UNIVERSITAT JAUME I
ESCOLA SUPERIOR DE TECNOLOGIA I CIÈNCIES EXPERIMENTALS
DEPARTAMENT DE QUÍMICA FÍSICA I ANALÍTICA
ÀREA DE QUÍMICA FÍSICA

UNDERSTANDING ACETYLCHOLINESTERASE INHIBITORS: COMPUTATIONAL MODELING APPROACHES

Érica Cristina Moreno Nascimento

CASTELLÓ DE LA PLANA
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TESIS DOCTORAL
Érica Cristina Moreno Nascimento

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2017

Juan M. Andrés Bort, Profesor Catedrático de la Universitat Jaume I de Castellón, y Mónica Oliva Domínguez, Profesora Titular de la Universitat Jaume I de Castellón,

CERTIFICAN

que la presente memoria correspondiente a la Tesis Doctoral ha sido realizada por Érica Cristina Moreno Nascimento bajo nuestra supervisión, y autorizan la presentación de la misma para su defensa.

En Castellón de la Plana, a 25 de julio de 2017.

Fdo. Juan M. Andrés Bort

Fdo. Mónica Oliva Domínguez

*Al pasado y al presente,
para que el futuro sea digno de vivir.
A la Felicidad.*

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LISTA ABREVIATURAS Y SIGLAS

5-HT: Serotonin
ACh: Acetylcholine
AChE: Acetylcholinesterase
AChEI-MAOI: Dual target inhibitor of acetylcholinesterase and monoamine oxidase
AChEIs: Acetylcholinesterase inhibitors
AD: Alzheimer's disease
APP: Amyloid precursor protein
ATP: Adenosine Triphosphate
A β : β -amyloid protein A/4
BACE-1: beta-secretase enzyme
BIE: Binding isotope effects
BuChE: Butyrylcholinesterase
CCAD: Current Computer-Aided Drug
ChEI: Cholinesterase inhibitors
CNS: Central nervous system
CP0: Camptothecin
DCV: Dichlorvos
DFT: Density functional theory
DMT: Bis(7)-tacrine
DNP: Donepezil
DOP: Dopamine
EDR: Edrophonium
EPI: Epinephrine
F11: Bis(5)-tacrine
FDA: Food and Drug Administration
FEP: Alchemical free energy perturbation methods
G3X: N-Piperidinopropyl-galanthamine
GL8: Galantamine bivalent derivative
GNT: Galantamine
HCA: Hierarchical cluster analysis
HUE: Huprine E
HUP: Huperzine A
HUX: Huprines
HUY: Huprine Y
HUR: Huprine
IPR: Iproniazide
ISO: Isocarboxazid
LAD: Ladostigil
LAD: Ladostigil
MAO: Monoamine oxidase

MAO-A: A isoform of Monoamine oxidase
MAO-B: B isoform of Monoamine oxidase
MD: Molecular dynamics
MM: Molecular mechanics
MOC: Moclobemide
MTB: Methylene blue
MTR: Metrifonate
ND: Neurodegenerative diseases
NIA: Nialamide
NMDA: N-methyl-D-aspartate receptor
NOR: Norepinephrine
OP-AChE: Organophosphoru-Acetylcholinesterase adduct
OPs: Organophosphorus compounds
PAS: Peripheral aromatic site
PBC: Periodic boundary conditions
PCA: Principal component analysis
PHZ: Phenelzine
PHS: Phenserine
PHY: Physostigmine
PMF: Potential of Mean Force
QM/MM: Quantum mechanics molecular mechanics
QM: Quantum mechanics
QSAR: Quantitative structure–activity relationship
RAS: Rasagiline
RIV: Rivastigmine
R-L: Receptor-Ligand
ROS: reactive oxygen species
SAR: Sarin gas
SCF: Self consistent Field
SEL: Selegiline
SG: Slow Growth
SEH: Catalytic triad of AChE- Ser200-Glu327-His440
SOM: soman gas
THA: Tacrine
TI: Thermodynamic Integration
TOL: Toloxatone
TRA: Tranylcypromine

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Chapter 1

Introduction

"I am among those who think that science has great beauty."
(Marie Skłodowska-Curie)

1. INTRODUCTION

1.1 General considerations

The age profile of the developed and the developing countries has changed over the centuries, consequence in application of human and economic resources in the promotion of a better quality of life. The change in the structure of the age pyramid in these countries shows that there is a marked increase in life expectancy of the population.

Figure 1. European Union age pyramid – 2014 census.¹

As shown in Figure 1, this scenery shows that the goal of prolonging human life has given positive results, in some countries the percentage of the population with people over 60 is already greater than 30%. However, with the increasing number of people in this age group, some health problems tend to occur more frequently, especially the diseases known as "age-dependent diseases".²

The occurrence of age-dependent diseases is also a result of poor psychosocial planning and public policy health targeting this strip age. Among age-dependent diseases stand out the dementias, diabetes and cardiovascular diseases.

There are several kinds of dementia and the most common to take place in the population above 60 years of age are Alzheimer's disease (AD), Parkinson's disease, vascular dementia, Lewy bodies disease, Pick's disease and others neurodegenerative diseases (ND).²⁻⁴

It is estimated that 45 million people worldwide exhibit some form of dementia, among those, 18 million have symptoms characteristic of Alzheimer's disease.⁴

Dementia is understood as a state of abnormal condition due to a progressive degeneration brain functioning that affects the cognitive functions as memory, behavior, emotional state and daily operational tasks of an individual.⁵ Dementias are considered as neurodegenerative diseases.²

Different risk factors to onset of AD are indicated even though the mechanism their trigger is not known. Therefore, other approaches are necessary in order to explain neurophysiologically and behavioral changes observed during the development of these neuropathologies. ND in general is an age-dependent condition but other reasons are related as well as to abnormal protein assemblies, oxidative stress in cellular environment, late-onset cell death in adulthood, inflammation (induced neurotoxicity), heredity, cardiovascular disease, diabetes, thyroid disease and head trauma could be also considered to be risk factors.^{2,5,6}

AD is a ND with unknown cause so for now no cure is possible. AD is a multifactorial disease characterized by progressive memory loss.^{7,8} Thus the disease treatment is focused in palliative solutions to help decrease the deleterious effects of this aggressive disease.⁹ A common physiological characteristic of patients with symptoms of AD is the suppression in the quantity of neurotransmitters responsible for the transmission of nerve impulses in the synaptic regions.¹⁰

The amount of neurotransmitters in the synaptic clefts rules an important function in the states of movement, mood, emotion, cognition, memory and learning,

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as they are the regulators of these functions. The increase and/or decrease in the amount of neurotransmitters in synaptic clefts can produce deleterious effects on the brain.¹¹⁻¹³

Many treatment strategies are applied to try to remedy the problem and bring improvement in the patient life. In most cases, these strategies are related with use of drugs acting on the central nervous system (CNS), specifically at synapses. These drugs are used to try to inhibit enzymes with specific function of the activation and deactivation of the neurotransmitters directly involved in the process of dementia onset.

In AD case, the strategies most used in AD treating consist in blocking the hydrolysis of acetylcholine (ACh) in synapse of cholinergic neurons. This strategy is called cholinergic hypothesis and nowadays is one of the main targets in the development of drugs able of promote chemical equilibrium concentration of acetylcholine in patients with AD.¹⁴⁻¹⁶ The hydrolysis of acetylcholine is prevented by blocking and inhibition of Acetylcholinesterase (AChE).

The research and development of drugs that act more effectively in the treatment of AD as well as the study of disease mechanisms are subject to several fields of knowledge as medicine, chemistry, biochemistry, molecular and cell biology, neuroscience, psychology and others. Brain is a system poorly studied due to its very complex structural nature. The complex system of the brain is the focus of biochemical and molecular biological studies of the XXI century.

Theoretical and computational chemistry plays an important role in the studies of chemical structure and reactivity of biological systems. This field of chemistry science is now maturing into a transdisciplinary research field with new approaches for understanding the integrated behaviors of systems composed of many interacting parts in a wide variety of application domains. In particular, applications of “realistic” quantum chemical systems have become possible and as such it is starting to become common practice in fields of, e.g., bioinorganic chemistry and biochemistry, to do experimental studies side-by-side with computational modeling.

Since 1960, the molecular docking simulations represent a huge development in several fields of sciences as physics, chemistry, biochemistry and computers. Docking of small molecules and its interactions with macromolecules is an essential technique in early stage of drug screening and protein-protein interactions.¹⁷ Combined quantum mechanics molecular mechanics (QM/MM) approach is the most powerful method to study such kind of system.¹⁸⁻²⁰ QM/MM schemes are among the best compromises between accuracy and tractability.^{21,22} A number of recent studies including empirical valence bond²³⁻²⁵ or QM/MM methods²⁶ have demonstrated the capability of quantitative approaches to predict the effect of mutations on enzyme catalysis. The use of quantum mechanics/molecular mechanics (QM/MM) methods in biomolecular reactions was reviewed recently by our group²⁷ In this regard, the Nobel Prize in Chemistry 2013 has been awarded to Karplus, Levitt, and Warshel “for the development of multiscale models for complex chemical systems”. The methods developed by the laureates couple classical and quantum mechanical theory and were the foundation in the use of hybrid methods such QM/MM methods.

1.2 The Central Nervous System

The central nervous system (CNS) is the highest anatomical complexity organ of human body. It is defined as a set of components of the nervous system contained in boxes bone (skull and spinal column). It is divided into the brain and spinal cord.¹¹

Figure 2. Graphical representation of CNS.

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The cerebral cortex (Figure 2) developed a huge quantity of complex functions. The sensory information (perceptions) is interpreted in the cerebral cortex, and in this region of the CNS occurs the planning, i.e., the commands programming to be sent to the spinal cord. The memory of emotions, and the physical actions, and the psychological reaction are also located in the cerebral cortex region. As the cerebral cortex, the cerebellum develops complex functions such as memory coordination procedures, sensory, emotional and cognitive functions.¹¹

The nervous system is a system of overall performance, works under high cooperation and synchronization between its different cellular elements. The cells of CNS are the neurons and the glial cells.²⁸ These two kind of cells are capable to generate signals leading locally and over long distances, after all transmitting this signals simultaneously to thousands of cells.

The cell nuclei of neurons differ according to the role they will play in the CNS. There are different of morphotypes of neurons, each type of neuron is correlated according with the enzymatic expression involved in the exerted neuronal function. The neurons that use acetylcholine as neuromediator are called cholinergic neurons, and are responsible for controlling the processes mediated by cholinergic receptors.¹¹

Table 1. Main brain's neurotransmitter and neuromodulators.¹¹

Neurotransmitter			Neuromodulator
<u>Amino acids</u>	<u>Amines</u>	<u>Purines</u>	<u>Peptides</u>
Glutamate	Acetilcholine (ACh)	Adenosine	Gastrin
Glycine	Epinephrine (EPI)	Adenosine	Cholecystokinin
Aspartate	Dopamine (DOP)	Triphosphate (ATP)	Vasopressin
γ -aminobutyric acid	Norepinephrine (NOR)		Oxitocin
	Serotonin (5-HT)		Insuline
	Histamine		β -endorphine
			Secretin
			Glucagon
			P substance
			K substance

The communication between neurons is led by neurotransmitters, which can carry information of electrical and chemical nature in order to make possible the process of communication between cells of the CNS. The contact structure formed by the extension of a presynaptic neuron and the postsynaptic neurons is called synapse. The mainly function of synapse is chemical transmission of information from the presynaptic neuron to the postsynaptic cell.

1.2.1 The synapse

The synapse is the focus of neural information processing, is also the site of action of many substances on the CNS. Synaptic transmission occurs when an action potential arrives at the presynaptic terminal and makes it depolarized.^{11,28} Figure 3 shows the steps of a general synaptic transmission:

Figure 3. Steps of synaptic transmission.

- 1: Synthesis and storage of the neuromediator in the synaptic vesicles (or neurotransmitter vesicles);
- 2: arrival of the action potential at the presynaptic terminal;
- 3: Ca^{2+} ion influx;

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4 - 6: exocytosis of neuromediator;

7: recognition and binding of the neuromediator to the receptor in the postsynaptic membrane,

8 - 9: generation of postsynaptic potential,

10: formation of new vesicles at the terminal membrane.

A vesicle stores the minimum quantity of neurotransmitter which can be released in a synaptic transmission. Vesicles are released in *quanta*. The amount to be released is determined by the duration of the depolarization in the membrane from the arrival of action potentials sequence. Thus, there is a direct relationship between the frequency of action potentials in the terminal and the amount of neuromediator released.

Once released the neurotransmitter it is established chemical concentration gradient, in the synaptic cleft, between the vicinity of the presynaptic membrane and the vicinity of the postsynaptic membrane.^{11,12,29} The transmission of synaptic signals fails when the delicate chemical equilibrium of the synaptic region is changed. Often, these defects can cause transient or permanent problems in the neurological and psychological status.

Many neuropathologies are associated with the decrease in the amount of neurotransmitter in the synapse, among them Alzheimer's disease where the drastic reduction of the neurotransmitter acetylcholine at cholinergic synapses causes cognitive impairment leading to dementia.

Hence, the study of the mechanisms that trigger this imbalance as well as the development of effective drugs that promote the concentration gradient of neurotransmitters at synapses are fundamental actions to mitigate and even, why not, heal the neuropathology on medium and long term.

1.3 The Alzheimer Disease

Alzheimer's disease was described at first time in the literature by the german psychiatrist Alois Alzheimer in 1906. After accomplished the autopsy of hundreds brains, Alzheimer noted that large part of his samples had decreased brain mass, as shown in Figure 4.³⁰ Those brains also possessed dark clusters and neurofibrillary tangles.³¹⁻³³

Figure 4. Post mortem brain anatomical structure in comparison: regular and AD brain.³⁴

AD was known erroneously as "sclerosis", sometimes it is defined as "evil of the century" or "silent epidemic". Despite the high social impact generated it is a neglected disease.³⁵ AD causes great changes in daily life of patients and their families. It is a disease of great social concern, because it is costly to society, economy and especially for the family nuclei.^{31,32} There is no cure to AD, and its incidence increases with increasing age of the population. AD is a degenerative and progressive disease.^{14,31,36-38}

There are several factors considered as risk for the onset of AD. Among them the most pronounced are:^{2,6,39}

- Age: is the most important risk factor for AD. People over 60 have at least 30% of chance to develop the disease. The statistics showing the range of risk for AD development is between 60 and 90 years.

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- Gender: the women are at higher risk of developing dementia, in particular the AD; this can be explained by estrogen deficiency in the last third part of life.
- Ethnicity and nationality: afro-american population above 65 years old has a higher frequency of the incidence of AD.
- Geography: the largest number of people with AD is observed in the northern hemisphere.
- Genetics and heredity: for 50-60% of cases that developed AD after 65 years there is a relative of 1st degree who also developed the disease before. As you get older the risk increases in the order of 5% for 70 years age, 10% at 80 years and 33% at 90 years of age. This was observed when there are cases of AD in relatives of 1st degree.
- Down syndrome: 95% of Down syndrome patients between 35 and 40 years old develop brain lesions characteristic of AD. It is suspected that a history of Down syndrome in the family can be a risk factor to develop AD.
- Intelligence Quotient (IQ) and language ability: The mental ability developed in childhood seems to modify the tendency to take dementia in senile age. Statistics show how much higher is the intellectual level developed in life, less likely to suffer AD in older age. Some suspicions indicate is during the childhood that AD begins to develop in the brain. Silently through years, forming senile plaques, modifying synaptic density and damaging neural plasticity until the external symptoms signalize the disease.
- Other factors: The AD may also be conditioned by environmental factors, number of social ties, unregulated power supply, rural life, premorbid personality, stress, depression, traumatic brain injury.

The main symptoms of this disease are gradual loss of memory and critical thinking; time and spatial disorientation; sudden changes in personality for no apparent reason; difficulty in learning and memorizing. The sharp drop in performing daily activities and the cognitive impairment symptoms are also observed in AD.^{32,40}

AD evolution is the cognitive loss of 5 to 15% per year, with an average period of eight years from its inception to the last stage. In the last stage of AD, the total

collapses of body functions occur, and consequently the death of the patient. It is usually divided into three phases: the initial, moderate and severe phases.^{34,35}

The first manifestations of Alzheimer's disease often go unnoticed, marked initially by memory lapses that will gradually worsen. There are reported cases in patients aged 40 years old diagnosed with the disease, but the AD effect markedly occurs after 60 years of age.³³

Several tests and a detailed medical history are necessary to diagnose AD since it is not possible to diagnose the illness with simple clinical analysis or psychological-cognitive checkup, because there are no tests to indicate AD separately. For now, only the post-mortem examination can confirm AD with 100% certainty.⁴¹

The main implication of the etiology of AD is the dramatic loss of brain cholinergic function through significant reduction of acetylcholine neurotransmitter and the choline acetyltransferase enzyme (which catalyzes the synthesis of acetylcholine in the cholinergic neurons) in neocortex and hippocampus.^{10,11} This is the most important psycho-biochemical abnormality on AD diagnosed brain, and it is an indicative of death of neurons in the basal nucleus of Meynert and surrounding areas.^{42,43}

1.3.1 AD causes

The AD causes are still unknown. Several hypotheses are launched to determine the disease's mechanisms. The histological and biochemical particularities that are intrinsically related with AD are listed below:

- senile neuritic plaques, composed mainly by β -amyloid protein A/4 ($A\beta$) which are accumulated in the neuron extracellular matrix of the cerebral cortex. These neuritic plaques are derived from the amyloid precursor protein (APP), its gene is located on chromosome 21. The accumulation of these plaques is considered neurotoxic;^{14,30,31,35,38,41,44,45}

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- formation of neurofibrillary tangles. These tangles are composed by filaments formed in the hyperphosphorylation of the tau protein within neurons (intracellular) and its branches projections (axons and dendrites) mainly in the cerebral cortex, specifically at pyramidal neurons, temporal lobe, the hippocampus and amygdale;^{14,30,31,35,38,44}
- sharp decrease in the amount of neurotransmitters in the synaptic regions. The acetylcholine (ACh), serotonin, dopamine, noradrenaline, and P substance are the neurotransmitters whose frequently diminish in AD processes.^{14,31,35,38,46,47}
- Presence of the E4 variant in the APOE gene: is present in 60% of registered AD cases.³⁹

In AD patients, acetylcholine concentration is markedly reduced in the synaptic clefts. This occur due to the low production of the neurotransmitter inside of neuronal cells, as a consequence cholinergic deficit is established, thereafter cognitive function is severely impaired.^{48,49}

In order to treat the disease was suggested the cholinergic hypothesis.^{30,46,50,51} Since causes and the complete mechanism that trigger AD is controversial and unclear, in addition to this hypothesis, several other proposals are being suggested as the amyloid hypothesis, the hypothesis of glutamate and the oxidative stress therapy.^{51,52}

In the amyloid hypothesis the deposit of senile plaques produced mainly by beta-amyloid protein can be avoided by using drugs that inhibit its excessive production in synapse.^{33,35,44,45,53} This hypothesis is viewed with certain caution, since tests on rats and mice have revealing is not always true that animals who develop amyloid plaques will develop AD.^{33,54,55}

The oxidative stress therapy proposes that metal (Fe, Cu, and Zn) ions may crucially participate in the pathological process of AD. These metal favor protein misfolding and aggregation (formation of A β plaques and neurofibrillar tangles). Beyond that, the metal catalyzes the production of reactive oxygen species (ROS) in

cortex brain, and induce oxidative damage associated with neuronal death. The level of metal ions in brains with AD increase 3-5 folds.^{44, 56}

In the glutamate hypothesis of AD, is assumed the high concentration of this neurotransmitter promotes the abnormal (excessive) entry of Ca^{2+} ions into the neuron cell, leading it to death. Nowadays drugs such as memantine are used in the treatment of AD patients to prevent the neurotransmitter (glutamate) from acting on glutamate receptors and preventing the uncontrolled flow of calcium ions.^{35,44}

1.3.2 Cholinergic hypothesis

The cholinergic hypothesis is the main target in AD treatment. It has generated a race in the drugs research that can bring benefits and significant improvement in cognitive functions of people with AD. In order to tackle the problem of the cholinergic deficit, several strategies have been launched by different research groups in drugs design that attenuate the deleterious effects of AD. Citing Sugimoto and co-workers:³¹

“Based on this theory, six classes of drugs have been developed to enhance cholinergic deficit in AD patient. These are:

- a) Cholinesterase inhibitors (ChEI), which block the AChE enzyme thereby invigorating cholinergic activity to enhance cognitive function.
- b) Choline precursors, such as phosphatidylcholine, aimed at increasing the bioavailability of choline.
- c) ACh releasers, which should facilitate the release of ACh from presynaptic end terminals.
- d) M1 and M3 receptor agonists, which mimic ACh on postsynaptic end terminal receptors.
- e) M2 and M3 receptor antagonists, generally presynaptic (auto receptors), which regulate ACh release via negative feedback.
- f) Nicotinic agonists or substances having nicotinic like effects, which should enhance ACh release.”

The first class of drugs cited as inhibitors of the enzyme acetylcholinesterase are called AChEIs. Nowadays constitute a major strategy for the treatment of AD patients, known as the cholinergic therapy. This therapy consists in the improvement of nerve impulse transmission in synapse.

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The prevention of ACh hydrolysis by inhibiting the enzyme (AChE) is carried out in the presence of drugs that interact with the enzyme allowing the maintenance of the gradient concentration of the neurotransmitter (acetylcholine) in the synaptic process. The correct use of medicines, aimed to increase the concentration of ACh in the synapse, have effectively delayed the progression of the disease.^{31,35}

There are four AChEI drugs approved by the FDA to AD treatment. In 1997 the first drug approved for AD treatment was the tacrine (THA, trade name Cognex®), nowadays this medicine is withdrawn due the severe side-effect during patient treatment. Nowadays three AChEIs, donepezil (DNP, trade name Aricept®), rivastigmine (RIV, trade name Exelon®), and galantamine (GNT, trade name Razadyne®) are currently in clinical practice in the moderate stage of AD. Another drug, memantine (Ebixa®), an NMDA antagonist is now available to treat AD in severe advanced stage.^{8,10,33,38,43,57,58}

Figure 5. ACh and FDA approved AChEIs 2D structure.

There are several other drugs in clinical trials, some in phase III as huperzine A (HUP), in phase II as ladostigil, and various derivatives of the first mentioned drugs. Besides these, there are several studies and research around new drugs that may act on the cholinergic neurons to ensure the maintenance of the levels of

acetylcholine in the synapse and thus maintain cognitive functions of patients in the best possible level.

The primary mechanism by which dopamine or cholinergic neurons degenerate in Parkinson's disease and Alzheimer's disease, are not well known. Nevertheless, many hypotheses and biochemical evidences have been provided to support a cascade of events that include an accentuated decreasing of the gradient of concentration of the neurotransmitters in synapses, as well the oxidative stress, inflammatory process, apoptosis, and lack of neurotrophic factors. Similar to neuropsychiatric diseases, neurodegenerative diseases are multifactorial situation.⁵⁹

Currently, many experts discuss which is the best way to treat neurodegenerative diseases. There is an agreement for many experts in several areas which such diseases require use of multidrug therapy approach in their treatment. The drugs ladostigil (LAD), rasagiline (RAS), selegiline (SEL) and rivastigmine fulfill the role of drug with multiple neuroprotective activities.^{8,59,60}

1.4 Acetylcholinesterase

The acetylcholinesterase (E.C. 3.1.1.7), is the most important molecular target for therapeutic intervention studies and developing drugs for treating various kind of disorders such as myasthenia gravis, AD and glaucoma. AChE have a highly specific biological function in consequence of this is an ultra-rapid-acting enzyme, operating at nearly diffusion-limited rates.⁶¹⁻⁶⁵

AChE 3D structure is shown in Figure 6. The AChE is an allosteric enzyme from the class of hydrolases, with 537 amino acid residues in its sequence. The enzyme is anchored in the postsynaptic membrane and hydrolyzes ACh in choline and acetate inside its catalytic site.¹⁴ The enzyme has its catalytic triad - Ser200-Glu327-His440 (SEH, in *Torpedo californica* numbering) - situated at the bottom of its active site.^{37,44,62,63,66,67} AChE is an enzyme with high dipole moment (800 - 1800 Debye) oriented along its active site.⁶⁸

Figure 6. 3D structure of monomeric form of *Torpedo californica* TcAChE/ACh complex (pdb: 2ACE⁶⁹ - 2,5 Å)

AChE is classified structurally under two forms: globular and asymmetric. The globular form of AChE is subdivided into monomeric (G1), dimeric (G2) and tetrameric form (G4). The G4 is the most profuse form in the brain, particularly in the cerebral cortex and hippocampus, and G1 is less abundant form in the brain. In the synapses of AD patients, the G4 form decreases sharply, as long as G1 quantity remains unchanged.^{61,70}

The active site of AChE is a deep canyon with approximately 20 Å depth, named gorge.^{44,62} The active site is characterized by the presence of multiple negative charges around the catalytic triad which would explain the attraction of ACh by SEH.^{65,71,72}

AChE is an enzyme with low flexibility despite possesses a large number of amino acids residues (more than 500). However, some important structural changes were observed in the residues of the active site pocket when enzyme is interacting with some inhibitors. The AChEIs bis(7)-tacrine (DMT), rivastigmine and huperzine A and B, induce significant conformational changes in the residues Trp279, Phe330, and in His440, and the residues of aromatic peripheral site (Figure 7).⁶²

An important factor for the biological activity of AChE is the presence of water molecules in the active site. Molecular dynamics studies showed the population of molecules of water in the gorge ranges from 16 to 22 molecules. The quantity of water in the gorge controls the entrance of ligands. The water molecules in the catalytic site lead the bond strength between catalytic residue Ser200 and ligands that enter the cavity.⁷³⁻⁷⁶

In addition of the cholinergic function, AChE also has a non-cholinergic function. The peripheral site gorge induces a conformational change in the structure of the amyloid precursor protein (APP) causing a breakdown of this protein and the appearance of amyloid fibers. The generated β -amyloid peptide is aggregate outside of neurons cells of β -amyloid during the initial phase of the AD.^{77,78}

Thus, drugs that inhibit the action of AChE not only in the region of SEH but also in the peripheral region of the gorge have great importance in the treatment of the disease; not only to promote the maintenance of cholinergic functions as well as to avoid the appearance of senile plaques that produce neuronal apoptosis. New studies have shown that AChE plays an important role in the formation of β -amyloid senile plaques.⁴¹

1.4.1 The active site Gorge

The gorge, represented in Figure 7, has four sub-sites. The first sub-site is the peripheral site located at the entrance of the gorge. The second one is the binding quaternary ammonium site located in the central region of the gorge. These two sub-sites consist of several aromatic residues (14 at total) and hence the whole area covered is termed peripheral aromatic site (PAS) of AChE.^{44,62,79} Others AChE sub-sites are the peripheral anionic site and the catalytic site located at the bottom of the acylation pocket where the hydrolysis of ACh occurs.

Figure 7. Gorge, the active site of AChE.

1.4.2 The acetylcholine hydrolysis mechanism

The ACh is the most abundant neurotransmitter in the brain and the main responsible for the propagation of nerve impulses in the synaptic clefts of cholinergic neurons. Its precursors choline and acetyl coenzyme A (acetyl-CoA) reacts producing acetylcholine within the nerve cells under the action of the acetylase enzyme, and the produced neurotransmitter is stored in the synaptic vesicles.^{12,61,80}

ACh is a naturally quaternary ammonium cation ester and is hydrolyzed in choline and acetic acid by action of AChE enzyme at the post-synaptic side.^{29,48}

Several mechanisms are proposed to determine the action of AChE on ACh. In general, the mechanism is divided into two stages: the acylation (Figure 8) and deacetylation (Figure 9) of AChE.

Figure 8. AChE mechanism of acylation of ACh.

Some AChE inhibitors such as organophosphorous derivatives act by competing with ACh. The interaction between AChE and organophosphorous compounds results in a phosphorylated enzyme through an effective covalent bond, and the irreversible inhibition of AChE. Others inhibitors as rivastigmine inhibit the acylation of the OH group of Ser200 residue forming a carbamoyl ester more stable than acetyl group of ACh and less apt to leave the active site, forming a pseudo-irreversible inhibition of the enzyme.^{61,81}

Figure 9. The AChE mechanism of deacylation of ACh.

1.5 The acetylcholinesterase inhibitors in Alzheimer's diseases (AChEIs)

The strategies most used in AD treating consist in blocking the hydrolysis of ACh in synapse of cholinergic neurons. This strategy is called cholinergic hypothesis and nowadays is one of the main targets in the development of drugs able of promote chemical equilibrium concentration of ACh in patients with AD.¹⁴⁻¹⁶ The hydrolysis of ACh is prevented by blocking and inhibition of AChE.

