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Article

Anticholinesterase activity of β-carboline-1,3,5-triazine hybrids

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The β-carboline-1,3,5-triazine hydrochlorides 8-13 were evaluated *in vitro* against acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). The analysed compounds were selective to BuChE, with IC_{50} values in the range from 1.0–18.8 μM being obtained. The *N*-{2-[(4,6-dihydrazinyl-1,3,5-triazin-2-yl)amino]ethyl}-1-phenyl-β-carboline-3-carboxamide (12) was the most potent compound and kinetic studies indicate that it acts as a competitive inhibitor of BuChE. Molecular docking studies show that 12 strongly interacts with the residues of His438 (residue of the catalytic triad) and Trp82 (residue of catalytic anionic site), confirming that this compound competes with the same binding site of the butyrylthiocholine.

Keywords: β-carboline. 1,3,5-triazine. Acetylcholinesterase. Butyrylcholinesterase.

INTRODUCTION

Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder (Larson, Kukull, Katzman, 1992), characterised by cognitive impairment, which is associated by means of cholinergic hypothesis, to the loss cholinergic of neurons and a decrease in levels of cholinergic neurotransmission (Francis *et al*., 1999; Pinto, Lanctôt, Herrmann, 2011). The hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetic acid, a reaction catalysed by enzymes of the cholinesterase family, is necessary to allow a cholinergic neuron to return to its resting state after activation (Čolović *et al*., 2013). The acetylcholinesterase (AChE) predominates in the healthy brain, while the butyrylcholinesterase (BuChE) is considered to play a minor role in the regulation of synaptic ACh levels (Silva *et al*., 2014). In fact, most of the current drugs used for AD treatment are based on this hypothesis, acting mainly by the inhibition of AChE (Silva *et al*., 2014; Čolović *et al*., 2013). However, studies have shown that as AD progresses, the BuChE activity progressively increases, while AChE activity remains unchanged or gradually decreases. When this occurs, the BuChE assumes function of metabolize the ACh in the synapse (Darvesh, Hopkins, Geula, 2003; Anand, Singh, 2013; Li *et al*., 2017). Thus, also inhibiting BuChE, cognitive improvements associated with the current cholinesterase inhibitors can be obtained. As a result, both AChE and BuChE can be considered as therapeutic targets in Alzheimer's disease treatment (Darvesh, Hopkins, Geula, 2003; Anand, Singh, 2013; Li *et al*., 2017).

The treatment of AD usually is performed with cholinesterase inhibitors (donepezil, rivastigmine and galantamine), which enhance cholinergic signalling in the central nervous system and cognitive symptoms. However, these drugs do not prevent the AD progression, and unfortunately there is still no cure for this disease (Silva *et al*., 2014; Pinto, Lanctôt, Herrmann, 2011; Čolović *et al*., 2013).

In recent years, several works have been carried out with the aim of obtaining new inhibitors for AChE and BuChE, for the treatment of AD (Anand, Singh, 2013; Li

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et al., 2017). In this context, several classes of heterocyclic compounds, including those containing the 1,3,5-triazine and β-carboline nucleus, were described as potential cholinesterase inhibitors (Veloso *et al.*, 2013; Jameel *et al.*, 2017; Maqbool *et al.*, 2016; Rook *et al.*, 2010; Jin-Shuai *et al.*, 2014; Horton *et al.*, 2017).

Studies have demonstrated that 1,3,5-triazine derivatives were found to act as multi-target anti-Alzheimer agents (Veloso *et al.*, 2013; Jameel *et al.*, 2017; Maqbool *et al.*, 2016; Trifunović *et al.*, 2017). Trisubstituted triazines (**I**, **Figure 1**), for example, inhibited important targets associated with AD, such as AChE, BuChE and Aβ aggregation (Veloso *et al.*, 2013). The triazine-triazolopyrimidine hybrid **II** (**Figure 1**) showed the inhibition of AChE (IC_{50} = 0.065 μM) and BuChE (IC_{50} = 1.88 μM) similar to donepezil (IC_{50} = 0.047 μM for AChE and 2.72 μM for BuChE), which is a potential candidate for anti-Alzheimer's drug (Jameel *et al.*, 2017). Also, cyanopyridine-triazine hybrids inhibit AChE and BuChE, and can reduce neuronal death induced by H_2O_2-mediated oxidative stress and $A\beta_{1-42}$ induced cytotoxicity (Maqbool *et al.*, 2016).

