are more deshielded than the axial hydrogens.^{25,26}

The mixture of isomers was used in the next step. Finally, the alcohols (**3a-3c**; **4a-4c**) were carbamoylated with *N,N*-dimethylcarbamoyl chloride to give compounds **5a-5c** and **6a-6c**. Further purification by column chromatography afforded the desired carbamates in moderate yields (30-46%), except the compound **5b** that was obtained as a mixture with the isomer **6b**.

Inhibition assays of AChE and BuChE

Inhibitory activities of the novel synthesized carbamates against cholinesterases (ChEs) from fresh human blood were evaluated by Ellman's modified²⁷ spectroscopic method. Since this method requires water soluble compounds, the respective hydrochlorides **5a'**, **5c'** and **6a'-6c'** were prepared. The assays were performed at five different concentrations (0.067, 0.13, 0.27, 0.67 and



Scheme 1. Synthesis of *cis*- and *trans*-3-arylamino-cyclohexyl *N,N*-dimethylcarbamates. Reagents and conditions: (i) arylamine, 1,8-diazabicyclo[5.4.0]-undec-7-en-8-ium acetate ([DBU][Ac]), r.t., 5 h; (ii) NaBH_4 , tetrahydrofuran (THF), r.t., 48 h; (ii') *N*-selectride, THF, $-80\text{ }^{\circ}\text{C}$, 4 h; (iii) NaH/THF , $80\text{ }^{\circ}\text{C}$, 8 h; *N,N*-dimethylcarbamoyl chloride, 16 h.

1.3 mmol L⁻¹) for compounds **5c'**, **6a'-6c'** and at four different concentration (0.05, 0.1, 0.2, and 0.5 mmol L⁻¹) for compound **5a'**. In these experiments, we could determine the inhibition potential of each compound tested against AChE (found in red cells) and BuChE (found in plasma). The inhibitory activity was expressed as the compound concentration that inhibits 50% of enzyme activity (IC₅₀). The results of the AChE and BuChE inhibition and IC₅₀ values, which were obtained from the curves of percent inhibition *vs.* concentration, are summarized in Table 1. First, we observed that all carbamate derivatives exhibited a dose-dependent inhibitory activity for both cholinesterases and showed selectivity for the BuChE inhibition. The compounds **5c'** and **6a'-6c'** displayed maximum inhibitory activity for BuChE of 85 to 88% at 1.3 mmol L⁻¹. Due to low solubility of compound **5a'**, the maximum inhibition for BuChE was 72% at 0.5 mmol L⁻¹. Derivatives **5c'** and **6c'** were the more active compounds, with an IC₅₀ of 0.11 mmol L⁻¹. The results suggest that there is no relevant difference in inhibition of BuChE activity between the *cis* and *trans* isomers and that the presence of the methoxyl group potentialized the anticholinesterase activity. The effect of methoxyl group is mainly noted in the lowest concentration tested (0.067 mmol L⁻¹). The compound **6c'** showed an inhibition of 40% against 30 and 25%

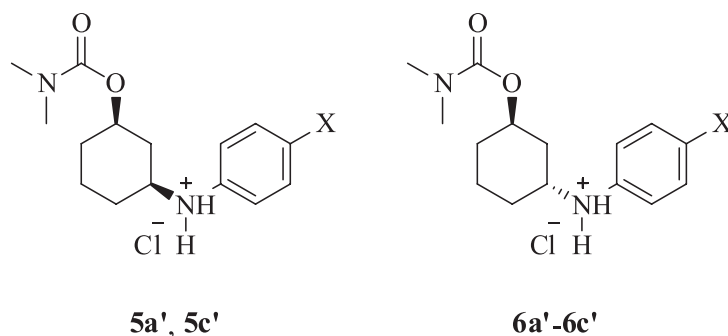
for compounds **6a'** and **6b'**, respectively. Relative to the AChE inhibition no tested compound inhibited 50% of the enzyme activity in any of the investigated concentrations. All compounds were weakly active against AChE with an inhibition between 12 and 48% in the maximum concentration measured.

Bagatin *et al.*²⁰ studied inhibitory properties of *cis*- and *trans*-2-arylamino-cyclohexyl *N,N*-dimethylcarbamates. Comparatively, the carbamates tested in this present study showed a significant improvement in the inhibitory activity against BuChE. The synthesized compounds showed IC₅₀ of 0.11 to 0.18 mmol L⁻¹ and all 1,3-disubstituted derivatives were more active than correlated 1,2-disubstituted compounds, displaying an improvement in the inhibitory activity of 30 to 50 times. These data indicate that the arylamine group in position 3 of cyclohexyl ring enhanced the inhibitory potential of the new carbamates.