Among AChEIs used in AD treatment stand THA,^{31,82} the first drug approved by the U.S. Food and Drug Administration (FDA) to treat of disease, DNP^{31,38,82} and GNT.³⁸ These drugs are indicated for treating mild and moderate stages, when the patient still has independent cognitive activity.

Other drugs have been clinically studied and tested for use in the treatment of AD, as physostigmine (PHY).^{38,82,83} Some drugs are promising candidates and are in the testing phase and approval, among them, HUP,^{64,81} metrifonate (MTR)³⁸ and phenserine (PHS).^{38,82} Recently, another class of molecules that are dual AChE ligands was studied. These drugs named huprines⁸⁰ in addition to the AChE inhibition also have a neuroprotective function, thus act more efficiently in the treatment of AD.^{37,78,80,84-86}

Figure 10. 2D structure of some AChEIs molecules.

Although AChEIs present common inhibition properties, the nature of their chemical structure and interaction are different, as shown in Figure 10. Generally, the AChEIs are divided into two categories depending on the type of interaction they form with the gorge.

The first group of molecules, displayed at the top of Figure 10, represents species that bind to the active site and inhibit its function using only non-covalent interactions. Bis(7)-tacrine (DMT), donepezil (DNP), huperzine A (HUP), galantamine (GNT), and tacrine (THA) all belong to this group. The second type of inhibitors, such as RIV, dichlorvos (DCV), and PHS (shown at the bottom of Figure 10), always form covalent bonds with the Ser200 residue of AChE.

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The rational design of AChEIs are given at two basic standards:^{15,78,87}

- i. drugs with molecular recognition in the catalytic site of AChE, preferably interacting at covalent bonds with hydroxyl of Ser200 serine residue. This bond may be reversible or pseudo irreversible;
- ii. drugs that are recognized by the catalytic and/or peripheral anionic site of the enzyme, to prevent the entry of the natural substrate in gorge.

1.5.1 Acridines

The 1,2,3,4-Tetrahydro-9-acridinamine, THA was the first drug approved by the FDA for the treatment of patients with AD.^{66,88} It is a derivative of acridine synthesized in 1945, applied first as an antimicrobial agent and monoamine oxidase inhibitor.^{89,90}

THA is a reversible inhibitor, non-competitive and non-selective for AChE. In patients with good tolerance to it, this drug increases moderately the cognitive function but, has high hepatotoxicity and high incidence of side effects⁶⁶ Although tacrine is not first drug of choice, it is taken as reference in search of more potent AChEIs.⁹⁰ THA exhibits a dual inhibitory activity: inhibits AChE enzyme and free radical scavenging activity.⁹¹

THA analogues are widely investigated and tested for inhibition of AChE. One of AChEIs derived from THA most studied is the Bis(n)-tacrine derivative, as shown in Figure 11, formed from two units of THA connected by a carbon skeleton with (CH₂)_n units.⁴⁴

Figure 11. 2D structure of Bis(n)-tacrine.

The compounds formed when $n=5$ and 7 , the Bis(5)-tacrine (F11) and DMT show greater selectivity and higher activity in the inhibition of AChE.⁹² DMT is a dual inhibitor, it inhibits AChE and MNDA activity in synapse.^{44,57,91}

There are several compounds from acridines derivatives proposed as AChEI candidate. The photosensitizer methylene blue (MTB)⁹³ a strong reversible AChEI, and the edrophonium (EDR) are two of them. Acridines derivatives from THA and DMT are largely studied in the pursuit of best AChEI to treat AD.⁴⁴

1.5.2 Alkaloids

Galantamine (GNT) was the fourth AChEI approved by the FDA in treatment of AD. It is an alkaloid present in several plants of the Amaryllidaceae family, particularly in the snow drop flowers.^{47,61} It is a selective, competitive and reversible acetylcholinesterase inhibitor and an allosteric modulator of nicotinic receptors in the brain and muscle.⁹⁴⁻⁹⁶ This alkaloid was approved in 2001 for the treatment of AD, especially for promoting significant improvements in cognitive state and inhibit the formation of tangles of beta-amyloid protein.^{45,53,61}

The GNT is an AChEI largely studied, due its dual biological activity. Several research groups developed year by year heterodimeric derivatives based in this AChEI, aiming the association of tolerability of GNT and potency of dual-target inhibitors in AD treatment.⁹⁷ The GNT derivatives with indole moiety,⁹⁸ bivalent derivative⁹⁹ as (GL8), and bis-functional derivatives⁴⁷ as the N-Piperidinopropyl-galantamine (G3X) are some of new alkaloids proposed as potent AChE inhibitors.

Huperzine A (HUP) is a naturally alkaloid found in the *Huperzia serrata* herbs and a potent reversible inhibitor of AChE. This alkaloid is a potential drug candidate for AD treatment, considering its prolonged action on the CNS, low toxicity, and protective action on neurons. HUP is efficient against glutamate excess that can lead to death of nerve cells.^{15,88} At normal concentrations, glutamate is a neurotransmitter necessary for brain functions such as learning and memory. However, at high

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concentrations the increased cellular activity caused by glutamate results in over-excitation of nerve cells, which eventually leads to cell death.¹⁰⁰ HUP demonstrate excellent performance in penetrate blood-brain barrier while comparing with others AChEIs profile. This inhibitor also has higher oral bioavailability and longer AChE inhibition in comparison with others alkaloids molecules.⁶¹

With synthetic route not yet defined to be produced on a large scale, the research to develop HUP derivative analogues has been intensified. Some of its analogs, the huprines proved to be 200 times more potent than HUP in the AChE inhibition.¹⁰¹

1.5.3 Carbamates

The molecules of this class are derivate compounds of carbamic acid (NH_2COOH). These compounds are used in different diseases as AD, myasthenia gravis, glaucoma, Parkinson's disease. Some carbamates as physostigmine (PHY) and rivastigmine (RIV) are reversible AChEIs.⁶¹

Physostigmine was the first drug clinically studied to be applied in the treatment of AD.⁴⁶ It extracted from beans, a legume native to West Africa known as bean-to-Calabar (*Physostigma venenosum*). PHY is a natural alkaloid belonging to the group of carbamates molecules.^{57,66}

The PHY is a non selective and pseudo-irreversible drug that despite having provided a good improved in cognitive functions, due to the low efficiency in the long-term clinical phases II and III was rejected by the FDA.^{31,61,66} This carbamate is a molecular reference object in the development of new analogues for the treatment of AD, even that is no longer used as a therapeutic drug.

In mid-1998 was approved in Europe rivastigmine (RIV), a drug derived from physostigmine by molecular modifications. RIV is a selective and pseudo-irreversible carbamate⁷⁰ but exhibits superior pharmacological profile than its precursor, with good selectivity and bioactivity in vivo. It is well tolerated by

patients, it presents neuroprotective properties and improves in cognitive function of AD patients.¹⁰²

RIV inhibits six times the G1 than the G4 form of AChE. This carbamate is therefore the primary drug indicated for the treatment of AD in mild and moderate stages.^{61,70} It was approved by the FDA in 2000. It is a potent neural protector while preserves the viability and neuronal structure in brain cortex.¹⁰³ RIV is also a dual inhibitor, its inhibits AChE and butyrylcholinesterase (BuChE) enzymes, presenting beneficial effects on AD treatment as well as its cognitive, functional and behavioral domains.⁹¹

The phenserine (PHS) is a phenyl carbamate analog of physostigmine developed for AD treatment. It is a highly selective and pseudo-irreversible AChEI.¹⁴ Nowadays it is in II clinical trials phase, PHS is a new promise considering its relative wide therapeutic window compared to other carbamate used for AD treatment.³⁷

1.5.4 Huprines

In the beginning of 2000s Camps and coworkers¹⁰¹ designed a new class of AChEIs provided from combination of two well-known inhibitors, the THA and HUP. By combining the carbobicyclic substructure of HUP with 4-aminoquinoline group of THA, the hybrid molecule called huprine was designed. This new class of AChEI provided the creation of various derivatives by the substitution of the position R₉ and the position R₃, see Figure 10.^{44,101}

Some huprines as HUX (chlorine at R₃ and ethyl at R₉) presented more affinity by AChE than others earlier AChEIs reported till 2000s. The huprines interacts with AChE as THA and HUP, in addition present some others important interactions with conservative residues of gorge as Asp72 and Glu199.^{44,101}

Following the tendency of other classes of AChEIs, a huge amount of multi-target heterodimeric derivatives of huprines were developed. The huprines HUX, and HUY (chlorine at R₃ and methyl at R₉) were connected by alkyl linkers to THA,

GNT, DNP, HUP and several other moieties resulting in a huge set of potent AChEIs. The new compounds presented multi-inhibitory properties as BACE-1, BuChE and AChE, inhibitors, and anti β -amyloid aggregation activity.^{44,104,105}

1.5.5 N-benzylpiperidines

In 1996 donepezil (DNP) the second AChEI for AD treatment was approved. This drug was developed using studies from molecular recognition QSAR.^{88,106} The DNP ((RS)-2-[(1-benzyl-4-piperidyl)methyl]-5,6-dimethoxy-2,3-dihydroinden-1-one) is a N-benzylpiperidine drug. DNP is highly selective AChEI, and a reversible and non-competitive drug.^{31,106,107} It was developed to overcome the disadvantages of the side effects produced by the THA.

The results when donepezil is taken in the treatment of mild and moderate AD shown significant improvements in compared treatment with THA.^{85,108} This AChEI is a dual site inhibitor being able to interact with CAS and PAS sites of AChE through hydrophobic interactions and hydrogen bonds with gorge residues.⁴⁴ DNP in addition its potent AChE inhibition properties, also shown good inhibitory power of the β -secretase, BACE-1, thus offering a neuroprotective effect to brain cells. AD patients treated with DNP shown ameliorated delay in cognitive and functional decline during the treatment.⁷⁷ The 7-ethyl-10-[4-(1-piperidino)-1-piperidino-] carbonyloxy-camptothecin (CP0) is an anticancer prodrug approved for the treatment of colon cancer, with known AChE inhibitory action. CP0 has a dipiperidine moiety, making similar interactions as the AChE-DNP complex.¹⁰⁹

Some others N-benzylpiperidines derivatives in which indanone moiety of DNP is replaced by different other groups was also proposed as AChEI. The bioisosteric N-benzyl piperidine benzisoxazoles presented higher selectivity and potency than DNP.⁴⁶

1.5.6 Organophosphates

The organophosphorus compounds (OPs) are esters or thiols derived from phosphoric, phosphonic and phosphinic acid. This class of compound has been used as chemical weapon agent, pesticides, plasticizers, pharmaceuticals and parasiticide in veterinary medicine.^{61,110} OPs are extreme toxic molecules acting directly in CNS and muscles cells of mammals and some other animals. The acute toxic effect of OPs is related to irreversible inactivation of AChE. This occurs through the phosphorylation of the enzyme to form a stable OP-AChE adduct by the covalent bond formed between phosphonyl group of OP and the –OH group of Ser200 residue.¹¹⁰ Some OPs were studied from the 1930s as potent irreversible inhibitors of AChE in very low concentrations.^{61,110-112}

Sarin (SAR) gas was the first OP molecule studied as AChE inhibitor.⁸¹ SAR is an organophosphorous compound also known as G-series ‘B’ nerve agent (GB), and a potent AChE irreversible inhibitor used as war chemical weapon. Nowadays SAR is still in use against civil citizens and innocents for some countries. This AChEI is a very potent nervous agent and acts on AChE producing a strong phosphonylated complex via effective covalent bond with the residue Ser200 of gorge.¹¹³⁻¹¹⁵

In same way of SAR, there is other organophosphorous compound used as chemical weapon, the soman gas (SOM), also known G-series ‘D’ nerve agent (GD). This AChEI is an extremely toxic nerve agent. The 2-(3,3-dimethylbuty) methylphosphonofluoridate, SOM is a non selective and irreversible cholinesterase inhibitor molecule, and inhibits potently both AChE and BuChE enzymes. SOM also forms an effective phosphonylated complex.¹¹⁵⁻¹¹⁷

Metrifonate (MTR) is a prodrug, and unique AChEI of OP studied as potential drug for the treatment of patients with AD. When metabolized, MTR produces the active metabolite 2,2-dichlorovinyl dimethylphosphate (DCV) that acts as selective and pseudo irreversible inhibitor on AChE with very slow rate of dephosphonylation.^{81,110} MTR was rejected on its FDA-approval because presented

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severe side effects in muscles and respiratory system for some patients in phase III of clinical trials.⁴⁶

The AChE-OP adducts formed by SAR, SOM, DCV, and other OPs have a very slow dephosphorylation reaction. In case of DVC it takes some days for completely dephosphorylation of AChE, but in other OPs as SAR the inhibition is considered irreversible. While acylated AChE adduct show very fast deacylation regenerating the enzyme in its free form, in other hand the dephosphorylation is very slow (or definitive) and the phosphorylated enzyme is unable to hydrolyze ACh.⁶¹ The irreversible inhibition of the AChE by OPs promotes accumulation of the neurotransmitter in the synaptic cleft. This accumulation generates the overstimulation of the nicotinic acetylcholine receptors in the central nervous system, causing the blocking the neurotransmission process. The dramatic side-effect of this overstimulation is known to be lethal.

1.5.7 Monoamino Oxidase Inhibitors

Mono amine oxidase (MAO, EC 1.4.3.4, amino-oxygen reductase) is a flavoenzyme localized at the inner of the outer mitochondrial membrane which is tightly bound through its C-terminal sequence. The biochemical function of MAO is the oxidative deamination of monoamine neurotransmitters as serotonin (5-HT), noradrenalin or norepinephrine (NOR), and dopamine (DOP).^{8,10} The enzyme presents two distinct isoforms, MAO-A and MAO-B. The isoform MAO-A preferentially deaminates 5-HT, epinephrine (EPI), and NOR, and selective inhibitors clorgyline. The MAO-B isoform metabolizes the neurotransmitters DOP and benzylamine (PHE), as well the inhibitors as rasagiline (RAS) and selegiline (SEL).

AChE, MAO-A and MAO-B are target enzymes in studies of different diseases as AD, Parkinson's disease, depression and glaucoma. A polypharmacological approach is desirable and aiming to treat and modify activities of these targets

simultaneously, particularly those associated with the progression of the diseases.⁷ In this way the development of multifunctional molecules is a tendency and crucial to treat different diseases that share some particularities as neurodegenerative disease.

A dual-action drug is a compound that combines two different desired pharmacological actions at a similar efficacious dose (or molar scale). Dual inhibitors possess two different biological activities, which may be due to inhibition of two different enzymes, neurotransmitters or biochemical target. Some examples of dual inhibitors are desipramine, dozepin, asenapine, amitriptyline, PHS, RIV, THA, HUP and ladostigil (LAD).⁹¹

The MAOIs began to be used as antidepressants in the mid-1950s. The first generation of MAOIs (Figure 12) is compound by drugs with non-selective and irreversible MAO-A and MAO-B inhibition. Drugs as tranylcypromine (TRA), isocarboxazid (ISO), iproniazide (IPR), and phenelzine (PHZ) were widely used in depression treatment. Currently its use is restricted only to cases where other drugs are not efficient, due the severe side effects caused during the depression treatment.¹⁰

To ameliorate the unwanted effects of first generation of MAOIs, a second generation of drugs has been developed for depression and Parkinson's disease treatment. These new inhibitors present selectivity for MAO isoforms inhibition. The well-known second generations of MAOIs are Nialamide (NIA), selective for MAO-A, and selegiline (SEL), and rasagiline (RAS) inhibitors of MAO-B.¹¹⁸⁻¹²¹

In a search for the reduction of side effects caused by first and second generations of MAOI in depression treatment, new reversible and selective MAO-A inhibitors (IRMAs) were developed. Drugs as moclobemide (MOC) and toloxatone (TOL), see Figure 12, are prescribed as antidepressants that inhibit only the MAO-A isoform. This allows the MAO-B isoform to perform the deamination of monoamines neurotransmitters and other toxic amines.

Recently it was identified the increasing in the level of MAO-B isoform in brain of elderly people.^{42,119-121} The increasing level of MAO-B in brain may

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contribute to onset of neurodegenerative diseases as AD and Parkinson's disease, through the formation of free radicals via MAO-B action.

Figure 12. 2D structure of first, second and third generations of MAOIs.

MAOIs play beneficial neuroprotective action in neurodegenerative diseases by reducing oxidative stress conditions, preventing neuronal damage and decreasing apoptosis in neural cells caused by MAO isoforms actions in synaptic clefts.¹⁰ Some drugs as SEL and RAS benefits AD patients.^{3,60,86} Therefore SEL, RAS and its derivatives were also studied as a potential AChEI for AD treatment and as multi-target drugs. The propargyl moiety in SEL and RAS may have intrinsic neuroprotective activity not related to its ability to bind covalently to MAO-B and inhibit it.⁵⁹

Association between AChEIs and MAOIs molecules has been studied aiming search multi-target drugs for neurodegenerative diseases. A more efficient molecule able to inhibit in the same concentration AChE and MAO isoforms is a special aim in biochemistry and computational chemistry field. A new class of multi-functional drugs - the AChEI-MAOI drugs is emerging.⁴⁴

Youdim et al. studied a large series of dual MAO-AChE inhibitors using the propargyl moiety (present in SEL and RAS) to composed the molecules. Their study was based in the molecular design of molecules combining at same molecular shape the propargylaminic fragment characterizing some well-known MAOIs as SEL and RAS, with carbamate moiety, which could add the AChE inhibitory property in the new molecule.^{10,59}

The Ladostigil (LAD) was designed as hybrid dual inhibitor AChE-MAO synthesized by linking directly the carbamate moiety from RIV (AChEI) at RAS (MAOI) molecule.^{8,59} Ladostigil, Figure 13, showing anti-depressant activity while inhibiting both MAO isoforms, and the ability to increase EPI and 5-HT levels on respective synaptic clefts. In addition LAD possesses multiple therapeutic activities as reduces apoptosis and stimulates the processing of APP protein.⁹¹ LAD nowadays is in II phase of clinic test for AD treatment.

Figure 13. 2D structure of LAD, a potent AChEI-MAOI molecule.

The high complexity of AD calls for huge effort to develop molecules able to modulate multiple CNS targets to promote a real improvement of AD therapy. The multi-factorial nature of the disease rules out the use of consolidated mono-therapy and put researches on the way of multipotent drugs hitting cooperatively different targets underlying the onset and/or progression of the disease.^{44,46,77} This approach is grounded on the idea that an AChEI could exhibit a more efficient therapeutic effect

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in AD when it is able to express an additional MAOI activity, and/or inhibiting other enzymes implicated in neurodegenerative diseases as BuChE. Other desirable property of an ideal AChEI is the ability to brake the production of the free radicals (the reactive oxidative species) provided from the oxidative stress in brain cells.¹⁰ Drugs which are selective against isoforms of particular biological target but nonselective against two or more targets are very useful in multifactorial diseases as AD and Parkinson's disease.¹²²

1.5.8 Other molecules

Several herbs extracts are studied as potential agents in the AD treatment. These herbs are widely known for the eastern population, and have been used for centuries to treat some dementias and evils that attack the CNS.

Herbs such as *Huperzia serrata*, *Panax ginseng*, *Ginkgo biloba*, *Salvia officinalis*, *Camellia sinensis*, *Pueraria lobata*, *Curcuma longa* L., *Rhodiola rosea* Linn, *Mimosa bahamensis* Benth, and many others are now the subject of studies in the research for natural compounds that act as neuroprotective molecules and AChE inhibitors.^{51,123-125}

The present work is a theoretical study of the process of inhibition of AChE by molecules that inhibit the enzyme blocking its activity by electrostatic, van der Waals, and π -stacking interactions, and/or forming covalent bonds with its residue Ser200. To a deeper understanding on the mechanism of AChE interacting with ligands that block the active site of the enzyme, a combination of computational methods such as QM/MM MD, QM/MM FEP, and QM/MM BIE has been employed. Thus, QM/MM calculations were performed to determine the binding free energy between the acetylcholinesterase and some inhibitors. In addition, to clarify how different molecules, with a wide variety of chemical structures, can act as AChE inhibitor, we employ a joint molecular docking *in silico* simulations and multivariate analysis study, based on the principal component analysis method and genetic

algorithm aggregation theory, to obtain the structural, electronic and topological properties that are capable to promote molecules in top as good inhibitors.

Outline, Scope, and Aims

*“Sé impecable con tus palabras...
No te tomes nada como personal...
No hagas suposiciones...
Haz lo máximo que puedas.” (Don Miguel Ruiz)*

2. OUTLINE, SCOPE, AND AIMS

In this Doctoral Thesis, the investigation of the role of the inhibitors of AChE that block the active site gorge, in the mechanism of inhibition of the enzyme, is presented. The chapter 2 presents the main aims of this Thesis. In the chapter 3 outlines the theoretical background, computational approaches and methods used in this Thesis. Chapter 4 establishes a theoretical framework for the systematic study of the mechanism of inhibition of AChE by some classical inhibitors used in AD treatment. Chapter 5 elucidates inhibition aspects of AChE by different molecules of a same family of AChEIs. Both 4 and 5 chapters aim to elucidate through QM/MM simulations the process of AChE inhibition for any molecule that block the gorge. In Chapter 6, the interaction between different classes of molecules with AChE is analyzed in order to formulate a consistent background to study new AChEIs. In Chapter 7, we sketch an interpretative scheme to elucidate the essential pharmacophore groups in drug design, aiming the development of more potent and efficient AD medicines. The last chapter, Chapter 8 summarizes the main conclusions and suggests future outlooks. This thesis is based in 4 papers, which have been published or are in via of submission to international journals and aim at addressing the issues and objective previously mentioned.

i. Chapter 4: Binding Analysis of Some Classical Acetylcholinesterase Inhibitors: Insights for a Rational Design Using Free Energy Perturbation Method Calculations with QM/MM MD Simulations.

Reference: J. Chem. Inf. Model., **2017**, 57 (4), pp 958–976
DOI:10.1021/acs.jcim.7b00037

ii. Chapter 5: Binding Free Energy Calculations to Rationalize the Interactions of Huprines with Acetylcholinesterase.

** Manuscript prepared to be submitted to the Journal of Chemical Information and Modeling.*

iii. Chapter 6: Systematic study of several Acetylcholinesterase Inhibitors through computational means: A molecular docking study.

** Manuscript prepared to be submitted to the European Journal of Medicinal Chemistry.*

iv. Chapter 7: Computational Modeling and Simulations on Acetylcholinesterase Inhibitors: Towards a Relationship among Ligands Properties, Pharmacophoric Profile, and Inhibition Mechanism.

**Manuscript in preparation.*

The main target of this research project is the study of the inhibition mechanism of AChE, focused in AD treatment. For this purpose 45 molecules with different chemical structures, belonging to 8 families were studied. The interactions between the inhibitors and AChE are studied by using a plethora of computational procedures and theoretical methods, i.e, molecular dynamic simulations based on QM/MM methods, molecular docking method, binding isotopic effects, and multivariate analysis methods.

The objectives proposed and addressed throughout this Doctoral Thesis can be summarized as follows:

- obtain the binding free energy of interaction of AChE inhibitors that do not acylate the enzyme, but block its catalytic site by non-covalent interactions through free energy perturbation methods based on QM/MM MD potentials.

- perform calculations of energy of interaction by residue to a set of molecules, thus obtaining the contribution of each residue in inhibition process,

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- calculations of BIEs using the QM/MM methodology, to identify important non-covalent interactions in the inhibition process,
- study of 8 different classes of molecules that inhibit the AChE using the molecular docking technique, and aiming to clarify how the inhibitory process occurs,
- exploratory search of the properties that correlate AChE inhibitors, performing electronic structure calculations, molecular docking, and principal components analysis and clustering algorithms. Thus, to propose an optimized pharmacophoric profile of AChEI able to block with efficiency the AChE.

Theoretical Background and Methods

*"If I have seen further than others,
it is by standing upon the shoulders of giants."
(Sir Isaac Newton)*

3. THEORETICAL BACKGROUND AND METHODS

3.1 Theoretical Background

The study how the enzymes interact with its natural substrate and how they interact with other molecules (e.g. ligands, and inhibitors) is essential to understand enzyme work. The study of proteomics is one of the central axes in the development of new drugs to treat and cure diseases.

Molecular modeling and theoretical chemistry methodologies are largely used to understand main process at atomic and molecular scales within the intracellular environment.^{19,126-128} Simulate reality in the cellular environment is not an easy task. Many approaches are necessary to study how a particular enzyme interacts with a molecule. For this purpose, the three-dimensional structure of the enzyme and molecules to be studied must be known.

Nowadays the best approaches to simulate reality in the intracellular environment are based on the combination of different methods and techniques of the theoretical and computational chemistry. The methods based on the molecular mechanics (MM) and quantum mechanics (QM) (the hybrid QM/MM methods), as well the alchemical free energy perturbation methods (FEP), beside the molecular docking algorithms (MDA), and the binding isotope effects (BIEs) methods can be considered as a set of tools in order to evaluate, together, the energy of binding and the study of the interactions between an enzyme (receptor - R) and a inhibitor or molecule (ligand - L).

In addition, several strategies as the quantitative structure–activity relationship (QSAR) can be employed in order to complement and support this kind of analysis. The QSAR method is an useful strategy for the systematic analysis of macromolecular system. This method combines low computational cost and reasonable accurate approach, by using structural and electronic properties of small molecules (ligands, inhibitors, analogues, etc) in the study of the R-L interactions.

The properties of the ligands can be determined by performing *ab initio* calculations using the density functional theory (DFT). The correlation between different ligands and the enzyme (receptor) can be determined using a combination of techniques as the principal component analysis (PCA) and Fuzzy K-means clustering methods, and then validated within cross-validation method.

It is well known that atoms, molecules and macromolecules are in constant movement into cellular environment or in an experimental procedure. In the simulation and study of enzymatic systems, it is necessary to consider some parameters like temperature and velocity of the molecules that compose this system. Thus, methods of molecular dynamics (MD) are applied, to simulate over time, how the enzymatic system behaves under a certain temperature and as the energy of microstates vary with time, providing information as structural conformations and the energy associated with these conformations. Therefore, the combination of methods QM/MM and MD is a powerful tool for modeling the enzymatic catalytic processes.^{126,127,129,130}

Another important point to be considered is the study of the free energies of interaction between enzymes and their ligands. This is a controversial matter and well studied since there are different approaches to determine the interaction energy in large systems. In general, the statistical thermodynamics principles are used to study and understand accurately the free energies of interaction between enzymes and ligands.^{127,131} Since the evaluation of the free energy of interaction of a receptor-ligand system is a very complex task, several techniques as the Potential of Mean Force (PMF),^{132,133} the Free Energy Perturbation (FEP),^{127,134} Slow Growth (SG)¹³⁵ and Thermodynamic Integration (TI) are used for this purpose. These methods take in account the free energy difference between two states of the system in order to calculate the binding energy of the R-L complex.

Docking techniques are very important for understanding chemical compounds.¹⁷ In systems as protein-ligand, protein-protein, and nanomaterials, docking is an useful computational methodology to predict the spatial orientation of

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small molecules when are forming complexes, adducts, or tight interactions with a receptor, as enzymes.^{136,137} This methodology is based in ‘lock and key’ concept of rational drug design, and offers an option in the determination of the interaction energy while with relatively low computational cost.

Over the last two decades, powerful molecular modeling tools have been developed which are capable of accurately predicting structures, energy, reactivity and other properties of molecules. Within the field of molecular modeling, the rational drug design is the area with high relevance of application. The use of the computational simulations in the prediction of a molecule with pharmacological activity, that consequently will become in a drug candidate for treating a disease, brings the huge expectation of reducing the search time and the cost in the molecular synthesis process during the development of medicines.¹³⁸

The R-L interactions establish the functionality of a given molecule. However, the study of the R-L interactions, by itself, does not provide enough parameters in the development of new ligand. Other important point of view to be considered is the mapping of the pharmacophoric profile of a molecule or a set of ligands which interact within a specific macromolecular target. The pharmacophoric profile is a group of chemical and geometrical parameters of a set of molecules that probably correlate them in a specific function, as the inhibition of a protein.

Basically, a pharmacophore describes the main molecular features regarding the recognition of a ligand by a biological macromolecule. Technically according Wermuth, a pharmacophore is the “largest common denominator shared by a set of active molecules to specific biological target”.¹³⁹ A pharmacophore is defined by the pharmacophoric descriptors which are a set of electronic, structural, electrostatic and topological properties of a system. Those descriptors can be obtained by experimental methods (e.g. X-Ray, crystallography, spectroscopic measurements, etc) and theoretical methods (e.g. *Born–Oppenheimer* approximation, DFT, Møller–Plesset Perturbation methods, etc).

