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Research Paper

Isoxazoles: synthesis, evaluation and bioinformatic design as acetylcholinesterase inhibitors

Margarita Gutiérrez[a], María Francisca Matus[a], Tomas Poblete[a], Jessica Amigo[a], Gabriel Vallejos[b] and Luis Astudillo[a]

[a]Laboratorio Síntesis Orgánica, Instituto de Química de Recursos Naturales, Universidad de Talca, Talca and [b]Laboratorio de Bio-Orgánica, Instituto de Ciencias Químicas, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile

Keywords
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Correspondence
Margarita Gutiérrez, Laboratorio Síntesis Orgánica, Instituto de Química de Recursos Naturales, Universidad de Talca, Casilla 747, Talca 3460000, Chile.
E-mail: mgutierrez@utalca.cl

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Abstract

Objectives Inhibition of acetylcholinesterase (AChE) is a common treatment for early stages of Alzheimer's disease. In this study, nine isoxazoles derivatives were tested for their in-vitro AChE activity. The molecular docking showed the interaction of the compounds with the active site.

Methods The isoxazoles were synthesized using 1,3-dipolar cycloaddition in the presence of sodium hypochlorite. They were also isolated and characterized by spectroscopic methods. The in-vitro activity was measured by an adapted version of Ellman's assay.

Key findings The isoxazoles are described as inhibitors of AChE. The most potent compound in the series exhibited a moderate inhibitory activity (50% inhibitory concentration = 134.87 μM). The design of new compounds was created by using the RACHEL module of the SYBYL software.

Conclusions Our research provided enough evidence of the efficacy of isoxazoles as AChE inhibitors. The isoxazoles were synthesized and evaluated as inhibitors of AChE. The docking study based on a novel series of complexes isoxazole with AChE from *Electroporus electricus* has demonstrated that the ligand bind is similar to the compounds used as reference. To find new candidates with the isoxazole core that act as inhibitors of AChE, part of the structure of the compound **9** was used for de-novo design. Molecular docking models of the ligand-AChE complexes suggest that the compound **10** is located on the periphery of the AChE active site.

Introduction

The heterocyclic compounds are widely distributed in nature and are essential to life in several ways. These compounds are important because of the wide variety of activity associated with this class of substances.[1] Heterocyclic rings are present in several compounds, for example, most of the members of vitamin B complex, antibiotics, chlorophyll, haemin, other plant pigments, amino acids and proteins, drugs, dye stuffs, enzymes and DNA. The isoxazole is an azole with an oxygen atom bonded to a nitrogen atom; these compounds are an important category of five-membered unsaturated heterocyclic compounds. They show numerous applications in diverse areas such as pharmaceuticals, agro-chemistry and industry among others. The isoxazoles are also found in natural sources such as insecticides, plant growth regulators and pigment functions.[2]

The isoxazole rings are found in natural products like the ibotonic acid – an active constituent of the psychotropic fly agaric mushroom *Amanita muscaria* – acting as ionotropic and metabotropic (G-protein linked) glutamate receptor subtypes.[3] (+)-Neopeltolide was isolated from a taxonomically uncharacterized sponge of the Neopeltidae family, order 'Lithistida' collected in deep water of the Jamaica's coast.[4] The compound was assigned structurally as macrolide with an isoxazole ring on a side chain and showed some similarities in the structure to the compound

previously known, leucascandrolide A.[5] The isoxazoles also form the basis for a number of specific drugs, including the cyclooxigenase-2 inhibitor valdecoxib (Bextra, Omaha, NE, USA).[6] Thus, the isoxazoles have been widely used and studied in the modern drug discoveries.[7]

The isoxazoles can be prepared in many ways, but the most widely reported and researched synthesis of isoxazoles is via [3 + 2] cycloaddition of a nitrile oxide and an alkyne. The conventional generation of a nitrile oxide requires the dehydration of a nitroalkane or a similar starting material. Other methods for generating nitrile oxides are by reaction of aldoximes with oxidizing agents or halogenating species.[8]

A series of 1,2-benzisoxazoles was synthesized, and found potent and selective inhibitors of acetylcholinesterase (AChE) *in vitro* against targets from different species.[9] New D-glyceraldehyde heterocyclic derivatives were synthesized and evaluated as potential inhibitors of AChE, the most active compounds possess the isoxazole ring.[10] Pyrroloisoxazole benzoic acid derivatives were synthesized and evaluated as potential AChE inhibitors for the management of Alzheimer's disease (AD).[11]

AD is a progressive neurodegenerative disorder associated with cognitive, functional and behavioural impairments as well as the most common form of dementia in the elderly.[12] It affects brain regions that control thoughts, memory, thinking, behaviour, emotion and language, and evolves to a devastating status for patients and caregivers. Nearly 50 million people worldwide have AD. Social and economic burdens of this disease are massive because of the estimated direct and indirect annual costs of $100 billion in patient care per year only in the USA.[13] These facts suggest that potent drugs are needed to slow down the progression of AD or even to prevent it.

