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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Phthalamide derivatives as ACE/AChE/BuChE inhibitors against cardiac hypertrophy: an in silico, in vitro, and in vivo modeling approach

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Abstract

Left ventricular hypertrophy (LVH) is a major adaptative response to the increase in the overload produced by hypertension, is a risk factor for myocardial infarction, stroke, and heart failure. Among the several factors involved in hypertension and in the progression to cardiac hypertrophy, the hyperactivity of the renin–angiotensin system (RAS) and the dysfunction of the neurovisceral/autonomic nervous system are the main mechanisms involved. Evidence demonstrates that the inhibition of RAS and the increase in parasympathetic activity reduce significantly LVH ameliorating cardiac function. The development of multi-target compounds is a relevant strategy for treating hypertension and cardiac hypertrophy. This study aimed to synthesize three phthalamide derivatives (M-01, M-02, and M-03) and evaluate them as three-target (ACE/AChE/BuChE) inhibitors with the possible dual effect of reducing hypertension and reverting cardiac hypertrophy. After in silico and in vitro experiments, one compound was tested in vivo on rats. All three phthalamides were synthesized in good yields, showing good competitive inhibition of the three-target enzymes in silico and in vitro. M-01 (10 mg/kg) significantly reversed cardiomyocite hypertrophy (by 87.3%; $p < 0.001$) in the heart of spontaneous hypertensive rat (SHR) model. It was at least 18-fold more potent than the reference drug (captopril), which provided only 32.7% reversion. Three-target inhibitory activity was herein demonstrated for M-01, M-02, and M-03 in vitro and in silico, each with a similar effect. The compound tested in vivo (M-01) exhibited great potency in reducing hypertension and reverting cardiomyocyte hypertrophy, making it a promising candidate for further research.

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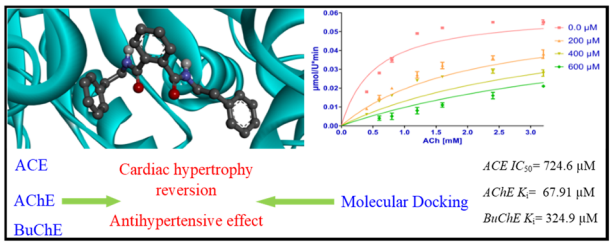
Graphical Abstract



Keywords Hypertension · Molecular docking · ACE inhibitor · AChE inhibitor · BuChE inhibitor · Cardiac hypertrophy reversion

Highlights

- The phthalamide derivatives are three-target inhibitors, acting on ACE, AChE, and BuChE.
- The test compounds showed potency in treating hypertension and reverting cardiomyocyte hypertrophy.
- M-01 was 18-fold more potent than captopril in reversing cardiomyocyte hypertrophy.

Introduction

Hypertension is a chronic, noncommunicable disorder of varied etiology, characterized by an increase in cardiac output and peripheral vascular resistance. It is a primary risk factor for heart disease (myocardial infarction and heart failure), strokes and sudden death [1], and a secondary risk factor for chronic kidney disease [2, 3]. Hence, lowering blood pressure should be a priority when treating patients with hypertension.

Despite the availability of a wide range of antihypertensive drugs nowadays, only 30% of treated patients are able to adequately control their blood pressure [4–6]. Thus, hypertension is the leading modifiable risk factor for disability and premature death from cardiovascular disease. Medical attention for this disease constitutes ~10% of healthcare spending globally [5, 7].

Among the several factors associated with the development of hypertension, two are key to its pathophysiology and progression to heart failure: the hyperactivity of the renin–angiotensin system (RAS) and the dysfunction of the neurovisceral/autonomic nervous system [8, 9]. Angiotensin II (Ang II) through its type 1 receptor (AT_1R) contributes to several physiological functions that regulate vasoconstriction, fluid volume, cardiac output, cell growth, and vascular wall integrity [10]. It also interacts with the sympathetic nervous system [11], which regulates vital cardiovascular functions such as heart rate acceleration, increased cardiac contractility, reduced venous capacitance, and peripheral vasoconstriction [12].

Contrarily, the parasympathetic nervous system affects the cardiovascular system by slowing the heart rate through vagal innervation [13]. An imbalance between these systems can trigger changes in cardiovascular functions. For example, high blood pressure may promote hypertensive heart disease characterized by myocardial alterations, structural and functional adaptations, fibrosis, and remodeling of the left ventricle (eventually capable of limiting compliance) [14]. In this context, the RAS and the autonomic system represent biological targets for the treatment of hypertension and its cardiac complications [15, 16].

Angiotensin-I-converting enzyme (ACE, EC 3.4.15.1), a zinc dipeptidyl carboxypeptidase, plays a vital role in blood pressure regulation. It cleaves the decapeptide angiotensin I (Ang I) to the octapeptide Ang II and also hydrolyzes bradykinin, a powerful vasodilator [1]. The excessive production of Ang II and degradation of bradykinin are known to contribute to high blood pressure and the development of hypertension [16]. Thus, Ang II inhibition is a useful strategy for hypertension therapy.

On the other hand, autonomic dysfunction implies a disturbance in the capacity of the sympathetic and parasympathetic systems to modulate cardiovascular functions and blood pressure. Alterations in inhibitory and excitatory mechanisms that affect brainstem vasomotor neurons could be involved in creating an imbalance in these two systems. Such a disruption in homeostasis may contribute to hypertension through the combination of abnormal activation of sympathetic tone [17] and diminished vagal activity, the latter resulting in a decline in parasympathetic control [18].

In the cardiovascular system, acetylcholine (ACh) induces vascular endothelium-dependent relaxation through the formation of nitric oxide (NO) [19], which regulates changes in heart rate and contractility in opposition to the influence of the sympathetic nervous system [20]. By prolonging the availability of ACh in cholinergic nerve

endings and enhancing the efficiency of cholinergic transmission, autonomic imbalance can be avoided. This will attenuate the inflammatory process and therefore reduce the tendency to increased pressure overload, left ventricular hypertrophy (LVH) and risk of heart failure [21].

Ach is hydrolyzed by cholinesterase, a specialized carboxylic ester hydrolase that breaks down esters of choline. Consequently, one way to prolong the availability of ACh is through the inhibition of cholinesterases. The two main kinds of cholinesterases are acetylcholinesterase (AChE), specific for ACh degradation, and butyrylcholinesterase (BuChE), which is a nonspecific enzyme with greater selectivity for butyrylcholine (BuCh) and/or propionylcholine [22].

The inhibition of AChE and/or BuChE has proven to be effective for decreasing cardiovascular risk [23, 24]. Vagal tone is improved and the blood pressure variability ameliorated [25]. Thus, drugs with high affinity for cholinesterases are an important therapeutic tool in preventing and treating cardiovascular disorders produced by hypertension. However, most of the synthetic compounds currently used to reduce hypertension and/or revert cardiac hypertrophy have a single target, and many of them cause significant side effects.