In order to enhance the insights to identify potential new medicinal drugs, computational strategies are widely used as previously described. Mathematics, statistics and quantum molecular modeling applied on bioinformatics are important examples of these strategies. The multivariate analysis methods (e.g. hierarchical cluster analysis – HCA; principal components analysis - PCA), quantum chemistry calculations (Density functional theory –DFT, etc), and molecular docking approach are some of the methods that when combined can led the identification of the main data required to describe the essence of the pharmacophore profile for determinate biochemical problem.

3.2 Theoretical Methods

3.2.1 Hybrid Method QM/MM

Classical mechanics could be used to simulate large molecules but not chemical reactions. Quantum mechanics could give realistic results for reactions but could not be used in large molecules problems, because the number of equations for such kind of system is computationally expensive and too much complex to model and solve it. The blended approach, which uses quantum mechanics only for key parts of molecules in the system (as bond formation and breaking, charge transfer, polarization, etc) and classical mechanics for the rest of the system, provides the accuracy of the quantum approach with manageable solutions from Newton's equations.^{20,128}

The application of this methodology requires the establishment of the definition (divisions) of the system in three distinct but inseparable regions. As shown in Figure 14, the QM is the lowest region of the parts comprising the smallest number of atoms (generally the ligand and the amino acid residues involved in enzyme catalysis), this part is studied by quantum mechanical methods. The MM is the second region, which is the remainder of the enzyme and waters, and is treated

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by force fields of classical molecular mechanics. The third part is the contour, that is a collection of restrictions (boundary conditions) applied to the system due to the fact that it is impossible simulate an infinite system.^{20,140}

Figure 14. Scheme of definition of a QM, MM and contour region.

The calculation of the energies and forces of the system is obtained by resolution of the time-independent Schrödinger's equation (equation 1) under proper approach.

$$\hat{H}_{eff} \Psi(r_i, R_\alpha, R_M) = E(R_\alpha, R_M) \Psi(r_i, R_\alpha, R_M) \quad (1)$$

Where r_i and R_α are the coordinates of the electrons and nuclei in QM region, R_M is coordinates of the atoms in MM part.

For this purpose, a Hamiltonian representing all parts of the system to be calculated (H_{eff}) is used as shown in the equation 2. This Hamiltonian is composed of the sum of four terms: The first term representing the QM region, the second one for the MM, third one to describe the interactions between regions QM/MM and the fourth term is the boundary conditions (contour) of the system.¹²⁸

$$\hat{H}_{eff} = \hat{H}_{QM} + \hat{H}_{MM} + \hat{H}_{QM/MM} + \hat{H}_{boundary} \quad (2)$$

The total energy, represented by the equation 3, is calculated for each position of the nuclei of the QM and the coordinates of MM atoms, using a self-consistent process (SCF, self-consistent Field).

$$E = \frac{\langle \Psi | \hat{H}_{eff} | \Psi \rangle}{\langle \Psi | \Psi \rangle} \quad (3)$$

The potential energy from H_{eff} Hamiltonian can be calculated by the sum of each term described in the equation 2. This energy is calculated as shown in equation 4.¹²⁸

$$E_{total} = \frac{\langle \Psi | H_{QM} + H_{QM/MM} + H_{boundary(QM)} | \Psi \rangle}{\langle \Psi | \Psi \rangle} \quad (4)$$

$$+ \sum_{\alpha M} \left\{ \frac{A_{\alpha} M}{R_{\alpha M}^{12}} - \frac{B_{\alpha} M}{R_{\alpha M}^6} \right\} + E_{MM} + E_{boundary(MM)}$$

Where the first term of equation 4 include the QM and QM/MM Hamiltonian. The second term is the Lennard-Jones potential to take in account the close-range exchange-repulsion terms and the longer-range dispersion contributions that are not explained using a pure Coulombic interaction. Other terms are the energy related with MM and the boundary part of the system.

The QM Hamiltonian represents the electronic part of the system in vacuum. It can be calculated through quantum mechanics with semiempirical methods as AM1,

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PM3, AM1/MNDO and/or *ab initio* methods as Hartree Fock, MP2 or DFT methods.¹²⁸

The QM/MM Hamiltonian corresponds to the Hamiltonian that describes the interactions between atoms treated with QM and with MM potentials. In the cases where there are no covalent bonds between atoms of the QM and MM regions, this Hamiltonian should be composed with the sum of the electrostatic term and the Lennard-Jones potential.

The MM Hamiltonian is determined by the positions of the atoms that constitute this region, and has the energy contributions of bonded and nonbonded terms. The bonded term of MM energy is composed of the bonds, angles and dihedral energy values. The nonbonded term is composed by Coulombic and van der Waals energies.^{19,128} These terms are represented by force fields which are a set of functions developed to be applied in molecular mechanics methods.

There are various forms of empirical potential functions used to calculate MM energy (see equation 5). Force field and most of potential functions are defined by two categories of terms which treat bonding and non-bonding interactions between atoms in a system.¹⁴⁰

$$U_{MM} = U_{\text{bonding}} + U_{\text{nonbonding}}$$

where,

$$U_{\text{bonding}} = U_{\text{bond}} + U_{\text{angle}} + U_{\text{dihedral}} + U_{\text{improper}} \quad (5)$$

and

$$U_{\text{nonbonding}} = U_{\text{Lennard-Jones}} + U_{\text{electrostatic}} + U_{\text{polar}}$$

The bonding terms in equation 5 are also called covalent terms, once define the covalent structure of the molecule. Bond and angle, equation 6 and 7, terms are defined as a harmonic form:

$$U_{bond} = \sum_{bonds} \frac{1}{2} k_r (r - r_0)^2 \quad (6)$$

Where, k_r is the force constant for the bond, r is the bond length between two atoms and r_0 is the equilibrium distance for the bond.

$$U_{angle} = \sum_{angles} \frac{1}{2} k_\theta (\theta - \theta_0)^2 \quad (7)$$

Where, k_θ is the force constant for the angle and θ_0 is the equilibrium value for the angle.

The dihedral term, equation 8, describes the energy changes in a molecule when there is torsion around of one of its bond. This term has a periodic form:

$$U_{dihedral} = \sum_{dihedrals} \frac{1}{2} v_n [1 + \cos(n\phi - \delta)] \quad (8)$$

In the equation 8, v_n is the force constant, n is the periodicity of the angle θ , and δ is the phase of the same angle.

The improper term, equation 9, describes the energy of out-of-plane motions as an improper dihedral angle ω .

$$U_{improper} = \sum_{impropers} \frac{1}{2} v_n [1 + \cos(n\omega - \delta)] \quad (9)$$

These four terms, equations 6-9, are the most important considerations to represent the bonding energy in a force field. There are others terms that can be add

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in a force field equation to obtain better agreement with experimental data, for example the cross-terms.¹⁴⁰

The non-bonding terms describe the energy of the intermolecular interactions in a system. The equation 10 represents the Lennard-Jones potential that mimics the long-range dispersion interactions and the short-range repulsive interactions between atom i and j .

$$U_{LJ} = \sum_{ij} \frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^6} \quad (10)$$

A_{ij} and B_{ij} are positive constants whose values depend upon the kind of the atom i and j .

The electrostatic term tries to describe the energy arising from the electrostatic interaction between atom i and j , more precisely the energy of charge distributions, q_i and q_j . This term has the form:

$$U_{electrostatic} = \frac{1}{4\pi\epsilon_0\epsilon} \sum_{ij} \frac{q_i q_j}{r_{ij}} \quad (11)$$

where ϵ is the dielectric constant and ϵ_0 is the permittivity of vacuum.

The polarization energy can be computed as

$$U_{polar} = - \frac{1}{2} \sum_{i=1}^N \mu_i^T E_i^q \quad (12)$$

Where μ_i is the dipole moment produced by the charge distribution of the environment in the presence of an electric field $\mathbf{E}_i$, and E_i^q is the electric field at each atom produced by the sum of the fields due to the charges

The boundary Hamiltonian represents the interaction between the system and the boundary region. This region is included in the calculations QM/MM in an attempt to describe the system in a solvent solution. Condensed phase systems have an infinite extension, which makes their simulation impracticable. So, to study the reaction in solvated environment, it must consider that only a small portion of the solvent is represented as explicit. For this discontinuity is necessary to employ methods that can mimic the rest of the solvated medium.

Figure 15. Three-dimensional Periodic conditions, representation of interactions between the unit cell (illustrated in white) and the replicated images.

Many approaches can be used to simulate the continuity of condensed phases, among the most common are the application of periodic boundary conditions (PBC) and deformable boundary conditions.^{140,141}

In the case of approaches by PBC methodology, the system is placed inside a box consisting of solvent molecules, which is replicated in all spatial directions generating a unit cell in a crystal lattice, Figure 15. For deformable boundary conditions, a radial potential is applied to certain atoms of the solvent molecules, so such molecules can continue reorienting but with translational movements restricted.

The QM/MM systems have generally a high number of atoms; hence the number of non-bonding interactions is huge. Computationally, it would be very expensive to calculate the energy contribution of all non-bonding pairs of atoms in a

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condensed system. To solve this problem, the cut-off methods are applied using truncations functions.¹⁴²

The directly truncated function uses an abrupt truncation ignoring all interactions that are beyond the cutoff distances. In this method, each term of non-bonding potential ($U_{non-bonding}$) shown in equation 5, is multiplied by the truncation function $S(r)$, of the form

$$S(r) = \begin{cases} 1 & r \leq r_c \\ 0 & r > r_c \end{cases} \quad (13)$$

There are inconveniences in such kind of abrupt truncation once the energy and its derivatives are no longer continuous functions. To solve this problem some other alternatives functions can be used as the smoothing functions $S(r)$. The switch function, equation 14, is a kind of smoothing function and is constructed so that its first derivative is continuous in the range $r \leq r_{off}$. This function computes all the interactions inside a radius (r_{on}), from which it is smoothed to zero at the outer cutoff (r_{off}).

$$S(r) = \begin{cases} 1 & r \leq r_{on} \\ \frac{(r_{off}^2 - r^2)^2 (r_{off}^2 + 2r^2 - 3r_{on}^2)}{(r_{off}^2 - r_{on}^2)^3} & r_{on} < r \leq r_{off} \\ 0 & r > r_{off} \end{cases} \quad (14)$$

The shift function shown in equation 15, is another type of smoothing function, that relies on just on cutoff distance and it modifies the form of the interaction throughout its entire range.

$$S(r) = \begin{cases} \left[1 - \left(\frac{r}{r_c}\right)^2\right]^2 & r \leq r_c \\ 0 & r > r_c \end{cases} \quad (15)$$

Figure 16. Plots of truncations functions with $r_c=r_{off}=10 \text{ \AA}$ and $r_{on}=5 \text{ \AA}$.

In Figure 16 the behavior of these truncations functions, $S(r)$, as function of distance r is shown.

3.2.2 Molecular Dynamics

The study of the mechanism of action of enzymes toward their substrates and inhibitors is the key on the development of new approaches in treatment and the search of the cure to several diseases. In macroscopic scale, it is possible to measure experimentally several properties, but estimate how the interaction between an enzyme and a molecule occurs at atomic and molecular level (nanoscale) is something that still is not possible to observe in an erlenmeyer.

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With the development of high-performance hardware, software and appropriate algorithms, now it is possible to simulate realistically the environment where the interactions and chemical reactions occur to explain how the macromolecular systems work in nanoscale.¹⁴³ In the macromolecular systems, the study of the behavior of atomic nuclei involved in the biochemical process, when it is necessary to take into account the time, is possible when Newton's equations of motion and dynamics (equation 16) are applied into computational methods developed with the aim to consider the time dependence.

$$F_i(t) = m_i \vec{a} = m_i \frac{\partial^2 \vec{r}_i}{\partial t^2} = - \frac{\partial U(r_i)}{\partial r_i} \frac{\vec{r}_i}{r_i} \quad (16)$$

The method of molecular dynamics (MD) simulations is the main tool for studying systems of biochemical interest in condensed phase. The MD is a method that allows us to estimate the structural conformations in an evolutionary time, calculate the thermodynamic and dynamic properties of biological molecules and their complexes. Although these systems are composed of a large number of atoms, it is necessary to increase the number of atoms to be studied to simulate the intracellular environment. The method of MD¹⁴³ allows us to simulate the enzymatic behavior: “The essence of the simulation is the use of the computer to model a physical system.”

The first simulations using MD methods were performed in the late 1950s by Alder and Wainwright, which studied the interaction of a system composed by rigid spheres,¹⁴⁴ providing important insights into interactions in liquid medium. Then, Rahman carried out first simulations using a realistic potential for liquid argon and also the first simulations of real molecular systems.¹⁴⁵ The inhibition mechanism of trypsin was the first protein simulated by MD, and it was performed by Karplus and coworkers in 1977.¹⁴⁶

Atoms are always in motion, with rare exceptions and extreme conditions, all the atoms and particles that make up the universe are in constant motion. Thus, the nuclei of the atoms that constitute an enzymatic system in a condensed phase or in solution are also moving continuously. The study of this movement by means of MD carried out over a period of time gives us a considerable amount of structural states (conformational configurations) and a huge quantity of information that are useful in predicting, estimate, calculate and determine the properties of the system.^{143,147}

The several conformational configurations obtained during a MD simulation have, due to small fluctuations in the geometry of the system, similar chemical and energetic information. One of the keys for an accurate simulation (e.g. enzymatic system) using MD is to get a suitable starting conformation for the system. On the other hand, to calculate the thermodynamic properties for the same system a considerable amount of information on the different structural states accessed by the system is required.

To obtain a suitable starting point to study enzymatic systems, some algorithms are applied in MD programs with aim to solve the terms of equation 16. The computation of thermodynamic properties through several states accessed by the system is lead for statistical mechanics.

The motion of the system through phase space is governed by the Hamiltonian of the system, equation 17, defined in function of momentum $\vec{p}_i$ and positions $\vec{r}_i$.

$$\begin{aligned} \hat{H}(\vec{p}_i, \vec{r}_i) &= \sum_{i=1}^n \frac{1}{2m_i} \vec{p}_i^2 + U(\vec{r}_i) \\ \vec{p}_i &= \frac{\partial \hat{H}}{\partial \vec{r}_i} = - \frac{\partial U}{\partial \vec{r}_i} = \vec{F}_i \\ \vec{r}_i &= \frac{\partial \hat{H}}{\partial \vec{p}_i} = \frac{\vec{p}_i}{m_i} \end{aligned} \quad (17)$$

where $U(r_i)$ is a potential energy function.

The MD simulations generate information at the microscopic level, for example, the position and velocity of the atomic nuclei of the system under study. The statistical mechanics is used to convert the information in the microscopic level in the macroscopic observables as pressure and energy. Thus, statistical mechanics principles are crucial for the study of enzymatic systems by MD.¹⁴⁷

Statistical mechanics gives a rigorous mathematical modeling to relate macroscopic properties to the distributions and motion of atoms and molecules of N-body system, this information connected with microscopic properties calculated from MD provides a powerful tool to study thermodynamics properties and/or kinetic phenomenon in protein and its ligands problem.

3.2.2.1 Some Molecular Dynamic Algorithms

The MD simulations programs perform a simple algorithm to solve the terms in equation 16 by determining the forces acting on each particle. In general, this algorithm has six steps that can be described as follows:

- i. Read the vector of coordinates of each atomic nuclei, $\mathbf{r}_i(t) = (x_i(t), y_i(t), z_i(t))$;
- ii. Read the initial velocities of each nuclei;
- iii. Integrate equation 16;
- iv. Calculate the force acting on each atomic nuclei;
- v. Calculate properties;
- vi. Return to step ii to calculate the next state.

The finite difference method using Taylor series expansion is one of the most efficient methods for solving differential equations as the equation 16.¹⁴⁸ Knowing the force that act on each nucleus is possible to calculate the new position that each atom will take after a time increment Δt , related with the position $\mathbf{r}_i(t)$ and the velocity $\mathbf{v}_i(t)$ at the previous instant (t). This is the main idea contained in the algorithm

developed by Loup Verlet in 1967. Equation 18 represents Verlet's algorithms applying to solution of equation 16 taking in account Taylor Series expansion of fourth order for positions $t+\Delta t$ and $t-\Delta t$.¹⁴⁹

$$r_i(t + \Delta t) = 2 \vec{r}_i(t) - r_i(t - \Delta t) + \frac{f(t)}{m_i} \Delta t^2 \quad (18)$$

$$\vec{v}_i(t) = \frac{1}{2\Delta t} \left(\vec{r}_i(t + \Delta t) - \vec{r}_i(t - \Delta t) \right)$$

The simplest algorithms used in MD are based on the Verlet's algorithm. The efficiency of the Verlet's algorithm can be improved employing the velocities of the last instant on the previous time rather than recalculating new velocities for each new time. This algorithm, equation 19, is called Velocity-Verlet.

$$\vec{v}_i(t + \Delta t) = \vec{v}_i(t) + \frac{\Delta t}{2m_i} \left(\vec{F}_i(t) + \vec{F}_i(t + \Delta t) \right) \quad (19)$$

The leapfrog algorithm is other kind of algorithm used in MD (equation 20), here the velocities are calculated at each $\Delta t/2$ and since the velocities obtained in time $t+\Delta t/2$ positions are updated at a time $t+\Delta t$.

$$\vec{v}_i\left(t + \frac{\Delta t}{2}\right) = \vec{v}_i\left(t - \frac{\Delta t}{2}\right) + \frac{f(t)}{m_i} \Delta t \quad (20)$$

$$\vec{r}_i(t + \Delta t) = \vec{r}_i(t) + \vec{v}_i\left(t + \frac{\Delta t}{2}\right) \Delta t$$

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Considering that Newton equations are conservative and the assurance that system will evolve the total energy (since is taken a time independent Hamiltonian), the linear and angular moments remain constant (Eckart conditions)¹⁴⁰ and allows us to believe that trajectories and forces obtained from these algorithm can be used in MD simulations.

3.2.2.2 Statistical Mechanics in MD

A system can be observed through its macroscopic and microscopic properties. According to statistical mechanics, the physical quantities are obtained by means of microscopic states (configurations) system, distributed according to certain set of statistics.¹⁴³

The set of parameters formed by thermodynamic properties (temperature, pressure and number of particles) stemmed of macroscopic observable of the system is known as thermodynamic state of the system. From equations of thermodynamic states other thermodynamic properties can be calculated.¹⁵⁰

The set of parameters consisting of microscopic properties (atomic coordinates and momentum) of a system is called a microscopic state or mechanical state. These can also be regarded as coordinates in a multidimensional space called phase space. The set of points in phase space satisfying the conditions of a particular thermodynamic state is called ensemble.¹⁴⁷

The MD simulations generate ensembles which correspond to different conformations of the system. An ensemble can be defined as the set of all possible systems that have different microstates, but share the same thermodynamic state.¹⁴⁷

In statistical mechanics, an “experimental” observable is assumed as the ensemble average. These averages (Ξ) values are defined as given by equation 21:

$$\langle \Xi \rangle_{ensemble} = \iint dp_i^N dr_i^N \Xi(p_i^N, r_i^N) \rho(p_i^N, r_i^N)$$

$$\rho(p_i^N, r_i^N) = \frac{1}{Q} e^{\left[\frac{\hat{H}(p_i^N, r_i^N)}{\kappa_B T} \right]} \quad (21)$$

$$Q = \iint dp_i^N dr_i^N e^{\left[\frac{\hat{H}(p_i^N, r_i^N)}{\kappa_B T} \right]}$$

Where $\hat{H}$ is the Hamiltonian, T is the temperature, κ_B is Boltzmann's constant and Q is the partition function of selected simulations conditions.

In MD simulations observables are assumed as time average of Ξ , shown in equation 22.

$$\langle \Xi \rangle_{time} = \lim_{\tau \rightarrow \infty} \frac{1}{\tau} \int_{t=0}^{\tau} \Xi(p_i^N(t), r_i^N(t)) dt \approx \frac{1}{M} \sum_{t=1}^M (p_i^N, r_i^N) \quad (22)$$

With τ defined as simulation time, M is the time steps in the MD and $\Xi(p^N, r^N)$ is the instantaneous value of Ξ .

As described above, the “experimental” and simulated observables have different way to be calculated. This difference can be solved by the fundamental axiom of statistical mechanics, the *ergodic hypothesis*, which consists in assuming that time average, $\langle \Xi \rangle_{time}$ is equal at the ensemble average, $\langle \Xi \rangle_{ensemble}$.

In the case of isolated systems where the number of atoms N , volume V and the energy are invariant we have a microcanonical ensemble (NVE). Systems where N , V and temperature T must remain constant are called canonical ensemble (NVT).^{140,147}

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The canonical ensemble (NVT) is the most useful ensemble to study enzymes and their ligands since the majority of chemical processes, in the condensed phase, occur under these conditions. In order to maintain the volume constant, the periodic boundary conditions are employed to guarantee the conservation of density of the medium during the simulation. The number of particles, N , is always constant.

Different kinds of approach may be used to maintain constant the temperature during the MD simulation, although the strategy for this purpose is generally the same: scaling the velocities or using a thermostatic bath in contact with the external system.

In a MD simulation is useful to be able to control the temperature of the system during the process. From the statistical thermodynamics, the temperature (T) can be defined as

$$T = \frac{2\langle K \rangle}{N_{df}\kappa_B} \quad (23)$$

Where $\langle K \rangle$ is the averaged kinetic energy and N_{df} is the number of degree of freedom. The instantaneous temperature T_i can be defined using the equation 23 but considering only the kinetic energy not its average.

The fixation of the temperature can be done scaling the velocities with the ratio between T_i and reference temperature of the system T_{ref} , as described below:

$$\vec{V}_{i,scaled}(t) = \vec{V}_i(t) \sqrt{\frac{T_{ref}}{T_i}} \quad (24)$$

The thermostatic bath method is implemented using algorithms derived from Newton's equations. These algorithms are simple and robust, and easy to implement in MD programs. The essential idea of the thermostatic bath method is considered

that in the simulation of a real system, this one is not isolated but interacts, or coupled, with an external bath. The most known methods of thermostatic baths are: Andersen, Berendsen, Nosé-Hoover and Langevin.^{140,151}

In Andersen's algorithm, instantaneous velocities of a group of randomly selected nuclei are reallocated using the Maxwell-Boltzmann distribution. The simulation obtained with this model is not fully useful once it produces discontinuous results. Berendsen and coworkers proposed an improvement in algorithm of Andersen including an additional term in the calculation of instantaneous velocities, associating it a friction force that is dependent of the reference temperature. The equation 25 describes this algorithm.

$$\vec{V}_i(t) = M^{-1} F + \frac{1}{2\tau_T} \left(\frac{T_{bath}}{T_i} \right) V \quad (25)$$

Where M^{-1} is the frictional force, T_{bath} is the reference that in the Berendsen algorithm is the temperature of the external thermal bath, τ_T is the coupling constant and V is the volume of the system.

The Langevin-Verlet (LV) integrator (equation 26) is a stochastic analogue of Newton's equation of motion in which random forces, dependent on the velocity (friction), are introduced so that coupling simulates external thermostat bath.^{140,141}

$$mdv(t) = -\nabla U(x(t))dt - \gamma mv(t)dt + \sqrt{2\gamma\kappa_B T_{bath}} m^{1/2} dD(t) \quad (26)$$

Where $x(t)$ is the position, $v(t)$ is the velocity, γ is the collision parameter.¹⁵² $D(t)$ is a vector of independent standard Wiener (Brownian motion) process, where ξ_j is considered as a sequence of independent, identically distributed random variables with mean 0 and variance 1. For each $n \geq 1$ define a continuous-time stochastic process $\{D_n(t)\}_{t \geq 0}$ by

$$D_n(t) = \frac{1}{\sqrt{n}} \sum_{1 \leq j \leq [nt]} \xi_j \quad (27)$$

D_n is a random step function with jumps of size $\pm \frac{1}{\sqrt{n}}$ at times $\frac{k}{n}$, where k is $\mathbf{Z}_+$. Since the random variables ξ_j are independent, the increments of $D_n(t)$ are independent.

The LV equation is employed to describe the dynamics of a particle interacting with a thermal bath.¹⁴⁰

3.3 Free Energy in MD

MD techniques explores the phase space fully, for this reason is possible to use it to determine thermodynamic quantities in a rigorously way. Several methods can be applied in the MD to calculate the free energies of certain kinds of chemical process either in gas or condensed phases.¹⁴⁰ In the calculating process of the free energy, using molecular dynamics is necessary to take into account several factors. One of them is the fact that during the MD simulations, at a specific temperature, only preferably states of lower energy are explored. Another factor is the interest in to express the free energy as a function of reaction coordinate.

Rewriting the partition function from the equation 21 is possible to obtain the partition function Q_{NVT} for a system with N indistinguishable particles at constant temperature and volume:

$$Q_{NVT} = \frac{1}{h^{3N} N!} \int dp_i^N \int dr_i^N e^{\left[-\frac{\hat{H}(p_i^N, r_i^N)}{\kappa_B T} \right]} \quad (28)$$

From the Hamiltonian described in equation 17, it is possible to perform the integration over the momentum, leaving an integral, called the configuration integral, that depends only on the position of the coordinates.¹⁴⁰ Thus the probability density distribution function given in equation 21 can be expressed in terms of the partition function Q_{NVT} .

$$\rho_{NVT} = \frac{1}{h^{3N} N!} \frac{e^{\left[-\frac{\hat{H}(p_i^N, r_i^N)}{\kappa_B T} \right]}}{Q_{NVT}} \quad (29)$$

In the free energy calculation, it is useful to consider the canonical ensemble (Q_{NVT}), whose observable for the free energy of the system is the Helmholtz (A) potential (equation 30), adequate to describe the system. In this case the simulation of a system is taken considering the NVT conditions.

$$A = -\kappa_B T \ln Q_{NVT} \quad (30)$$

Q_{NVT} can be considered being a property that depends on the energy of each configuration, and rewriting it as an ensemble average:

$$Q_{NVT} \propto \frac{1}{\left\langle e^{\frac{U}{\kappa_B T}} \right\rangle} \quad (31)$$

Despite of being attractive the idea to obtain the energy of the system from the calculation of the Q_{NVT} , this is unfeasible due, as described above, in a MD simulation just some lowest energy states are explored during the simulation process. Some

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strategies are used attempting calculate the free energy combining the Helmholtz energy, the partition function derivations and other considerations.

In the field of computational chemistry and computer-aided drug design (CADD), and in enzymatic catalysis systems, several studies to determine the binding free-energy of enzymatic systems with high accuracy and good agreement with experimental data are being carrying out.^{153,154} For this purpose a rigorous formal theoretical framework and powerful approach techniques as the free energy perturbation FEP,^{127,155} the potential mean force (PMF)¹⁷, and thermodynamic integration (TI)¹⁵⁶ are applied. In addition, the analysis of atomistic molecular dynamics or Monte Carlo simulations to determine the free-energy difference between two related ligands through a chemical or alchemical pathway¹⁵³ are frequently performed.

3.3.1 Potential of Mean Force

The potential mean force (PMF) is one of the strategies to calculate free energy in MD simulations. The PMF can be obtained as a function of one or more degrees of freedom (ξ_0) of the system. The ξ is defined as function of the Cartesian coordinates of the atoms, or as distance or angle, or other properties chosen according the process being studied.^{135,140}

In order to calculate the free energy of a system using PMF, it is appropriate to express it in terms of the ensemble average of the probability distribution function of the coordinate $\rho(\xi_0)$ as shown in equation 32:

$$\langle \rho(\xi_0) \rangle = \frac{\int dr \delta(\xi(r) - \xi_0) e^{\left[-\frac{U(r)}{\kappa_B T} \right]}}{\int dr e^{\left[-\frac{U(r)}{\kappa_B T} \right]}} \quad (32)$$

Where δ is the Dirac delta, and ensures that only the chosen reaction coordinate, ξ_0 is taken into account. Thus, the PMF is defined as

$$W(\xi_0) = c' - \kappa_B T \ln \langle \rho(\xi_0) \rangle \quad (33)$$

where c' is an arbitrary constant.

The problem to use the equation 33 is that performing free dynamics for a system, at given temperature, it will evolve minimum values of energy. An elegant solution for this problem is to use the Umbrella Sampling technique. With this technique it is possible get the average of thermodynamic magnitude from "guided" observations (biased).^{140,157}

For a correct calculation of $\rho(\xi_0)$ it is necessary to perform a series of calculations with a potential as Umbrella, to concentrate sampling in different regions of space (windows). Thus, the system may access to areas of higher potential energy. The trajectories obtained for each window will come together to complete a valid distribution function for the full range of the coordinate ξ_0 . The Umbrella potential chosen is a harmonic potential as:

$$\Omega_{Umb}(\xi_0) = \frac{1}{2} k_{Umb} (\xi_0 - \xi_{ref})^2 \quad (34)$$

Where the k_{Umb} is the force constant of the potential and the ξ_{ref} is the reference value of the reaction coordinate.