The current therapies for AD, either symptomatic or palliative, rely mainly on the restoration of acetylcholine levels[14] and on the partial antagonism of the N-methyl-D-aspartate receptor.[15] AChE is the enzyme that terminates the transmission of nerve impulses in cholinergic synapses by hydrolysing the neurotransmitter acetylcholine to acetic acid and choline. Because cholinergic transmission is involved in a variety of physiological systems, some inhibitors are used to treat various disorders, such as myasthenia gravis, and as a symptomatic approach to the management of AD.[16]

This paper describes the work undertaken to study the effectiveness of synthesis, biological activity and the mechanism of interactions by computational analysis. The results can be highly useful and may provide a convenient platform for the development of new inhibitors of AChE towards the treatment of AD, which can generate a massive reductions development time of drugs.

Materials and Methods

Chemistry

All solvents used were of analytical grade. Melting points were determined by open capillary methods on a Buchi apparatus and are uncorrected. Infrared (IR) spectra, KBr pellets, 500–4000 cm^{-1} were recorded on a Thermo Nicolet NEXUS 670 FT-IR spectrophotometer (Madison, WI, USA), with a 0.125 cm^{-1} spectral resolution. The ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra were recorded on a Bruker AM 400 instruments (Rheinstetten, Germany). Chemical changes are expressed as values relative to tetramethylsilane as internal standard. Multiplicities are designated as singlet (s), doublet (d), triplet (t), quadruplet (q) and multiplet (m). High-resolution mass spectrometry electrospray ionization mass spectrometry (ESI-MS) and ESI-tandem mass spectrometry (ESI-MS/MS) analysis were conducted in a high-resolution hybrid quadrupole (Q) and an orthogonal time-of-flight (TOF) mass spectrometer (Waters/Micromass Q-TOF micro, Manchester, UK), with a constant nebulizer temperature of 100°C. The experiments were carried out in positive ion mode, and the cone and extractor potentials were set at 10 and 3.0 V, respectively, with a scan range of *m/z* 100–600. MS/MS experiments were carried out by mass selection of a specific ion in Q1, which was then submitted to collision-induced dissociation with helium in the collision chamber. The product ion MS analysis was accomplished with the high-resolution orthogonal TOF analyser. The samples were directly infused into the ESI source via a syringe pump at flow rates of 5 μl/min through the instrument's injection valve. The elemental analysis has been obtained using a LECO CHNS-900 and Thermo Finnigan FlashEA1112 CHNS-O (STIUJA, Hemel, Hempstead, UK) elemental analysers. TLC was done on precoated silica gel 60 F254 plates (Merck, Darmstadt, Germany).

Synthetic methodologies

The isoxazole derivatives were efficiently synthesized according to the protocol outlined in Figure 1. This protocol can be divided in two steps:

Step 1: preparation of oxime derivatives

An aqueous solution of hydroxylamine (NH_2OH 50%) 24 ml (12.0 mmol) and sodium acetate 1.0 g (12.2 mmol) were dissolved. The aldehyde (10 mmol) was dissolved in ethyl alcohol (10 ml); both solutions were mixed and kept from 30 to 40 min at room temperature. Then the oxime was extracted with dichloromethane (3 × 15 ml) and separated from the aqueous layer, and the volume was reduced half under decreased pressure. The formation of oxime was analysed for TLC.

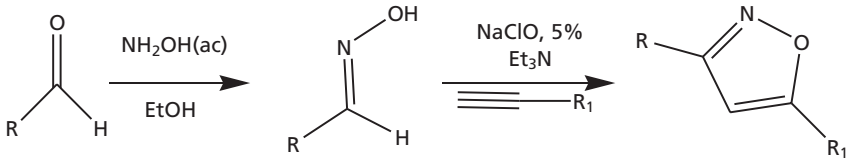


Figure 1 Protocol for synthesis of isoxazole derivatives.