Kinetic studies of AChE and BuChE inhibition

The type of AChE and BuChE inhibition was investigated from the kinetics studies using the modified Ellman's method²⁷ and compound **6c'**, the most potent inhibitor of both enzymes. To assess the kinetic parameters, we measured the initial rate of enzyme activity at

Table 1. ChEs inhibitory activity of compounds **5a'**, **5c'** and **6a'-6c'**



Compound	X	IC ₅₀ / (mmol L ⁻¹)		Inhibition / %			
		AChE ^a	BuChE	Minimum ^b	Maximum ^c		
				BuChE	AChE	BuChE	
	H	5a'	n.a.	0.18 (± 0.06)	12	12	72
	OCH ₃	5c'	n.a.	0.11 (± 0.01)	33	32	88
	H	6a'	n.a.	0.14 (± 0.01)	30	32	85
	F	6b'	n.a.	0.14 (± 0.01)	25	38	85
	OCH ₃	6c'	n.a.	0.11 (± 0.01)	40	48	86
	Rivastigmine		29 μmol L ⁻¹	0.32 μmol L ⁻¹	–	–	–

^an.a.: Not active (IC₅₀ > 1.3 mmol L⁻¹); ^bthe minimum concentration measured was 0.067 mmol L⁻¹ for **5c'** and **6a'-6c'** and 0.05 mmol L⁻¹ for **5a'**; ^cthe maximum concentration measured was 1.3 mmol L⁻¹ for **5c'** and **6a'-6c'** and 0.5 mmol L⁻¹ for **5a'**. IC₅₀: Compound concentration that inhibits 50% of enzyme activity; AChE: acetylcholinesterase; BuChE: butyrylcholinesterase.

different concentrations of substrate acetylthiocholine (0.05 to 1.0 mmol L⁻¹) or butyrylthiocholine (0.75 to 1.0 mmol L⁻¹) in the absence and presence of the inhibitor **6c'**. Lineweaver-Burk plots (Figure 1) were built by the reciprocal of the initial rate (V_0^{-1}) against the reciprocal of substrate concentrations ($[S]^{-1}$) for the different inhibitor concentrations resulting from the substrate-velocity curves for AChE and BuChE.

Graphical analysis of Lineweaver-Burk plots and the kinetic parameters of AChE activity (Figure 1a) showed a practically unchanged K_m value with increasing inhibitor concentration and increasing slopes with increasing inhibitor concentration, i.e., V_{max} is reduced. This pattern indicates a non-competitive-type inhibition.²⁸ However, Lineweaver-Burk plots and the kinetic parameters of BuChE activity (Figure 1b) showed both increasing slopes (lower V_{max}) and intercepts (higher K_m) with higher inhibitor concentration, i.e., the increasing inhibitor concentration reduces V_{max} and increases K_m value. This pattern indicates a mixed-type inhibition of BuChE.²⁸ It is shown that the compound **6c'** could interact with both the free enzyme and the complex enzyme substrate, which could explain the higher activity against BuChE than AChE. These results also suggest that although carbamates occupy a significant fraction of the catalytic gorge, they do not compete for the same binding site as the substrate. The dissociation constant (K_i) values obtained are consistent with inhibitory activity. Compound **6c'** was found to be more potent inhibitor of BuChE (K_i 0.21 mmol L⁻¹) than AChE (K_i 0.69 mmol L⁻¹).

Molecular modeling studies

The molecular docking calculations were performed using AutoDock 4.2.3²⁹ program implemented at the PyRx 0.9³⁰ interface in order to evaluate the enzyme-inhibitor interactions of the newly synthesized compounds. The compound **6c'** was docked in the active site of AChE

(Protein Data Bank (PDB) 1ACJ) and BuChE (PDB 4BDS) derived from complex of the enzymes with tacrine obtained from the PDB. The best docked poses, i.e., the lowest energy conformer in the most populated cluster of conformers were subjected to energy minimization by NAMD³¹ program and analyzed to explain interactions between ligands and the target enzyme. Figure 2a shows the compound **6c'** complexed with BuChE. In active site gorge, both hydrogen atoms of the protonated amino group are likely to form an H-bond with an imidazolyl nitrogen atom of His438 and an oxygen atom at carbon 5 of Glu197 with a distance of 1.6 and 1.8 Å, respectively. The positively-charged nitrogen of ligand made a cation- π interaction with the imidazole ring of His438. Carbamate group is stabilized by H-bond between carbonyl oxygen and indole NH of Trp82 with a distance of 3.0 Å. Also, cyclohexyl ring is stabilized by van der Waals interaction with aromatic rings of Trp82. It is possible to observe that the aromatic ring of **6c'** is surrounded by the aromatic rings of Trp231 and Phe398 forming T-stacking type interaction. In addition, *N*-methyl group of ligand interacts with Tyr332 and the aromatic ring of compound **6c'** forms T-stacking type interaction with Phe329. Studies performed by Macdonald *et al.*³² with inhibitor competition and BuChE mutant species indicate that Tyr332 and Phe329 are amino acid residues important for binding inhibitors at the peripheral anionic site (PAS) of BuChE. This binding mode is in agreement with mixed-type inhibition of **6c'**, in which the ligand is able to interact with both active gorge site and PAS.