The reconstruction of all distribution functions separately for each window is a key step in the calculations performed with Umbrella Sampling method.^{140,158} The Weighted Histogram Analysis Method (WHAM) is an efficient procedure for this. In the WHAM, the probability distribution function is expressed as a linear combination of a number P of distribution functions for different values of reaction coordinate $\xi_{0,i=1,P}$.

$$\langle \rho(\xi_0) \rangle = \sum_i^P c_i \langle \rho(\xi_{0,i}) \rangle \quad \left\{ \begin{array}{l} \sum_i^P c_i = 1 \\ \langle \rho(\xi_0)^2 \rangle - \langle \rho(\xi_0) \rangle^2 \quad \text{Minimum} \end{array} \right. \quad (35)$$

Where the c_i coefficients are chosen to minimize the random error. Then, molecular dynamics with different values of $\xi_{0,i}$ (windows) are carried out to calculate the correspondence distribution functions values $\langle \rho(\xi_{0,i}) \rangle$ or histograms as shown in Figure 17.

Figure 17. Representation of the (a) Umbrella Sampling technique; and (b) WHAM method.

3.3.2 Free Energy Perturbation

The alchemical free energy perturbation method (FEP) is another approach used in MD simulations to get binding free energies. In cases where the simulation is impossible to be done or reproduced in laboratory, the FEP is applied in order to obtain free energies of such kind of system.^{127,140,159}

In this way are possible proceed calculations of free energy differences between states of a system where the number and type of atoms are modified

(alchemical). This method is also called by Gao *et al.* the double annihilation method.^{140,159}

The FEP method is based on thermodynamic cycles in which the interaction of the ligand with its surroundings, whether it is the protein or bulk solution, is progressively annihilated. In FEP technique, the perturbation of the system does not have corresponding physical process. Thus, it is to possible apply a series of nonphysical overlapping states using FEP method descriptions.^{127,140,159,160}

In FEP simulations, if two state are taken in consideration, the calculation of free energy difference between these states associated with the equation of Helmholtz is performed as:^{140,159}

$$\Delta Y_{I \rightarrow II} = Y_{II} - Y_I \quad \therefore \quad \Delta Y_{I \rightarrow II} = -\kappa_B T \ln \frac{Q_{II}}{Q_I} \quad (36)$$

Expressing Y_I and Y_{II} in terms of Q_{NVT} from equations 28 and 31

$$Y_I = -\kappa_B T \ln \left\langle e^{-\frac{\hat{H}_I}{\kappa_B T}} \right\rangle \quad \text{and} \quad Y_{II} = -\kappa_B T \ln \left\langle e^{-\frac{\hat{H}_{II}}{\kappa_B T}} \right\rangle \quad (37)$$

Thus $\Delta Y = Y_{II} - Y_I$ is given as

$$\Delta Y(I \rightarrow II) = -\kappa_B T \ln \left\langle e^{-\frac{\hat{H}_{II} - \hat{H}_I}{\kappa_B T}} \right\rangle_I \quad (38)$$

In these equations, the Hamiltonian $\hat{H}$ can be decomposed into potential (dr) and kinetic (dp) energies, thereby is possible integrating the kinetic energy separately. The useful result is the change in the free energy to go from Υ_I to Υ_{II} states can be obtained from the average of the exponential of the energy difference ($H_{II}-H_I$, or potential $U_{II}-U_I$), under the conditions given from state $\Upsilon(I)$.¹⁴⁰

Sometimes the $H_{II}-H_I$ difference is very large, due the configurational space of one state was not properly defined. Thus, this difference does not contribute significantly to the average. To solve this problem, it is possible to introduce an unlimited number of intermediate states between Υ_I and Υ_{II} , considering that the thermodynamic pathway route to go from state 1 to state 2 is not necessarily a physical path.¹⁴⁰ Thus, the free energy difference is calculated as a sum of small amounts:

$$\Delta\Upsilon = \Upsilon_{II} - \Upsilon_I = (\Upsilon_{II} - \Upsilon_{N-1}) + (\Upsilon_{N-1} - \Upsilon_{N-2}) + \dots + (\Upsilon_2 - \Upsilon_I)$$

$$\Delta\Upsilon = \kappa_B T \sum_{i=1}^{N-1} \ln \left\langle e^{-\frac{\hat{H}_i - \hat{H}_{i+1}}{\kappa_B T}} \right\rangle_{i+1} \quad (39)$$

A way of defining the states is to introduce a coupling or perturbation parameter of the system, λ , allowing a continuous transformation from the Hamiltonian of the Υ_I to Υ_{II} state.

$$\hat{H}(p, r, \lambda) = (1 - \lambda) \hat{H}_I(p, r) - \lambda \hat{H}_{II}(p, r) \quad (40)$$

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Where $\lambda=0$ define the Υ_I state and $\lambda=1$ the Υ_{II} state. A straightforward extension of the equation 40 gives the nonlinear scheme:

$$\hat{H}(p,r,\lambda) = (1-\lambda)^n \hat{H}_I(p,r) - \lambda^n \hat{H}_{II}(p,r) \quad (41)$$

Thus, the free energy difference between two intermediate states with different values of the perturbation parameter λ_I and λ_{II} can be calculated. Again, taking the kinetic energy terms cancel out, the difference is

$$\Delta\Upsilon_{i \rightarrow j} = -\kappa_B T \ln \left\langle e^{\left(- (U(r,\lambda_j) - U(r,\lambda_i)) / \kappa_B T \right)} \right\rangle_{\lambda_i} \quad (42)$$

The sum of the individual free-energy differences between the intermediates states on going from Υ_I to Υ_{II} states gives the total free energy difference

$$\Delta\Upsilon_{I \rightarrow II} = \sum_{i=0}^{N_w} \Delta\Upsilon_{i \rightarrow (i+1)} \quad (43)$$

Where N_w is the number of intermediates states (windows) and the states $i=0$ and $i=N_w+1$ refer the Υ_I to Υ_{II} states

A practical advantage of the FEP method is that simulations conducted by state I can be used to calculate the free energy difference in both senses of a chemical process. The value of the two differences senses should be equal in magnitude and opposite in sign. Thus the value of the two differences¹⁴⁰ is given by:

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$$\Delta Y_{I \rightarrow II} = - \Delta Y_{II \rightarrow I}$$

$$\Delta Y(I \rightarrow II) = - \kappa_B T \ln \left\langle e^{-\frac{\hat{H}_{II} - \hat{H}_I}{\kappa_B T}} \right\rangle_I$$

and

$$\Delta Y(II \rightarrow I) = - \kappa_B T \ln \left\langle e^{-\frac{\hat{H}_I - \hat{H}_{II}}{\kappa_B T}} \right\rangle_{II}$$
(44)

The thermodynamic cycle is an excellent support in an attempt to modeling the studies involving the calculations of free energy differences between two states.

Figure 18. Scheme of the Thermodynamic cycle.¹⁴⁰

In a thermodynamic cycle as shown in Figure 18, the process of calculating the relative binding affinities between the ligand A and B to a host molecule Z is given by the sum of the individual free energies around the cycle. This sum is equal to zero because the free energy is a thermodynamic state function. This means that the free energy difference depends only upon the nature of the states.

$$\Delta Y_{AZ} + \Delta Y_{AZBZ} - \Delta Y_{BZ} - \Delta Y_{AB} = 0$$
(46)

According to equation 46 the relative binding affinity of the ligands A and B can be determined as

$$\Delta Y_{AZ} - \Delta Y_{BZ} \quad \text{or} \quad \Delta Y_{AB} - \Delta Y_{AZBZ} \quad (47).$$

During this thesis, a series of QM/MM MD and alchemical FEP simulation have been carried out in the gorge and in a box of water molecules (solvent) in order to evaluate the AChE-Inhibitors interaction free energy of binding.

The potential energy used is based in the approach implemented in fDynamo by Moliner and coworkers,¹²⁷ defined as

$$E_{QM/MM} = \left\langle \Psi \left| \hat{H}_0 \right| \Psi \right\rangle + \left(\sum \left\langle \Psi \left| \frac{q_{MM}}{r_{e,MM}} \right| \Psi \right\rangle + \sum \sum \frac{Z_{QM}q_{MM}}{r_{QM,MM}} \right) + E_{QM/MM}^{vdW} + E_{MM} \quad (48)$$

$$E_{QM/MM} = E_{vacuum} + E_{QM/MM}^{elec} + E_{QM/MM}^{vdW} + E_{MM}$$

Where E_{vacuum} is the gas phase energy of the polarized QM region, $E_{QM/MM}^{elec}$ is the electrostatic interaction energy which includes the coulombic interaction of the QM nuclei (Z_{QM}) and the electrostatic interaction of the polarized electrons wave-function Ψ with the protein charges (q_{MM}), $E_{QM/MM}^{vdW}$ is the van der Waals interaction energy between QM and MM part.

To compute free-energy difference from the alchemical FEP perturbations, two parameters λ and γ were introduced in the electrostatic and van der Waals QM/MM interaction terms in the total energy as shown in equation 49.

$$E_{QM/MM}(\lambda) = \left\langle \Psi \left| \hat{H}_0 \right| \Psi \right\rangle + \lambda \left(\sum \left\langle \Psi \left| \frac{q_{MM}}{r_{e,MM}} \right| \Psi \right\rangle + \sum \sum \frac{Z_{QM} q_{MM}}{r_{QM,MM}} \right) + \quad (49)$$

$$\gamma E_{QM/MM}^{vdW} + E_{MM}$$

In this scheme, the λ parameter varies mildly between the values from 0 to 1. The parameter λ value equal 1 correspond to a full MM charge–wave function interaction; and when λ is equal to 0 represents no electrostatic interaction with the force field. The γ values varies also between 0 to 1, when it is equal to 1 corresponding to a full QM/MM van der Waals interactions (once the electrostatic charges of the ligand in QM region are annihilated); and when γ is equal 0 represents no van der Waals interaction with the force field.¹²⁷

The calculation of free-energy differences of two consecutive windows between two different states, and the annihilation of charges and the van der Waals parameters is performed through FEP methods. In this calculation, the total free energy variation is taken by the sum of all windows covering the full transformation from the initial and final states. This procedure is done in two steps showed in the equations 50 and 51:

$$\Delta G_{QM/MM}^{elect} = -\frac{1}{\kappa_B T} \left[\sum \ln \left\langle e^{-\kappa_B T [E(\lambda_{i+1}) - E(\lambda_i)]} \right\rangle_{\lambda_i} \right]_{\gamma=1} \quad (50)$$

and

$$\Delta G_{QM/MM}^{vdW} = -\frac{1}{\kappa_B T} \left[\sum \ln \left\langle e^{-\kappa_B T [E(\gamma_{i+1}) - E(\gamma_i)]} \right\rangle_{\gamma_i} \right]_{\lambda=1} \quad (51)$$

The thermodynamic cycle to compute enzyme-inhibitors binding free energy by alchemy FEP methods is described in Figure 19.

Figure 19. Thermodynamic cycle from alchemy FEP method.

Where $(L-E)_w$ is the complex enzyme (E) and inhibitor (L) in solution, $(E')_w$ is the apo form of the enzyme, $(L)_o$ is the inhibitor in the gas phase. According to the scheme shown in Figure 19 it is possible to calculate the binding free energy of a ligand and compare the predicted value with the experimental data (using the inhibition constants K_i). The ΔG can be calculated applying the equations 50 and 51, in water and in the enzyme respectively. So, the binding free energy is expressed as:

$$\Delta G = \Delta G_E - \Delta G_w \quad (52)$$

In practice, this calculation is performed in two steps yielding the electrostatic and van der Waals terms, ΔG_{elec} and ΔG_{vdW} . The total binding free energy is obtained by adding these two terms:

$$\Delta G_{bind} = \Delta G_{elec} + \Delta G_{vdW} \quad (53)$$

3.3.3 Thermodynamic Integration (TI)

The thermodynamic integration (TI) is a method used to calculate free energy differences between two states of a system. This method uses the same coupling parameter λ approach as FEP methods.

TI method uses the identity expressed by the following equation

$$\Delta Y_{I \rightarrow II} = \int_0^1 d\lambda \frac{\partial Y}{\partial \lambda} \quad (54)$$

Again, expressing Y_I and Y_{II} in terms of Q_{NVT} from equations 28 and 31, the determination of the derivatives of the free energy is straightforward:

$$\Delta Y_{I \rightarrow II} = \int_0^1 d\lambda \left\langle \frac{\partial \hat{H}}{\partial \lambda} \right\rangle_\lambda \quad (55)$$

The integral of equation 54 is determined performing simulations to calculate the ensemble average in the integrand at several values of λ and applying numerical integration technique to the obtained values.

3.3.4 Slow Growth

In this technique, the coupling parameter changes slowly during the simulation. However, this technique has been criticized because the system never has time to be balanced once uses a slowly and continuously variable Hamiltonian.¹⁶¹

The expression of slow growth method (SG) to calculate free energy can be derived from the FEP or TI equations. Using a Taylor expansion for one state, the SG is calculated as:

$$\Delta Y = -\kappa_B T \sum_{\lambda=0}^{\lambda=1} \ln \left\langle e^{\frac{-\left[\hat{H}(\lambda+\Delta\lambda) - \hat{H}(\lambda)\right]}{-\kappa_B T}} \right\rangle_{\lambda} \quad (56)$$

The SG techniques is not so efficient, because if in the end of the simulation is concluded that it is necessary to increase the simulation time, it will be necessary to repeat the simulation from the beginning.

3.4 Binding Isotopic Effect (BIE)

Binding isotope effects (BIEs)¹⁶² can be a useful tool to identify important non-covalent interactions occurring during binding inhibitor to the active site. Changes in normal-mode force constants surrounding the isotopic substitution between free (usually dissolved in water) and bonded inhibitor, give rise to a binding preference for light or heavy isotope in the bound state and hence a measurable BIE.¹⁶¹

Considering the process of binding of an inhibitor, from solution to the formation of the inhibitor–enzyme complex, BIE would be defined as the ratio between the binding equilibrium constant of the light (L), or natural abundant isotope species, and the heavy (H) or isotopically substituted one,

$$BIE = \frac{K_L}{K_H} \quad (57)$$

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BIEs can also be expressed in terms of the binding free energy of the light and heavy species, considering the relationship between the equilibrium constant and the binding free energy:

$$BIE = e^{-(\Delta G_L + \Delta G_H)/RT} \quad (58)$$

Where ΔG_L and ΔG_H are the binding free energies for the light and heavy isotopomers, respectively, R is the universal gas constant and T the temperature.

$$G_i = U_i - RT \ln Q_i + ZPE_i \quad (59)$$

$$\begin{aligned} \Delta G &= G_e - G_w \\ &= (U_e - U_w) + (-RT \ln Q_e + RT \ln Q_w) \\ &\quad + (ZPE_e - ZPE_w) \\ &= \Delta U + RT \ln \frac{Q_w}{Q_e} + \Delta ZPE \end{aligned} \quad (60)$$

$$BIE = \frac{\left(\frac{Q_e}{Q_w}\right)_L}{\left(\frac{Q_e}{Q_w}\right)_H} e^{-1/RT(\Delta ZPE_L - \Delta ZPE_H)} \quad (61)$$

3.5 Molecular Docking

Computational automated molecular docking is a primary essential computational tool applied in CCAD focused in the interactions of receptor-ligand complexes. Docking simulations is a methodology centered in determines the binding energy of biomolecular complexes. This methodology is based in the union of two methods: a searcher explorer of conformational space available, the score function; and a force field to evaluate and estimate the energy of binding in each conformation available.^{17,163} The center key of a molecular docking implementation is located in the kind of the force field implemented and the score function used to predict the interaction energy and ligand pose score.

Since 1995, a large number of molecular docking algorithms have been developed (e.g. FlexX, GOLD, FLOG, AutoDock, DOCK) permitting during simulation a rigid and flexible ligand and also some residues of the proteins. Computational low cost with well-suited relative rankings as AutoDock, and others docking implementation more potent as AutoDock Vina with a statistical score function to improve the accuracy of prediction and speed of calculation are available. DrugScoreX is other implementation with high accuracy in prediction. In this row is valid cite the LigScore that uses distance-dependent statistical scoring function providing a relatively high accuracy in energy bidding prediction.¹⁶⁴

In general manner, the molecular docking AutoDock program take the approach performing free binding energy of R-L systems using a simple hybrid force field and exploring a wider region of conformational space using a grid strategy, without loose the power of the predict binding energy with a low computational cost. AutoDock combines an empirical free energy force field and a Lamarckian Genetic algorithm, to increase the velocity of prediction of the bound conformation and its correlated free energy in a R-L interaction.

The last version of AutoDock, the AutoDock4 has a semiempirical free energy force field implemented in it. The AutoDock4 force field is based in thermodynamic model where are incorporated intramolecular energies into the predict free energy of

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binding. In addition, the AutoDock4 has implemented a full desolvation model, and monitor of the direction of hydrogens bonds as well a term to evaluate the conformational entropy by the sum of the torsional degrees of freedom. In this docking algorithm the water molecules are taken as implicitly and allows fully flexible modeling of elected portions of the protein and ligand.¹⁶⁵

The semiempirical force field of AutoDock4 is formed by six pair-wise terms (V) and a term to estimate the conformational entropy (ΔS_{conf}) with the representations: L ligand, P protein, and $P-L$ protein-ligand complex.

$$\begin{aligned} \Delta G_{bind} = & (V_{bound}^{L-L} - V_{nonbound}^{L-L}) + (V_{bound}^{P-P} - V_{nonbound}^{P-P}) + \\ & + (V_{bound}^{P-L} - V_{nonbound}^{P-L} + \Delta S_{conf}) \end{aligned} \quad (62)$$

The pair-wise (V) is composed by dispersion/repulsion, hydrogen bonding, electrostatic and desolvation terms. The κ weighting constants are the optimized calibrate values based on a set of experimentally characterized complexes.¹⁶³

$$\begin{aligned} V = & \kappa_{vdW} \sum_{ij} \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^6} \right) + \kappa_{h-bond} \sum_{ij} E(t) \left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^6} \right) + \\ & + \kappa_{elec} \sum_{ij} \frac{q_i q_j}{\epsilon(r_{ij}) r_{ij}} + \kappa_{solv} \sum_{ij} (S_i V_j + S_j V_i) e^{r_{ij}^2 / 2\sigma^2} \end{aligned} \quad (63)$$

3.6 DFT method

The receptor-ligand interactions establish the functionality of a given molecule. However, only the studies of the binding free energy of interactions do not provide enough parameters to the development of a new ligand. The properties of a molecule can be obtained by theoretical calculations using classical mechanics and quantum mechanics. In the molecular mechanics approach, the classical mechanics deals with the problem without taking into account the electrons. Thus, electronic properties can only be treated by methods of electronic structure by means of quantum mechanical calculations. Thus, the main properties that describe a molecule are classified according to how it is defined spatially.

The DFT is a theoretical computational quantum mechanics method used to investigate the electronic structure of many-body systems, like atoms and molecules.¹⁶⁶ This method can be used to calculate electronic and structural properties of molecules with biological activity, like new drugs to treat a disease. The DFT method applies a functional spatially dependent on the electronic density of multi-electronics systems.

DFT is the most widely used method for the study of the electronic structure of molecular condensed systems. This method includes in its scope a term for the electron correlation energy, which is the main advantage of this method in relation to the Hartree-Fock method. Kohn and Sham gave DFT a new meaning and the results of calculations obtained with the DFT are in many cases closer to the experimental data with a lower computational cost. In addition to provide a more representative value for energy, the DFT still provides a higher speed in performing the calculations compared to other methods that calculate the electron correlation energy of multielectronic systems.^{166,167}

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The DFT relates the states of minimum energy of a set of electrons under the influence of an external electric field (Coulombic) as a function of the electronic density of the system. The wave function of the fundamental state of a system and consequently the properties of this state are, in the DFT, functions of the electronic density, an observable.

The DFT is grounded on the Hohenberg-Kohn theorem; it considers that the electronic energy of the fundamental state of a non-degenerate system is completely and uniquely determined by its electronic probability density, $\rho(r)$. Thus, the electronic energy of the ground state is a functional defined by:

$$E = E[\rho(r)] \quad (64)$$

Similar to the other quantum chemistry methods, in DFT the functional energy can be described using three functional:

$$E(\rho) = T(\rho) + V_{ne}(\rho) + V_{ee}(\rho) \quad (65)$$

where $T(\rho)$ term is the kinetic energy of non-interacting electron, $V_{ne}(\rho)$ is the term of correlation between nuclei and electrons, and $V_{ee}(\rho)$ is the term of interelectronic repulsion.

The equation 64 can be interpreted in terms of a not known functional $F(\rho)$, which is independent of the external potential applied

$$E(\rho) = \int \rho(r)r(r)dr + F(\rho)$$

$$\text{where, } F(\rho) = T(\rho) + V_{ee}(\rho) \quad (66)$$

In the term $V_{ee}(\rho)$ are implied two contributions: the classical Coulomb interaction $V_H(\rho)$, and the exchange correlation functional, $V_{xc}(\rho)$.^{166,167} The problem of the DFT theory is that although the exchange and correlation potential is unique, it has not been explicitly defined. The election of the functional that will be used in the solution of the equation 66 is what differ each DFT methods.

Hohenberg and Kohn developed a variational theorem that in conjunction with the approach proposed by Kohn and Sham become the DFT applicable to problems of chemical interest. The Hohenberg and Kohn's variational theory proposes to apply a proof density function that satisfies the condition:

$$\int \rho_{pr}(r) = n \text{ and also } \rho_{pr}(r) \geq 0 \quad (67)$$

Thus, for each r is valid to state:

$$E_0 = E_v(\rho) \quad (68)$$

Where, ρ is the real electronic density in the fundamental state of the system that minimizes the energy functional of Equation 68 as a function of ρ_{pr} .

In this way, using the Hohenberg-Kohn variational theorem is possible to obtain the energy of a system by rewriting the Equation 66:

$$\begin{aligned} T(\rho_{pr}) + V_{ee}(\rho_{pr}) + \int \rho_{pr} v(r) dr &\geq E_v(\rho) \\ \rightarrow E_v(\rho_{pr}) &\geq E_v(\rho) \end{aligned} \quad (69)$$

Equation 69 demonstrates that none electronic density of proof can generate ground state energy greater-than the true electronic density of the ground state. Notwithstanding, this theorem allows calculating all the properties of a molecular system from ρ in the ground state without to know the wave function of this system.

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The exact expression of the molecular electron energy of Kohn-Sham is given by:

$$E(\rho) = V_{Ne} + T_s(\rho) + J + V_{NN} + E_{XC}(\rho) \quad (70)$$

Choosing a good functional exchange-correlation energy allows to perform DFT calculations with greater accuracy.

In order to increase the accuracy and reliability of the calculated values for properties and energies of molecular systems several exchange-correlation functional have been developed and proposed. Among the exchange-correlation functionals highlight the local density approximation (LDA), local spin density approximation (LSDA).

Other well-known functional are: the generalized gradient approximation (GGA). In this type of approach developed by Becke, Perdew and Wang were proposed the gradient-corrected ex-change (PWx86), (PWx91), and Becke (B88) functionals. Others GGA derivatives functionals as gradient-corrected correlation are proposed, and the most commonly used are Becke (Bc95), Lee-Yang-Parr (LYP) and Perdew (Pc86).^{150,168,169}

The most common functionals used for electronic structure calculations are hybrid functionals, where a GGA exchange functional can be associated with any GGA correlation functional. The most common are the BLYP hybrid functionals (B88 and LYP functional association) and the most popular is the hybrid functional B3LYP.¹⁵⁰

3.7 Principal component Analysis

Searching for the pharmacophoric profile based on the concepts of chemical and structural contribution of the receptor active sites, as well as receptor ligand interactions, are fundamental for the development of new potential drugs for several

different diseases and body dysfunction such as degenerative brains disorders, Alzheimer's disease, Parkinson's, diabetes Mielittus, cancer and many others known sickness conditions.^{138,170}

In modern computational chemistry, pharmacophores are used to define the essential features of one or more molecules with the same biological activity. In the past few years, it has been common that the drug discovery has a contribution from pharmacophore modeling to identify and develop new potential molecules with desired biological effect.¹⁷¹

In the study of chemical systems there are many variables, of a diversified nature, from properties external to this system to the properties intrinsic to it. However, not all the variables obtained during the study and in the calculations of molecular systems are relevant to represent this system. Generally, the choice of important variables that can represent a chemical system is based on the experience or the chemical intuition, subjective criteria that can neglect important variables.^{172,173}

With the purpose of treating systems with many variables, statistical techniques are applied to the studies of chemical systems to select in a proper way the main properties that represent it. Multivariate analysis techniques, like the Principal Component Analysis (PCA), are employed in chemometrics studies to analyze data sets containing several variables.^{172,174,175} The PCA is an exploratory multivariate data technique, introduced in 1901 by Karl Pearson, later substantiated in Harold Hotelling's 1933 article.¹⁷⁶

PCA method has four main aims:¹⁷⁵

- i) extract the most important information from a set of data;
- ii) decrease variable sets in the problems of multivariate data;
- iii) select the best properties (linearly independents) that describe the system (principal components);
- iv) analyze the structure of the observations and the variables of the system.

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According to Jolliffe¹⁷⁷ the central idea of PCA analysis “*is to reduce the dimensionality of a data set in which there are a large number of interrelated variables, while retaining as much as possible of the variation present in the data set*”. This reduction is achieved by transforming to a new set of variables, the principal components, which are uncorrelated, and which are ordered so that the first few retain most of the variation present in all the original variables. Computation of “*the principal components reduces to the solution of an eigenvalue-eigenvector problem for a positive-semidefinite symmetric matrix*”.¹⁷⁷

The main objective of PCA is to explain the variance and covariance structure of any vector, composed of random p-variables by means of the linear combination of the original n-variables, called principal components (PCs).¹⁷⁸

The PCA study is capable to obtain from a very large number of properties a smaller set of variables that represents the main contributions. In other words, it allows reducing the original space of the variables, keeping all the relevant information of the studied system.^{172,175,177}

Article 1

4. Binding Analysis of Some Classical Acetylcholinesterase Inhibitors: Insights for a Rational Design Using Free Energy Perturbation Method Calculations with QM/MM MD Simulations.

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*“Confía en tí mismo y defiende tus ideales.
Dios nunca te abandona.” (Santa Rita da Cascia)*

Binding Analysis of Some Classical Acetylcholinesterase Inhibitors: Insights for a Rational Design Using Free Energy Perturbation Method Calculations with QM/MM MD Simulations

Érica C. M. Nascimento,^{*,†,‡,§} Mónica Oliva,[†] Katarzyna Świderek,^{†,§} João B. L. Martins,[‡] and Juan Andrés^{*,†}

[†]Department of Analytical and Physical Chemistry, Jaume I University, 12071 Castellón, Spain

[‡]Institute of Chemistry, University of Brasília, 70910-000, Brasília-DF, Brazil

[§]Institute of Applied Radiation Chemistry, Lodz University of Technology, 90-924 Lodz, Poland

Supporting Information

ABSTRACT: In the present study, the binding free energy of some classical inhibitors (DMT, DNP, GNT, HUP, THA) with acetylcholinesterase (AChE) is calculated by means of the free energy perturbation (FEP) method based on hybrid quantum mechanics and molecular mechanics (QM/MM) potentials. The results highlight the key role of the van der Waals interaction for the inhibition process, since the contribution of this term to the binding free energy is almost as decisive as the electrostatic one. The analysis of the geometrical parameters and the interaction energy per residue along the QM/MM molecular dynamics (MD) simulations highlights the most relevant interactions in the different AChE–ligand systems, showing that the charged residues with a more prominent contribution to the interaction energy are Asp72 and Glu199, although the relative importance depends on the molecular size of the ligand. A correlation between the binding free energy and the number of cation– π interactions present in the systems has been established, DMT being the most potent inhibitor, capable of forming four cation– π interactions. A layer of water molecules surrounding the inhibitors has been observed, which act as bridges along a network formed by the ligands and the residues of the gorge and also between different residues. Although several hydrogen bonds between ligands and AChE do appear, no significant values of BIEs have been recorded. This behavior can be accounted for by the special features of AChE, such as the presence of several subsites of different natures in the gorge or the existence of several water molecules that act as bridges in the electrostatic interactions.