Step 2: preparation of 3,5-disubstituted isoxazoles

Oxime (10 mmol) in dichloromethane (20 ml) at 0°C was dropwise added to a mixture of alkyne (10 mmol), triethylamine 1.5 ml (11 mmol) and 5% of aqueous sodium hypochlorite (20 ml) after stirring for 60 min in iced water. Then, the mixture was extracted with dichloromethane (3×15 ml) and separated from the aqueous layer. Organic extracts were combined, dried over Na_2SO_4 and the solvent was removed under vacuum. The solid obtained was suspended in hot ethyl alcohol and crystallized, obtaining the isoxazole. TLC checked the reaction completion.

Biological activity

In the 96-well plates, 50 ml of the sample dissolved in phosphate buffer (8 mmol/l K_2HPO_4, 2.3 mmol/l NaH_2PO_4, 150 mmol/l NaCl, and 0.05% Tween 20 at pH 7.6) as well as 50 ml of the AChE solution (0.25 unit/ml), from *Electroporus electricus*, in the same phosphate buffer were added. The assay solutions except the substrate were preincubated with the enzyme for 30 min at room temperature. After preincubation, the substrate was added. The solution substrate consists of Na_2HPO_4 (40 mmol/l), acetylthiocholine (0.24 mmol/l) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (0.2 mmol/l, Ellman's reagent). The absorbance of the yellow anion product due to the spontaneous hydrolysis of substrate was measured at 405 nm for 5 min on a Microtiter plate reader (Multiskan EX, Thermo, Finland). The AChE inhibition was determined for each compound. The enzyme activity was calculated as a percentage compared with a control using only the buffer and enzyme solution. The compounds were assayed in the dilution interval of 500–15 μg/ml, and the galantamine alkaloid was used as the reference compound. Each assay was run in triplicate, and each reaction was repeated at least three independent times. The 50% inhibitory concentration (IC50) values were calculated by means of regression analysis.

Statistical analysis

Three or more independent experiments were performed. The data were analysed and expressed as mean ± standard error of the mean (SEM) using Statistical Product and Service Solutions version 17th (SPSS, Inc., Chicago, IL, USA). IC50 of isoxazole derivatives against AChE was calculated with a regression analysis. Statistical analyses were performed by one-way analysis of variance (ANOVA). Significant differences between groups were determined at $P < 0.05$.

Docking studies

Molecular docking studies on acetylcholinesterase

The computational process of searching for a ligand that is able to fit both geometrically and energetically the binding site of a protein is called molecular docking.[17] Docking studies were performed using Glide[18] version 5.5. Glide docking uses a series of hierarchical filters to find the best possible ligand binding locations in a receptor grid space previously built. The filters include a systematic searching approach, which samples the positional, conformational and orientational space of the ligand before evaluating the energy interactions of the ligand with the protein. The Glide program is contained in Maestro 9.0 suite (New York, NY, USA). The protein coordinates were extracted from the X-ray crystal structure of AChE from the *E. electricus* organism that was acquired from the Protein Data Bank (PDB ID: 1EEA). Extra-precision module of Glide was used. The docking hierarchy begins with the systematic conformational expansion of the ligand, followed by the placement in the receptor site. Then, minimization of the ligand in the field of the receptor is carried out using the OPLS-AA[19] force field, with a distance-dependent dielectric 2.0. Subsequently, the lowest energy poses are subjected to a Monte Carlo procedure that samples nearby torsional minima. The best pose for a given ligand is determined by the Emodel score while different compounds are ranked using Glide Score (a modified version of the Chem Score function of Eldridge *et al.*[20]) that includes terms for buried polar groups and steric clashes.

De-novo design

This technique enables design inhibitors from the binding site on the target or the pharmacophore, that is, the information of spatial points relevant of the receptor–ligand interaction. Once knowing both, the binding mode and the structural relationship between the compound **9** and AChE

and their experimental activity, we proceeded to the optimization of the compound **9** through the RACHEL module of SYBYL (TRIPOS, St Louis, MO, USA). Starting from a ligand/receptor complex, RACHEL performs automated combinatorial optimization of lead compounds by systemically derivatizing the user-defined sites on the ligand. These compounds are conformationally searched within the active site and then evaluated; only those that bind tightly with the receptor are retained. Afterwards, this new population of compounds is processed to form the next generation of derivatives. Over time, a lead compound is iteratively refined into a set of high-affinity structures.