The new non-covalent interaction (NCI) approach developed by Yang and co-workers³³ enables simultaneous analysis and graphical visualization of a wide range of these interactions in real space surface. Thus, the network of interactions was identified using the NCI index, which defines the regions of attractive or repulsive interactions, as well as their strength.³³ In what follows, the enzyme-ligand interactions revealed by the NCI analysis are color

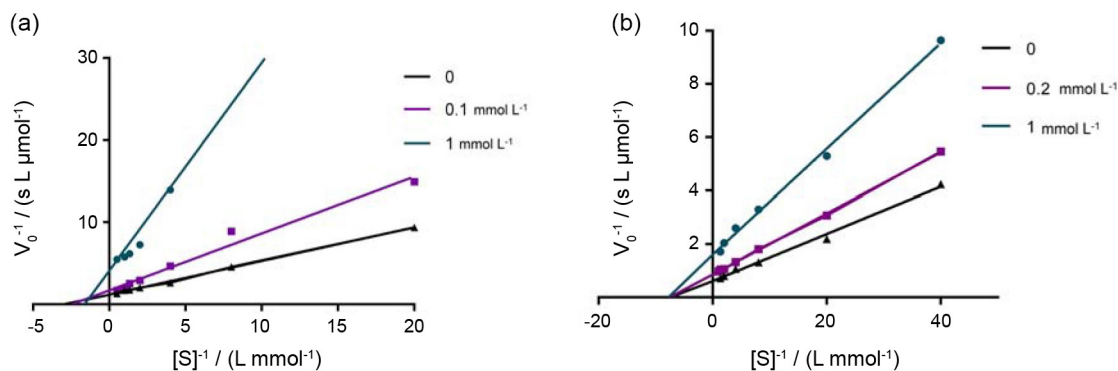


Figure 1. Lineweaver-Burk plot for the inhibition of (a) AChE with different acetylthiocholine concentrations (0.05 to 1.0 mmol L⁻¹) in the absence and presence of compound **6c'** in concentrations of 0.2 and 1 mmol L⁻¹; and (b) BuChE with different butyrylthiocholine concentrations (0.75 to 2.0 mmol L⁻¹) in the absence and presence of compound **6c'** in concentrations of 0.1 and 1 mmol L⁻¹.

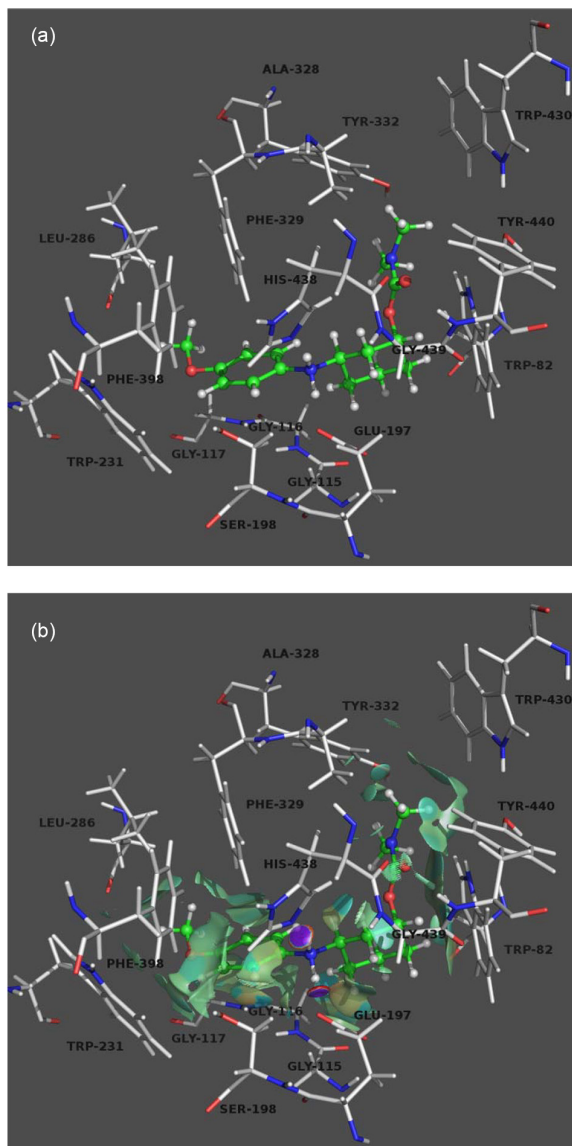


Figure 2. (a) Binding mode of **6c'** and BuChE. The compounds are rendered in green ball-and-stick models, and the residues are rendered in grey colored sticks. (b) Non-covalent interaction (NCI) isosurfaces (blue to violet: strongly attractive; green: weakly attractive; orange to red: repulsive).

coded from violet for strongly attractive weak interactions (hydrogen bonds) to red for repulsive ones (steric clashes). In between, the very weak van der Waals interactions appear in green. The gradient isosurface (Figure 2b) indicates two attractive interactions (violet disks) corresponding to strong H-bonds and several intermolecular van der Waals interactions (in green) that are not atom-specific and occupy broader regions in space.³³ Strongly repulsive interactions, which would appear in red, were not observed, showing that the ligand is well stabilized in active site of BuChE.