Therefore, hard work is being made to find novel agents with better efficacy and lower toxicity [26]. In previous work, our group demonstrated the antihypertensive activity of a phthalamide derivative in an in vivo animal model. The molecules were designed following some important characteristics of the molecular targets, in order to inhibit ACE, the molecules have phenylethylamine moieties which is necessary to interact with the ACE, moreover to be able to inhibit cholinesterases, the molecules possess in their structure ethylamine moieties as well as carbonyl groups, which allows to adequately inhibit cholinesterases. This contribution aimed to synthesize and test three phthalamides as possible multi-target inhibitors of ACE, AChE, and BuChE in order to reduce hypertension and revert cardiac hypertrophy. Additionally, the inhibitory potency and mechanism of action of these enzymes were studied in silico, in vitro, and in vivo.

Materials and methods

Docking

A semiempirical method (PM3) was employed for the conformation analysis of all ligands. Physiological conditions (pH 7.4) were assumed when determining the corresponding protonation state on GaussView 5.0.9 and Gaussian 09 [27] software. A hybrid Lamarckian Genetic Algorithm [28] and a population size of 100 were used. Gasteiger charges for ligands and Kollmann partial charges for the protein were established with Raccoon [29] and AutoDock tools 1.5.4 [30] software. The crystal structures of *Homo sapiens* butyrylcholine esterase (*Hu*BuChE) and *Electrophorus electricus* acetylcholine esterase (*Ee*AChE) were obtained online from the Protein Data Bank (PDB code 4BDS and 1C2O). The grid box was set at 126 Å × 126 Å × 126 Å, with a mesh separation of 0.375 Å and grid center coordinates of $X = 138.889$, $Y = 123.537$, and $Z = 38.703$ for *Hu*BuChE, and $X = 88.481$, $Y = 109.042$, and $Z = 83.026$ for *Ee*AChE. AutoDock4 in Fedora 22 as the operative system was used to perform the molecular docking, finding the following values: Gibbs free energy (ΔG), $-\log_{(10)}$ dissociation constant (p*Kd*), dissociation constant (K*d*), binding distance, number, and type of interactions.

Synthesis and characterization

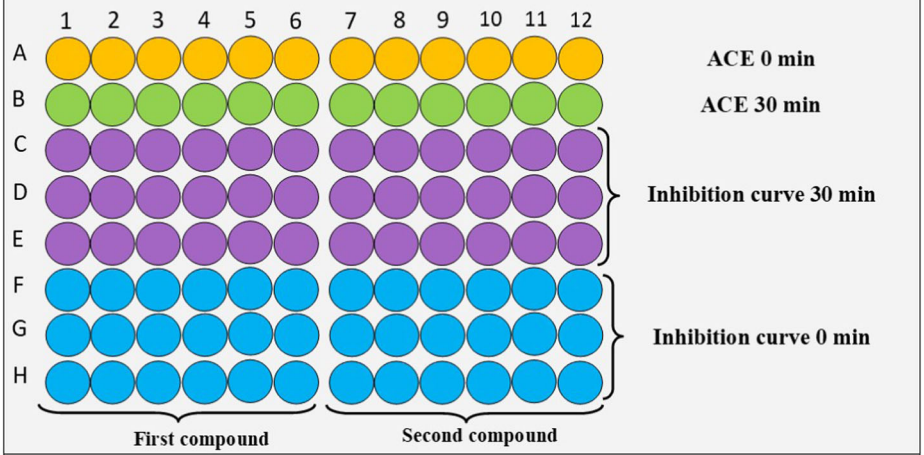
The compounds were synthesized as previously reported by our group [31]. Briefly, in a 100 mL round-bottom oven-dried flask were placed 3 mmol of the corresponding amine and 1.5 mmol of phthalic anhydride. The reaction was gently heated to the melting point of the reagents for 10–15 min under constant stirring with a magnetic stirring bar. After the reaction was cooled to room temperature (rt), purification was carried out with ethyl acetate (40 mL), and sonicated until a precipitate formed. Finally, the white powder was purified by washing it four times with basic water (pH 13).

All solvents and reagents were utilized as received from Sigma-Aldrich (St. Louis, MO, USA). The compounds were characterized by melting point (uncorrected) using a Stuart® SMP40 automatic apparatus, infrared spectrometry by utilizing a 100 FT-IR spectrometer (Perkin-Elmer), nuclear magnetic resonance was recorded on a Varian Mercury 300 spectrometer (^{1}H, 300 MHz; ^{13}C, 75 MHz) with TMS as the internal reference. Finally, a Bruker micrOTOf-Q-II instrument was used to perform the electrospray ionization high-resolution mass spectrometry.

Solutions for the angiotensin-I-converting enzyme activity assay

For the phosphate buffer solution (PBS, 0.1 M, pH 8.3), 8.07 g of K_2HPO_4 and 0.5 g of KH_2PO_4 were weighed and added to a volumetric flask. Subsequently, 8.76 g of NaCl and 28.4 g of Na_2SO_4 were added to reach a 0.3 M and 0.4 M solution, respectively. Finally, distilled water was added to give a final volume of 500 mL. ACE was obtained from the serum of 10-week-old male Wistar rats (250–300 g) previously euthanized under procedures approved by the Bioethics Committee. Serum was stored at −70 °C to await processing. Equal volumes of serum and

ACE 0 min	ACE 30 min	Inhibition curve 30 min	Inhibition curve 0 min
Buffer 140 µl	Buffer 140 µl	Buffer 140 µl	Buffer 140 µl
DMSO 1% 20 µl	DMSO 1% 20 µl	Test Compound 20 µl▲	Test Compound 20 µl▲
ACE 20 µl	ACE 20 µl	ACE 20 µl	ACE 20 µl
	HHL 20 µl	HHL 20 µl	
Incubate 30 min at 37 °C			
HHL 20 µl			HHL 20 µl
Take 150 µL of each well to a microtube with 50 µL of TT to stop the reaction			
Mix for 5 min			
Centrifuge for 8 min at 6000 rpm			
Take 80 µL of each microtube to a 96-well plate			
Read at 382 nm			



▲ Using the corresponding concentrations of test compound

Scheme 1 Representation of the improved method for determining ACE activity carried out in a 96-well microplate. This pattern was followed for evaluating the enzymatic inhibitory activity of phthalamide derivatives

phosphate buffer solutions were mixed immediately before use. The substrate N-Hippuryl-His-Leu (HHL code H1635, Sigma-Aldrich, St. Louis, MO, USA.) was dissolved in phosphate buffer to afford a 5 mM solution. For the 2,4,6-Trichloro-1,3,5-triazine solution (TT code C95501, Sigma-Aldrich, St. Louis, MO, USA.), 2.72 g were weighed and added to a volumetric flask to provide a final volume of 100 mL with 1,4-dioxane (2.7%).