Results

Nine isoxazoles disubstituted, with yield around 80%, were synthesized using cycloadittion 1,3-dipolar of aldehyde and alkyne with hydroxylamine, triethylamine and sodium hypochlorite. Structures of all compounds with alkynes and aldehydes used for synthesis are showed in Table 1.

Structural identification

2-(5-Phenyl-isoxazol-3-yl)pyridine (**1**): Yield 87%. Mp80-82°C. ^{1}H NMR δppm ($CDCl_3$-400 MHz): 8.73 (1H, ddd, J = 8.0; 4.8; 1.2 Hz); 8.15(1H, d, J = 8.0); 7.87 (2H, dd, J = 8.0; 1.6 Hz), 7.84 (1H, dt, J = 7.6, 1.6), 7.49 (3H, m), 7.38 (1H, ddd, J = 7.5, 5.2, 1.2 Hz), 7.20 (1H, s). ^{13}C δppm ($CDCl_3$-100 MHz): 170.64; 163.76; 149.71; 148.55; 136.91; 130.24; 129.02; 127.24; 125.84; 124.52; 121.66; 98.31. MS *m/z* (EI): 222.98 (M^++1). IR (KBr)/cm: 3413; 1649;1456. Anal. Calcd for $C_{14}H_{10}N_2O$:C, 75.66; H, 4.50. Found: C, 75.67; H, 4,51.

3-(5-Phenyl-isoxazol-3-yl)pyridine (**2**)**:** Yield 88%. Mp134-135°C. ^{1}H NMR δppm ($CDCl_3$-400 MHz): 9.09 (1H, d, J = 1.2 Hz), 8.72 (1H, dd, J = 4.8, 1.6 Hz), 8.22 (1H, dt, J = 8.0, 2.0 Hz), 7.86 (2H, dd, J = 8.0, 1.2 Hz), 7.51 (3H, m), 7.44 (1H, dd, J = 8.0, 4.8 Hz), 6.89 (1H, s). MS *m/z* (EI): 222.98 (M^++1). IR (KBr)/cm: 3417; 2106; 1642; Anal. Calcd for $C_{14}H_{10}N_2O$: C, 75.66; H, 4.50. Found: C, 75.67; H, 4,51.

4-(5-Phenyl-isoxazol-3-yl)pyridine (**3**): Yield 82%. Mp123. ^{1}H NMR δppm ($CDCl_3$-400 MHz): 8.68 (2H, d, J = 8.0 Hz), 8.63 (2H, d, J = 8.0 Hz), 8.58 (2H, d, J = 8.0 Hz), 7.71 (1H, t, J = 8.0 Hz), 7.66 (2H, d, J = 8.0 Hz), 6.76 (1H, s). MS *m/z* (EI): 222.98 (M^++1). IR (KBr)/cm:3442; 2102; 1632; 998. Anal. Calcd for $C_{14}H_{10}N_2O$: C, 75.66; H, 4.50. Found: C, 75.67; H, 4,51.

2-(5-Thiophen-3-yl-isoxazol-3-yl)pyridine (**4**): Yield 78%. Mp119°C. ^{1}H NMR δppm ($CDCl_3$-400 MHz): 8.72 (1H, ddd, J = 8.0, 4.8, 1.2 Hz); 8.13 (1H, d, J = 8.0),7.83 (2H, m), 7.47 (1H, t, J = 6.0 Hz), 7.45 (1H, dd, J = 5.0, 3.2 Hz), 7.37 (1H, ddd, J = 7.5, 4.8, 1.2 Hz),7.06 (1H, s).MS *m/z* (EI): 228.93 (M^++1). IR (KBr)/cm: 3433; 2078; 1641; 997. Anal.Calcd for $C_{12}H_8N_2OS$: C, 63.14; H, 3.53. Found C, 63.16; H, 3.51.

3-(5-Thiophen-3-yl-isoxazol-3-yl)pyridine (**5**): Yield 81%. Mp198-199°C. ^{1}H NMR δppm ($CDCl_3$-400 MHz): 9.16 (1H, d, J = 1.6 Hz), 9.06 (1H, d, J = 1.6 Hz), 8.83 (1H, dd, J = 5.0, 1.2 Hz), 8.71 (1H, dd, J = 4.8, 1.6 Hz), 8.20 (2H,m), 7.86 (1H, dd, J = 2.6, 1.2 Hz), 6.74 (1H, s). MS *m/z* (EI): 228.93 (M^++1). IR (KBr)/cm: 3438; 2072; 1648, 78. Anal. Calcd for $C_{12}H_8N_2OS$: C, 63.14; H, 3.53. Found C, 63.17; H, 3.54.