The orientation of the compound **6c'** in AChE (Supplementary Information Figure S2) is quite different

from the orientation in BuChE, ergo, the interactions between the ligand and AChE are also different. The carbamate group does not exhibit any significant interaction with residues. Both hydrogen atoms of the protonated amino group form an H-bond with the indole nitrogen atom of Trp84 and oxygen atom of Gly441 with a distance of 2.3 and 1.8 Å, respectively. The cyclohexyl ring is stabilized by van der Waals interaction with aromatic rings of Trp84. Finally, the aromatic ring of **6c'** can interact with Tyr442 and Phe330 via T-stacking and π - π stacking interaction, respectively. The isosurfaces of AChE (Figure S3) are in agreement with the molecular docking.

Conclusions

This study has resulted in a series of new selective BuChE inhibitors, with IC_{50} values of 0.11 to 0.18 mmol L⁻¹. The compounds **5c'** and **6c'** were the most active in each series, showing that the *cis-trans* isomerism does not influence the activity of the compounds significantly and that the methoxy group potentialized the anticholinesterase activity.

In general, the newly synthesized carbamates showed a significant improvement in the inhibition of BuChE activity when compared to their 1,2-substituted analogs, indicating that the presence of arylamine group in position 3 of cyclohexyl ring enhanced the activity against BuChE. Preliminary kinetics studies for the most active inhibitor (**6c'**) indicated a non-competitive-type inhibition for AChE and mixed-type inhibition against BuChE which is compatible with our molecular modeling studies. Molecular docking combined with NCI approach was shown to be quite efficient in analyzing the inhibition mode of the new carbamates against AChE and BuChE.

Experimental

Chemicals

Starting materials and reagents were purchased from Sigma-Aldrich and Acros. For column chromatography, silica gel 60, 230-400 mesh (Merck) was used. Melting points were determined with a Micro-Química apparatus model MQAPF-301 and are uncorrected. Nuclear magnetic resonance (NMR) spectra were acquired with Varian Mercury Plus BB 300 MHz and Bruker Avance III HD 300 and 500 MHz. The spectra were recorded in 20 mg cm⁻³ solutions of CDCl₃, with a probe temperature of ca. 300 K and tetramethylsilane (TMS) as reference. High-resolution mass spectrometry (HRMS) analyses were performed for new 3-arylamino-cyclohexyl *N,N*-dimethylcarbamates. The products were dissolved in a solution of 50% (v/v)

chromatographic grade acetonitrile (Tedia), 50% (v/v) deionized water and 0.1% formic acid. The solutions were infused directly individually into the electrospray ionization (ESI) source by means of a syringe pump (Harvard Apparatus) at a flow rate of 150 $\mu\text{L min}^{-1}$. ESI(+)-MS and tandem ESI(+)-MS/MS were acquired using a hybrid high-resolution and high accuracy (5 $\mu\text{L L}^{-1}$) microTof (quadrupole time-of-flight (Q-TOF)) mass spectrometer (Bruker Scientific) under the following conditions: capillary and cone voltages were set to +3500 and +40 V, respectively, with a de-solvation temperature of 100 °C. For ESI(+)-MS/MS, the energy for the collision induced dissociations (CID) was optimized for each component. For data acquisition and processing in QTOF-control data analysis software (Bruker Scientific) was used. The data were collected in the m/z range of 70-800 at the speed of two scans *per* second, providing the resolution of 50,000 full width at half maximum (FWHM) at m/z 200.

General procedure for the synthesis

3-Arylamino-cyclohexanones (**2a-2c**)

To a mixture of 2-cyclohexen-1-one (20 mmol) and arylamine (20 mmol) was added ionic liquid [DBU][Ac] (0.3 eq). The reaction was stirred at room temperature for 5 h. After completion of the reaction, 5 mL of water and 20 mL of ethyl acetate were added to the reaction mixture. The aqueous phase was separated and stored for the recovery of ionic liquid. To the organic phase were added 10 mL of water and a solution of NaOH (0.1 mol L^{-1}) until pH 12. The organic phase was washed with NaCl solution, dried over anhydrous Na_2SO_4 and the solvent removed under vacuum evaporation. The crude product was purified by recrystallization from hexane/ethyl acetate. The recrystallized solid was washed with cooled hexane and dried under vacuum at room temperature.

cis-3-Arylamino-cyclohexanols (**3a-3c**)

To a solution of NaBH_4 (26 mmol) in anhydrous tetrahydrofuran (THF) (30 mL) was slowly added 3-arylamino-cyclohexanone **2a**, **2b** or **2c** (10.5 mmol). The reaction was stirred at room temperature under nitrogen atmosphere for 48 h. A solution of 1% HCl was added for the formation of a white salt. The mixture was extracted with ethyl acetate (3 \times 30 mL), the organic phase was dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure.

trans-3-Arylamino-cyclohexanols (**4a-4c**)