Solutions for the acetylcholinesterase and butyrylcholinesterase activity assay

The PBS (0.1 M, pH 8), sodium hydroxide solution (4.2 M), and hydroxylamine solution (2.4 M) were prepared following our previously reported methodology for AChE and BuChE activity assays [32, 33]. For the alkaline hydroxylamine solution was prepared immediately before use, using equal volumes of NaOH and NH_2OH solution. For ferric chloride solution (0.75 M), 101.55 g $FeCl_3{\cdot}6H_2O$ were slowly mixed with an HCl solution (7.5 M) to reach a final volume of 500 mL. After elaborating the ACh solution (ACh iodide, 256 mM) and BuCh solution (BuCh iodide, 100 mM), numerous dilutions were made for the experiment (Ach 48, 32, 24, 16, 12, 8, 6, and 4 mM; BuCh 32, 28, 24, 16, 12, 8, 6, and 4 mM). The stock AChE solution (1 U/mL, *Electrophorus electricus* AChE, Sigma Chemical C3389), as well as, the stock BuChE solution (0.4 U/mL, *human* BuChE, Sigma Chemical C-9971) were prepared with the corresponding freeze-dried powder. All the test and reference compounds were prepared by adding distilled water or DMSO (<1%) as the vehicle. The solutions were kept at −20 °C.

Angiotensin-I-converting enzyme activity assay

For enzyme kinetic experiments, twenty microliters of each solution (HHL, inhibitor, or ACE) were placed in a 96-well microplate following the pattern shown in Scheme 1 to construct the standard curve for ACE and the inhibitory curves. The microplate was incubated at 37 °C for 30 min in a water bath. Upon completion of this period, the reaction was stopped with 50 µL TT solution. The microplate was then kept in the dark at rt for 5 min prior to reading optical density at 382 nm in a microplate reader (Epoch

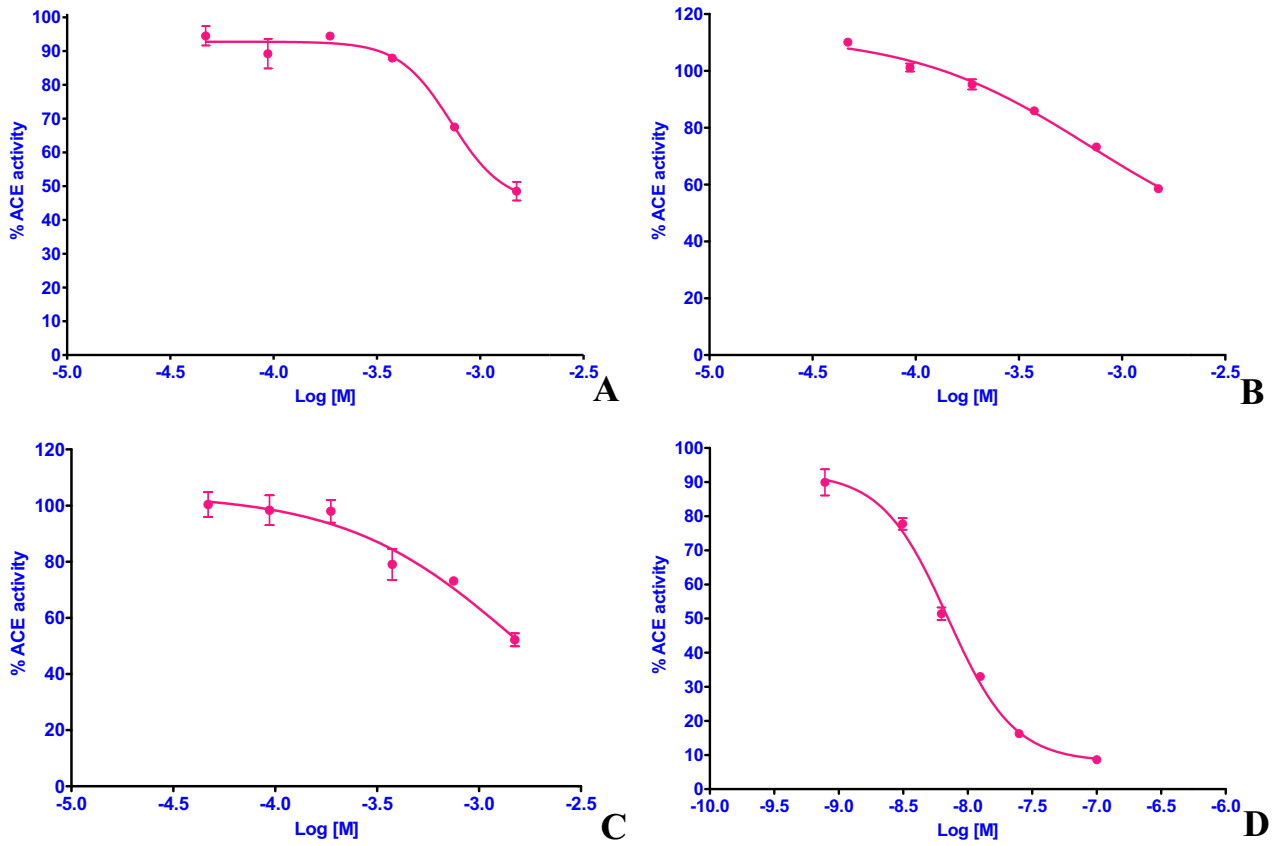


Fig. 1 Inhibitory plots of the test and reference compounds on *Rattus norvegicus* ACE: (**A**) M-01; (**B**) M-02; (**C**) M-03, and (**D**) captopril. Nonlinear regression analysis was carried out using the IC_{50} module

equipment). The evaluation of ACE activity was repeated in triplicate for each test and reference compound and the resulting data averaged to obtain the final value. The IC_{50} analysis was made with a nonlinear regression, a 95% interval confidence was considered for the experiments.

Acetylcholinesterase and butyrylcholinesterase activity assay

The inhibitory effect produced on AChE and BuChE was measured with an improved version of the corresponding Bonting and Featherstone method previously reported by our group [32, 34, 35]. These procedures follow the hydroxamic acid method. Briefly, 20 µL of each solution of AChE, BuChE, the corresponding inhibitors, ACh and BuCh were placed in the wells (containing buffer solution) of a 96-well microplate to afford a final volume of 160 µL. The microplates for AChE and BuChE were incubated for 20 and 55 min, respectively, at 37 °C in a water bath (Felisa), then stopped by adding 40 µL of alkaline hydroxylamine solution. A microplate reader (Accuris MR9600) was used to read the optical density at 540 nm after 100 µL of ferric chloride solution (premixed during 30 s) was added to each well. The standard curve for Ach, BuCh, AChE, and BuChE was built by following the same procedure without the inhibitor. All assays were performed in triplicate.