INTRODUCTION

Alzheimer's disease (AD) is currently the most frequent form of dementia in the elderly. The most commonly used strategies to treat AD are focused on hindering the hydrolysis of acetylcholine (ACh) in cholinergic neurons and, as a result, preventing synaptic depression. This strategy is called the cholinergic hypothesis¹ and nowadays is the one used in the development of drugs capable of promoting chemical equilibrium in the gradient of the concentration of ACh in patients with AD.^{2–4} Acetylcholinesterase (AChE) plays a key role in ACh hydrolysis in the nervous system.^{5,6} The hydrolysis of ACh is prevented by blocking the functions of AChE. For this purpose several AChE inhibitors (AChEIs) are used in the treatment of Alzheimer's disease. Among them tacrine^{1,7} (THA) was the first drug approved by the U.S. Food and Drug Administration (FDA), followed by donepezil⁸ (DNP), rivastigmine,^{1,7} and galantamine (GNT).⁸ These drugs are indicated for treating mild and moderate stages of AD, when the patient still enjoys independent cognitive activity. Other drugs, such as physostigmine, have been clinically studied and tested for use in the treatment of AD.^{7–9} Some drugs, like

huperzine A (HUP),^{10,11} metrifonate,⁸ and phenserine^{7,8} are promising candidates which are currently in the testing and approval phases. Recently, different classes of molecules that are dual-target AChEIs have been studied, for instance bis(7)-tacrine (DMT), composed of two tacrine units linked by a (CH₂)₇ chain, is a selective AChE that strongly inhibits AChE and also interacts with nicotinic cholinergic receptors (nACh receptors).^{5,12,13} Other dual-bind inhibitors are the huperzines:^{14,15} a class of tacrine–huperzine A hybrid acting as a potent and selective AChEI molecule.¹⁵ Interestingly, in addition to AChE inhibition, the dual-target AChEIs also have a neuroprotective function and thus act more efficiently in the treatment of AD.^{15–20}

Although AChEIs present common inhibition properties, the nature of their chemical structure and interaction are different, as shown in Figure 1. Generally, the AChEIs are divided into two categories depending on the type of interaction they form with the active site of AChE, called gorge. The first group of

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Figure 1. 2D structure of some AChEIs molecules.

molecules, displayed at the top of Figure 1, represents species that bind to the active site and inhibit its function using only nonbonding interactions. DMT, DNP, HUP, GNT, and THA all belong to this group. The second type of inhibitors, such as rivastigmine, dichlorvos, and phenserine (shown at the bottom of Figure 1), always form covalent bonds with the Ser200 residue of AChE.

The gorge (see Figure 2) presents a depth of 20 Å and is composed of four subsites.²¹ The first subsite, located at the

Figure 2. 2D schematic representation of the AChE active site (gorge).

entrance of the gorge, is called the peripheral site, while the second subsite is the quaternary ammonium binding site represented by residues Tyr70, Asp72, Trp84, and Phe330. Both subsites are composed of several aromatic residues, and the whole area is called the peripheral aromatic site (PAS, residues in blue in Figure 2). According to Dvir et al.,²¹ since the gorge has a small amount of acidic residues and a huge quantity of aromatic residues, there is an increase in hydrophobicity in this region, consequently loading a high effective local charge to induce the natural substrate, ACh, to enter inside the active site of AChE.^{21,22} The other two subsites of AChE are located deeper in the cavity: the anionic subsite (oxyanion hole) described by residues in green, and the catalytic subsite composed of the catalytic triad of residues

Ser200–Glu327–His440, where the hydrolysis of ACh occurs. These two subsites are known as the catalytic acylation site (CAS).

In all crystallographic structures of AChE available in the Protein Data Bank, a certain number of water molecules are detected inside the gorge. These water molecules play a key function in the catalysis, substrate and inhibitors displacement, and the inhibition process of AChE.^{23–25} According to Henschman and co-workers, the number of water molecules in the gorge is not constant and is within the range of 16 to 22 molecules.²⁴ Water molecules at the active site are capable of forming networks of hydrogen bonds that lead to some strong binding interactions between AChE residues and inhibitors or natural substrate.²⁵ During the process in which the ligand enters into the gorge, the flux and reorganization of these water molecules facilitate their diffusion.²⁵ The fluctuation of the water molecules in the gorge also contributes to the size-selectivity of the ligand binding in AChE.²⁴

Reliable prediction of protein–ligand binding energies is one of the great challenges in the field of computer-aided drug design and accurate predictions could help accelerate drug discovery efforts. In order to guide a rational design of AChEIs, two different cases are considered. In the first, the focus is on molecules that are able to form covalent bonds with the residue Ser200, through reversible or pseudo irreversible interactions. In the second approach, new proposed inhibitors should be able to interact with PAS or CAS subsites of AChE in such a way as to block the entrance of ACh into the gorge.^{4,17,26}

Accurately computing the free energy of ligand binding to a protein is crucial to be able to design and obtain the extraordinary physical and chemical properties of biological systems with diverse applications.^{27–30} Due to the key role of AChE inhibitors throughout the treatment of AD, several theoretical procedures have been used to calculate the binding energy of different ligands: molecular dynamics–linear response (MD/LR),^{31,32} the molecular mechanics Poisson–Boltzmann/solvent accessible (MM-PB/SA) protocol,^{4,31,33} the solvent interaction energy (SIE) technique,^{34,35} which is a variant of the MM-PB/SA method, and the free-energy thermodynamic integration (TI)^{15,31,36,37} method. The TI approach estimates the differences in binding free energies

for related compounds, but only one pair of inhibitors are compared in a single calculation. Conversely, less demanding computational methods, such as MM-PB/SA, are not suitable for discriminating compounds with a very similar inhibitory potency.

Here we present a study, grounded on molecular dynamics (MD) simulations within hybrid quantum mechanics/molecular mechanics (QM/MM) potentials, aimed at gaining an understanding of the binding process of some classical inhibitors (DMT, DNP, GNT, HUP, THA) that interact with AChE blocking the gorge. The alchemical free energy perturbation (FEP) method is employed to estimate the binding free energy of these ligands. FEP using MD sampling is among the most suitable approaches to achieve accurate binding free energy predictions, due to the rigorous statistical framework of the methodology, correct representation of the energetics, and thorough treatment of the important degrees of freedom in the system (including explicit waters). This method is based on a rigorous and formal theoretical framework and represents a good compromise between computer resources and quality of results.^{30,38} Since ligands are described quantum mechanically, errors in the choice of force field parameters are avoided and ligand polarization upon binding is included. This strategy has a potential high-value application in computer-aided drug design to provide an accurate prediction of protein–ligand binding affinities. Finally, binding isotope effects³⁹ (BIE) have been calculated to analyze interactions between ligands and the gorge of AChE.

The article is structured in the following way: the **Concepts and Methods** section carries a very brief theoretical background, where the alchemical FEP method is discussed and the protocol to be used to calculate the interactions energy by residue and binding isotope effects is explained. The **Materials and Protocols** section contains a discussion of the system setup, together with the protocols used throughout the QM/MM MD simulations. In the next section, the results are presented and discussed. Finally, in the **Conclusions** section, we have summarized the main results of all our work.

■ CONCEPTS AND METHODS

Alchemical Free Energy Perturbation Method. In this work, a series of QM/MM MD simulations are carried out at the gorge site and in a box of water molecules in order to evaluate the binding free energy of AChE–ligand complexes by means of alchemical FEP. The starting structures in FEP calculations were the equilibrated AChE–ligand after 1 ns QM/MM MD simulations and the water–ligand after 300 ps QM/MM MD simulations.

To compute the free-energy difference from the alchemical FEP method, two parameters λ and γ were introduced into the electrostatic and van der Waals QM/MM interaction terms in the total energy, as shown in eq 1.

$$E_{\text{QM/MM}}(\lambda, \gamma) = \langle \Psi | \hat{H}_0 | \Psi \rangle + \lambda \left(\sum \left\langle \Psi \left| \frac{q_{\text{MM}}}{r_{e,\text{MM}}} \right| \Psi \right\rangle + \sum \sum \frac{Z_{\text{QM}} q_{\text{MM}}}{r_{\text{QM,MM}}} \right) + \gamma E_{\text{QM/MM}}^{\text{vdW}} + E_{\text{MM}} \quad (1)$$

In this scheme, the λ changes smoothly between the values of 1 and 0. The parameter λ equal to 1 corresponds to a full MM charge–wave function interaction, and when λ is equal to 0 it represents no electrostatic interaction with the force field. The γ values are also comprised between 1 and 0 (once the electrostatic charges of the ligand in the QM region are annihilated); when it is equal to 1 it corresponds to a full QM/MM van der Waals interactions, while the value equal to 0 refers to no van der Waals interaction with the force field.³⁸

The calculation of free-energy differences of two consecutive windows between two states and the annihilation of charges and the van der Waals parameters are performed through FEP method. The total free energy variation is taken by the sum of all windows covering the full transformation from the initial to the final states. This procedure is performed in two steps, as represented in eqs 2 and 3:³⁸

$$\Delta G_{\text{QM/MM}}^{\text{elect}} = -\frac{1}{\beta} \left[\sum \ln \langle e^{-\beta[E(\lambda_{i+1}) - E(\lambda_i)]} \rangle_{\lambda_i} \right]_{\gamma=1} \quad (2)$$

and

$$\Delta G_{\text{QM/MM}}^{\text{vdW}} = -\frac{1}{\beta} \left[\sum \ln \langle e^{-\beta[E(\gamma_{i+1}) - E(\gamma_i)]} \rangle_{\gamma_i} \right]_{\lambda=0} \quad (3)$$

The thermodynamic cycle used to compute the binding free energy of enzyme inhibitors by alchemical FEP methods is presented in Figure 3: where $(L-E)_w$ is the enzyme $(E)_w$ and

Figure 3. Thermodynamic cycle from alchemical FEP methods.

inhibitor $(L)_w$ complex in solution, $(E')_w$ is the apo form of the enzyme, and $(L)_o$ is the inhibitor in the gas phase. The binding free energy of a ligand was calculated according to the scheme shown in Figure 3, and the predicted value was compared with the experimental data (using the inhibition constants K_i). The ΔG can be calculated by applying eqs 2 and 3, in water (W) and in the enzyme (E), so the free energy is expressed as

$$\Delta G = \Delta G_E - \Delta G_W \quad (4)$$

In practice, this calculation is performed in two steps yielding the electrostatic and van der Waals terms, ΔG_{elec} and ΔG_{vdW} . Finally, the total binding free energy is obtained by adding the two terms:

$$\Delta G_{\text{bind}} = \Delta G_{\text{elec}} + \Delta G_{\text{vdW}} \quad (5)$$

A total of 50 windows were used to evaluate the electrostatic interaction energy term, from $\lambda = 0$ (no electrostatic interaction) to $\lambda = 1$ (full interaction), in steps $(\delta\lambda)$ of 0.02. The same number of windows were used to calculate the van der Waals energy interaction term, from $\gamma = 0$ (no interaction) to $\gamma = 1$ (full interaction) in steps $(\delta\gamma)$ of 0.02.³⁸ In order to compute and analyze the consistence of the free binding energy of interaction values, a total of 10 ps of relaxation followed by 10, 20, 50, and 100 ps of production of QM/MM MD

simulation were performed using the NVT ensemble at the reference temperature of 300 K.

Interaction Energy per Residue. The main interest in studying the AChE–inhibitor system is the rational design of drugs capable of blocking the enzyme activity in the hydrolysis of acetylcholine in cholinergic neurons. Thus, knowledge about the single contribution of each residue of the enzyme to the interaction energy between the inhibitor and the environment can shed light on the process. To calculate the interaction energy, the difference between the QM/MM energy and the energies of the noninteracting QM and MM subsystems has to be computed for the same system geometry. The interaction energy was obtained by decomposition of the interaction energy per residue. In this case, the residues (i) of interest were turned on and off, thus obtaining the contribution of each of them to the interaction energy as expressed in eq 6:

$$\begin{aligned} E_{\text{QM/MM},i}^{\text{Int}} &= E_{\text{QM/MM},i}^{\text{elect}} + E_{\text{QM/MM},i}^{\text{vdW}} \\ &= \sum_{\text{MM} \in i} \left[\left\langle \Psi \left| \frac{q_{\text{MM}}}{r_{e,\text{MM}}} \right| \Psi \right\rangle + \sum_{\text{QM}} \frac{Z_{\text{QM}} q_{\text{MM}}}{r_{\text{QM},\text{MM}}} \right] \\ &\quad + \sum \sum 4e_{\text{QM},\text{MM}} \left[\left(\frac{\sigma_{\text{QM},\text{MM}}}{r_{\text{QM},\text{MM}}} \right)^{12} - \left(\frac{\sigma_{\text{QM},\text{MM}}}{r_{\text{QM},\text{MM}}} \right)^6 \right] \quad (6) \end{aligned}$$

Structures obtained from the QM/MM MD simulation trajectories over the last 500 ps of the 1 ns dynamics simulations were used to calculate the average interaction energy per residue.

Binding Isotope Effects (BIEs). Binding isotope effects (BIEs)³⁹ can be a useful tool to identify important nonbonding interactions occurring during the binding of the inhibitor to the active site. Changes in normal-mode force constants surrounding the isotopic substitution between free (usually dissolved in water) and bound inhibitors give rise to a binding preference for a light or a heavy isotope in the bound state and hence a measurable BIE.⁴⁰

Considering the binding process of an inhibitor, from solution to the formation of the inhibitor–enzyme complex, BIE would be defined as the ratio between the binding equilibrium constant of the light (L), or naturally abundant, isotope species and the heavy (H), or isotopically substituted, one:

$$\text{BIE} = \frac{K_L}{K_H} \quad (7)$$

BIEs can also be expressed in terms of the binding free energy of the light and heavy species, considering the relationship between the equilibrium constant and the binding free energy:

$$\text{BIE} = e^{-(\Delta G_L + \Delta G_H)/RT} \quad (8)$$

where ΔG_L and ΔG_H are the binding free energies for the light and heavy isotopomers, respectively, R is the universal gas constant, and T is the temperature.

$$G_i = U_i - RT \ln Q_i + \text{ZPE}_i \quad (9)$$

$$\begin{aligned} \Delta G &= G_e - G_w \\ &= (U_e - U_w) + (-RT \ln Q_e + RT \ln Q_w) \\ &\quad + (\text{ZPE}_e - \text{ZPE}_w) \\ &= \Delta U + RT \ln \frac{Q_w}{Q_e} + \Delta \text{ZPE} \quad (10) \end{aligned}$$

$$\text{BIE} = \frac{\left(\frac{Q_e}{Q_w} \right)_L}{\left(\frac{Q_e}{Q_w} \right)_H} e^{-1/RT(\Delta \text{ZPE}_L - \Delta \text{ZPE}_H)} \quad (11)$$

In this study, 11 structures of each inhibitor (ACh, DMT, DNP, GNT, HUP, and THA), bound in the active site of AChE and dissolved in aqueous solution, were selected in order to calculate BIEs. Those structures were selected from the last 200 ps of our QM/MM MD simulations.

MATERIALS AND PROTOCOLS

System Setup. The starting geometries for QM/MM MD calculations were obtained from X-ray structures of the complex of *Torpedo californica* AChE (TcAChE) with acetylthiocholine as the ligand available in the Protein Data Bank (PDB code 2CSG). This structure was chosen for the calculations, because it is the one with the best crystallographic resolution (1.95 Å) available in the PDB data bank for TcAChE inhibitors. This PDB file is the one with the fewest missing residues and atoms, specifically only the three initial residues (Asp1, Asp2, His3) and the last one (Ala536). The structure (comprising the protein and structural water molecules) to be used as a reference for the construction of the protein–inhibitor complex systems was obtained after removing acetylthiocholine, PEG, and NAG molecules in the 2CSG PDB file.

The optimum position coordinates of five protonated AChE inhibitors and natural substrate (acetylcholine) were put in the gorge. They were obtained by superimposing the AChE structure (modified 2CSG) with X-ray structures of the complex, between the protein and each corresponding ligand, namely: bis(7)-Tacrine from the structure with PDB ID 2CKM, donepezil from the structure of PDB ID 1EVE, galantamine from the structure of PDB ID 1DX6, (–)-huperzine A from the structure of PDB ID 1VOT, tacrine with PDB ID 1ACJ, and acetylcholine with PDB ID 2ACE. All structural water molecules located inside the gorge were kept with the same coordinates as found in the corresponding inhibitor structure of the original X-ray. The coordinates of residues Phe330, Trp279, and Tyr70 were replaced in the new coordinate systems according to the original X-ray inhibitor structures. Moreover, the coordinates of some residues of the active site were adjusted according to the corresponding inhibitor structures, especially the ones involving a flip in the oxyanion hole residues on the huperzine A complex. Superimposition was performed with the DaliLite program.⁴¹ The RMSD range of values was between 0.18 Å (to THA) and 0.34 Å (to HUP), and thus, the structures obtained after superimposition were assumed to be suitable for this study.

Since the standard pK_a values can be modified by the local effects of the protein environment,⁴² the protonation state of all titratable residues at pH = 7.0 has been characterized for AChE–inhibitor systems using the programs Propka 3.1⁴³ and also the “cluster method”,⁴² implemented by Field and co-

workers (personal communication). The results indicate that the protonation states of most residues remain unchanged. The results also present variations in the state of protonation of residues Glu199, His440, Glu443, and Glu445 when comparing both methods and the literature. According to Wlodek and co-workers,³⁶ there are probably two lowest energy states to the AChE–THA complex, where residues His440 and Glu443 can assume protonated and nonprotonated states. The predictions put forward by Propka and the cluster method reproduced both states. However, Wlodek found that the protonation state yielding the lowest energy in pK_a calculation presents unfavorable interactions in the AChE–tacrine system due to electrostatic effects.

In order to clarify the uncertainty regarding the protonation state, some brief and preliminary binding energy calculations were performed. The results indicate that the tritable state yielding the most favorable binding energy involves nonprotonated His440 and Glu199, and protonated Asp392, Glu443, and His471. These results are in agreement with Wlodek and co-workers.³⁶

Hydrogen atoms were incorporated into the structure, and the energy minimization of these atoms was performed as implemented in the fDynamo library. Subsequently the system was neutralized by adding sodium counterions in optimal electrostatic positions around the protein (never closer than 10.5 Å to any atom and not less than 5 Å from another counterion, using a regular grid of 0.5 Å). The number of counterions added to the systems was a minimum of 4 and a maximum of 5 sodium atoms, depending on the inhibitor.

Afterward, the system was placed inside a previously relaxed box of water (100 Å × 100 Å × 100 Å). All water molecules within a distance of 2.8 Å from the protein were removed.

In order to work with such large enzyme molecular systems, the hybrid QM/MM potential was used, where a small part of the system, the ligand, was described at the quantum level using the semiempirical AM1⁴⁴ method, while the protein and the solvent were represented by a classical OPLS-AA⁴⁵ and TIP3P⁴⁶ force field, respectively. All procedures used were implemented in the fDynamo library,^{47,48} with modifications made by our group.^{38,49,50}

After energy minimization of the system, Langevin–Verlet (NVT) MD simulations were carried out, with QM/MM MD simulations conducted at the AM1/MM level with a bath temperature of 300 K and a collision frequency for each atom of 100 ps. The time step was 1 fs. Dynamics and structures for later analyses were stored every 100 steps. In order to reduce the computational time, 300 ps of QM/MM MD simulation, considering all atoms as being free, were followed by a 1 ns QM/MM MD simulation, where part of the system was frozen. In this case, all residues located at a distance greater than 25 Å from the OG atom of the residue Ser200 were frozen, ensuring that all residues of the gorge were within a mobile sphere. As stated before, the QM region comprises the substrate or inhibitor molecule and the MM part contains all the protein residues, water (crystallographic and solvent) and the counterions. The cutoffs applied to nonbonded interactions by a switch function were $r_{\text{on}} = 14$ Å and $r_{\text{off}} = 16$ Å. Periodic boundary conditions were applied to the systems.

Inhibitor–solvent systems were also required to compute the AChE–ligands binding free energy, and thus several systems were built by placing each inhibitor in the prerelaxed box of water. AM1/MM MD simulations for the inhibitor–solvent system were also conducted taking into account two steps. The

first step of the inhibitor–solvent system simulation was performed considering all atoms to be free for 100 ps. Subsequently, the second step was carried out with part of the system frozen. Thus, all water molecules located at a distance greater than 25 Å from the most central heteroatom of each inhibitor were frozen and the equilibration simulation time was 300 ps.

RESULTS AND DISCUSSION

Geometric Parameters. In order to understand the influence of the geometry on the inhibition process of AChE, the average value of the important distances, angles, and dihedral measured during the trajectories of QM/MM MD simulations in each AChE–ligand system will be discussed. The selected geometrical parameters are reported as the average of the last 500 ps structures from the 1 ns QM/MM MD simulation trajectories, once the system was equilibrated. The interactions between the main residues of the gorge and the ligands are reported below.

Here, we also interpret the key role of some water molecules of the gorge that interact directly with the ligands. These water molecules, in some cases, act as a bridge in important interactions between ligands and the protein active site.

ACh. The molecular recognition of ACh by AChE is highlighted in the literature, where the quaternary nitrogen of the neurotransmitter has a strong electrostatic interaction with the carboxyl group of residues Asp72 (recognition residue) and Glu199.¹⁰ Glu199 is cited as an important residue that is directly involved in ACh hydrolysis. The main function of this residue seems to be related with the ordering and conformation of water molecules in the gorge, acting as a tap which opens and closes the traffic of water molecules in the catalytic and inhibition processes of AChE.^{24,51} According to Harel et al., residues Trp84, Glu199, and Phe330 generate a concave binding site that makes a close fit with the ACh quaternary ammonium group, interacting through cation– π interaction.⁵² It is also reported that residues Trp84 and Phe330 have an auxiliary function in the stabilization of the Me_3N^+ charge due to the cation– π interaction with the choline group of ACh.⁵¹

Figure 4 shows the structure of the Michaelis complex obtained after 1 ns QM/MM MD simulations to relax the AChE–ACh system and depicts some of the average distances. Here, the COO^- group of the residue Glu199 (represented in

Figure 4. Michaelis complex structure of AChE–ACh. Some selected average distances in angstroms are depicted.

CPK) is directed to the charged center of the Me_3N^+ group, acting as a counterion to stabilize the positive charge of ACh.

Average distances between the C10 atom of ACh, belonging to one of the methyl groups from Me_3N^+ close to the condensed aromatic rings of residue Trp84, indicate the formation of cation- π interaction between these groups. The evolution of these distances is reported in the [Supporting Information](#). Distances between the aromatic ring of Phe330 and the C21 atom of the other methyl group are similar but longer because phenylalanine forms less effective cation- π interactions than tryptophan.⁵³

THA. Studies of molecular dynamics between AChE and THA^{36,54} have shown that the inhibitor is recognized in the gorge but in different subsites, more precisely: in the anionic site through hydrophobic interactions; in the peripheral site of the enzyme⁵⁴ by interactions as “weak” van der Waals forces and charge transfer by cation- π ; and in the catalytic site by hydrogen bonds with the residue His440 and sandwich stacking-type interactions with residues Trp84 and Phe330, specifically in the formation of a charge transfer complex with Trp84.⁵⁴

An analysis of the results presented in [Figure 5](#) shows that the most significant favorable interactions take place between

Figure 5. AChE-THA main interactions.

THA and residues Trp84, Glu199, Phe330, and His440 and some structural water molecules. THA presents a very strong stable hydrogen bond between positively charged regions located in the pyridine ring and the main chain O:His440 atom (1.92 ± 0.14 Å). In the same region, THA forms a π - π and a cation- π charge transfer interaction with Trp84 rings. These two interactions are important for the stabilization of the charge of the inhibitor in the inhibition process.

A nonclassical C-H...O hydrogen bond between H22 and OE1:Glu199 (2.71 ± 0.18 Å) is highlighted. This residue is also implicated in the stabilization of the positive charge of THA, as a counterion in long-range interaction at the average distance (6.22 ± 0.22 Å).

The NH_2 group of THA establishes hydrogen bonds with two water molecules of the gorge (for given average values of $\text{H28}\cdots\text{OH2}$ [2.41 ± 0.21 Å] and $\text{H29}\cdots\text{OH2}$ [2.34 ± 0.27 Å], see [Figure 5](#)).

As shown in [Figure 5](#), THA has a parallel π -stacking interaction with residues Trp84 and Phe330, forming a sandwiched π - π stacking. The distance evolution of some atoms of the Trp84 pyridine ring and THA are reported in the

[Supporting Information](#). The average values of this range given throughout the simulation time vary between the $\text{C10}\cdots\text{CG}:\text{Trp84}$ (3.53 ± 0.16 Å) and $\text{C11}\cdots\text{CZ3}:\text{Trp84}$ (4.10 ± 0.20 Å). The short distance between $\text{N7}\cdots\text{CE2}:\text{Trp84}$ (3.97 ± 0.21 Å) indicates a strong-dependence cation- π charge transfer interaction between the protein and the inhibitor.

As represented in [Figure 5](#), the benzene ring of THA forms a π - π parallel stacking with Phe330 benzene ring atoms. The average range of values of these interactions is between $\text{C1}\cdots\text{CD1}:\text{Phe330}$ (3.49 ± 0.18 Å) and $\text{C4}\cdots\text{CE2}:\text{Phe330}$ (4.97 ± 0.49 Å).

The data presented in this work suggest that residues Trp84, Glu199, Phe330, and His440 make important contributions to the interaction between AChE and THA.

GNT. Galantamine is a selective, competitive, and reversible alkaloid used in the treatment of AD. GNT is an AChEI approved by the FDA with a better pharmacological profile and fewer side effects in the liver of the patients than THA.⁹

Greenblatt et al. obtained the X-ray crystallographic structure of AChE complexed with galantamine, where it is possible to observe how it interacts with the residue Ser200 mainly by hydrogen bonding interactions, and with the residue Trp84 by π - π interactions.⁵⁵ Phe330 and Phe331 are involved in the cation- π interactions between GNT nitrogen and the aromatic ring of the residues.

The residue Asp72 has an important function in AChE recognition and catalytic action by acting as a bracer to trap cationic-charged ligands at the entrance of the gorge.⁵⁶ As shown in [Figure 6](#), the CH_3HN^+ group of the ligand is directed

Figure 6. AChE-GNT main interactions.

to the negative resonance set of OD1/OD2 atoms of Asp72. According to Greenblatt et al. the *N*-methyl group C21 atom has a nonclassical hydrogen bond⁵⁵ interaction with OD2:Asp72 atom (3.69 ± 0.27 Å) and also with a structurally conserved water molecule of the gorge (3.14 ± 0.26 Å). These distance values are in agreement with Bartolucci and co-workers, who observed $\text{C21}\cdots\text{OD2}:\text{Asp72}$ at 3.6 Å and a W803 water molecule at 3.2 Å.⁵⁶

[Figure 6](#) shows the main interactions between GNT and Glu199. Geometrical analysis over the last 500 ps structures of the 1 ns QM/MM MD simulation reveals an important strong hydrogen bond between atom H39 of GNT and the OE1 and OE2 atoms of the residue Glu199, (1.79 ± 0.14 Å) and (3.00 ± 0.24 Å) respectively. As previously reported^{56,57} OE1:Glu199 and O20 GNT are interacting by a hydrogen bond at 2.72 Å, and this value is in complete agreement with the results found

in our QM/MM MD simulations (2.62 ± 0.10 Å). The negatively charged residue Glu199 also presents other important hydrogen bonds, the nonclassical h-bond with the nonpolar H25 hydrogen atom of GNT at (3.13 ± 0.19 Å) and also with a structural water molecule (2.10 ± 0.51 Å). All these interactions point to residue Glu199 being extremely important in the AChE–ligand inhibition process, as will be shown in the following sections.

GNT presents a kind of oxygen crown, that is, a region with high electronic density composed of O20 ~ O7 ~ O19 atoms. In this region important hydrogen bonds are formed between GNT and the AChE residues. The O20 atom of the inhibitor interacts with the H:Gly118 (3.27 ± 0.32 Å) atom and, as stated above, with Glu199. The O7 atom presents a strong h-bond with the catalytic residue His440 (2.63 ± 0.16 Å). As shown in Figure 6, there is a structural water molecule that forms special tetra-coordinated interactions by strong hydrogen bonding with all oxygen atoms of the GNT [O20 (2.19 ± 0.23 Å), O7 (2.87 ± 0.26 Å), and O19 (2.60 ± 0.28 Å)], and also with the catalytic residue OG:Ser200 (2.61 ± 0.21 Å). The strong interactions that take place in this area contribute significantly to the interaction between AChE and GNT, as is stated below.

There are interactions due to van der Waals forces between the cyclohexane ring of GNT and the indole ring of Trp84 through π -stacking interactions (see Figure 6). The main interactions between GNT and residues Phe330 and Phe331 take place by cation– π and π - π . As can be observed in Figure 6, the cation– π interaction between the inhibitor and Phe330 is intermediated by a water molecule. This water molecule has the same function as the terminal hydroxyl group of the PEG molecule present in the original PDB complex structure 1DX6).^{9,55} The interactions with residues Phe330 and Phe331 play an important key role in the orientation and stabilization of the cation ligand charge.