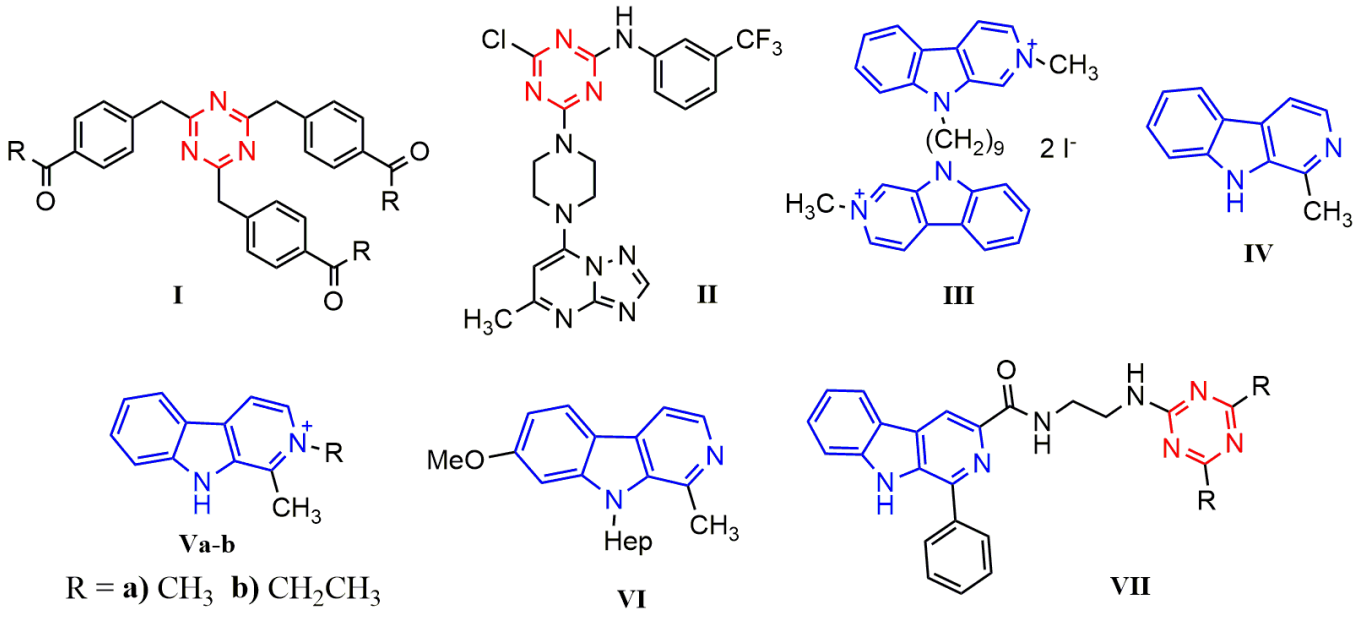


FIGURE 1 - Structures of 1,3,5-triazine (**I** and **II**) and β-carboline (**III**, **IV** and **Va,b**) derivatives with anticholinesterase activity, of DYRK1A inhibitor **VI** and of β-carboline-1,3,5-triazine hybrid **VII**.