In vivo evaluation

Twenty-week-old male spontaneously hypertensive rats (SHR, 250–300 g) and Wistar Kyoto (WKY), acquired from the Institute of Cell Physiology (ICP-UNAM), were used for the cardiomyocyte hypertrophy reversion experiment. Compound M-01 (10 mg/kg) and Captopril (40 mg/kg) were administered intragastrically every day for 30 days, distilled water was used as the vehicle (Veh). Four groups were formed: the normotensive control (WKY + Veh), the hypertensive control (SHR + Veh), the reference group (SHR + captopril), and the experimental group (SHR + M-01). Rats had food and water ad libitum, and the conditions were 22 ± 2 °C of temperature, 40–60% of humidity, and 12/12 h light/dark cycle. Bioethics Committee of our institution approved all procedures with animals, the protocol complied with the technical specifications of the Mexican Official Norm for the production, care, and use of laboratory animals (NOM-062-ZOO-1999, Ministry of Agriculture).

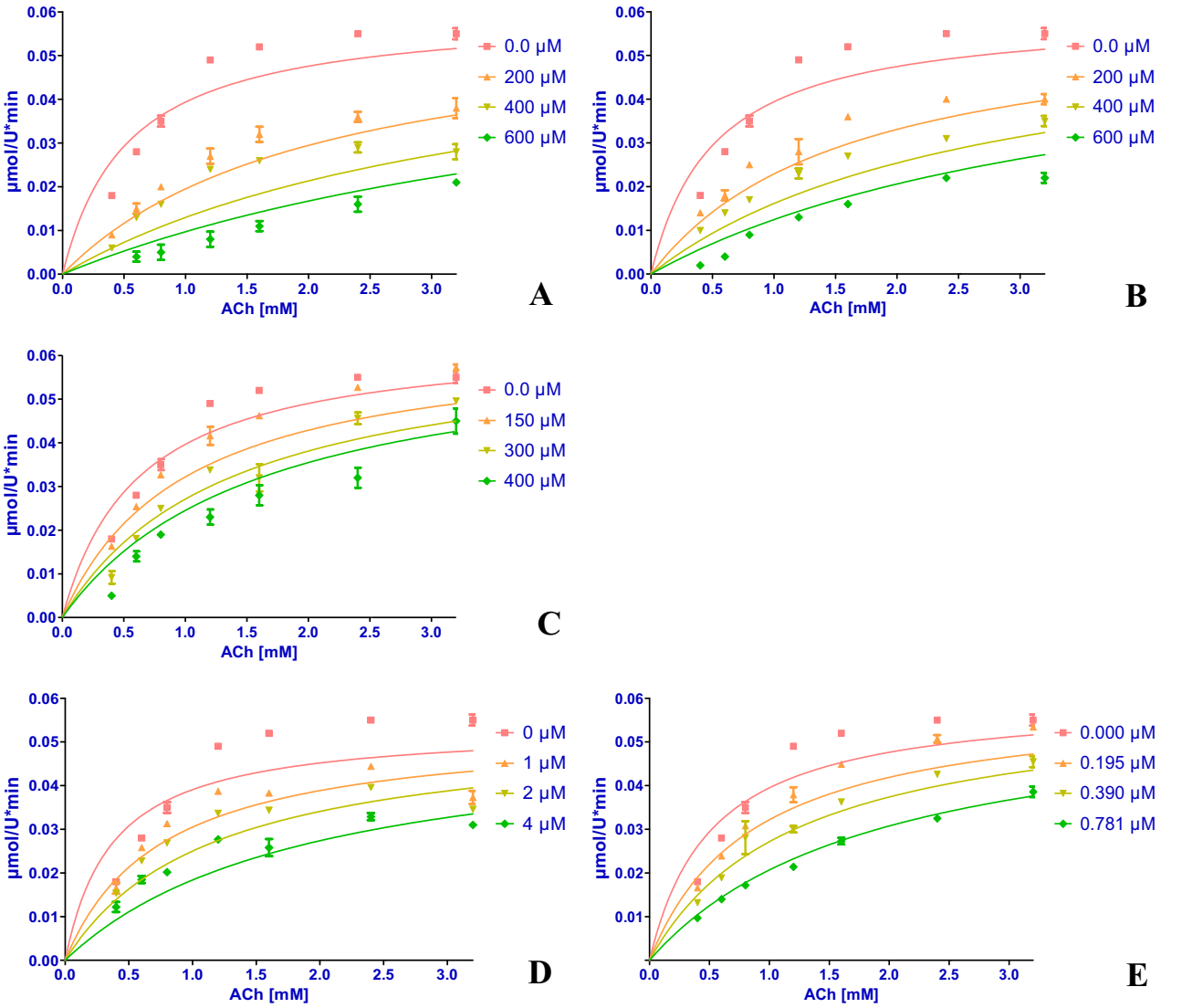


Fig. 2 Inhibitory plots of the test and reference compounds on *Electrophorus electricus* AChE: (**A**) M-01; (**B**) M-02; (**C**) M-03; (**D**) galantamine, and (**E**) neostigmine. Nonlinear regression analysis was carried out using a kinetic module

Histological evaluation

Histological analysis was conducted on cardiomyocytes of the hearts of the hypertensive groups (SHR + Veh, SHR + M-01, and SHR + captopril) and normotensive control (WKY + Veh). Rats were euthanized with 50 mg/kg of sodium pentobarbital (ip). Subsequently, the heart from each animal was removed, dissected and fixed in 4% phosphate-buffered formaldehyde solution for 24 h ($n =$ 3–4 animals per group), and then dehydrated, cryopreserved (with 30% sucrose during 24 h) and embedded in tissue-tek (Sakura Finetek, USA) before being frozen with dry ice and acetone. Hearts were sectioned in 5 µm slices and stained with hematoxylin–eosin. The area (µm^2) of cardiomyocytes was evaluated in different fields, measuring the major and minor transverse axes to estimate the cross-sectional area on ZEN 2012 software (Carl Zeiss Laser Scanning Systems LSM 510, Oberkochen, Germany) [36].

Statistical analysis

All data from enzyme kinetic experiments are expressed as the mean ± 95% confidence intervals. GraphPad Prism software was used to carry out the statistical analysis with the IC50 module and the enzyme kinetic module. The results from the cardiomyocyte hypertrophy reversion are expressed as the mean ± standard error of the mean. One-way analysis of variance and Bonferroni post hoc test was used to determine statistical significance ($p < 0.001$).