4-(5-Thiophen-3-yl-isoxazol-3-yl)pyridine (**6**): Yield 83%. Mp162-163°C. ^{1}H NMR δppm ($CDCl_3$-400 MHz): 8.76 (2H, d, J = 6.0 Hz), 7.88 (1H, s), 7.74 (2H, d, J = 6.0 Hz), 7.48 (2H, m), 6.75(1H, s). MS *m/z* (EI): 228.93 (M^++1). IR (KBr)/cm: 3438; 2075; 1640; 948. Anal. Calcd for $C_{12}H_8N_2OS$: C, 63.14; H, 3.53. Found C, 63.16; H, 3.51.

2-(5-Cyclohex-1-enyl-isoxazol-3-yl)-pyridine (**7**): Yield 85%. Oil. ^{1}H NMR δppm ($CDCl_3$-400 MHz): 8.66 (1H, d, J = 8.0 Hz), 8.06 (1H, d, J = 8.0 Hz) 7.77 (1H, t, J = 7.6 Hz), 7.32 (1H, t, J = 7.6 Hz), 6.73 (1H, s); 6.67 (1H, brs), 2. 31 (4H, m) 1.72 (4H, m). ^{13}Cδppm ($CDCl_3$-100 MHz): 171.78, 163.12, 149.56, 148.79, 136.77, 130.17, 125.32, 124.26, 121.52, 96.94, 25.36, 25.07, 22.01, 21.63. MS *m/z* (EI): 226.94 (M^++1). IR (KBr) cm^{-1}3435; 2102; 1670; 864. Anal. Calcd for $C_{14}H_{14}N_2O$: C, 74.31; H, 6.24. Found C, 74.33; H, 6.20.

3-(5-Cyclohex-1-enyl-isoxazol-3-yl)-pyridine (**8**): Yield 83%. Mp186-187°C. ^{1}H NMR δppm ($CDCl_3$-400 MHz): 9.36 (1H, s), 8.91 (2H, m), 8.03 (1H, t, J = 8.0), 6.74 (2H, brs), 2.34 (4H, m), 1.75 (4H,m). MS*m/z* (EI): 226.95 (M^++1). IR(KBr)/cm: 3397; 2097; 1644; 949. Anal. Calcd for $C_{14}H_{14}N_2O$: C, 74.31; H, 6.24. Found C, 74.32; H, 6.23.

4-(5-Cyclohex-1-enyl-isoxazol-3-yl)-pyridine (**9**): Yield 78%. Oil. ^{1}H NMR δppm ($CDCl_3$-400 MHz):8.41 (2H, d, J = 8.0 Hz), 7.92 (2H, d, J = 8.0 Hz), 6.31 (1H, brs), 6.25 (1H, s), 2.02 (4H, m), 1.39 (4H, m). MS*m/z* (EI): 227.054 (M^++1). IR(KBr)/cm 3418; 2130; 1649; 832. Anal. Calcd for $C_{14}H_{14}N_2O$: C, 74.31; H, 6.24. Found C, 74.34; H, 6.25.

Biological results

The concentration of the compound required for 50% enzyme inhibition (IC50) was calculated by means of regression analysis. All tabulated results in Table 2 are expressed in micrograms per millilitre and micromoles per litre as means ± SEM, and was compared by using ANOVA analysis. A P-value of less than 0.05 was considered significant. Details for pharmacological experiments are described in Materials and Methods as well as in previous works.[21] The series of compounds with 1-ethynylcyclohexene used as alkyne (**7**–**9**) showed the highest activity with IC50 values upper to 200 μM. Although the activity of compounds is deficient comparable with the reference employed, it is

Table 1 Aldehydes and alkynes required for the synthesis of compounds **1–10** and structure to obtain