A solution of 3-arylamino-cyclohexanone **2a**, **2b** or **2c** (8 mmol) in anhydrous THF (30 mL) was cooled to

$-80\text{ }^\circ\text{C}$ and 1.0 mol L^{-1} *N*-selectride (16 mL, 16 mmol) was added. The mixture was stirred at low temperature ($-80\text{ }^\circ\text{C}$) under nitrogen atmosphere for 4 h. The reaction mixture was allowed to attain room temperature and then was hydrolyzed with cooled water (3 mL) and ethanol (9 mL). The organoborane was oxidized with 6 mol L^{-1} NaOH (6 mL) and 30% H_2O_2 (9 mL). The mixture was extracted several times with ethyl acetate. The organic phase was dried with anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The resultant crude product was purified by recrystallization from hexane/ethyl acetate. The recrystallized product was washed with cooled hexane and dried under vacuum at room temperature.

Spectral data of compounds **2a-2c**, **3a-3c** and **4a-4c** are available in Supplementary Information.

3-Arylamino-cyclohexyl *N,N*-dimethylcarbamates (**5a-5c**; **6a-6c**)

To a solution of 3-arylamino-cyclohexanol **3a**, **3c**, **4a**, **4b** or **4c** (3.3 mmol) in anhydrous THF (30 mL) was added sodium hydride (6.7 mmol). The resulting mixture was stirred to 80 °C under nitrogen atmosphere for 8 h. After this time, dimethylcarbonyl chloride (5.4 mmol) was slowly added and the reaction was stirred under reflux for 16 h. The reaction mixture was allowed to attain room temperature, poured into a cold solution of 1% NaHCO_3 and extracted with ethyl acetate (3 \times 30 mL). The organic phase was dried with anhydrous Na_2SO_4 and the solvent removed under vacuum evaporation. The residue was purified by column chromatography (hexane/ethyl acetate at increasing gradient polarity) to give the pure product.

cis-3-(Phenylamino)cyclohexyl *N,N*-dimethylcarbamate (**5a**)

Crystal solid; m.p. 101.2-101.5 °C; yield: 46%; $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.17 (m, 2H, H3', H5'), 6.68 (m, 1H, H4'), 6.59 (m, 2H, H2', H6'), 4.74 (dddd, 1H, *J* 10.1, 10.1, 4.1, 4.1 Hz, H1), 3.60 (sl, 1H, NH), 3.41 (dddd, 1H, *J* 10.5, 10.5, 3.8, 3.8 Hz, H3), 2.90 (s, 6H, 2Me), 2.41 (m, 1H, H2e), 2.02 (m, 2H, H4e, H6e), 1.82 (m, 1H, H5e), 1.36 (m, 1H, H5a), 1.33 (m, 1H, H4a), 1.25 (m, 1H, H2a), 1.15 (m, 1H, H6a); $^{13}\text{C NMR}$ (75 MHz, CDCl_3) δ 156.2 (C7), 147.1 (C1'), 129.5 (C3', C5'), 117.4 (C4'), 113.4 (C2', C6'), 72.3 (C1), 50.4 (C3), 38.8 (C2), 37.6 (C8), 32.5 (C6), 31.9 (C4), 21.4 (C5); HRMS (ESI+) calcd. for $\text{C}_{15}\text{H}_{23}\text{N}_2\text{O}_2$ [$\text{M} + \text{H}$] $^+$: 263.1754; found: 263.1734.

cis-3-(4-Methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate (**5c**)

Brown oil; yield: 42%; $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 6.78 (d, 2H, *J* 5.0 Hz, H3', H5'), 6.59 (d, 2H, *J* 5.0 Hz, H2', H6'), 4.73 (dddd, 1H, *J* 10.1, 10.1, 4.1, 4.1 Hz, H1),

3.76 (s, 3H, OMe), 3.32 (dddd, 1H, *J* 10.0, 10.0, 3.8, 3.8 Hz, H3), 2.91 (s, 6H, 2Me), 2.41 (m, 1H, H2e), 2.03 (m, 2H, H4e, H6e), 1.86 (m, 1H, H5e), 1.38 (m, 1H, H5a), 1.33 (m, 1H, H6a), 1.23 (m, 1H, H2a), 1.11 (m, 1H, H4a); ¹³C NMR (75 MHz, CDCl₃) δ 156.5 (C7), 152.6 (C4'), 141.5 (C1'), 115.3 (C2', C6'), 115.2 (C3', C5'), 72.6 (C1), 56.2 (OMe), 51.8 (C3), 39.5 (C2), 35.9 (C8), 32.8 (C6), 32.2 (C4), 21.8 (C5); HRMS (ESI+) calcd. for C₁₆H₂₅N₂O₃ [M + H]⁺: 293.1860; found: 293.1827.

trans-3-(Phenylamino)cyclohexyl *N,N*-dimethylcarbamate (**6a**)