Studies focusing on the properties of *β*-carbolines concerning neurodegenerative diseases have also been intensified in recent years, and several researches have highlighted these alkaloids as a new class of anti-Alzheimer agents. β-Carboline derivatives showed activities in neurological disorders associated with AD, acting as potent inhibitors of AChE and BuChE (Rook *et al.*, 2010; Jin-Shuai *et al.*, 2014; Horton *et al.*, 2017; Torres *et al.*, 2012), dual specificity tyrosine phosphorylation regulated kinase-1A (DYRKA) (Drung *et al.*, 2014; Rüben *et al.*, 2015) and monoamine oxidase (MAO) (Santillo *et al.*, 2014). The bivalent β-carboline derivative **III** (**Figure 1**) showed potent anticholinesterase activity, displaying AChE inhibition (IC_{50} = 0.5 nM) higher than the reference drug tacrine (IC_{50} ≅ 45 nM), and approximately the same activity as that of tacrine for BuChE (IC_{50} = 5 nM) (Rook *et al.*, 2010). On the other hand, the harmane (**IV**, **Figure 1**) and its β-carbolinium derivatives **Va** and **Vb** (**Figure 1**) exhibited greater selectivity towards BuChE over AChE. The compounds **Va** and **Vb** (IC_{50} = 0.23 and 0.637 μM) were more active to BuChE than physostigmine (IC_{50} = 3.7 μM) making them suitable prototypes in the search for anti-Alzheimer drugs (Torres *et al.*, 2012). Also, β-carbolines with an extended aromatic ring system were

highly active and selective for BuChE, and it was found that over 60% of the studied compounds showed a better inhibitory activity of BuChE than the drug galantamine (Horton *et al.*, 2017).

In our previous work, we investigated the properties of *β*-carbolines related to neurodegenerative diseases, which led us to identify compound **VI** (**Figure 1**) as a potent DYRK1A and MAO-A inhibitor (Drung *et al.*, 2014). By continuing our research, and taking in account the related proprieties of β-carbolines and 1,3,5-triazines, in this work we evaluated the anticholinesterase activity of β-carboline-1,3,5-triazine hybrids **VII** (**Figure 1**) against AChE and BuChE. Additionally, kinetic and molecular docking studies were carried out for the most potent compound, aiming to evaluate its inhibition mode against BuChE.

MATERIAL AND METHODS

Synthesis of β-carboline-1,3,5-triazine hydrochlorides (8-13)

The β-carboline-1,3,5-triazine hybrids were synthesised as described for Baréa *et al.* (2018). The hydrochloride salts **8-13** were prepared from the treatment of β-carboline-1,3,5-triazine hybrids (1 mmol) with hydrochloric acid (12 M) in methanol, at room temperature for 4 h. Compounds **8-13** were obtained in yields in the range from 50–80%. Elemental analysis for compound **12**, calculated for $C_{23}H_{23}N_{11}O.5HCl$: C 42.38, H 4.33, N 23.64, found: C 43.26, H 4.76, N 20.20.

In vitro assays

In vitro inhibition studies on AChE and BuChE

AChE (from electrophorus electricus, type VI-S, lyophilised powder, lot 041M7009V), BuChE (from equine serum, lyophilised powder, lot SLBB2114V). 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent), acetylthiocholine iodide, and S-butyrylthiocholine iodide were purchased from Sigma Aldrich. Absorbance measurements were taken using a Molecular Devices FlexStation 3 Microplate Reader with Softmax Pro 5.3 software.

Anticholinesterase activities of β-carboline-1,3,5-triazine hydrochlorides against AChE and BuChE were evaluated according to Ellman's modified method (Ellman *et al.*, 1961). Stock solutions of the tested compounds **8-13** were prepared in milli-Q water. The tests were performed in polystyrene 96-well plate, with 125 µL of DTNB (0.5 mM), 50 µL of buffer solution of phosphate (pH 8), 25 µL of sample solution at different concentrations and without the inhibitor (control), and 25 µL of enzyme solution of AChE (0.23 U mL^{-1} prepared in buffer solution of phosphate) or BuChE (0.23 U mL^{-1} prepared in buffer solution of phosphate) were added to each well. The plate was incubated at 30°C for 15 minutes under stirring, and then absorbance was measured at a wavelength of 412 nm. After this time, 25 µL of substrate (acetylthiocholine or butyrylthiocholine, 5 mM, prepared in milli-Q water) was added to each well. The plate was kept at 30°C, under stirring, and the absorbance was measured again at the same wavelength after 4 minutes. The tests were performed in triplicate.