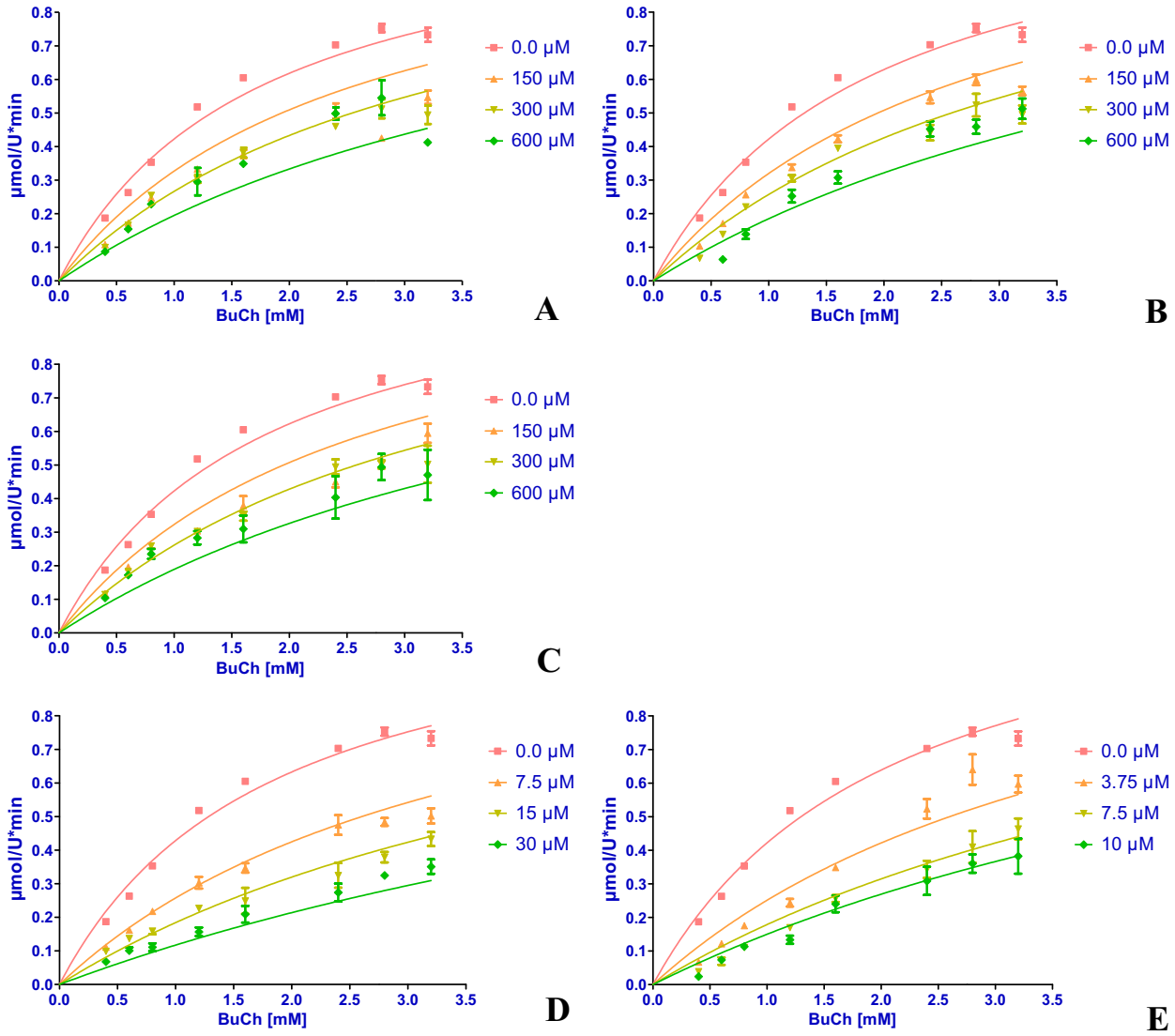


Fig. 3 Inhibitory plots of the test and reference compounds on human BuChE: (**A**) M-01; (**B**) M-02; (**C**) M-03; (**D**) galantamine, and (**E**) neostigmine. Nonlinear regression analysis was carried out using a kinetic module

Results and discussion

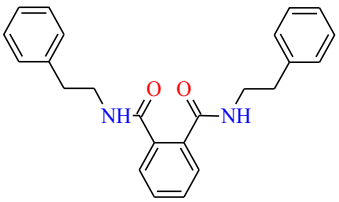
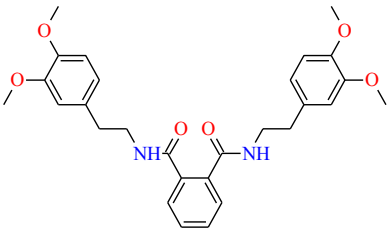
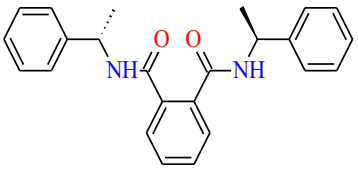
Synthesis

Three compounds were synthesized in good yields according to our previously reported method without modification [31]. The three compounds N,N′-bis(2-phenylethyl)phthalamide (M-01), N,N′-bis[2-(3,4-dimethoxyphenyl)ethyl]phthalamide (M-02), N,N′-bis[(1S)-1-phenylethyl)phthalamide (M-03) were characterized by NMR, infrared, mass spectroscopy, the results are in agreement with our previous reported [31].

Enzyme activity assays (ACE, AChE, and BuChE)

The inhibitory capacity of the three phthalamide derivatives (M-01, M-02, and M-03) was tested on the activity of ACE, AChE, and BuChE (Table 2, Figs. 1–3). According to a previous study by our group, the oral administration of compound M-01 reduces the blood pressure of SHR in vivo model, being ~7-fold more potent than the reference (captopril), a well-known ACE inhibitor [31]. However, it was beyond the scope of that investigation to evaluate the reversion of cardiac hypertrophy or explore the mechanism of the antihypertensive effect. The phthalic anhydride

Table 1 Structure of the synthesized compounds evaluated as ACE, AChE, and BuChE inhibitors in vitro and in silico