Yellow oil; yield: 36%; ¹H NMR (300 MHz, CDCl₃) δ 7.16 (m, 2H, H3', H5'), 6.67 (m, 1H, H4'), 6.58 (m, 2H, H2', H6'), 5.09 (m, 1H, H1), 3.63 (dddd, 1H, *J* 10.1, 10.1, 3.8, 3.8 Hz, H3), 3.51 (sl, 1H, NH), 2.95 (s, 6H, 2Me), 2.18 (m, 1H, H2e), 2.04 (m, 1H, H6e), 1.80 (m, 1H, H4e), 1.68 (m, 2H, H5a, H5e), 1.55 (m, 1H, H4a), 1.41 (m, 1H, H2a), 1.24 (m, 1H, H6a); ¹³C NMR (75 MHz, CDCl₃) δ 156.0 (C7), 147.4 (C1'), 129.5 (C3', C5'), 117.3 (C4'), 113.0 (C2', C6'), 70.8 (C1), 47.9 (C3), 37.5 (C2), 36.3 (C8), 32.5 (C6), 30.2 (C4), 20.0 (C5); HRMS (ESI+) calcd. for C₁₅H₂₃N₂O₂ [M + H]⁺: 263.1754; found: 263.1731.

trans-3-(4-Fluorophenylamino)cyclohexyl *N,N*-dimethylcarbamate (**6b**)

Brown oil; yield: 42%; ¹H NMR (300 MHz, CDCl₃) δ 6.86 (m, 2H, H3', H5'), 6.50 (m, 2H, H2', H6'), 5.07 (m, 1H, H1), 3.54 (dddd, 1H, *J* 10.0, 10.0, 3.8, 3.8 Hz, H3), 2.94 (s, 6H, 2Me), 2.18 (m, 1H, H2e), 2.03 (m, 1H, H6e), 1.81 (m, 1H, H4e), 1.66 (m, 2H, H5a, H5e), 1.53 (m, 1H, H4a), 1.43 (m, 1H, H2a), 1.22 (m, 1H, H6a); ¹³C NMR (75 MHz, CDCl₃) δ 157.3 (C7), 155.2 (d, *J* 235.6 Hz, C4'), 143.6 (d, *J* 1.9 Hz, C1'), 116.0 (d, *J* 22.3 Hz, C3', C5'), 114.1 (d, *J* 7.3 Hz, C2', C6'), 70.9 (C1), 48.7 (C3), 39.5 (C2), 36.3 (C8), 32.5 (C6), 30.3 (C4), 20.2 (C5); HRMS (ESI+) calcd. for C₁₅H₂₂FN₂O₂ [M + H]⁺: 281.1660; found: 281.1672.

trans-3-(4-Methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate (**6c**)

Brown oil; yield: 40%; ¹H NMR (300 MHz, CDCl₃) δ 6.77 (d, 2H, *J* 9.9 Hz, H3', H5'), 6.55 (d, 2H, *J* 9.9 Hz, H2', H6'), 5.08 (m, 1H, H1), 3.74 (s, 3H, OMe), 3.54 (dddd, 1H, *J* 10.0, 10.0, 3.8, 3.8 Hz, H3), 2.94 (s, 6H, 2Me), 2.17 (m, 1H, H2e), 2.06 (m, 1H, H6e), 1.79 (m, 1H, H4e), 1.67 (m, 2H, H5a, H5e), 1.53 (m, 1H, H4a), 1.43 (m, 1H, H2a), 1.21 (m, 1H, H6a); ¹³C NMR (75 MHz, CDCl₃) δ 157.3 (C7), 152.2 (C4'), 141.5 (C1'), 115.2 (C3', C5'), 114.8 (C2', C6'), 71.0 (C1), 56.0 (OMe), 49.0 (C3), 37.8 (C2), 36.3 (C8), 32.7 (C6), 30.4 (C4), 20.2 (C5); HRMS (ESI+) calcd. for C₁₆H₂₅N₂O₃ [M + H]⁺: 293.1860; found: 293.1828.

3-Arylamino-cyclohexyl *N,N*-dimethylcarbamate hydrochlorides (**5a'**, **5c'**; **6a'-6c'**)

A solution of appropriate carbamates **5a**, **5c** and **6a-6c** (1.0 mmol) in dichloromethane was cooled in ice bath (0-5 °C) and 37% hydrochloric acid (0.5 mL) was added, keeping the solution under stirring for 5 min. The solution was dried over anhydrous Na₂SO₄ and the solvent removed under vacuum evaporation.

cis-3-(Phenylamino)cyclohexyl *N,N*-dimethylcarbamate hydrochloride (**5a'**)

White solid; yield: 96%; ¹H NMR (300 MHz, CDCl₃) δ 11.3 (sl, NH), 7.55 (m, 2H, H3', H5'), 7.39 (m, 3H, H2', H4', H6'), 4.47 (m, 1H, H1), 3.29 (m, 1H, H3), 2.84 (s, 6H, 2Me), 2.27 (m, 1H, H2e), 2.23 (m, 1H, H4e), 1.91 (m, 2H, H6a, H6e), 1.75 (m, 2H, H2a, H5e), 1.25 (m, 1H, H4a), 1.23 (m, 1H, H5a); ¹³C NMR (75 MHz, CDCl₃) δ 155.7 (C7), 134.0 (C1'), 130.0 (C2', C6'), 129.2 (C4'), 124.1 (C3', C5'), 71.3 (C1), 60.5 (C3), 40.6 (C2), 36.4 (C8), 31.2 (C6), 28.6 (C4), 21.7 (C5).