The bridge between GNT and residue Phe330, formed by a solvent water molecule, suggests that this interaction also leads to the inhibitory power of the ligand, as is reflected in the contribution of this residue to the interaction energy (see below). The distance of the interactions between this solvent water molecule and all the atoms of the Phe330 benzene ring is given within the range H1:HOH...CD1:Phe330 (2.88 ± 0.55 Å) and H1:HOH...CZ:Phe330 (3.59 ± 0.77 Å). This water molecule also forms a very strong hydrogen bond with the GNT H43 (1.91 ± 0.18 Å) atom.

The distances selected as functions of QM/MM MD time between GNT and some residues are reported in the Supporting Information.

HUP. MD theoretical studies conducted by Camps et al. suggested that the HUP amino group has cation– π interactions with the five-member ring of Trp84 and the phenyl ring of the residue Phe330.³³ In another study, it was noted that the carbonyl and NH groups of the inhibitor interact by hydrogen bonds with the OH group of the residue Tyr130.³¹ In the present work we confirmed all those interactions and other significant interactions in the inhibition of AChE by HUP.

As shown in Figure 7, residues Glu199 and Tyr130 play a crucial role in the inhibitory process, where the presence of a strong hydrogen bond with HUP is observed, while the aromatic residues Trp84 and Phe330 also play an important function in the binding interaction of the AChE–HUP system.

The interactions between the charged residues Glu199 and Asp72 are given by medium-length and long electrostatic

Figure 7. Main AChE–HUP interactions. Some average distances are depicted.

contacts with the positively charged region of the inhibitor, generally intermediated by structural water molecules.^{58,59} In the case of Asp72, the interaction with HUP is not direct. In fact it is intermediated by two water molecules of the gorge that form strong short hydrogen bonds between the residue and water that are no longer than (1.85 ± 0.10 Å). Residue Glu199 interacts with HUP directly and is also intermediated by a water molecule, as shown in Figure 7.

A very strong hydrogen bond was found between the HUP O2 atom and HH:Tyr130 (1.94 ± 0.13 Å). The OH:Tyr130 atom also forms hydrogen bonds with H19 of HUP (3.04 ± 0.20 Å) and with the H1 atom of a structural water molecule (2.15 ± 0.27 Å). This water molecule is shared by both residues, Tyr130 and Glu199, and also interacts directly with the H19 atom at (2.10 ± 0.23 Å) (see Figure 7).

A nonclassical hydrogen bond O...H–C11 is noted between the main chain O:His440 catalytic residue and the ethylidene methyl group of HUP.^{23,59} The average distance between O:His440...C11 is (3.06 ± 0.14 Å).

The aromatic residues Trp84 and Phe330 develop cation– π and π - π interactions with HUP.^{58,59} The evolution of the distances between the five-member ring atoms of Trp84 and the RNH_3^+ charged group of HUP are shown in the Supporting Information. The aromatic ring of the residue Phe330 interacts with the double bond of C11=C12 atoms of HUP (see the Supporting Information for the distance evolution along the QM/MM MD). According to Dvir and co-workers, these interactions are around 4.4 Å, while we found values between (3.70 ± 0.25 Å) and (4.13 ± 0.27 Å) for these interactions. HUP also interacts with residues Tyr70, Tyr121, and Phe331 (not shown).

DNP. According to the literature, the ligand DNP has its phenyl ring presenting a π -stacking interaction with residue Trp84.^{31,60,61} The indanone ring of DNP stacks against the indole ring of residue Trp279 by parallel π - π interactions.^{31,60} The DNP piperidine ring interacts with residues Tyr70, Tyr121, Phe330, and Tyr334. The interactions between the aromatic residues Phe330 and Trp279 are responsible for the selectivity and affinity between the enzyme and DNP.⁶¹ These data support the results obtained in our work.

It has also been reported^{1,10} that the distance between the O24:DNP and N14:DNP atoms is critical for the high inhibition of AChE, because it mimics the corresponding O...Me₃N⁺ intramolecular distance in ACh. In our work, we monitored this distance throughout the last 500 ps of the QM/

MM MD simulation, and the average value of O24:DNP...N14:DNP was (6.66 ± 0.16 Å).

The interaction between residue Asp72 and the inhibitor is due to the electrostatic charge counterion effect between N14 and OD1:Asp72 atoms (see shaded region in Figure 8).

Figure 8. Average AChE–DNP aromatic cage interactions.

Furthermore, this interaction is enhanced by the presence of structural water molecules, which form a bridge between the inhibitor and the residue. Asp72 has water molecules around the residue that interact by hydrogen bonding with DNP and Tyr334. In turn, the HH:Tyr334 atoms present a strong interaction with OD1:Asp72 residue (1.73 ± 0.15 Å).

The DNP benzyl ring, as shown in Figure 8, interacts directly with Trp84 as face-to-face (parallel) stacking interactions. The average distance between these two rings is (3.84 ± 0.24 Å), in agreement with the literature (3.6 – 3.83 Å).^{60,62}

The three aromatic residues Tyr121, Trp279, and Phe330 are responsible for stabilizing DNP inside the gorge. These residues interact with the ligand through cation– π and π – π forces providing sufficient energy to stabilize the AChE–DNP complex.^{51,63} The short distance between DNP and the residue Phe330 is the key to promoting a favorable cation– π interaction. The evolution of some selected distances between the inhibitor and these residues as a function of the QM/MM MD simulation time is reported in the Supporting Information. As depicted in Figure 8, a water molecule acts as a bridge in the interaction between Tyr121 and DNP.

According to the literature, DNP presents several hydrogen bonds with structural water molecules inside the gorge.^{31,60} As shown in Figure 8 and also reported in the Supporting Information, some structural water molecules are important in the interaction of AChE with DNP. The O24, O25, and O27 atoms of the methoxyl groups interact with water molecules by strong hydrogen bonds. We also note that all heteroatoms in DNP interact with structural water molecules by strong hydrogen bonding. These hydrogen bonds are responsible for the interaction among the aromatic residues Trp84, Tyr121,

Trp279, and Tyr334 of AChE and the nonaromatic rings of DNP.^{60,63,64}

DMT. Rydberg et al. studied DMT⁵ and its electronic density map experimentally by X-ray techniques. They concluded that this inhibitor interacts with the gorge by means of cation– π and π – π stacking interactions between its aromatic rings and the aromatic rings of residues Trp84 and Phe330 located inside the gorge. Furthermore, as DMT comprises two units of THA connected by a carbon chain with 7 units of CH_2 , in the other THA unit of DMT the same kind of interactions occur with residues Tyr70 and Trp279 at the entrance of the gorge.

DMT is the most potent inhibitor studied here. This ligand interacts with a huge number of gorge residues (Figure 9a and b). It also interacts with the catalytic residue His440 by a strong hydrogen bond involving the H79 DMT atom; here, the tacrine unit of DMT acts as a hydrogen donor to carbonyl oxygen of His440.

As shown in Figure 9a, two strong hydrogen bonds between the H79 atom and the main chain O:His440; and the H78 atom and OH_2 of the structural water residue $\text{H}_2\text{O}:871$ are present. The structural water $\text{H}_2\text{O}:871$ is linking the inhibitor and residue Glu278 through a hydrogen bond bridge $\text{Glu278:OE2}\cdots\text{H}_2\text{:HOH871}$. The DMT H63 atom interacts with a strong hydrogen bond with the OH_2 atom of the structural water molecule $\text{H}_2\text{O}:341$. This water also acts as a bridge between the inhibitor and the Asp72 residue $\text{OD1:Asp72}\cdots\text{H}_2\text{:HOH341} = 1.74 \pm 0.13$ Å. Another unusual hydrogen bond is observed between the nonpolar H58 atom of DMT and Glu199. The evolution of some selected distances along the QM/MM MD simulation are reported in the Supporting Information.

As observed in Rydberg's studies,⁵ DMT forms two cation– π interactions. These interactions were also observed in this work (see Figure 9b), and they occur between positively charged N^+ atoms of DMT (N35 and N37) and the indole ring of the aromatic residues Trp84 and Trp279. The residues Tyr70 and Phe330 also interact with DMT by cation– π and π – π interactions. Residues Trp84 and Phe330, as well as Trp279 and Tyr70, perform sandwich parallel stacking interactions, thus contributing to a stronger interaction in an instantaneous dipole moment between the negative charge density in the center of the aromatic ring of the residues and the positive charge of each tacrine unit of the inhibitor. The aromatic residue Tyr121 also interacts with DMT by T-stacking interactions. Distances between DMT and aromatic residues as a function of QM/MM MD simulation time are available in the Supporting Information.

An analysis of the results shows that for all inhibitors there are both short and long-range interactions with different residues of AChE. Another relevant fact is that these interactions are sometimes reinforced by gorge water molecules. The structural water molecules act as a leader in the interaction between the residues and the inhibitor.

Interaction Energy per Residue. The average interaction energy (IE) between the ligand and the environment was computed and evaluated per AChE residues. Structures obtained from 1 ns QM/MM MD simulation trajectories over the last 500 ps were used in the calculations, and the average IE for these structures is reported. Results for some target residues of the gorge involved in AChE–ligand interactions studied in this work are shown in Figures 10–13.

Negatively Charged Residues of the Gorge. Figure 10 shows the average IE of the negatively charged residues of the

(a)

(b)

Figure 9. (a) Average AChE-DMT charged residue interactions. (b) Average AChE-DMT aromatic residue interactions.

gorge: Asp72, Glu199, Glu278, and Glu327. These residues and His440 present the most significant favorable interactions among all the ligands studied and the AChE residues. Asp72 and Glu199 present the most favorable values of IE for all AChE–ligand systems. The highest IE value for Glu199 is observed in ACh ($-48.8 \pm 2.6 \text{ kcal}\cdot\text{mol}^{-1}$) and Asp72 at DMT ($-42.0 \pm 2.7 \text{ kcal}\cdot\text{mol}^{-1}$).

From Figure 10 it is possible to conclude that there are three patterns in the profile of IE per residue between the natural substrate and the inhibitors. The first pattern involves HUP,

Figure 10. Average IE of leading negatively charged residues in the AChE–ligand systems (all values are taken in kilocalories per mole).

THA, and GNT, which have the same energy profile as that found for ACh. This similarity may be related with the recognition between the specific residue Asp72 of the enzyme and the inhibitors. As commonly accepted in the literature systems, Asp72 is directly involved in ACh recognition. In the AChE–ACh system, the contribution of Asp72 is important but quantitatively not decisive. The contribution of Asp72 to the IE increases from THA to GNT. Figure 11 shows some selected geometrical parameters related to the distance between the positively charged nitrogen of the inhibitor and Asp72 and Glu199. It can be seen that the $\text{Asp72:OD}_x\cdots\text{N}^+$ distance is quite large to make a decisive contribution in the binding interaction, but it is not negligible. GNT represents an intermediate case, where the contribution of Asp72 becomes greater due to the shorter $\text{Asp72:OD}_x\cdots\text{N}^+$ distance, which promotes a more effective charge–charge interaction.

The second pattern occurs when the first pattern is disturbed, and more specifically when the inhibitor is quite a lot larger than the ACh, as occurs in DMT and DNP. In these two cases Asp72 presents a more significant contribution than Glu199. On analyzing the distances in Figure 11, this can seem contradictory in the case of DMT, but as can be seen in Figure 9a, the secondary amine group of this inhibitor interacts with Asp72 by hydrogen bonding.

The third pattern is related with the highest individual value of the interaction energy. The residue with the highest individual energy value always interacts with the inhibitor through hydrogen bonding or charge–charge interactions.

As is well-known, AChE has a large permanent dipole moment, pointing at the gorge, and thus, a positively charged molecule can easily enter in the gorge by electrostatic attractions.^{23,25} A particular and peculiar charge distribution, which implies a composed dipole relation, is observed in all AChE–ligand complexes (Figure 11). The positively charged N^+ atom of the molecules is pointing toward residues Glu199 and Asp72. As represented in Figure 11, an $[\text{OD}_x^-/\text{N}^+/\text{OE}_y^-]$ composed dipole is represented in a triangular relationship.

DMT properly mimics the ACh dipole (Michaelis complex model). This can be verified by the larger distance b in relation to c as well as the value of the angle α , whereas the THA dipole relation is quite similar to acylated AChE–ACh (ACh^*) due to the larger distance b in relation to c as well as the value of angle α . This can also be a good explanation for the fact that acridines interact with AChE, since this class of molecule is completely different from the natural substrate.

Other inhibitors present some differences in the dipole relations, since in DNP and GNT the distance c is larger than

Figure 11. Geometrical parameters in the relationship between the recognition residue Asp72 of AChE, charge-leading residue Glu199, and N⁺ atom of the ligand in AChE–ligand complex interactions. Average values from the last 500 ps of the QM/MM MD simulation. ACh* represents the acylated AChE–ACh complex.

b. HUP represents an intermediate situation due to the fact that distances *b* and *c* are almost equal.

Taking into account the IE results and comparing with the previous geometrical analysis, it can be concluded that the larger the inhibitor, the more prominent the individual contribution of Asp72 to IE. The same behavior is observed for residue Glu278 (see Figure 10). Some short-range interactions are observed between these residues and large inhibitors, thereby explaining the high contribution to IE.

As analyzed in the previous section, Glu199 is interacting by hydrogen bonding (directly or mediated by water molecules) with all the inhibitors, thus accounting for the significant individual contribution of this residue to the IE. In comparison with other systems, DNP is an exception to this rule, as reflected in the lower contribution of Glu199 in this set of inhibitors.

Catalytic Triad. The set up of the catalytic triad of AChE, composed of residues Ser200-Glu327-His440 (S200-E327-H440), is an important framework in the protein binding interaction.⁵ The hydrogen bonds established between the residues HG:Ser200...NE2:His440 (d1) and OE1:Glu327...HD21:His440 (d2) should be stabilized during the catalysis and inhibition of AChE. The distances d1 and d2 are represented in the Supporting Information.

Figure 12 presents the strong contribution of residues His440 and Glu327 in the IE with all ligands. As is well-known in the literature, there is a direct electrostatic interaction

Figure 12. Average interaction energy of catalytic triad residues in the AChE–ligand systems binding interactions (values in kilocalories per mole).

between His440 and THA (-12.4 ± 1.0 kcal mol⁻¹) and between His440 and DMT (-13.2 ± 1.3 kcal mol⁻¹) that stabilizes these ligands at the entrance of the CAS pocket of the AChE active site. The values of the energy of interaction between these two inhibitors are in the same range as the substrate value (-13.6 ± 1.7 kcal mol⁻¹). This shows us that this kind of inhibitor mimics the same energy profile for this residue.

The larger contribution of His440 in THA and DMT systems seems to be related to the strong hydrogen bond established between histidine and the charged nitrogen of the inhibitor (see Figures 5 and 9a). As reported in the previous sections, there are other interactions between this residue and GNT and HUP through hydrogen bonds. However, no direct interaction is observed in the DNP system, which reflects the lowest contribution to the IE. Nevertheless, in spite of the significant contribution of His440 to the IE in the ACh system, no direct interaction with ACh is observed.

Favorable interactions are observed between Glu327 and the inhibitors. This residue does not present direct interactions with the ligands, but its contribution to the IE is quite significant. Yet, the contribution of Glu327 to the IE is dependent on that of His440 and seems to be related to the strong interaction between the two residues. Therefore, the distance between OE1:Glu327 and HD21:His440 atoms seems to be a key geometrical parameter associated with the stabilization in the catalytic triad setup. In all AChE inhibitor systems this interaction is due to a very strong h-bond, with ranges of 1.53–1.63 Å (see the Supporting Information).

The catalytic residue Ser200 presents an unfavorable interaction with all ligands, including the natural substrate (Figure 12). A possible explanation for this unfavorable contribution is the strong interaction between His440 and the ligand, which seems to be disturbing the interaction between Ser200 and His440. The HG:Ser200...NE2:His440 distance presents values under 2.50 Å in all ligand systems, but the stronger the His440 individual energy interaction, the longer the d1 distance (see the Supporting Information).

Aromatic Residues of the Gorge. The high aromaticity of the gorge composition, the 14 conservative aromatic residues, plays a key role in the binding and recognition of ACh and other ligands.²³ Figure 13 shows the average interaction energies of some gorge aromatic residue obtained from large 1 ns QM/MM MD simulation trajectories over the last 500 ps. These residues contribute strongly to AChE–ligand system interactions through van der Waals forces, stacking, π – π , cation– π , and hydrogen bonds.

The cation– π interaction is an important kind of interaction in different systems, such as organic molecules, supramolecules,

Figure 13. (left) Average interaction energy of leading gorge aromatic residues in the AChE-ligand system (all values are shown in kilocalories per mole). (right) Indicative gorge aromatic residues involved in the cation- π interaction in the AChE-ligand system.

and proteins. Cation- π is influenced by intermolecular noncovalent forces, such as charge-quadrupole, charge-dipole, dispersion forces, and hydrophobic forces. Moreover, the electrostatic term contributes strongly to the cation- π , since it

interacts with the permanent quadrupole moment of the resonant structure of the aromatic ring.^{53,65,66} Tryptophan, tyrosine, and phenylalanine residues participate in a huge number of cation binding sites. Tryptophan is the most

Table 1. Terms of Electrostatic and van der Waals Interaction Free Energy in Protein–Ligand and Water–Ligand Based on QM/MM MD Simulations (kcal mol^{−1})

	Electrostatic							
	AChE–ligand ($\Delta G_{\text{elec-E}}$)				water–ligand ($\Delta G_{\text{elec-w}}$)			
	10 ps	20 ps	50 ps	100 ps	10 ps	20 ps	50 ps	100 ps
ACh	−46.03	na	na	−43.98	−27.70	na	na	−27.78
DMT	−81.71	−78.85	−77.98	−77.48	−41.57	−40.87	−40.71	−40.46
DNP	−58.38	−57.80	−54.95	−53.28	−29.83	−30.06	−29.42	−29.05
GNT	−52.60	−52.54	−51.90	−51.55	−32.17	−31.92	−31.38	−30.96
HUP	−61.36	−59.66	−58.72	−58.12	−41.87	−41.02	−41.29	−40.99
THA	−40.74	−41.43	−41.69	−40.64	−22.64	−22.74	−22.46	−22.32
	van der Waals							
	AChE–ligand ($\Delta G_{\text{vdW-E}}$)				water–ligand ($\Delta G_{\text{vdW-w}}$)			
	10 ps	20 ps	50 ps	100 ps	10 ps	20 ps	50 ps	100 ps
ACh	−26.00	na	na	−26.40	−15.98	na	na	−15.42
DMT	−64.63	−64.16	−64.63	−65.82	−40.04	−39.57	−40.54	−42.18
DNP	−51.66	−51.66	−51.74	−51.76	−33.92	−33.67	−33.88	−33.93
GNT	−39.68	−39.68	−40.28	−40.23	−24.44	−24.51	−24.37	−24.29
HUP	−39.38	−38.72	−39.1	−39.69	−18.56	−18.40	−19.14	−20.17
THA	−30.47	−29.82	−30.27	−30.47	−18.37	−18.01	−18.46	−18.71

prominent residue used by proteins in this kind of interaction. Tyrosine and phenylalanine are sequentially the other two residues participating in these interactions.⁵³

Interactions involving aromatic residues are an important point in AChE catalysis and inhibition.⁶⁷ The aromatic amino acids Trp84, Tyr121, and Phe330 are critical for the ACh binding acylation step, which is promoted through cation– π interaction between these aromatic residues and the quaternary ammonium cation of ACh. These cation– π interactions seem to support the stabilization of the positive charge of ACh.⁶⁷

As shown in Figures 10–13, Trp84, Glu199, Glu327, and His440 play an important role in molecular recognition and the total interaction energy in the AChE–ACh system. Figure 13 shows the most significant favorable interactions (negative values) that take place between ACh and the aromatic residues Trp84, Tyr130, Phe330, and Phe331.

The systems where the enzyme interacts with small molecules (size ~ 11 Å)⁶⁸ such as ACh, THA, HUP, and GNT present a similar pattern in the energy of interaction per residue. As can be observed in Figure 13, these ligands generally exhibit a strong favorable interaction with Trp84 and Phe330. These two residues are involved in the strong noncovalent binding force cation– π interaction in AChE–ligand systems. This interaction is responsible for the binding of ACh⁶⁹ and its hydrolysis by AChE. Trp279 and Tyr121 have also shown a significant favorable interaction with DMT and DNP, the largest molecules.

Figure 13 represents the most favorable THA interactions, in descending order: Trp84, Tyr130, Phe330, and Phe331. As stated above, the benzene ring of THA forms a sandwiched π – π stacking with residues Trp84 and Phe330. However, Trp84 leads the contribution to the IE due to the cation– π interaction established between them.

The most important favorable GNT interactions, in descending order, are Trp84, Phe330, Phe331, Tyr121, Tyr130, and Tyr70. As represented in Figure 6, the cyclohexane ring of GNT and the indole ring of Trp84 interact through π –stacking. Residues Phe330 and Phe331 establish cation– π and π – π interactions with GNT.

Figure 13 presents the most important favorable interactions of HUP with the aromatic gorge residues, which are, in descending order: Trp84, Tyr130, Phe330, Phe331, Tyr70, and Tyr121. As can also be seen in Figure 13, the interaction between HUP and residues Trp84 and Tyr130 are relevant in comparison with the other systems. Our results are in agreement with Kitisripanya⁵⁷ and co-workers, who predicted that most significant residues in terms of the contribution to AChE inhibition by HUP are Trp84 (-7.07 kcal mol^{−1}) and Tyr130 (-8.08 kcal mol^{−1}). The difference in quantitative values can be understood by considering that they used a small rigid molecular model with the inhibitor in the neutral form and a higher level of calculation.

In the AChE–HUP complex, the large favorable interactions between the inhibitor (at N15 cation) with the residue Trp84, and O2:HUP with the residue Tyr130 indicate the importance of the interaction of aromatic residues in the inhibitory process of AChE. These cation– π (Trp84) and h-bond (Tyr130) interactions are reported in the previous section, and highlighted in the literature as a strong indication of the interactions of this system.^{57–59,70}

In Figure 13 we can observe that Phe330, Tyr121, and Trp279 have a prominent influence on the affinity between AChE and DNP. As stated in the Geometric Parameters section, these residues interact with the ligand through cation– π forces. Trp84 also makes a noticeable contribution to the IE because the DNP benzyl ring forms a parallel stacking interaction with Trp84.

The prominent contribution of residues Trp84, Phe330, Trp279, and Tyr70 to the IE in the AChE–DMT system is a result of the cation– π interactions established between these residues and the inhibitor. As mentioned above, Trp84 and Phe330 as well as Trp279 and Tyr70 perform sandwiched parallel stacking interactions with each tacrine unit of DMT. The residue Tyr121 contributes to the IE due to a T-stacking interaction with DMT (see Figure 9).

Figure 13 also shows, schematically, the cation– π interactions between AChE and all the ligands studied here. DMT forms four cation– π interactions with gorge aromatic residues Trp84, Phe330, Trp279, and Tyr70. DNP forms three

Table 2. Electrostatic (ΔG_{elec}) and van der Waals (ΔG_{vdW}) Terms of the Water–Ligand ($_{\text{W}}$) and Protein–Ligand ($_{\text{E}}$) Interaction Free Energy and Differences in Interaction between the Binding Site of the Protein and Water (ΔG_{elec} , ΔG_{vdW}) and the Binding Free Energy (ΔG_{bind})^a

ligand	W(water)		E(enzyme)		ΔG		ΔG_{bind}
	$\Delta G_{\text{elec-W}}$	$\Delta G_{\text{vdW-W}}$	$\Delta G_{\text{elec-E}}$	$\Delta G_{\text{vdW-E}}$	ΔG_{elec}	ΔG_{vdW}	calculated
ACh	−27.78	−15.42	−43.98	−26.4	−16.2	−10.98	−27.18
DMT	−40.46	−42.18	−77.48	−65.82	−37.02	−23.64	−60.66
DNP	−29.05	−33.93	−53.28	−51.76	−24.23	−17.83	−42.06
GNT	−30.96	−24.29	−51.55	−40.23	−20.59	−15.94	−36.53
HUP	−40.99	−20.17	−58.12	−39.69	−17.13	−19.52	−36.65
THA	−22.32	−18.71	−40.64	−30.47	−18.32	−11.76	−30.08

^aBased on 100 ps FEP simulation, values in kilocalories per mole.

cation– π interactions with Tyr121, Phe330, and Trp279. In the case of the inhibitors HUP and GNT there are two cation– π interactions, but the Trp84/Phe330 pair shows more efficiency in stabilizing the positively charged inhibitor than the Phe330/Phe331 pair. Finally, THA only presents one cation– π interaction. The number of cation– π interactions is tightly correlated with the power of inhibition, as will be verified by comparing these data with the binding free energy reported in the next section.

FEP Results. The binding free energy terms of the electrostatic and van der Waals contributions were calculated among ligands at the active site of AChE, as well as in aqueous solution. These calculations were performed according to the time dependence of the QM/MM MD simulations, and the results are shown in Table 1.

The values of the electrostatic term depend on the simulation time for the AChE–ligand system (Table 1). Results of the simulation time of 10 and 20 ps in the enzyme are inherently unreliable, typically overestimating more reliable values. Therefore, more precise and refined results are required due to differences larger than 3 kcal mol^{−1} among some derived values, such as the values of the electrostatic term in the enzyme–ligand for DMT and DNP. In order to pursue a more precise calculation, and also to understand the dependence of binding free energy on the simulation time, we have extended the calculations of the electrostatic and van der Waals terms to 100 ps with FEP methods (Table 1).

An analysis of the results presented in Table 1 shows that over a change in simulation time from 10 to 50 ps, the electrostatic term changes by 3.7, 3.4, and 2.6 kcal mol^{−1}, for DMT, DNP, and HUP, respectively. Otherwise, changes from 50 to 100 ps are significantly reduced to 0.5 (for DMT), 1.7 (for DNP), and 0.6 kcal mol^{−1} (for HUP). Results for simulations with a time extended to 100 ps present meaningfully smaller changes with respect to those obtained with a time shorter than or equal to 50 ps. Differences are below 2 kcal mol^{−1} for all the ligands studied. Therefore, it is reasonable to consider that the values obtained in the simulations of 50 and 100 ps are adequate for a comparative analysis. Interestingly, previous studies on other systems applying the same method used the same or even lower simulation time.^{38,40} However, in previous cases inhibitors were smaller molecules than those studied herein and the convergence of the results was obtained with a shorter dynamic simulation time.

The corresponding values of FEP obtained using eq 4 (ΔG_{elec} , ΔG_{vdW}) are shown for this simulation time and reported in Table 2. The binding energy (ΔG_{bind}) obtained by means of eq 5 is also reported in Table 2. It is clear that the electrostatic energy component contributes significantly to the

binding free energy for all inhibitors. However, the van der Waals component generally also makes a significant contribution, in particular in the case of HUP, where this component is responsible for 53% of all interaction energy with AChE.

Although it is valid to notice that, overall, the van der Waals term makes a significant contribution to the binding energy of these systems, as shown in Figure 14, contributing over 39%

Figure 14. Binding free energy in kilocalories per mole. The contribution (as a percentage) of electrostatic and van der Waals terms are depicted in red and blue, respectively.

(DMT and THA) to the inhibition process of AChE, the van der Waals term makes quite a large contribution to the total energy in HUP (53%) and GNT (44%). Since AChE is an enzyme with very high catalytic efficiency,²⁵ the aromaticity of the gorge, together with the electrostatics steering and the particular properties of the gorge water molecules, are the main factors in AChE–ligand catalysis and inhibition.^{23,25}

In Table 3 the inhibition constant values, K_i , and the experimental binding free energy (that corresponds to this K_i) of each inhibitor are presented together with the theoretical binding free energy calculated by means of eq 5.

As shown in Figure 14 and Table 3, the values for the binding free energy calculated by FEP as well as the

Table 3. Experimental and Computed Values of K_i and ΔG_{bind} of Some AChEIs

inhibitor	K_i^a (nM)	ΔG_{bind}^b (exp; kcal mol ^{−1} , at 300 K)	ΔG_{bind} (calc; kcal mol ^{−1} , at 300 K)
DMT ⁵	77×10^{-6}	−18.0	−60.7
DNP ⁶¹	3.0	−11.7	−42.1
HUP ⁵⁹	175	−9.3	−36.7
GNT ⁷¹	190	−9.2	−36.5
THA ⁷¹	230	−9.1	−30.1

^a*Torpedo californica* AChE. ^bCalculated from K_i experimental values.

Figure 15. Correlation between experimental and calculated values of $\Delta G_{\text{binding}}$.

experimental data point to DMT^{5,72} as the most efficient inhibitor of AChE.