Structure	ID
	M-01
	M-02
	M-03

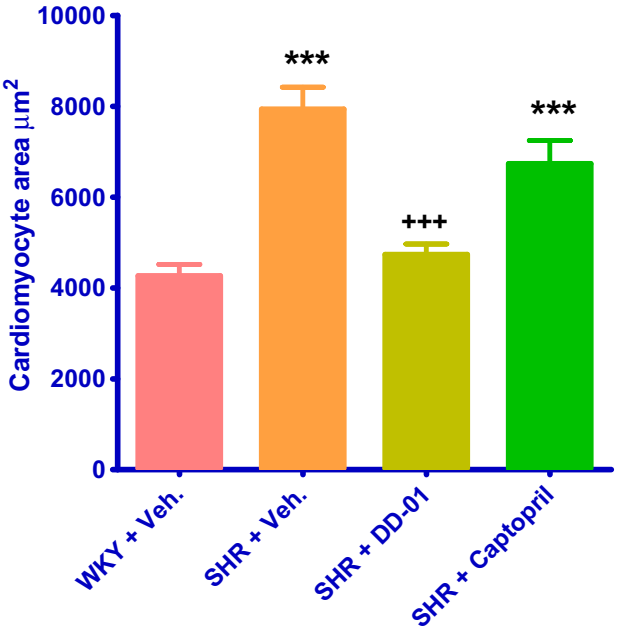


Fig. 4 Effect on the reversion of cardiomyocyte hypertrophy in hypertensive rats by molecule M-01. *** Means a significant difference versus the normotensive control (WKY + Veh); +++ means a significant difference versus the hypertensive control (SHR + Veh). The data are expressed as the mean ± standard error of the mean. Statistical significance was determined with one-way ANOVA and the Bonferroni post hoc test ($p < 0.001$)

moiety has been found to produce important biological activity [31, 35], thought to stem from multi-target inhibition of ACE, AChE, and BuChE.

In the current contribution, each of the derivatives inhibited the three enzymes, showing the following order of potency: AChE > BuChE > ACE. The type of inhibition of AChE and BuChE is competitive for all compounds. The in silico experiments suggest that the inhibition of ACE is also competitive. The average *Ki* for AChE and BuChE was 90.2 and 314.9 µM, respectively, while the mean IC_{50} for ACE was 706.5 µM. In addition to the test compounds, some reference drugs were assessed (captopril, galantamine, and neostigmine), not only to validate the results but also as a point of comparison for the phthalamide derivatives. According to the in vitro experiments, none of the test compounds were better inhibitors than the reference drugs. M-01 was more than a thousand-fold less potent than captopril ($IC_{50} = 1.79–15.1$ nM [37] vs. 6.98 nM). Nevertheless, it provided approximately 6.85-fold greater potency than captopril in regard to the in vivo antihypertensive effect, presumably due to its three-target activity. On the other hand, M-01 provided a very potent effect for cardiac hypertrophy reversion, while captopril had no significant effect.

As described in earlier reports, cholinesterase inhibitors improve autonomic and cardiac function in models of cardiovascular disease by attenuating the sympathovagal imbalance, ischemia-induced arrhythmia, inflammation, oxidative stress, and apoptosis [38, 39]. Moreover, the inhibition of cholinesterase enhances the availability of ACh in the central nervous system and the formation of NO peripherally. The latter molecule is known to regulate vascular resistance and blood pressure [40]. Cholinesterase inhibitors provide benefits by balancing autonomic dysfunction, but only a few drugs are able to increase the efficiency of cholinergic transmission. Since M-01 carried out both of these functions, it is an excellent candidate for further research into its capacity to treat and/or prevent cardiovascular diseases.

Histological evaluation of cardiomyocytes

Histological analysis demonstrated a significantly larger size of the isolated cardiomyocytes of the hypertensive control group (SHR + Veh) than those of the normotensive control (WKY + Veh; $p < 0.001$). Thus, the SHR animals herein proved to have hypertrophied cardiomyocytes, similar to the results found in previous studies [41]. It has been proposed that cardiac hypertrophy may be due to an over activation of the sympathetic nervous system and the RAS [42–44]. The administration of M-01 in SHR rats decreased the area of cardiomyocytes by 87.3%, reaching a

Table 2 Inhibitory concentration 50 (IC_{50}), inhibition constant (K_i), and type of inhibition of phthalamide derivatives and reference drugs for the angiotensin-I-converting enzyme (ACE), acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)

ACE (*Rattus norvegicus*)			AChE (*Electrophorus electricus*)			BuChE (*Homo sapiens*)		
	IC_{50} (μM)	Interval 95%	K_i (μM)	Interval 95%	Type of inhibition	K_i (μM)	Interval 95%	Type of inhibition
M-01	724.6	538.4–899.9	67.91	50.44–85.38	Competitive	324.9	264.4–385.3	Competitive
M-02	698.4	465.5–1048	94.06	70.62–117.5	Competitive	307.7	265.3–350.0	Competitive
M-03	696.7	303.5–1090	108.8	77.5–140.1	Competitive	312.1	260.3–363.9	Competitive
Captopril	6.98 nM	6.03–8.08 nM	–	–	–	–	–	–
Galantamine	–	–	0.96	0.54–1.37	Competitive	7.29	6.50–8.06	Competitive
Neostigmine	–	–	0.29	0.38–0.65	Competitive	3.74	3.28–4.19	Competitive

Table 3 Values obtained for the main affinity parameters of the interactions between the phthalamides and reference compounds with the cholinesterases (AChE and BuChE)

*Ee*AChE				*Hu*BuChE			
	ΔG_0 (kcal/mol)	K_d (μM)	pK_d		ΔG_0 (kcal/mol)	K_d (μM)	pK_d
M-01	−9.87	0.058	7.2	**M-01**	−9.4	0.12855	6.89
M-02	−9.09	0.217	6.66	**M-02**	−8.720	0.407	6.39
M-03	−10.72	0.138	6.86	**M-03**	−8.960	0.270	6.57
Acetylcholine	−4.54	461.23	3.34	**Acetylcholine**	−4.6	453.9	3.34
Galantamine	−6.6	17.41	4.76	**Galantamine**	−6.7	14.6	4.84
Neostigmine	−5.73	70.2	4.15	**Neostigmine**	−5.69	73.1	4.14

Table 4 Amino acid residues involved in the interaction inhibitor-enzyme obtained by molecular docking, as well as the type of interaction