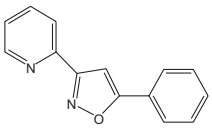
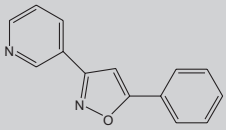
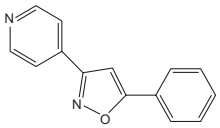
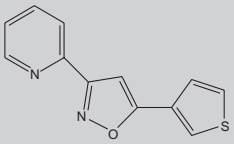
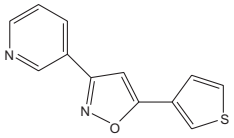
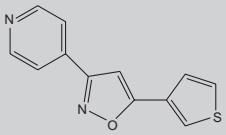
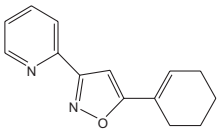
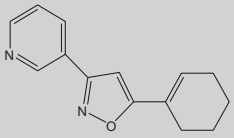
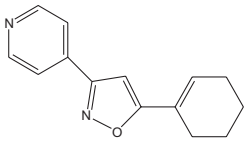
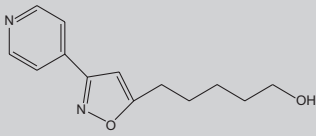
Compound	Aldehyde (R)	Alkyne (R_1)	Structure
1	Pyridine-2-carbaldehyde	Phenylacetylene	
2	Pyridine-3-carbaldehyde	Phenylacetylene	
3	Pyridine-4-carbaldehyde	Phenylacetylene	
4	Pyridine-2-carbaldehyde	3-ethynylthiophene	
5	Pyridine-3-carbaldehyde	3-ethynylthiophene	
6	Pyridine-4-carbaldehyde	3-ethynylthiophene	
7	Pyridine-2-carbaldehyde	1-ethynylcyclohexene	
8	Pyridine-3-carbaldehyde	1-ethynylcyclohexene	
9	Pyridine-4-carbaldehyde	1-ethynylcyclohexene	
10	Pyridine-4-carbaldehyde	5-hexyn-1-ol	

Table 2 IC50 of isoxales derivatives and galantamine on acetylcholinesterase evaluated by a microplate assay

Compound	IC50 (μg/ml)	IC50 (μM)
1	69.19	311.67 ± 2.54
2	79.65	358.78 ± 2.85
3	205.06	923.69 ± 3.07
4	>250	
5	81.46	357.28 ± 0.17
6	>250	
7	44.53*	197.04 ± 0.28*
8	41.53*	183.76 ± 0.44*
9	**30.48***	**134.87 ± 0.29***
Galantamine	1.1	0.54 ± 0.10

Values are average ± SEM from three experiments. Bold values show the most active compound. *$P < 0.05$.

show as interesting pharmacophores to be used for the design of new biologically active compounds against enzymes related with neurodegenerative diseases. Compound **9** showed the major activity against AChE, with IC50 of 134.87 μM. With the aim of knowing the location and interactions generated with the active site of the enzyme, compound **9** was subjected to molecular docking, and then, these compounds were used from de-novo design.

Docking studies on acetylcholinesterase

Figure 2 depicts the compound **9** (gray representation) docked at the active site of AChE using Glide software. The binding energy of the best pose obtained from docking was –9.32 kcal/mol. Compound **9** to establish a hydrogen bond (HB) interaction with the residue E199 (yellow representation), the amine group of the aldehyde derivative portion in the compound **9** is the HB donor group, and the carbonylic oxygen in this residue is the HB acceptor group. Furthermore, the residues H440 and W84 (cyan representation) established π-π stacking interactions with this portion, and the residue F330 established π-π stacking interactions with the isoxazolic ring. The residues S200 and F331 (green representation) are key residues in the AChE active site. These residues do not establish any bond, but they were found to be less than 3 Å.

De novo design

Compound **10** was the most potent compound (score: 11.75) found. This novel compound is a derivative of isoxazol **9**, with modifications on the cyclohexen ring. Molecular docking studies showed better affinity with active site of AChE. The binding energy of the best pose obtained from docking was –8.45 kcal/mol. Figure 3 depicts the most important interactions between this inhibitor and AChE. Compound **10** was able to established HB interactions with residues E199 and F330 (yellow representation). In residue E199, the carbonylic oxygen is the HB acceptor, and the amino group of the aldehyde derivative portion in compound **10** is the HB donor group. In residue F330, the carbonylic oxygen is the HB acceptor, and the hydroxyl group in the compound **10** is the HB donor group. The residues H440 and W84 (cyan representation) established π-π stacking interactions with the aldehyde derivative portion and with the isoxazolic ring. The residues S200 and F331 (green representation) are key residues in the active site of AChE. These residues do not establish any bond, but they were found to be less than 3 Å, similarly to the compound **9**-AChE complex.