The rates of reactions were calculated using appropriate software (Origin 6.1). The inhibition percentages were calculated by comparing of control reaction rate with the sample reaction rate using Eq.1:

%inhibition = ((control reaction rate-sample reaction rate)/control reaction rate) x 100 (1)

The inhibition curve was obtained by plotting an inhibition percentage graph versus the logarithm of the inhibitor concentration.

Kinetic analysis of BuChE inhibition

Kinetic studies were carried out by Ellman's modified method (Ellman *et al.*, 1961) for compound **12**, using a 0.23 U mL^{-1} solution of BuChE from equine. The test was performed without the inhibitor, in 0.3 and 3.0 µM concentrations of inhibitor **12** for BuChE. Butyrylthiocoline iodine was used as substrate of the reaction in the following final concentrations: 0.05, 0.125, 0.50, 0.75, 1.0 and 2.0 µM. The absorbance was measured in 10 s for 6 min. The obtained data were used to create substrate-velocity curves which were transformed using the Origin 6.1 program into Linerweaver-Burk plots.

Molecular Modelling

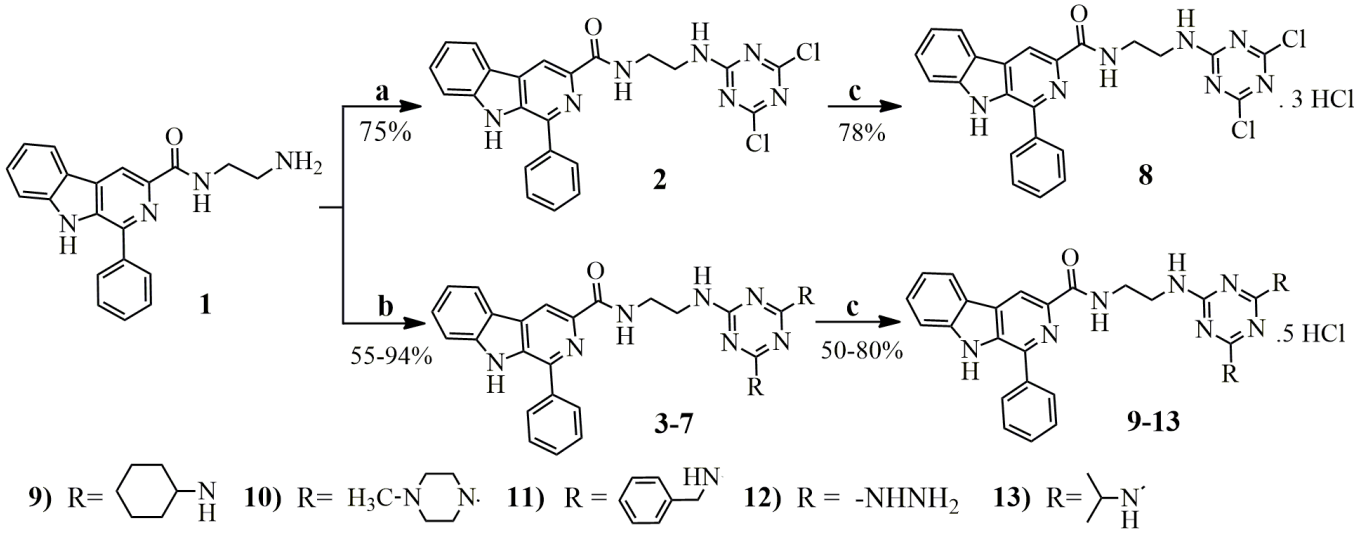
The crystal structure of BuChE complexed with butyrylcholinesterase (code ID: 1P0P) (Nicolet *et al.*, 2003) was obtained from the Protein Data Bank and the *N*-{2-[(4,6-dihydrazinyl-1,3,5-triazin-2-yl)amino] ethyl}-1-phenyl-β-carboline-3-carboxamide (**12**) structure was drawn using the Marvin Sketch Version 14.8.25 ChemAxon. The molecular docking studies were performed using the AutoDock Vina program (Trott, Olson, 2010) implemented at the interface PyRx 0.9 (Wolf, 2009) using default parameters. For each PDB file, some molecules of water and other ligands (except butyrylcholinesterase) were removed. The box dimensions were set at 25 × 22 × 22 Å and the center of the grid box was placed at coordinates x = 133.7, y = 115.1, z = 41.0.