ID	Hydrophobic	π–π interaction	H-bond	π–cation	Other
*Hu*BuChE					
M-01	Leu125	Trp231, Phe329, Trp82	Try128, Glu197		Glu197 (π–anion) Gly116 (π–amide)
M-02	Trp82, Phe329	Trp82, Trp231, Phe329	Tyr128, Tyr332, Thr120	–	Gly116 (π–amide)
M-03	Pro84, Ile69	Tyr440, Trp82	Glu197, Thr120		Gly116 (π–amide)
Butyrylcholine	–	Trp231	Ser198	–	Glu197 (electrostatic)
Neostigmine	Glu197	Trp82, His438	Tyr128	–	Glu197 (electrostatic)
Galantamine	Trp82	Tyr128	Glu197, Gly116, Thr120	–	–
*Ee*AChE					
M-01	Trp86	Gly121	Asp74	His447	Ser203, Ser125
M-02	Trp286, Trp86, Tyr332	Trp86, His447, Tyr341	Tyr124, Tyr337	His447	
M-03	Phe338, Tyr341	His447, Tyr337, Tyr341, Phe297, Tyr124	Ser125	His447	Glu202 (π–anion)
Acetylcholine	Tyr124	His447	Ser203, Trp86	Tyr337	Glu202 (electrostatic)
Neostigmine	Trp86	Trp86	Ser125	Tyr337	Glu202 (electrostatic)
Galantamine	Tyr124	Trp286, Tyr341	Ser203, Arg296	–	–

value similar to the normotensive control (WKY + Veh; Fig. 4). Contrarily, captopril only showed a tendency, but no significant difference, in the reduction of the area of cardiomyocytes (32.7%) in relation to the normotensive control. M-01 turned out to be at least 18.28-fold more potent than captopril (mole to mole comparison), considering the captopril dose needed to reach a similar effect.

Treatments with ACE inhibitors are reported to prevent hypertension, LVH, and vascular remodeling in young SHRs [41]. Additionally, they lower blood pressure and

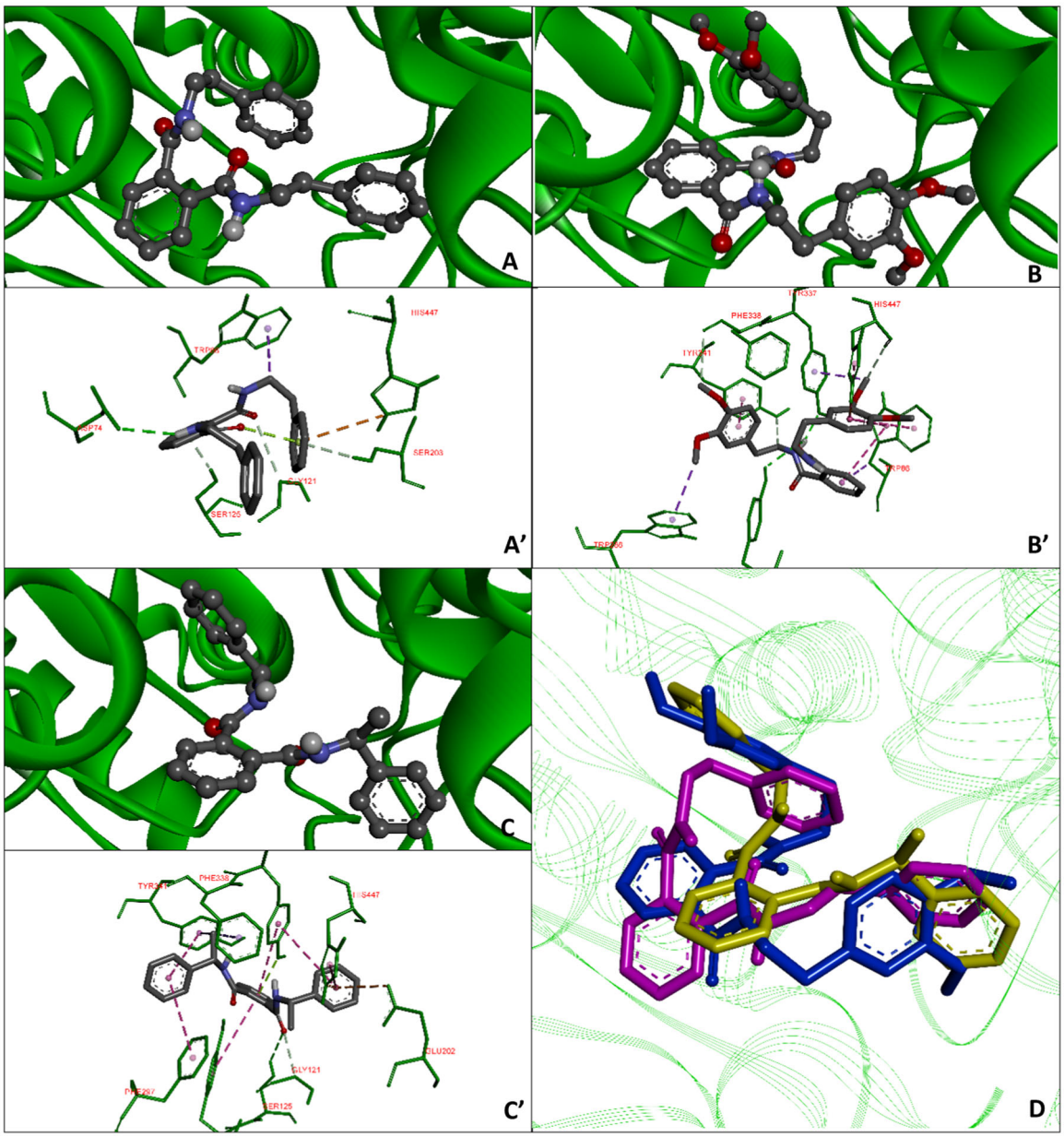


Fig. 5 Docking simulation for the ligands on *Electrophorus electricus* acetylcholine esterase: (**A**) binding mode of molecule M-01; (**A′**) amino acid residues involved in the interaction with molecule M-01; (**B**) binding mode of molecule M-02; (**B′**) amino acid residues involved in the interaction with molecule M-02; (**C**) binding mode of molecule M-03; (**C′**) amino acid residues involved in the interaction with molecule M-03; (**D**) Compounds M-01, M-02, and M-03 show similarity in their binding mode

diminish fibrosis, cardiac weight, and hypertrophy in older SHRs [45, 46]. At the same time, cardiomyocytes have their own functional cholinergic machinery that compensates for adrenergic overstimulation, avoiding cardiac hypertrophy, molecular changes, and alterations in calcium signaling. Therefore, the inhibition of AChE, which leads to greater bioavailability of ACh and NO, apparently modifies cholinergic signaling in the heart to counteract or partially neutralize the hypertrophic effects of adrenergic stimulation [47]. Consequently, the current data suggest that the pronounced in vivo activity of M-01 resulted from the sum of the inhibitory effects produced on the target enzymes (ACE, AChE, and BuChE).