cis-3-(4-Methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate hydrochloride (**5c'**)

Brown solid; yield: 93%; ¹H NMR (300 MHz, CDCl₃) δ 11.3 (sl, NH), 7.42 (d, 2H, *J* 9.9 Hz, H3', H5'), 6.84 (d, 2H, *J* 9.9 Hz, H2', H6'), 4.49 (m, 1H, H1), 3.79 (m, 3H, OMe), 3.22 (m, 1H, H3), 2.84 (s, 6H, 2Me), 2.30 (m, 1H, H2e), 2.26 (m, 1H, H4e), 1.96 (m, 1H, H6e), 1.78 (m, 1H, H5e), 1.71 (m, 1H, H2a), 1.55 (m, 1H, H4a), 1.27 (m, 1H, H6a), 1.26 (m, 1H, H5a); ¹³C NMR (75 MHz, CDCl₃) δ 158.3 (C7), 154.6 (C4'), 125.9 (C1'), 123.6 (C2', C6'), 113.9 (C3', C5'), 70.2 (C1), 58.8 (OMe), 54.5 (C3), 35.3 (C8), 33.6 (C2), 30.1 (C6), 27.6 (C4), 20.1 (C5).

trans-3-(Phenylamino)cyclohexyl *N,N*-dimethylcarbamate hydrochloride (**6a'**)

Yellow solid; yield: 91%; ¹H NMR (300 MHz, CDCl₃) δ 11.3 (sl, NH), 7.55 (m, 2H, H3', H5'), 7.39 (m, 3H, H2', H4', H6'), 5.02 (m, 1H, H1), 3.52 (m, 1H, H3), 2.83 (s, 6H, 2Me), 2.21 (m, 1H, H6e), 2.20 (m, 1H, H2e), 1.83 (m, 1H, H2a), 1.79 (m, 2H, H4e, H6a), 1.59 (m, 2H, H5a, H5e), 1.55 (m, 1H, H4a); ¹³C NMR (75 MHz, CDCl₃) δ 155.5 (C7), 133.8 (C1'), 130.0 (C2', C6'), 129.4 (C4'), 124.3 (C3', C5'), 69.5 (C1), 58.9 (C3), 36.3 (C8), 33.2 (C2), 29.0 (C6), 28.7 (C4), 19.3 (C5).

trans-3-(4-Fluorophenylamino)cyclohexyl *N,N*-dimethylcarbamate hydrochloride (**6b'**)

Gray solid; yield: 95%; ¹H NMR (300 MHz, CDCl₃) δ 11.3 (sl, NH), 7.56 (m, 2H, H3', H5'), 7.09 (m, 2H, H2', H6'), 5.07 (m, 1H, H1), 3.48 (m, 1H, H3), 2.85 (s, 6H,

2Me), 2.23 (m, 1H, H4e), 2.22 (m, 1H, H2e), 2.21 (m, 1H, H6e), 1.83 (m, 1H, 2a), 1.72 (m, 1H, 4a), 1.63 (m, 1H, H5e), 1.54 (m, 1H, H5a), 1.39 (m, 1H, H6a); ^{13}C NMR (75 MHz, CDCl_3) δ 162.8 (d, J 235.6 Hz, C4'), 155.4 (C7), 129.5 (d, J 1.9 Hz, C1'), 126.4 (d, J 22.3 Hz, C3', C5'), 117.1 (d, J 7.3 Hz, C2', C6'), 69.3 (C1), 59.3 (C3), 36.3 (C8), 33.1 (C2), 29.0 (C6), 28.6 (C4), 19.3 (C5).

trans-3-(4-Methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate hydrochloride (**6c'**)

Brown solid; yield: 92%; ^1H NMR (300 MHz, CDCl_3) δ 11.2 (s, NH), 7.47 (d, 2H, J 9.9 Hz, H3', H5'), 6.86 (d, 2H, J 9.9 Hz, H2', H6'), 5.05 (m, 1H, H1), 3.80 (s, 3H, OMe), 3.46 (m, 1H, H3), 2.85 (s, 6H, 2Me), 2.26 (m, 1H, H4e), 2.20 (m, 1H, H2e), 1.83 (m, 1H, H6e), 1.81 (m, 1H, H2a), 1.72 (m, 1H, H4a), 1.61 (m, 2H, H5a, H5e), 1.25 (m, 1H, H6a); ^{13}C NMR (75 MHz, CDCl_3) δ 159.3 (C7), 155.6 (C4'), 127.6 (C1'), 124.6 (C3', C5'), 114.9 (C2', C6'), 71.2 (C1), 59.9 (OMe), 55.5 (C3), 36.4 (C2), 36.3 (C8), 28.6 (C4), 28.0 (C6), 21.1 (C5).