Re-docking simulations were performed to validate the parameters that had been chosen and were repeated four times which gave a RMSD values below 0.5 Å. The best results were submitted to energy minimisation with the NAMD2 program (Phillips *et al.*, 2005). The force field adopted for proteins was CHARMM C35b2-C36a2, and for the ligands, they were generated in the same format as the SwissParam server (Zoete *et al.*, 2011). The results were shown with the CCP4 Molecular Graphics software (McNicholas *et al.*, 2011).

RESULTS AND DISCUSSION

Chemistry

The β-carboline-1,3,5-triazine hybrids **8-13** (**Scheme 1**) were synthesised as described for Baréa *et al.* (2018). Briefly, the β-carboline intermediate **1**, obtained from *L*-tryptophan commercial, was subjected to reaction with cyanuric chloride in the absence or presence of different amines, under basic medium. The hydrochloride salts were prepared from the treatment of compounds **8-13** with hydrochloric acid in methanol (**Scheme 1**).



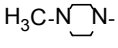
SCHEME 1 - Synthesis of compounds **8-13**. Reagents and conditions: (a) Cyanuric chloride, NaOH (1M), H_2O, CH_3CN, THF, 0ºC, 1 h. (b) 1) Cyanuric chloride, NaOH (1M), H_2O, CH_3CN, THF, 0ºC, 1h; 2) Amine (cyclohexylamine for **9**; 1-methylpiperazine for **10**; benzylamine for **11**; hydrate hydrazine for **12**; isopropylamine for **13**), 70°C, 48 h; (c) MeOH, HCl 12 M, rt., 4 h.

Anticholinesterase activity

The anticholinesterase activities of β-carboline-1,3,5-triazine hydrochlorides **8-13** and donepezil (reference compound) were evaluated according to the Ellman method (Ellman *et al*., 1961). Firstly, the compounds **8-13** were evaluated *in vitro* at concentrations of 10 μM and 100 μM against AChE and BuChE and their percentages of inhibition were determined (**Table I**). All compounds showed less than 50% or no inhibition for AChE, and their IC_{50} (50% Inhibitory Concentration) values were not determined for this enzyme. On the other hand, all hybrids inhibited the BuChE at a concentration of 100 μM, showing inhibition percentage in the range from 67.3–91.9%, and most of them also inhibited this enzyme at concentration of 10 μM (40.5–82.7%). Thus, the IC_{50} values were determined for compounds that inhibited more than 40% of this enzyme, at a concentration of 10 μM (**Table I**). The obtained IC_{50} values ranged from 1.0–18.8 μM, with derivative **12**, containing the hydrazinyl group at 6- and 4-positions of 1,3,5-triazine ring, the most active among the tested compounds for BuChE.

In summary, our results show that the analysed β-carboline derivatives were selective to BuChE. This selectivity can be explained based on the volume of the BuChE active site gorge, which is approximately 200 $Å^3$ larger than the AChE gorge (Saxena *et al*., 1997; Johnson, Moore, 2012; Masson, Carletti, Nachon, 2009), allowing the accommodation of tested β-carboline-1,3,5-triazine hybrids. Moreover, other compounds of the β-carboline class with a flexible linker also exhibited selectivity for BuChE (Zhao *et al*., 2018). The authors explain that folded molecules are suitable for the relatively spherical and large cavity of BuChE, but not stretched and slender enough to fit the narrow gorge of AChE (Zhao *et al*., 2018). Therefore, the presence of the flexible *N*-aminoethyl-carboxamide group between the β-carboline and 1,3,5-triazine moieties in **8-13** probably corroborated the obtained selectivity.