Discussion

These coupling reactions were performed under mild conditions (room temperature, 24 h) in the presence of aqueous sodium hypochlorite (5%). The isoxazoles products were obtained in good yield with almost no by-products. All isoxazoles were purified by recrystallization process.

Isoxazoles **1–9** were characterized by ^{1}H-NMR, ^{13}C-NMR and mass spectra. ^{1}H-NMR spectra of all isoxazoles synthesized were quite similar and characterized by the presence of three groups of signals (aromatics protons, protons near heteroatoms and aliphatic protons), which resonated in different zones. All ^{1}H-NMR spectra showed a characteristic proton singlet of the isoxazole ring with shift near seven.

The mass spectra showed similar fragmentation patterns between compounds, all showing the molecular ion $[M + H^{+}]^{+}$ and the gain or loss of small fragments of low molecular mass.

All compounds were evaluated as AchE inhibitors showed values.

All compounds were evaluated as inhibitors of AChE, showing the differences statistically significant of compounds **7–9** with the rest of the analysed groups. Compound **9** was the one with the most activity; with IC50 of 134.87 μM, the results suggest that rational modifications of the substituent in the scaffold isoxazoles would provide a rational basis for the development of novel AChE inhibitors. Compound **10** showed to be an interesting compound to block this protein. This molecule (as compound **9**) was able to establish interactions and bonds with key residues that belong to the active site of AChE. The interactions with these residues have been reported before[22] in AChE – the galantamine complex. Although isoxazol**9** showed better binding energy than **10,** this difference is not significant. Compound **10** was able to establish more numbers of H-bond, which explains that this molecule would be closer related to AChE than the compound **9**. It is important to analyse the differences of the alkyne derivative portion in both compounds. Compound **9** has a ring, which estab-

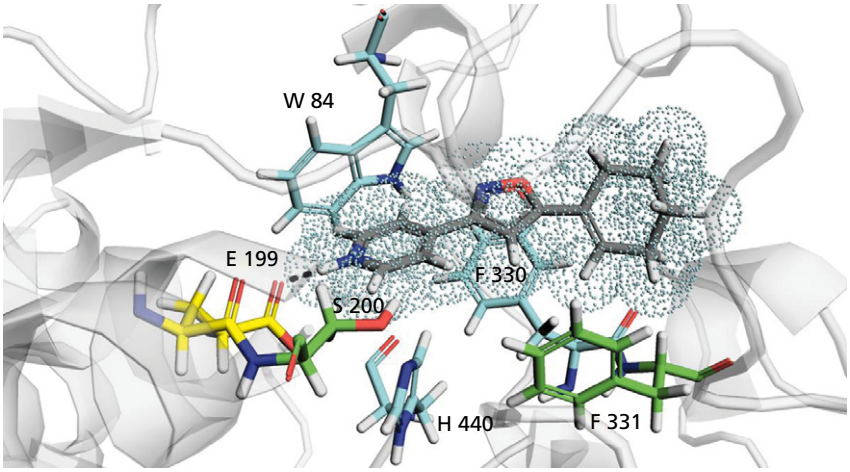


Figure 2 Molecular docking models for compounds **9** within the AChE binding site highlighting the protein residues that form the main interactions with the inhibitors.

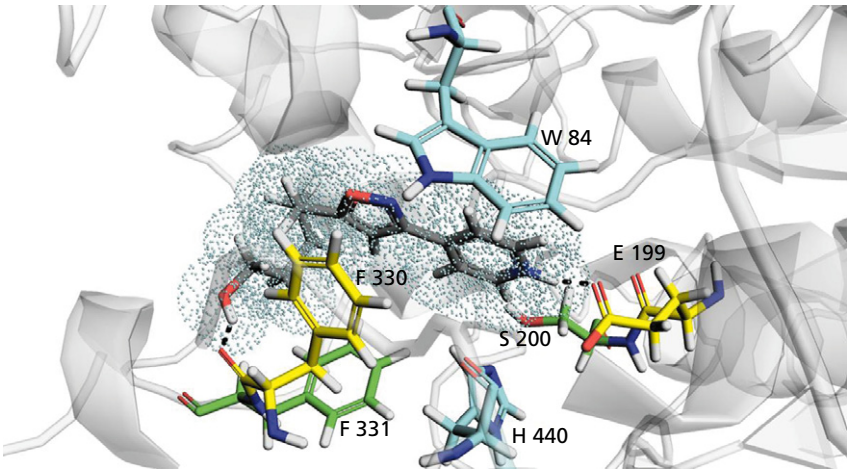


Figure 3 Molecular docking models for compounds **10** within the AChE binding site highlighting the protein residues that form the main interactions with the inhibitors.