Moreover, as can be observed in Figure 15, where the correlation between the experimental and calculated values of $\Delta G_{\text{binding}}$ is plotted, there is good agreement between the experimental data and our theoretical results. The experimental K_i values used to compare with our results are the latest published for *Torpedo californica* AChE (the enzyme form we used).

Several theoretical binding studies for some AChE inhibitors have been reported previously. The calculated energy of electrostatic interaction for the AChE-THA complex was obtained with the Poisson-Boltzmann method,³⁶ which estimated a $\Delta G_{\text{bind}} = -2.86 \text{ kcal mol}^{-1}$. Han and co-workers⁷³ calculated absolute binding free energy of several tacrine derivatives to AChE in order to evaluate a method based on finite difference thermodynamic integration. They found that results qualitatively ranked the order of affinity of the compounds, although they obtained a value of $\Delta G_{\text{bind}} = -262.98 \text{ kcal mol}^{-1}$ for tacrine. This represents an overestimation of 26 times the experimental value, which ranges from -11.0 to $-9.1 \text{ kcal mol}^{-1}$.

Kitisripanya et al.⁵⁷ obtained the binding energy, by means of the ONIOM3[MP2/6-31G(d,):B3LYP/(6-31G(d,p):PM3] method, for the neutral form of GNT and HUP in the binding pocket of AChE. The study was performed using a high computational level of theory, but it was based on a fairly rigid reduced molecular model. Calculations were performed using a single structure, and only 23 residues of the gorge plus the inhibitor were included in the model. The authors obtained $\Delta G_{\text{bind}} = -17.0 \text{ kcal mol}^{-1}$, $-28.4 \text{ kcal mol}^{-1}$ for GNT and HUP, respectively.

Luque et al. estimated the binding energy for THA, HUP, and huprine X by using MD/LR³² and MM-PB/SA³³ their results showing that HUP is more active than THA. This is in agreement with our results and with the updated experimental data reported in Table 2. The MD/LR and MM-PB/SA were also used to compute the binding affinities of several huprine inhibitors, but neither of the methods was able to reproduce the experimental results completely.³¹ Nevertheless, the differences in inhibitory activity for huprines were successfully predicted by free energy TI calculations.^{14,33}

Quantitative agreement between the theoretical and experimental binding free energy values is something difficult to achieve. However, as shown in Figure 15, the qualitative agreement of the order of the inhibition between theoretical and experimental values can be achieved by means of the alchemical FEP method that was applied. This method has been employed and yielded reliable values of binding free energies at a reasonable computational cost.^{30,38,40} Even though the inhibitors studied here have somewhat different molecular sizes, the correlation recorded is quite good. The use of quantum mechanics to describe the ligands is probably a key point, due to the polarizable nature of the ligands and the high aromaticity of the gorge. Unfortunately, all residues in direct contact with the inhibitor plus all aromatic residues in the gorge cannot be treated quantum mechanically, as the computational cost would be prohibitive. Even though the AM1 semiempirical method is not powerful method, AM1/MM calculations represent a good compromise between computer resources and quality of results, as have been previously reported.^{38,39}

Binding free energy calculated by means of alchemical FEP based on hybrid QM/MM potentials reproduces the experimental inhibitory tendency. Moreover, these results highlight the significant contribution of the electrostatic as well as the van der Waals terms to the inhibition process. The nature of the AChE gorge and the analysis of geometrical parameters and interaction energy per residue support the results. As mentioned above, the interactions among residues Asp72, Glu199, Glu327, and His440 and the ligands are critical to trap the inhibitor in the gorge. Nevertheless, the contribution of the aromatic residues of the gorge is not negligible. One of the most important observations from Figure 13 is that DMT and DNP, the most efficient inhibitors according to the FEP results and experimental data, present favorable interactions with all aromatic residues. It seems that the more stacking interactions there are, the greater inhibitory efficiency is, even if the magnitude of each individual interaction is moderate. All these data support the nature of the inhibitory action of these ligands, where the van der Waals contribution to the binding energy is as important as the electrostatic contributions.

BIEs. Binding isotope effects for inhibitors isotopically labeled by ^{13}C , ^{15}N , and ^{18}O atoms were computed in this work using the QM/MM method based on 11 optimized

Figure 16. BIEs of AChEIs and AChE natural substrate.

structures of inhibitors dissolved in water and bound in the active site of AChE. Average BIE values obtained from these structures are depicted in Figure 16.

A BIE value equal to 1 means that no binding isotope effect is observed. The average values <1 present an inverse BIE, and average values >1 mean a direct BIE. In other words, inverse BIE (<1) means that the isotopically substituted atom has a stronger interaction in the enzyme than in water.^{39,40}

It is important to note that average values of BIEs other than 1 were expected only in the case of important electrostatic enzyme–ligand interactions being present in the gorge. However, no significant BIEs were identified in the AChE–ligand systems. Previous studies⁴⁰ have shown that binding isotope effects can be a useful tool to distinguish between hydrophobic and hydrophilic binding sites in a protein. AChE is a particular case since its active site is quite large, with several aromatic rings (hydrophobic regions) and some essential charged residues such as glutamic and aspartic (hydrophilic areas). However, the importance of the hydrogen bond interactions is not evidenced in the AChE BIE results. Frequently, in the inhibitors studied here, these interactions are mediated by structural water molecules. Moreover, as reported in the literature^{23–25} and also observed throughout the QM/MM MD simulation carried out in this paper, there is a layer of water molecules surrounding the inhibitors in contact

with the residues of the gorge. Such a situation masked the importance of these interactions in the BIE results.

Galantamine presents an inverse BIE of 0.985 in the oxygen (O20) of the hydroxyl group. This group forms two hydrogen bonds: a strong hydrogen bond between atom H39 of GNT and OE1 of residue Glu199 (1.79 ± 0.14 Å) and another one between the O20 atom of the inhibitor and the H:Gly118 (3.27 ± 0.32 Å) atom. Inverse BIE observed in an oxygen atom indicates that this atom forms stronger hydrogen bonds with the gorge than with water molecules while it is dissolved in water.

The FEP results emphasize the importance of the electrostatic and van der Waals terms in the binding energy of the systems. And an analysis of the interaction energy per residue and distances along the 1 ns QM/MM MD trajectory reveals that these inhibitors have important interactions with aromatic residues and with some hydrophilic residues. The nature of the hydrophobic interactions and the similar intensity of the hydrophilic interactions in water and enzyme lead to no remarkable BIEs.

CONCLUSIONS

Furthering our understanding of the interaction behavior of acetylcholinesterase (AChE) inhibitors using QM/MM MD simulations is an ongoing initiative in the authors' research group, which has as its ultimate aim the development of an

alternative and computationally cost-effective approach to exploring interesting biological phenomena. In this study we calculated the binding free energy, using hybrid QM/MM MD simulations, of the inhibitors (DMT, DNP, GNT, HUP, THA) of acetylcholinesterase (AChE) with the enzyme blocking the gorge.

The main conclusions can be summarized as follows:

- (i) Satisfactory results have been obtained, since binding free energy correlates properly with available inhibition constants.
- (ii) Present results highlight the importance of the van der Waals interaction for the inhibition process, since the contribution of this term to the binding free energy is almost as decisive as the electrostatic one. This fact reflects the special features of AChE, i.e., a large gorge with several subsites with different residue compositions.
- (iii) Ligand polarization upon binding has been considered, since the simulations are based on hybrid QM/MM potentials. This fact can be decisive for successful results, due to the nature of the inhibitors and the gorge.
- (iv) The analysis of the geometrical parameters and the interaction energy per residue highlight the most relevant interactions in the different AChE–ligand systems. Results are in agreement with previous studies reported in the literature. Nevertheless, a comparative analysis for several ligands has the additional advantage of establishing patterns to highlight important differences.
- (v) Charged residues with a more prominent contribution to the interaction energy are Asp72 and Glu199, although the relative importance depends on the molecular size of the ligand. The contribution made by Asp72 prevails in the larger inhibitors, whereas the contribution of Glu199 predominates in the smaller ones. The contribution of Glu278 stands out in the AChE–DMT system due to a strong hydrogen bond mediated by a water molecule.
- (vi) The aromatic residues with a significant contribution to the interaction energy in all AChE–ligand systems are Trp84, Phe330, and Phe331. In addition, Tyr121 and Trp279 also make a noticeable contribution to the interaction energy in the larger ligand systems, DMT and DNP, respectively. Another important contribution to the interaction energy between AChE and DMT is also associated with Tyr70. A particular feature of AChE–HUP is the importance of Tyr130 due to the formation of a hydrogen bond between this residue and the inhibitor.
- (vii) A correlation between the binding free energy and the number of cation– π interactions present in the systems has been established. DMT, the most potent inhibitor, forms four cation– π interactions with gorge residues, whereas THA only makes one.
- (viii) A layer of water molecules surrounding the inhibitors has been observed in all the AChE–ligand systems throughout the QM/MM MD simulations. These water molecules act as bridges in an interaction net between the ligands and the residues of the gorge, and also between different residues.
- (ix) Although several hydrogen bonds are established between ligands and AChE, no significant BIEs are found. Special features of AChE, such as the presence of several subsites with different natures in the gorge or the

existence of several water molecules that act as bridges in the electrostatic interactions, account for this behavior.

- (x) This theoretical procedure can be expected to have a positive impact because it would be a useful tool for predicting the binding free energy of new molecules, and thus for selecting the best ones for synthesis.
- (xi) The present explicit interaction protocol will hopefully lay the foundations for extensive theoretical as well as experimental research to verify all the emerging aspects of the present study. In particular, in the field of relative binding free energy calculations applied to pharmaceutically relevant targets, but significantly more validation work is needed. This is a line which we are pursuing through additional retrospective validation and, importantly, through prospective applications in real-world drug discovery projects.

■ ASSOCIATED CONTENT

5 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jcim.7b00037.

Distance evolution along the QM/MM MD time, distances in the AChE triad set up, RMSD plots, number of water molecules in contact with the negatively charged residues of the gorge, and hydrogen bond between Asp/Glu residues and inhibitor (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Tel.: +34 964 728067. Fax: +34 964 728066. E-mail: nascimen@uji.es (E.C.M.N.).

*E-mail: andres@uji.es (J.A.).

ORCID

Érica C. M. Nascimento: 0000-0003-1365-177X

Mónica Oliva: 0000-0001-6651-7852

Katarzyna Świderek: 0000-0002-7528-1551

João B. L. Martins: 0000-0001-8677-3239

Juan Andrés: 0000-0003-0232-3957

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Notes

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■ ABBREVIATIONS

DMT, bis(7)-tacrine; DNP, donepezil; GNT, galantamine; HUP, huperzine A; THA, tacrine; AChE, acetylcholinesterase; QM/MM, hybrid quantum mechanics and molecular mechanics method; MD, molecular dynamics; AD, Alzheimer's disease; ACh, acetylcholine; FDA, U.S. Food and Drugs Administration; AChEIs, acetylcholinesterase inhibitors; PAS, peripheral aromatic site; CAS, catalytic acylation site; MD/LR, molecular dynamics-linear response theoretical approach method; MM-PB/SA, molecular mechanics Poisson–Boltzmann/solvent accessible protocol; SIE, solvent interaction energy; TI, free-energy thermodynamic integration method; FEP, alchemical free energy perturbation method; IE, interaction energy per residue; BIEs, binding isotope effects; TcAChE, acetylcholinesterase of *Torpedo californica* specie; PDB, protein data bank; PEG, polyethylene glycol; NAG, N-acetylglucosamine; RMSD, root-mean-square deviation; AM1, semiempirical method for the quantum calculations of molecular electronic structure in computational chemistry; OPLS-AA, optimized potential for liquid simulation all atoms force field; QM, quantum mechanics; MM, molecular mechanics; Me3N⁺, quaternary ammonium cation; CPK, Corey–Pauling–Koltun model; HOH, water molecule; OD_α[−], delta oxygen of aspartic acid residue; OE_γ[−], epsilon oxygen of glutamic acid residue

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Supporting Information

Binding Analysis of Some Classical Acetylcholinesterase Inhibitors: Insights for a Rational Design Using FEP Calculations with QM/MM MD Simulations.

Érica C. M. Nascimento^{*†}, Mónica Oliva[‡], Katarzyna Świderek^{‡§}, João B. L. Martins[‡] and Juan Andrés^{*†}

[†]Department of Analytical and Physical Chemistry, Jaume I University, 12071 Castellón, Spain; [‡] Institute of Chemistry, University of Brasília, 70910-000, Brasília-DF. Brazil and [§]Institute of Applied Radiation Chemistry, Lodz University of Technology, 90-924 Lodz, Poland

Author whom correspondence should be addressed: E-mail: nascimen@uji.es, andres@uji.es

Figure S1. Distance evolution of some atoms of the residue Trp84 and ACh along the MD simulation time.

(a)

(b)

Figure S2. (a) Distance evolution of some atoms of Trp84 and THA along the MD simulation time; (b) Distance evolution of some atoms of THA and residues Glu199 and His440 along the MD simulation time.

(a)

(b)

(c)

(d)

Figure S3. (a) Distance evolution of some atoms of GNT and residue Glu199 along the MD time; (b) Distance evolution of some atoms of GNT and residue Trp84 along the MD time; (c) Distance evolution of some atoms of GNT atoms and residue Phe330 along the MD time; (d) Distance evolution of some atoms of GNT and residue Phe331 along the MD time.

(a)

(b)

(c)

(d)

Figure S4. (a) Distance evolution of some atoms of HUP and residue Tyr130 along the MD time; (b) Distance evolution of some atoms of HUP and residue Glu199 along the MD time; (c) Distance evolution of some atoms of HUP and residue Trp84 along the MD time; (d) Distance evolution of some atoms of HUP and residue Phe330 along the MD time

Figure S5. (a) Distance evolution of some atoms of DNP and residue Trp84 along the MD time; (b) Distance evolution of some atoms of DNP and residues Phe330 and Phe331 along the MD time; (c) Distance evolution of some atoms of DNP and residue Tyr121 along the MD time; (d) Distance evolution of some atoms of DNP and several water molecules along the MD time.

(c)

(d)

Figure S6. (a) Distance evolution of some atoms of DMT and residue His440 along the MD time; (b) Distance evolution of some atoms of DMT and residues Asp72, Glu199, and Glu278 along the MD time; (c) Distance evolution of some atoms of DMT and residue Trp84 along the MD time; (d) Distance evolution of some atoms of DMT and residue Trp279 along the MD time.

Figure S7. Distance evolution between residues Glu327 and His440 along the MD in the different AChE-ligand systems.

Figure S8. Schematic representation of **d1**, **d2**, and **d3** distances in AChE triad set up.

Figure S9. (a) Root mean square deviation (RMSD) evolution of the AChE backbone and (b) total energy evolution for the AChE-Ligand systems (Ligands: DMT, DNP, HUP, GNT and THA) as

function of the AM1/MM MD simulation time. Variation coefficient (VC) for the total energy is also included.

Table S1. Number of water molecules in contact with the negatively charged residues of the gorge: D72 (Asp72), E199 (Glu199), and E278 (Glu278); **Distance**: between N⁺ atom of the ligand and gorge residues. **H-bond**: hydrogen bond between O⁻ (Glu/Asp residues) and hydrogen atoms of inhibitors. Values from last minimized structure of QM/MM MD simulation at 1 ns.

Ligand	D72	E199	E278	E327	Distance(Å)	H-bond (Å)
ACh*	1	1	6	0	3.68 ^(E199)	-
ACh	4	2	6	1	3.83 ^(E199)	-
DMT	2	3	4	1	5.97 ^(E199)	H58 [‡] : 2.68 ^(E199)
					4.70 ^(E278)	H38 [‡] : 2.70 ^(E278)
DNP	4	4	6	1	5.82 ^(D72)	H42 [‡] : 3.49 ^(D72)
						H49 [‡] : 2.59 ^(E199)
GNT	5	2	5	1	5.51 ^(D72)	H39: 1.70 ^(E199)
HUP	3	2	6	1	7.60 ^(E199)	H19: 3.24 ^(E199)
THA	4	2	4	1	6.05 ^(E199)	H22 [‡] : 2.55 ^(E199)

‡: non polar hydrogen.

Article 2

5. Binding Free Energy Calculations to Rationalize the Interactions of Huprines with Acetylcholinesterase.

*“Los obstáculos son enseñanzas para tu espíritu.
Nunca te des por vencido.” (Santa Rita da Cascia)*

Binding Free Energy Calculations to Rationalize the Interactions of Huprines with Acetylcholinesterase

Érica C. M. Nascimento, Mónica Oliva* and Juan Andrés

Departament de Química Física i Analítica, Universitat Jaume I, 12071 Castelló, Spain

ABSTRACT: In the present study, the binding free energy of a family of huprines with acetylcholinesterase (AChE) is calculated by means of free energy perturbation method (FEP), based on hybrid quantum mechanics and molecular mechanics (QM/MM) potentials. A good correlation with the experimental data has been obtained for a family of inhibitors with moderate differences in the enzyme affinity. The analysis of the geometrical parameters and interaction energy per residue reveals that Asp72, Glu199 and His440 contribute significantly to the network of interactions between active site residues, which stabilize the inhibitors in the gorge. It seems that a cooperative effect of the residues of the gorge determines the affinity of the enzyme for these inhibitors, where Asp72, Glu199 and His440 have a prominent contribution. Binding free energy calculations and the analysis of the geometrical parameters highlight the importance of the stereochemistry of huprines in AChE inhibition.

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INTRODUCTION

Alzheimer's disease (AD) is mainly characterized by the formation of deposits of extracellular amyloid plaques and the intraneuronal neurofibrillary tangles of hyperphosphorylated tau protein.¹ Modifications of several neurotransmitter systems have also been observed, with the most characteristic being the relatively selective loss of cholinergic neurons.¹ The depletion of cholinergic neurotransmission in the brain of AD patients gave rise to the cholinergic hypothesis of AD,²⁻⁴ which afforded the rational basis from which most of the currently approved anti-Alzheimer drugs have derived. Indeed, four out of the five approved drugs are inhibitors of the enzyme acetylcholinesterase (AChE). This enzyme is responsible for the hydrolysis of acetylcholine, and therefore its inhibition can restore to some extent the cholinergic transmission. Apart from the acetylcholinesterase inhibitors (AChEIs), memantine, a glutamate NMDA receptor antagonist, was approved for AD treatment.⁵ While a number of drug candidates deriving from other hypotheses, prominently the amyloid hypothesis, have not yet provided satisfactory results in clinical trials, AChEIs constitute the basis of AD treatment and the design of novel AChEIs is still an area of very intensive research.

Some AChEIs, such as rivastigmine,⁶ form covalent bonds with the Ser200 (*Torpedo californica* AChE numbering) residue of AChE, whereas most inhibitors, including the approved drug tacrine,⁷ donepezil⁸ and galantamine,⁹ block the active site of the enzyme through non-covalent reversible interactions. Prediction of the binding affinities of AChEIs can be very useful for the development of novel inhibitors. This is one of the great challenges of computational chemistry, and several methods have been applied trying to get a reliable prediction of protein-ligand binding energies.¹⁰⁻¹²

As far as AChEIs concerned, several protocols have been used to estimate the binding affinity of numerous ligands.¹³⁻²¹ Unfortunately, the methods with less computational cost were not always able to reproduce the experimental results.¹⁸

Recently, we have published a paper where the binding free energy of some classical inhibitors of AChE has been calculated, obtaining good results with an acceptable computational cost.²² In that work, the studied ligands had quite different protein affinities. However, it is interesting to compare inhibitors having similar inhibition constant. To verify if our protocol is adequate in the latter circumstances, we have decided to apply it in a set of huprines, a known class of AChEIs, which feature similar characteristics and affinity.

Figure 1: Schematic representation of the huprines considered in this study.

Huprines are a group tacrine-huperzine A hybrids that simultaneously feature the 4-aminoquinoline system of tacrine and the carbobicyclic bridged system of huperzine A.^{14,16,23,24} These compounds are racemic species, which can be separated into their enantiomers (Figure 1). The levorotatory enantiomers of these hybrid compounds ((-)-(7*S*,11*S*)-huprines) are much more active (eutomers) for AChE inhibition than the dextrorotatory forms (distomers), and the most active derivatives are those substituted at position 3 with a chlorine atom (Table 1).^{14,16,21,23}

Huprine X binds to human AChE with an affinity 40-fold higher than that of donepezil, 180-fold higher than that of (-)-huperzine A, and 1200-fold higher than

that of tacrine.^{19,23} The simultaneous occupancy by huprines of the binding zone of tacrine and a part of the binding zone of (-)-huperzine A seems to be responsible for their high inhibitory potency.²⁴ Pharmacological studies demonstrated that huprine X exhibits an agonistic action on muscarinic M₁ and nicotinic receptors.^{25,26} Moreover, results obtained in efficacy studies in mice strongly suggest that the treatment with huprine X improves the cognitive performance of the animals and induces some neurochemical changes which could contribute to the beneficial effects observed.²⁷ The outstanding pharmacological profile of huprines together with the work by Pang, Carlier and Han on tacrine dimers as dual binding site AChE inhibitors, inspired the synthesis of new heterodimeric compounds based on huprine, such huprine-tacrine heterodimers or donepezil-huprine heterodimers, among others.^{21,24}

Table 1: AChE inhibitory activity (IC₅₀ values, nM) and affinity (K_i values, nM) of the huprines considered in the study, for the racemic mixture, (-)-(7*S*,11*S*)-enantiomer and (+)-(7*R*,11*R*)-enantiomer. Data taken from Refs. (14, 16, 21, 23).

Compound	R ₃	R ₉	IC ₅₀ rac(nM)	IC ₅₀ (-) (nM)	IC ₅₀ (+) (nM)	K _i (nM) ²³
HUX	Cl	Ethyl	2.77±0.75 ^{a,16} 0.67±0.05 ^{b,21}	1.30±0.26 ^{a,16} 0.27±0.02 ^{b,21}	402±36 ^{a,16} 6.30±0.50 ^{b,21}	0.026±0.002 ^{b,23}
HUY	Cl	Methyl	4.23±0.86 ^{a,16} 0.61±0.03 ²¹	1.15±0.11 ^{a,16} 0.43±0.03 ^{b,21}	36.1±3.6 ^{a,16} 13.6±1.5 ^{b,21}	0.033±0.003 ^{b,23}
HUE	H	Ethyl	38.5±4 ^{a,16}	27.4±3.1 ^{a,14}	888±141 ^{a,14}	
HUM	H	Methyl	65±15 ^{a,16}	47.1±6.3 ^{a,14}	329±58 ^{a,14}	

^a values determined from inhibition of bovine erythrocyte AChE, ^b values determined from inhibition of human AChE.

As a continuation of the previous paper,²² here we present a study, aimed at gaining an understanding of the binding process of huprines. Binding free energies are calculated by means of the alchemical free energy perturbation (FEP) method. FEP is a recognized method for the prediction of ligand binding poses and affinities.^{28,29} The contributions of each residue of the gorge to the interaction energy between the inhibitors and the enzyme have been analyzed, and these results have been connected with the geometrical parameters obtained in our simulations. Finally, binding isotope effects³⁰ (BIE) have been calculated to further analyze interactions between ligands and the gorge of AChE.

COMPUTATIONAL METHODS

System setup

The system setup was performed following the same protocol used in our previous work on the classical AChE inhibitors.²² In this way, we used the same structure for the protein and structural water molecules (PDB code 2C5G), and the coordinates of (-)-huprine X were put in the gorge by superimposing the AChE structure complexed with huprine X (PDB ID 1E66) using the DaliLite program.³¹ All structural water molecules in the gorge, as well as the coordinates of residues Phe330 and Trp432 were replaced in the new coordinate systems according to the 1E66 pdb file. The root mean square deviation (RMSD) was 0.18 Å, and thus the structures obtained after superimposition were assumed to be suitable for this study. The other (-)-huprines-AChE systems were obtained after minor changes in positions 3 and/or 9 of (-)-huprine X. With regard to (+)-huprines, the initial coordinates for these systems were obtained after docking the corresponding (+)-huprines in the *Torpedo californica* AChE (TcAChE) 2C5G pdb file using AutoDock 4.2 program.³² The coordinates of Phe330 and Trp432 were also adjusted according to the 1E66 pdb file. Regarding the protonation state of the residues, the enzyme was modeled with neutral His440 and deprotonated Glu327

and Glu199. The standard ionization state at neutral pH was considered for the rest of ionizable residues with the exception of Asp392 and Glu443, which were neutral, and His471, which was protonated, in accordance with our previous work²² and in agreement with Wlodek and co-workers.¹³

Hydrogen atoms were incorporated into the structure, and the energy minimization of these atoms was performed as implemented in the fDynamo library.^{33,34} Next, 5 sodium counterions were added in optimal electrostatic positions around the protein (never closer than 10.5 Å to any atom and not less than 5 Å from another counterion, using a regular grid of 0.5 Å) to have a total charge equal to zero. Subsequently, the system was placed inside a previously relaxed box of water (100×100×100 Å³). All water molecules within a distance of 2.8 Å from the protein were removed.

In the present study, all calculations were performed with the hybrid Quantum Mechanics/Molecular Mechanics (QM/MM) method as implemented in the fDynamo library.^{33,34} The huprines were treated using the semi-empirical AM1 method,³⁵ and the rest of the system (protein and solvent) were described using OPLS-AA³⁶ and TIP3P³⁷ force fields, respectively. After energy minimization of the systems, 300 ps of AM1/MM molecular dynamics (MD) simulations were carried out where all atoms were free to move. In order to reduce the computational cost of the calculations, these simulations were followed by 1 ns AM1/MM MD where the residues and water molecules 25 Å apart from the OG atom of the residue Ser200 were kept frozen. In both cases, Langevin-Verlet (NVT) MD at 300 K with a time step of 1 fs were performed, applying periodic boundary conditions and cutoff using a switching scheme within a range radius from 14 to 16 Å.

Inhibitor-solvent systems were also required to compute the AChE-ligands binding free energy, and thus several systems were built by placing each inhibitor in the pre-relaxed box of water. AM1/MM MD simulations for the inhibitor-solvent system were also conducted. First the inhibitor-solvent system simulations

were performed considering all atoms to be free for 100 ps. Subsequently, 300 ps of AM1/MM MD were carried out where all water molecules 25Å apart from the carbon atom that holds the amine group were frozen, and the equilibration simulation time was 300 ps.

Alchemical Free Energy Perturbation (FEP) Method

The starting structures in FEP calculations were the equilibrated AChE-huprine and water-huprine systems described in the previous section. In order to evaluate the huprine-protein binding free energy, a series of AM1/MM MD simulations have been carried out at the gorge and in the box of solvent, introducing the parameters λ and γ into the electrostatic and van der Waals QM/MM interaction terms, as shown in Equation 1

$$E_{QM/MM}(\lambda, \gamma) = \left\langle \Psi \left| \hat{H}_0 \right| \Psi \right\rangle + \lambda \left[\sum \left\langle \Psi \left| \frac{q_{MM}}{r_{e,MM}} \right| \Psi \right\rangle + \sum \sum \frac{Z_{QM} q_{MM}}{r_{QM,MM}} \right] + \gamma E_{QM/MM}^{vdW} + E_{MM} \quad (1)$$

In this equation, λ changes smoothly between the values of 1 and 0. The parameter λ equal to 1 corresponds to a full MM charge–wave function interaction, whereas a value of λ equal to 0 represents no electrostatic interaction with the force field. The γ values are also comprised between 1 and 0 (once the electrostatic charges of the ligand in the QM region are annihilated); when it is equal to 1 it corresponds to a full QM/MM van der Waals interaction, while the value equal to 0 refers to no van der Waals interaction with the force field.³⁸

A total of 50 windows were used to evaluate the electrostatic interaction energy term, from $\lambda=1$ (full interaction) to $\lambda=0$ (no electrostatic interaction), in steps ($\delta\lambda$) of 0.02. The same number of windows were used to calculate the van der Waals energy interaction term, from $\gamma=1$ (full interaction) to $\gamma=0$ (no interaction)

in steps ($\delta\gamma$) of 0.02. A total of 10 ps of relaxation followed by 100 ps of production of AM1/MM MD simulation were performed using the NVT ensemble at the reference temperature of 300 K. This simulation time provided suitable results in previous papers based on the same protein, where the consistence of the free binding energy as a function of the simulation time was analyzed,²² as well as on other proteins.^{38,39} The calculation of free-energy differences of two consecutive windows of the two stages, annihilation of charges and the van der Waals parameters, was performed through the FEP method. The total free energy variation is calculated through the sum of all windows covering the full transformation from the initial to the final states. This procedure is done in two steps, as represented in Equations 2 and 3:³⁸

$$\Delta G_{QM/MM}^{elect} = -\frac{1}{\beta} \left[\sum \ln \left\langle e^{-\beta[E(\lambda_{i+1})-E(\lambda_i)]} \right\rangle_{\lambda_i} \right]_{\gamma=1} \quad (2)$$

$$\Delta G_{QM/MM}^{vdW} = -\frac{1}{\beta} \left[\sum \ln \left\langle e^{-\beta[E(\gamma_{i+1})-E(\gamma_i)]} \right\rangle_{\gamma_i} \right]_{\lambda=0} \quad (3)$$

The thermodynamic cycle used to compute the binding free energy of enzyme-inhibitors by alchemical FEP methods is presented in Figure 2. In this way, free energy is calculated by applying Equations 2 and 3, in water (W) and in the enzyme (E)

$$\Delta G = \Delta G_E - \Delta G_W \quad (4)$$

This calculation is performed in two steps yielding the electrostatic and van der Waals terms, ΔG_{elec} and ΔG_{vdW} . Finally, the total binding free energy is obtained by adding the two terms:

$$\Delta G_{bind} = \Delta G_{elec} + \Delta G_{vdW} \quad (5)$$

Figure 2. Thermodynamic cycle to compute enzyme-huprine binding free energies. E is the enzyme with huprine (L) in its binding site, E' is the apo form of enzyme, and (L)₀ is the ligand in gas phase.