TABLE I - Inhibition percentages of compounds **8-13** against AChE and BuChE and theirs IC_{50} values for BuChE

		% inhibition AChE		% inhibition BuChE		BuChE IC_{50} (μM)
Comp.	**R**	**10 μM**	**100 μM**	**10 μM**	**100 μM**	
8	Cl	NI	6.5 ± 3.4	44.9 ± 5.4	80.9 ± 2.2	10.2 ± 0.3
9		NI	0.5 ± 0.1	NI	67.3 ± 9.9	N.D.
10		NI	45.9 ± 2.8	40.5 ± 2.9	83.4 ± 1.6	12.0 ± 1.4
11		NI	NI	49.3 ± 5.4	83.0 ± 2.2	18.8 ± 3.8
12	$NHNH_2$	7.6 ± 0.2	29.2 ± 6.5	82.7 ± 0.3	91.9 ± 5.5	1.0 ± 0.1
13		9.7 ± 3.3	17.1 ± 1.5	71.6 ± 3.5	80.1 ± 6.2	5.8 ± 4.1

NI = No Inhibition. ND = Not Determined. Donepezil was used as positive control ($IC_{50\ AChE}$ = 10.8 ± 3.0 nM; $IC_{50\ BuChE}$ = 2.9 ± 0.5 μM).

Enzyme kinetics

Due to the potent activity observed for compound *N*-{2-[(4,6-dihydrazinyl-1,3,5-triazin-2-yl)amino] ethyl}-1-phenyl-β-carboline-3-carboxamide (**12**), this compound was submitted to kinetics studies to investigate its type of BuChE inhibition. The kinetics studies were performed using the modified Ellman's method (Ellman *et al.*, 1961). To assess the kinetic parameters, we measured the initial rate of enzyme activity at different concentrations of substrate butyrylthiocholine (0.05 to 2.0 mM) in the absence and presence of the compound **12**. Lineweaver-Burk plots (**Figure 2**) were generated by plotting the reciprocal of the initial rate ($1/V_0$) against the reciprocal of substrate concentrations (1/[S]) for the different concentrations of **12**, resulting from the substrate–velocity curves for BuChE.

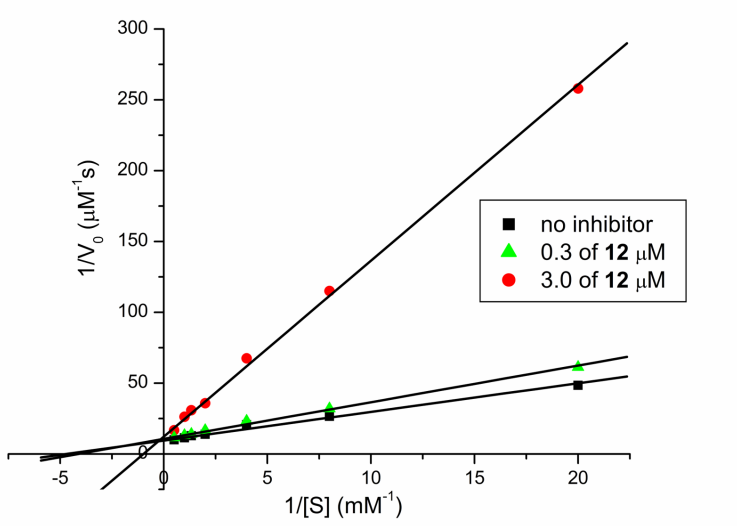


FIGURE 2 - Lineweaver-Burk plot for the inhibition of BuChE with different butyrylthiocholine concentrations (0.05 to 2.0 mM) in the absence and presence of compound **12** at concentrations of 0.3 and 3.0 μM.