Molecular docking

To provide insights into the outcome of the present in vitro and in vivo assays, the three phthalamide derivatives (Table 1) were modeled and docked on *Ee*AChE and *Hu*BuChE. The values of ΔG, K_d, and pK_d were determined for the molecular interactions between the enzymes and the phtelamides (Table 2). The docking simulations demonstrated

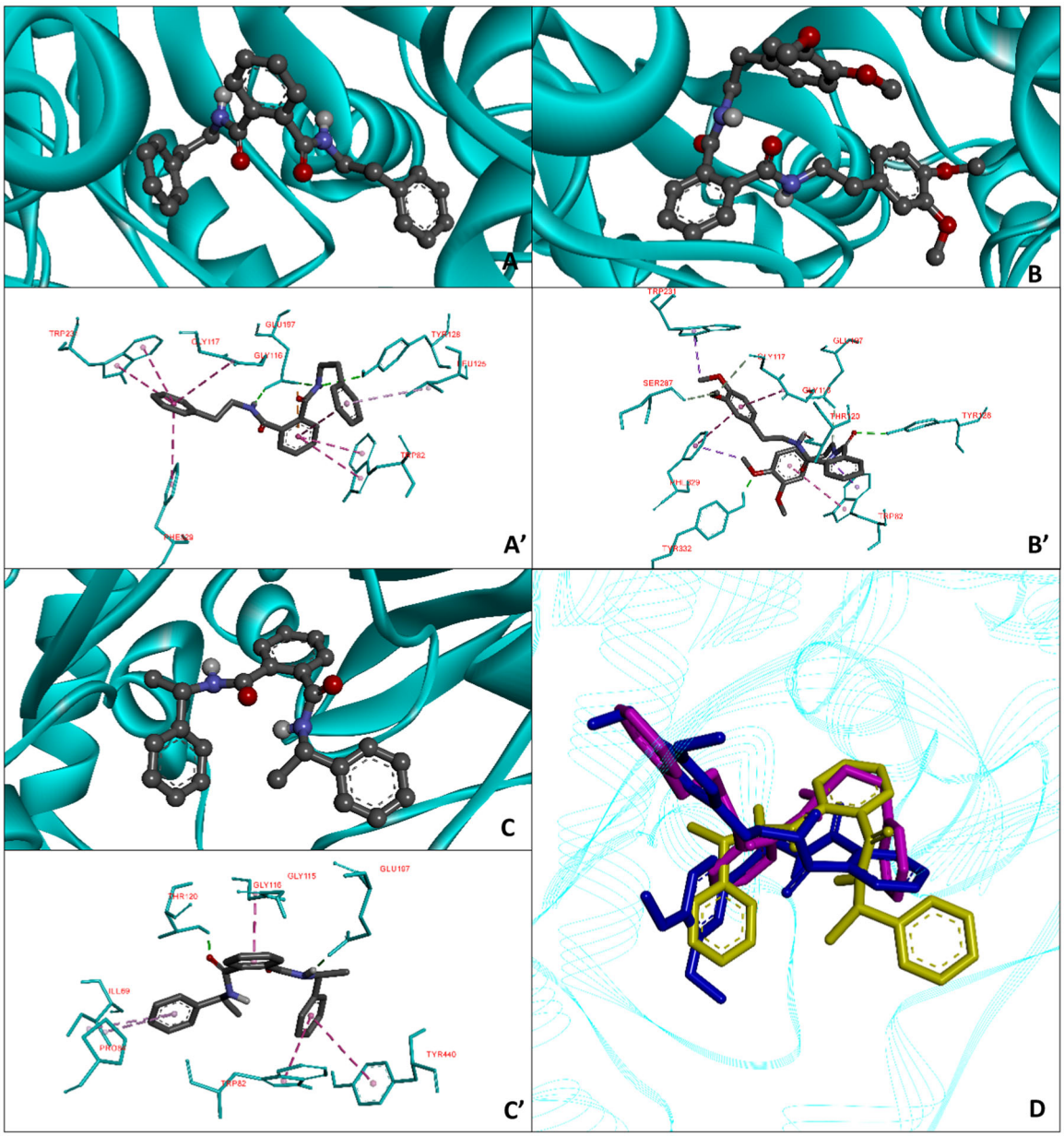


Fig. 6 Docking simulation for the ligands on *Homo sapiens* butyrylcholine esterase: (**A**) binding mode of molecule M-01; (**A′**) amino acid residues involved in the interaction with molecule M-01; (**B**) binding mode of molecule M-02; (**B′**) amino acid residues involved in the interaction with molecule M-02; (**C**) binding mode of molecule M-03; (**C′**) amino acid residues involved in the interaction with molecule M-03; (**D**) Compounds M-01, M-02, and M-03 show similarity in their binding mode

good affinity of the phthalamide derivatives for both enzymes (*Ee*AChE and *Hu*BuChE), concurring with the in vitro results (Table 3).

Some amino acid residues were observed in the interaction ligand-receptor for both enzymes, as well as the type of interaction, all the above is shown in Table 4. The main interactions for *Ee*AChE are π–π, owing to the number of aromatic rings in the structure. The amino acids that participated in the interactions include Trp86, His447, Glu202, and Ser203 (Table 4 and Fig. 5), the same ones involved in binding to the reference drugs (e.g., neostigmine and galantamine). Thus, the test compounds are capable of binding to two important sites of the enzyme: the stearic and the anionic site. Regarding *Hu*BuChE, on the other hand, the amino acid residues Gly116, Trp82, Glu 197, and Trp231 take part in recognition (Table 4 and Fig. 6). For both enzymes, the competitive inhibition evidenced by the in vitro experiments is consistent with the docking simulations.

Molecular docking for the ligands on ACE was previously reported. Briefly, the three phthalamide derivatives showed good affinity for ACE, interacting with Lys511,

His353, Tyr523, Glu384, His387, and Zn^{+2}, located in the catalytic active site of ACE. Moreover, the binding mode of the test compounds turned out to be similar to that of the reference drugs, suggesting competitive inhibition.

Conclusions

The development of new multi-target molecules is a relevant strategy for improving the treatment of hypertension, especially if the effect of reverting cardiac hypertrophy is involved. After three phthalamide derivatives (M-01, M-02, and M-03) were synthesized, they were evaluated in vitro as inhibitors of three-target enzymes (ACE, AChE, and BuChE). M-01 was then tested on the SHR a model for heart failure with the aim of reverting cardiac hypertrophy. In old rats, the compound produced a potent effect against cardiomyocyte hypertrophy, which was ~18-fold greater than that of captopril. The reversion of cardiomyocyte hypertrophy caused by the reference drug was not significant versus the hypertensive control. The potent effect of M-01 is probably due to a multi-target inhibitory activity. Since M-02 and M-03 have the same in vitro and in silico pattern as M-01, they would likely furnish the same in vivo effect. The apparent dual pharmacological activity against hypertension and cardiac remodeling is promising for M-01 and perhaps the other two test compounds. Further studies are needed to explore the potential of these molecules for treating cardiovascular diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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