ChEs inhibition assays

AChE and BuChE inhibitory activities were evaluated in heparinized fresh human blood by spectrophotometrical Ellman's modified method²⁷ and rivastigmine was used as reference compound. AChE inhibitory activity was determined from total cholinesterase (erythrocyte + plasma) in a reaction cuvette. For measurement, a solution of 10 μL of human blood in 10 mL of a 100 mmol L^{-1} phosphate buffer at pH 8.0 was used. Firstly, 3 mL of this solution and 40 μL of inhibitor solution were preincubated for 10 min at 30 $^\circ\text{C}$. Then, 50 μL of 10 mmol L^{-1} 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) solution in 100 mmol L^{-1} phosphate buffer at pH 7.0 containing 0.17 mmol L^{-1} NaHCO_3 (50 μL) and 20 μL of 75 mmol L^{-1} acetylthiocholine iodine solution were added. Enzyme activity was determined by measuring the increase of absorbance at 412 nm for 5 min at 30 $^\circ\text{C}$ with a Shimadzu UV-1061PC apparatus. An inhibitor-free sample was used (100% enzyme activity) as a reference. The assays were performed at five different concentrations (0.067, 0.13, 0.27, 0.67 and 1.3 mmol L^{-1}) for compounds **5c'**, **6a'-6c'** and at four different concentration (0.05, 0.1, 0.2, and 0.5 mmol L^{-1}) for compound **5a'**, in triplicate. IC_{50} values were calculated by nonlinear regression analysis using Excel. BuChE inhibitory activity was assessed similarly using a solution of 20 μL human plasma in 12 mL of a 100 mmol L^{-1} phosphate buffer at pH 8.0. A volume of 3 mL of this solution and 40 μL of inhibitor solution were preincubated for 10 min at 30 $^\circ\text{C}$. Next, 25 μL of 10 mmol L^{-1} DTNB solution in 100 mmol L^{-1} phosphate

buffer solution at pH 7.0 containing 0.17 mmol L^{-1} NaHCO_3 were added. Enzyme activity was measured as for total cholinesterase.

Kinetic studies of ChEs inhibition

Kinetics studies were carried out by Ellman's modified method for compound **5c'**, using a 0.1 U mL^{-1} solution of AChE from electric eel (500 U) and BuChE from horse serum (1200 U). The test was performed without the inhibitor, in 0.2 and 1.0 mmol L^{-1} concentration of the inhibitor for AChE and 0.1 and 1.0 mmol L^{-1} concentration for BuChE. Acetylthiocholine iodine and butyrylthiocholine iodine were used as substrate of reaction in the following final concentrations: 0.025, 0.05, 0.125, 0.50, 0.75, 1.0 and 2.0 mmol L^{-1} . The absorbance was measured in 6 s for 5 min. The obtained data were used to create substrate-velocity curves which were transformed in GraphPad Prism program to Lineweaver-Burk plots.

Molecular modeling

The crystal structures of AChE complexed with tacrine (ID: 1ACJ) and BuChE complexed with tacrine (ID: 4BDS) were obtained from the Protein Data Bank and the molecular docking studies were performed using the AutoDock 4.2.3 program²⁹ implemented at the PyRx 0.9 interface.³⁰ For each PDB file, a few molecules of water and other ligands (except the main ligand tacrine) were removed.

The compound structures were drawn and optimized with the Gaussian 09 program.³⁴ The box dimensions were set at 50 \times 46 \times 46 \AA^3 and all maps were calculated with 0.375 \AA spacing between grid points. The center of the grid box was placed at the following coordinates AChE [4.24, 69.34, 65.34]; BuChE [132.5, 116.0, 40.7]. The Lamarckian genetic algorithm (LGA) was used as a standard protocol of 50 poses obtained for the ligand, an initial population of 150 random individuals, a maximum number of 2.5×10^5 energy evaluations and a maximum of 2.7×10^4 generations. The docked results within a root mean square deviation (RMSD) of 2.0 \AA were clustered and the final results of each ligand were selected considering both the embedded empirical binding free energy evaluation and the clustering analysis.

Redocking simulations were performed to validate the parameters that had been chosen and were repeated four times which gave RMSD values below 0.5 \AA . The hydrogens of amino acid residues of each enzyme were added by Gromacs program version 4.6-beta1.³⁵ The best results were submitted to energy minimization with the NAMD2 program.³¹ The force field adopted for proteins

was CHARMM C35b2-C36a2, and for the ligands, they were generated in the same format by the SwissParam server.³⁶ The results are shown with the PyMOL molecular graphics system, version 1.7.4 (Schrödinger LLC).

NCIs were plotted using NCIPLOT version 3.0³³ with the coordinates from the minimized structure by E-Babel.³⁷ Three-dimensional representations were generated using PyMOL.

Supplementary Information

Supplementary information is available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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