lishes π-π stacking interactions with some residues. However, this ring structure leads to the ligand to be anchored to the active site in a stiffer way. In contrast, compound **10** has a more flexible tail in this part, which establishes H-bonds with some key residues. Therefore, the whole structure has more mobility, which suggests that if the protein suffers a conformational change, this tail could be adapted and fit better than the compound **9** into the active site because of its greater numbers of freedom of degrees, maintaining this interaction over the time.

Values of IC50 obtained for the compound **9** of this series may be comparable with other reported in literature for active ingredients of commercial pharmaco as galantamine, donepezil, rivastigmine and tacrine.[23] In addition, it will be expected to obtain IC50 values for the compound **10** lower than those reported in this work for the compound **9**. Moreover, the goal of the search for new inhibitors aims has a decline in side effects showed by the current drugs used in the treatment of this disease;[24,25] as well as based in previous reports for compounds with isoxazolic ring as effective drugs in other diseases treatments with low side effects.[26,27] It is expected a decreased of these to analyse *in vivo*.

Values of IC50 show some grade of relation between the substituents that possess the molecule and its biological activity; for instance, the presence of structures with a lower degree of saturation promotes interaction with the active site, whereas the position of the nitrogen in the pyridine ring seems not to influence the activity of the compounds. The presence of rings with heteroatoms or aromatics rings

in the portion derived from alkyne used is unfavourable for the interactions with active site of the enzyme. This is corroborated by de-novo design because of compound **10** obtaining a greater flexibility and thus facilitating the entry into the active site as well as the rapprochement between the compound and the amino acidic residues.

Our analysis also shows that synthetic isoxazoles could be the potential target molecules for the inhibition of AChE. Hence, the more active of a series could be used as the template for designing therapeutic lead molecule. We strongly hope that the ingenuity and success of the computational efforts discussed above bode well for the future prospects of finding new inhibitors, which could result into massive reductions in therapeutics development time.

Conclusions

In summary, a series of 1,3-disubstituted isoxazoles derivatives were designed, synthesized and evaluated as AChE inhibitors. Structure–activity studies showed that the AChE inhibitory activity of compounds was influenced by the substituent pattern at position 3 of isoxazol. The most potent compound (**9**) contains a ciclohexil group in this position. The molecular modelling study of compound **9** indicated that this is accommodated inside the active-site gorge of AChE, adopting the same interactions that were described for the AChE–galantamine complex.

Evidence was acquired demonstrating that isoxazoles 1,3-disubstituted is a potential scaffold for the design of novel AChE inhibitors. A detailed analysis of the experimental results obtained so far allows us to conclude that the affinity or selectivity of the isoxazoles towards AChE may be greatly influenced by the nature of their substituents. Compound **9** exhibits the best performance and can be considered as the starting point for the design and synthesis of new AChE inhibitors. The results suggest that rational modifications of the substituent in the scaffold 4-(5-cyclohex-1-enyl-isoxazol-3-yl)-pyridine would provide a rational basis for the development of novel AChE inhibitors. Further efforts aimed to develop potent AChE inhibitors based on the modification of compound **9** will be continued in our laboratory. This group of compounds could be an interesting lead for AChE inhibitors.

Currently, our working group is synthesizing new isoxazoles with the substitution pattern of compound **10** obtained by de-novo design to assess whether improvements are experimentally observed in the activity. Compound **10** can be synthesized from pyridine-4-carbaldehyde and 5-hexyn-1-ol. This compound could be suggested as a new possible inhibitor of AChE depending on the experimental results. In addition, this compound could be suggested as a member of a promising class of next-generation derivatives, improving even more its features against AChE.

To conclude, in this report, an innovative method for the synthesis of isoxazoles, a biologically important heterocyclic scaffold, was described. This synthesis is based on cycloaddition 1–3 dipolar in presence of sodium hypochlorite, which offers a faster, cheaper and safer way to construct isoxazoles with biological activity. This study shows the effectiveness that may have the use of bioinformatics tools for the rational design of drugs, which implies a reduction in the time devoted to the synthesis and the available resources.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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