Graphical analysis of Lineweaver-Burk plots (**Figure 2**) and the kinetic parameters of BuChE activity showed a practically unchanged V_{max} value (V_{max} = 0.11, 0.10 and 0.11 μMs^{-1} in the absence and presence of 0.3 and 3.0 μM of **12**, respectively) and an increasing K_m value (K_m = 0.28, 0.30 and 1.58 mM in the absence and presence of 0.3 and 3.0 μM of **12**, respectively) with increasing inhibitor concentrations, *i.e.* increasing slopes and the same intercepts on the y-axis ($-1/V_{max}$). This pattern indicates a competitive type of inhibition (Copeland, 2000). It is shown that compound **12** and substrate (butyrylthiocholine) compete for the same active site, *i.e.* the inhibitor interacts with the same binding site as the substrate.

For hybrid **12**, the dissociation constant (K_i) value obtained was 0.55 μM while the K_m value of butyrylthiocholine iodide for BuChE was 0.28 mM, which shows that the binding capacity of **12** with BuChE is approximately 509-fold that of the substrate. In addition, the hybrid **12** exhibited a K_i value similar to derivative **Vb** (**Figure 1**, K_i = 0.64 μM for BuChE) (Torres *et al.*, 2012) and showed a binding capacity that was approximately 164-fold greater than that of harmane (**IV**, **Figure 1**, K_i = 90 μM for BuChE) (Torres *et al.*, 2012).

Molecular modelling studies

The molecular docking calculations were performed using AutoDockVina program (Trott, Olson, 2010) implemented at the interface PyRx 0.9 (Wolf, 2009). Compound **12** was docked in the active site of BuChE (PDB: 1P0P) (Nicolet *et al.*, 2003) derived from the

complex of the enzymes with butyrylcholinesterase obtained from the Protein Data Bank (PDB). The best docked poses, *i.e.* the lowest energy conformer in the most populated cluster of conformers, were subjected to energy minimisation by NAMD (Phillips *et al.*, 2005) program and analysed to explain interactions between ligands and the target enzyme. **Figure 3** shows that compound **12** is oriented in active site gorge of BuChE. The hydrogen atom of protonated triazine moieties interacts with the carboxylate oxygen atom of Gly115 via an H-bond. The oxygen atom of the carbonyl group forms an H-bond with OH group of Thr120 and NH group also forms an H-bond with the carboxylate oxygen atom of Gly116. The protonated nitrogen of hydrazine moieties interacts with Tyr440 and Trp82 by π-cation interaction. Both hydrogen atoms of the protonated hydrazine moieties are therefore likely to form an H-bond with the carboxylate oxygen atoms of His438 and Gly439 and the other protonated hydrazine moieties also interact with Glu197 by H-bond interactions. This strong interaction with His438 (residue of the catalytic triad) (Nicolet *et al.*, 2003) and the π-cation interaction with Trp82 (residue of catalytic anionic site) (Nicolet *et al.*, 2003) confirm that the compound competes with the same binding site of the butyrylthiocholine.

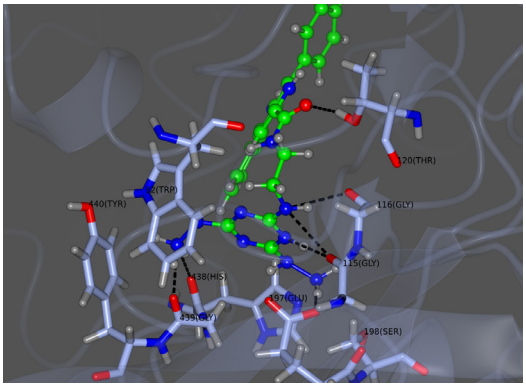


FIGURE 3 - Binding mode of **12** and BuChE. The compound is rendered in green ball-and-stick models, and the residues are rendered in grey coloured sticks.

CONCLUSION

In conclusion, we evaluated the anticholinesterase activity of the β-carboline-1,3,5-triazine hybrids against AChE and BuChE. All of the compounds showed significant activity and selectivity for BuChE. The kinetics and molecular docking studies for the most active hybrid **12** indicate that this compound inhibited BuChE via a competitive type of inhibition.

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