Interaction Energy per Residue

The interaction energy between the huprine and the protein is calculated as the difference between the QM/MM energy and the energies of the non-interacting QM and MM subsystems for the same system geometry. This interaction energy can be decomposed in a sum over the residues, the residue (i) is turned on and off, thus obtaining the contribution of each of them to the interaction energy as expressed in Equation (6):

$$\begin{aligned}
 E_{QM/MM,i}^{Int} &= E_{QM/MM,i}^{elect} + E_{QM/MM,i}^{vdW} \\
 &= \sum_{MM \in i} \left[\left\langle \Psi \left| \frac{q_{MM}}{r_{e,MM}} \right| \Psi \right\rangle + \sum_{QM} \frac{Z_{QM} q_{MM}}{r_{QM,MM}} \right] \\
 &\quad + \sum \sum 4\epsilon_{QM,MM} \left[\left(\frac{\sigma_{QM,MM}}{r_{QM,MM}} \right)^{12} - \left(\frac{\sigma_{QM,MM}}{r_{QM,MM}} \right)^6 \right]
 \end{aligned} \tag{6}$$

The average interaction energy per residue has been obtained using the 1ns AM1/MM MD simulations for each huprine-AChE system. Structures of the last 500 ps have been considered for these calculations.

Binding Isotope Effects (BIEs)

Binding isotope effects (BIEs) can be a useful tool to identify important non-bonding interactions occurring during the binding of the inhibitor to the active site. Changes in normal-mode force constants surrounding the isotopic substitution between inhibitor dissolved in water and inhibitor in the active site of the enzyme, give rise to a binding preference for a light or a heavy isotope in the bound state and hence a measurable BIE.³⁰

Considering the binding process of an inhibitor, from solution to the formation of the inhibitor-enzyme complex, BIE would be defined as the ratio between the binding equilibrium constant of the light (L), or naturally abundant, isotope species and the heavy (H), or isotopically substituted, one:

$$\text{BIE} = \frac{K_L}{K_H} \quad (7)$$

BIEs can also be expressed in terms of the binding free energy of the light and heavy species, considering the relationship between the equilibrium constant and the binding free energy:

$$\text{BIE} = e^{-(\Delta G_L + \Delta G_H)/RT} \quad (8)$$

where ΔG_L and ΔG_H are the binding free energies for the light and heavy isotopomers, respectively, R is the universal gas constant, and T is the temperature. Considering the definition of free energy of a state, as a function of the internal energy, the total partition function and the zero point vibrational energy, the binding isotope effect can be calculated using equation (9):

$$\text{BIE} = \frac{\left(\frac{Q_e}{Q_w}\right)_L}{\left(\frac{Q_e}{Q_w}\right)_H} e^{-1/RT(\Delta\text{ZPE}_L - \Delta\text{ZPE}_H)} \quad (9)$$

In equation (9), Q_e is the total partition function (the product of translational, rotational, and vibrational partition functions) for the inhibitor in the enzyme, and Q_w is the total partition function for the inhibitor in water. ΔZPE_L and ΔZPE_H are the differences in zero point vibrational energies from water to the gorge of AChE, for the inhibitor with the light and heavy isotope, respectively.

BIEs were calculated by selecting eleven structures from the 1ns AM1/MM MD simulation for each AChE-huprine system, and eleven structures from the 300ns AM1/MM MD simulation for each huprine in solution. These structures were obtained from the last 200ps of these simulations.

RESULTS AND DISCUSSION

FEP results

The huprine-AChE binding free energies (ΔG_{bind}) and their decomposition into electrostatic and van der Waals terms computed from FEP method are reported in Table 2. The electrostatic terms of protein-ligand ($\Delta G_{\text{elec-E}}$) and water-ligand ($\Delta G_{\text{elec-W}}$) are calculated by means of equation 2, and the corresponding van der Waals terms of protein-ligand ($\Delta G_{\text{vdW-E}}$) and water-ligand ($\Delta G_{\text{vdW-W}}$) systems are calculated using equation 3. The differences between electrostatic terms in the enzyme and water provide the electrostatic contribution to the binding process (ΔG_{elec}), which ranges from -17.04 to -25.21 kcal·mol⁻¹, for the least and most potent inhibitors, respectively. For the (-)-(7*S*,11*S*)-huprines this term correlates with the binding free energy, whereas for the (+)-(7*R*,11*R*)-huprines the value is substantially lower, around -17.5 kcal·mol⁻¹, and no trends are observed. Regarding the differences in van der Waals terms in the protein and in solution, the van der Waals contribution (ΔG_{vdW}) to the binding energy is always smaller than the corresponding electrostatic one for each inhibitor. However, the van der Waals term is not negligible and contributes significantly to the binding free energy (Figure 3). This contribution accounts for around the 45% of the total binding energy in most systems, except for (-)-HUY and (+)-HUM systems where this term represents the 38 and 40% of the total binding free energy, respectively. It is interesting to note that for most inhibitors, the value of ΔG_{vdW} is between -15 and -17 kcal·mol⁻¹, but for (-)-HUX (the most potent inhibitor) ΔG_{vdW} is -19.58 kcal·mol⁻¹, and for the less effective inhibitors the value is close to -12.5 kcal mol⁻¹.

Table 2. Electrostatic (ΔG_{elec}) and van der Waals (ΔG_{vdW}) terms of the water-ligand ($_{\text{W}}$) and protein-ligand ($_{\text{E}}$) interaction free energy, and differences in interaction between the binding site of the protein and water (ΔG_{elec} , ΔG_{vdW}) and the binding free energy (ΔG_{bind}). Values in kcal mol⁻¹.

	W(water)		E(Enzyme)		ΔG		ΔG_{bind}
Ligand	$\Delta G_{\text{elec-W}}$	$\Delta G_{\text{vdW-W}}$	$\Delta G_{\text{elec-E}}$	$\Delta G_{\text{vdW-E}}$	ΔG_{elec}	ΔG_{vdW}	<i>calculated</i>
(-)-HUX	-19.51	-23.31	-44.72	-42.89	-25.21	-19.58	-44.79
(-)-HUY	-20.82	-23.34	-45.58	-38.36	-24.76	-15.02	-39.78
(-)-HUE	-19.32	-23.23	-40.49	-40.12	-21.17	-16.89	-38.06
(-)-HUM	-19.56	-23.03	-40.31	-38.99	-20.75	-15.96	-36.71
(+)-HUE	-19.68	-23.73	-37.02	-39.66	-17.34	-15.93	-33.27
(+)-HUX	-19.35	-22.58	-36.96	-38.00	-17.61	-15.42	-33.03
(+)-HUM	-17.81	-21.62	-35.81	-33.75	-18.00	-12.13	-30.13
(+)-HUY	-21.00	-23.27	-38.04	-35.82	-17.04	-12.55	-29.59

Figure 3. Binding free energy in kcal·mol⁻¹. The contribution of electrostatic and van der Waals terms are depicted in red and blue, respectively. The percentage of these contributions to the total energy is also indicated.

The affinity of (-)-huprines for AChE is considerably greater than that of the (+)-huprine, in agreement with the experimental data (Table 1).^{14,16,21} Figure 4 shows the correlation between our results and experimental values of $\Delta G_{\text{binding}}$, corresponding to the IC_{50} values determined from inhibition of bovine erythrocyte AChE.^{14,16} As it can be seen in the figure, there are some discrepancies between these experimental data and our results. According to the FEP results the most potent inhibitor is (-)-HUX, but the experimental IC_{50} for (-)-HUX and (-)-HUY are 1.30 ± 0.26 and 1.15 ± 0.11 nM, respectively. Although the order of inhibition does not match, it falls within the experimental margin of error. More striking is the discrepancy in the (+)-huprines, since the experimental values of IC_{50} show a great difference between (+)-HUY and the rest of (+)-huprines.

Figure 4. Correlation between experimental^{14, 16} and calculated values of $\Delta G_{\text{binding}}$.

Previous free energy calculations¹⁶ performed using thermodynamic integration (TI) coupled to MD found free energy differences of $-4.0 \text{ kcal} \cdot \text{mol}^{-1}$ from (-)-HUE to (-)-HUX, $-3.8 \text{ kcal} \cdot \text{mol}^{-1}$ from (-)-HUM to (-)-HUY, $-0.7 \text{ kcal} \cdot \text{mol}^{-1}$ from (-)-HUY to (-)-HUX, and $-0.5 \text{ kcal} \cdot \text{mol}^{-1}$ from (-)-HUM to (-)-HUE. The greater discrepancy between these data and our results is observed in the enhancement of the inhibitory potency by changing the methyl group by an ethyl group in huprines (Table 2). This discrepancy can be associated with the fact that

our results are based on quantum mechanical simulations, which enables description of the polarization of the ligand upon binding.

More recent data for the inhibition of human recombinant AChE by both enantiomers of huprines X and Y are available in the literature.²¹ Unfortunately, to the best of our knowledge there are no more recent data for the rest of huprines herein studied. A plot of the correlation between these last experimental data and our results is displayed in Figure 5 in order to verify the goodness of our results. Quantitative agreement between the theoretical and experimental binding free energy values is something difficult to achieve. However, as shown in Figure 5, the qualitative agreement of the order of the inhibition between theoretical and experimental values can be achieved by means of the alchemical FEP method. Previously, we found that this method was able to reproduce the experimental affinity order for several AChE inhibitors that exhibit quite different K_i values.²² Now, we have verified that the alchemical FEP method is capable of reproducing the tendency on the inhibition power of a family of molecules with relatively close IC_{50} values. Therefore, this method represents a good compromise between computer resources and quality of results.

Figure 5. Correlation between experimental²¹ and calculated values of $\Delta G_{\text{binding}}$.

Analysis of the Geometric Parameters and Interaction Energy per Residue

To explain the different affinity of these huprines for AChE, an analysis of the geometric parameters and the interaction energy (IE) per residue between the ligand and the environment was performed. These parameters are reported as the average of the last 500 ps structures from the 1 ns AM1/MM MD simulation trajectories, once the system was equilibrated. In the supporting information (Figure S1), the potential energy evolution and RMSD evolution of the AChE backbone for the AChE-huprine systems as function of the simulation time are reported.

(-)-Huprines

(b)

Figure 6. Plots of the main interactions between (-)-HUY and the subset of residues forming the binding pocket. Some selected average distances in Å are depicted.

The main interactions between the residues of the gorge and huprine (-)-HUY are depicted in Figure 6, and selected geometrical parameters for (-)-huprines are reported in Table 3. As it can be seen in Figure 6a, the polar residues of the gorge are connected between them, and with the inhibitor by a network of hydrogen bonds mediated by a layer of water molecules. A similar network was previously observed for several classical AChE inhibitors²² and also reported in the literature.^{19,40-43} These water molecules reinforce the interactions between the residues of gorge and the inhibitors, as the interaction between Asp72 and the primary amino group of (-)-huprines. This residue contributes significantly to the stabilization of the inhibitor at the active site of the enzyme (Figure 7), and its IE

lowers from (-)-HUX to (-)-HUM. The distance between the carboxylate group of Asp72 and the hydrogen of the amino group of (-)-HUX is considerably shorter than in the other (-)-huprines.

Figure 7. Average IE of polar residues in the AChE-huprine systems (all values are given in $\text{kcal} \cdot \text{mol}^{-1}$).

Table 3: Selected averaged geometrical parameters for (-)-huprines (values of the distances are given in Å).

Distance (Å)	(-)-HUX	(-)-HUY	(-)-HUE	(-)-HUM
OD1(Asp72)-H12'₁) Huprine	3.86 ± 0.19	4.56 ± 0.30	4.86 ± 0.52	4.60 ± 0.26
O (His440) – H5 Huprine	1.98 ± 0.18	2.10 ± 0.18	1.96 ± 0.17	2.09 ± 0.19
OE2(Glu199) – N5 Huprine	5.99 ± 0.33	6.30 ± 0.24	5.61 ± 0.22	6.20 ± 0.35
OD1(Asp72) – N5 Huprine	8.04 ± 0.17	8.72 ± 0.21	9.04 ± 0.47	9.00 ± 0.26
CG(Phe 330) – C4 Huprine	3.54 ± 0.15	3.47 ± 0.14	4.01 ± 0.24	3.77 ± 0.21
CD2(Phe330) – N5 Huprine	3.94 ± 0.27	3.69 ± 0.21	4.39 ± 0.29	3.88 ± 0.25
CE1(Phe330) – C1 Huprine	3.49 ± 0.19	3.48 ± 0.20	3.48 ± 0.18	3.55 ± 0.20
CD1(Phe330) – C3 Huprine	3.42 ± 0.14	3.47 ± 0.14	3.98 ± 0.26	3.79 ± 0.21
CG(Trp84) – C12 Huprine	3.65 ± 0.17	3.78 ± 0.14	3.56 ± 0.14	3.87 ± 0.19
CE2 (Trp84) – N5Huprine	3.94 ± 0.19	4.39 ± 0.29	3.85 ± 0.21	4.02 ± 0.20

Another key residue to stabilize inhibitors in the active site of the enzyme is His440. As previously reported in the crystal structure of (-)-HUX-AChE complex,¹⁹ the aromatic nitrogen of the huprine is hydrogen-bonded with the main-chain carbonyl oxygen of His440. This interaction is conserved in all (-)-huprines (Figure 6a), and similar interaction energies of His440 in (-)-huprines-AChE systems are observed (Figure 7). IE values are slightly more favorable for (-)-huprines with an ethyl group in R₉, which correlates with shorter distances between the hydrogen of the aromatic nitrogen and the histidine oxygen.

Glu199 is the residue that most contributes to the interaction energy in most huprines (Figure 7), in agreement with previous studies.⁴⁴ Glu199 presents several interactions with the surrounding residues mediated by water molecules. It also

presents non classical C-H...O hydrogen bonds, in particular with the hydrogen of one chiral carbon atom of the molecule, namely the bridgehead carbon atom at position 7. These interactions take place in the surrounding of the aromatic nitrogen of the huprines, and the IE of Glu199 is related to the distance between its carboxylic group and this nitrogen (Table 3 and Figure 7). A similar relationship is established between the IE of Asp72 and the distance of its carboxylic group to the aromatic nitrogen of (-)-huprines.

Due to the nature of the forces involved in the interaction between the ligand and the aromatic residues, the analysis of the interaction energy per residue is not evident in this case (Figure S3 of the supporting information). Therefore, an analysis has been made considering the contribution of these residues in an additive way. Huprines undergo parallel cation- π interactions with residues Trp84 and Phe330 (Figure 6b). We have added the IE of Trp84 and Phe330 (Figure 8a) to evaluate the effect that substituents in R₃ and R₉ have in the modulation of the π - π stacking interactions. The (-)-huprine that has a better interaction is (-)-HUM, which features a methyl group in R₉ and a hydrogen atom in R₃. When a chlorine atom is added in R₃, the IE of the aromatic residues involved in the cation- π interaction decrease. This fact can be explained because the relative position between Phe330 and the quinoline unit of (-)-HUY changed, approaching the benzyl group of the residue to the charged nitrogen of (-)-HUY. Substitution of methyl by ethyl at R₉, without the presence of chlorine at R₃ [(-)-HUE], also affects adversely to the cation- π interaction. The presence of a bulkier group in R₉ displaces the quinoline unit of huprine relative to its position between Trp84 and Phe330, hindering their interaction (Table 3). In this case, there is a parallel shift of Phe330 towards the amino group of (-)-HUE, so this displacement is in the opposite direction to that which takes place in (-)-HUY. Surprisingly, by replacing the hydrogen of position R₃ with chlorine in (-)-HUE, thus converting it in (-)-HUX, the cation- π interaction improves. As in the previous case, the ethyl group in R₉ displaces the huprine, but here another bulky group in R₃ restrains this

movement. This is reflected in the interaction of the (-)-huprines with the catalytic triad (Figure 8b), a bulkier group in R₉ displaces the huprine towards His440 (see distances in Table 3) improving this interaction. However, this is less pronounced when there is another bulky substituent in R₃. The IE profile is smoothed when the interactions due to the catalytic triad are added to the cation- π ones (Figure 9c).

Figure 8. Average values of the sum of interaction energies (in kcal·mol⁻¹) of (a) aromatic residues involved in the cation- π interaction, (b) residues of the catalytic triad and, (c) residues of the catalytic triad plus aromatic residues involved in the cation- π interaction.

The effect of chlorine atom in R_3

According to the experimental data^{14,16,21} and to the FEP results, (-)-huprines having a chlorine at position R_3 are the most potent inhibitors. However, the chlorine atom affects adversely the cation- π interaction, as seen above. It has been proposed that the contribution of the chlorine atom to the interaction between these inhibitors and the enzyme is due to nonspecific close spatial contacts with the hydrophobic pocket.¹⁹ Therefore, the contribution to the IE of the residues surrounding the chlorine atoms should be larger in the systems with a chlorine atom in R_3 in relation to their counterparts with hydrogen atom in this position. As depicted in Figure 6b and reported in Table S1 of the supporting information, the closest residues to the chlorine atoms are Phe330, Trp432, Met436, and Ile439. However, the major contribution of Phe330 to this IE is due to the cation- π component. The total contributions of these residues to the IE, including and excluding Phe330, are reported in Figure S4 of the supporting information. Values that involve Phe330 do not represent solely the interaction with chlorine, and if we do not take into account the Phe330 contribution the results are clearly unfavorable.

Figure 9. Average values of the addition of interaction energies (in $\text{kcal}\cdot\text{mol}^{-1}$) of (a) Trp432 + Met436 + Ile 439 + Tyr442 + Tyr334, (b) Asp72 + Trp432 + Met436 + Ile 439 + Tyr442 + Tyr334, and (c) Asp72 + Trp84 + Phe 330 + Trp432 + Met436 + Ile 439 + Tyr442 + Tyr334, in the (-)-huprine-AChE systems.

The pocket where the chlorine atom is accommodated includes a network of aromatic residues that are connected by π -stacking interactions and hydrogen bonds. Thus, Trp432 is hydrogen bonded to Tyr442, which is also hydrogen bonded to Trp84 (see Table S1 in the supporting information). Moreover, there is another tyrosine residue (Tyr334) interacting with Phe330 and hydrogen bonded to Asp72. In this way, Tyr334 and Tyr442 connect the residues that directly interact with chlorine atom with those involved in the cation- π interaction and with Asp72. Therefore, the effect of this cluster of residues in the IE is analyzed in an additive way (Figure 9): first, by including the contribution of Tyr334 and Tyr442 to the interaction energy due to the residues that are closest to the chlorine atom (not including Phe330); later, expanding this group with the contribution of Asp72 residue; and finally, also introducing the effect of the residues involved in the cation- π interaction. By adding the effect of both tyrosine residues to the interaction energy of Trp432, Met436, and Ile 439, the total interaction energy of these residues is now negative. However, there is not proof that the contribution of the chlorine atom is due to close contacts, because the difference in IE in the systems with a chlorine atom in R₃ in relation to their counterparts with hydrogen atom in the same position is less than 1 kcal·mol⁻¹. Adding the effect of Asp72 to the previous contribution shows that the difference in IE between (-)-HUX and (-)-HUE is around -8 kcal·mol⁻¹, and between (-)-HUY and (-)-HUM is around -6 kcal·mol⁻¹. If we also add the effect of the residues involved in the cation- π interaction, these differences become -9 and -0.36 kcal·mol⁻¹, respectively. These results suggest that the effect of chlorine atoms is more noticeable in a residue that is quite far at 8 Å (as Asp72) than in the closest ones (Trp432, Met436, and Ile439). On the other hand, the contribution due to the cation- π interaction compensates this effect since the presence of a chlorine atom has an unfavorable effect on the interaction, especially in the huprines with a methyl group in R₉.

The effect of ethyl group in R₉

The ethyl group in R₉ fills a hydrophobic pocket formed by Tyr121, Phe290, Phe330 and Phe331. The total contribution of these residues to the IE doesn't explain the greater affinity of the (-)-huprines having an ethyl group in R₉ with respect to the huprines having a methyl group in this position. However, we should keep in mind that the major contribution of Phe330 to the IE is due to the cation- π interaction, and an ethyl group in R₉ does not profit from this interaction. By removing the contribution of Phe330 of this IE, contradictory results are obtained for compounds with chlorine and those without chlorine. The methyl/ethyl group in R₉ is close to residues of the oxyanion hole (Gly118, Gly119), but the effect that these later residues have on the IE is not significant. Nevertheless, residues of the hydrophobic pocket form a network of interactions with Phe288, Ser122, His440, Ser200 and Glu199. The IE obtained by adding the contribution of these residues is consistent with the higher affinity of the enzyme for inhibitors having ethyl group at R₉ relative to those having a methyl group. Finally, residues forming the cation- π interaction (Phe330 and Trp84) do not modify this tendency. Thus, it seems that the effect of the ethyl group in position R₉ cannot be explained by short-range interactions, but as in the case of the chlorine atom in R₃, it is due to a network of interactions in the gorge. In these networks aromatic residues, as well as polar residues, and several water molecules are involved. This is consistent with the binding free energy results obtained by means of the alchemical FEP method, where the contribution of the van der Waals term was almost as important as the electrostatic term.

Figure 10. Average values of the addition of interaction energies (in kcal·mol⁻¹) of (a) Tyr121 + Phe290 + Phe330 + Phe331, (b) Tyr121 + Phe290 + Phe331, (c) Gly118 + Gly119 + Ser122 + Tyr121 + Glu199 + Ser200 + Phe288 + Phe290 + Phe331 + His440, and (d) Trp84 + Gly118 + Gly119 + Ser122 + Tyr121 + Glu199 + Ser200 + Phe288 + Phe290 + Phe330 + Phe331 + His440, in the (-)-huprine-AChE systems.

(+)-Huprines

The arrangement of (+)-huprines in the gorge differs from that of the (-)-huprines (Figure 11): the primary amino group of (+)-huprines is hydrogen bonded to the backbone oxygen of His440, and the protonated aromatic nitrogen of the inhibitor is interacting with Ser122 through a water molecule. (+)-Huprines also undergo parallel cation- π interactions with residues Trp84 and Phe330. However, Asp72 does not interact with the amino group of (+)-huprines, since this group is located on the other side of the molecule with respect to the residue. In the complex (+)-HUE-AChE, Asp72 interacts with two water molecules, with Tyr334 and with H3 of the inhibitor (Figure 11). The interactions with tyrosine and water molecules are retained in the other (+)-huprine systems, although the distances with the water

molecules are longer, especially in the case of huprines with a chlorine atom in R₃. This is evident in the case of (+)-HUX, where the presence of chlorine disrupts these interactions and there is only one water molecule in this position. In the latter case, the lowest value of the Asp72 interaction energy is obtained (Figure 7). The amino group of (+)-huprines interacts with the carbonyl oxygen of His440. Nevertheless, these distances are longer than the distances between (-)-huprine aromatic nitrogen and histidine oxygen, which is reflected in the values of IE for His440 (Figure 7). The presence of an ethyl group at R₉ favors this interaction, as seen in the IE and the nitrogen-oxygen distances. In the (+)-HUX-AChE system, the repulsion between the chlorine and Asp72 pushes away the inhibitor towards the histidine 440, which leads to a shorter distance between the amino group of inhibitor and the histidine and, hence, to a more favorable IE. The geometrical parameters in the relationship between residue Asp72, residue Glu199, and N⁺ atom of the ligand are reported in Figure S2 of the supporting information. The longer distance between Asp72 and the aromatic nitrogen of the inhibitor in (+)-HUX-AChE system relative to other (+)-huprine-AChE complexes evidences this displacement. Glu199 forms non-classical C-H...O hydrogen bonds with hydrogen atoms of the carbobicyclic ring of the huprines and this residue also forms a hydrogen bond with Tyr130. Moreover, both residues are involved in a network of water-mediated hydrogen bonds with other residues and the inhibitor (Figure 11a). However, the relationship between the Glu199 IE and the geometrical parameters is not evident for (+)-huprines. Paradoxically, the contribution of Ser122 to the IE is relatively small even though this residue is forming a hydrogen bond mediated by a water molecule with the charged aromatic nitrogen.

Figure 11. Plot of the main interactions between (+)-HUE and the subset of residues forming the binding pocket. Some selected average distances in Å are depicted.

BIEs

Binding isotope effects for inhibitors isotopically labeled with ^{13}C , ^{15}N and ^{18}O atoms were computed in this work using the QM/MM method based on 11 optimized structures of inhibitors dissolved in water and bound in the active site of AChE. As stated above, these structures were selected each 20ps from the last 200ps of the long QM/MM MD simulations.

A BIE value equal to 1 means that no binding isotope effect is observed. The average values <1 indicate an inverse BIE, which means that the isotopically substituted atom has a stronger interaction in the enzyme than in water. The average values >1 indicate a direct BIE.^{30,39} It is important to note that average values of BIEs other than 1 were expected only in the case of important electrostatic enzyme-ligand interactions being present in the gorge.

In (-)-huprine-AChE systems no significant BIE are observed. This is consistent with our previous study where no significant BIEs for tacrine or huperzine A were observed.²² Since (-)-huprines are tacrine-huperzine hybrids that retain most significant interactions with AChE, no significant BIEs are expected. In the (+)-huprines-AChE systems most substitutions lead to non-representative BIEs. However slight direct BIEs, namely 1.011 ± 0.002 and 1.011 ± 0.003 , are detected in the nitrogen of the amino group of (+)-HUE and (+)-HUX, respectively. This result indicates that the hydrogen bonds established between the amino group of these huprines and the water molecules of the solvent are more favorable than the interactions with the enzyme, despite the low value of the BIE.

AChE is a singular enzyme, whose active site is a deep cavity of 20 Å composed by several aromatic residues interacting between them and with the inhibitors by π -stacking interactions. This cleft is filled of water molecules that enable a network of hydrogen bond interactions between the polar residues of the gorge and the inhibitor. Moreover, due to their hydroxyl group, some tyrosine

residues bridges that connect the bulk of aromatic and polar residues of AChE. All these particularities result in the singular interactions between the enzyme and the inhibitors. The nature of the hydrophobic interactions and the similar intensity of the hydrophilic interactions in water and enzyme lead to no remarkable BIEs.

CONCLUSIONS

Alchemical free energy perturbation method, based on MD simulations within hybrid QM/MM potentials, has been used to calculate the binding free energy of a set of huprines with AChE. Previously, it was verified that this method was appropriate to distinguish AChE ligands that present large inhibitory activity differences at a reasonable computational cost.²² (-)-Huprines have been found to be more effective inhibitors than their corresponding enantiomers, in agreement with the experimental results. Present results reproduce the experimental inhibitory tendency²¹ for both huprine X and huprine Y enantiomers. The present study points to this method as a suitable approach to distinguish between inhibitors of similar potencies. Findings also reveal the importance of the van der Waals interaction in the inhibition process since the contribution of this term is almost as important as the electrostatic one. The analysis of the geometrical parameters and interaction energy per residue confirm these data and highlight the nature of this singular enzyme. BIE results also corroborate the distinctive features of AChE, since no significant BIEs were observed in systems with some important electrostatic interactions. This can be attributed to the large number of water molecules involved in the interactions between the inhibitors and the enzyme.

Asp72, Glu199, and His440 are the residues that most contribute to the interaction energy with the inhibitors. The amino group of (-)-huprines interacts with Asp72 through one or two water molecules whereas the aromatic nitrogen forms a direct hydrogen bond with the carbonyl oxygen of His440. These residues

stretch the inhibitor in opposite directions and the balance of forces is conditioned by the substituents at R₃ and R₉. A bulky group at R₉ (ethyl) displaces the inhibitors towards His400, thereby promoting this interaction, albeit at the expense of disturbing the cation- π interaction with Phe330 and Trp84. On the contrary, if there is a chlorine atom at R₃, the distance between His440 and the inhibitor increases and the His440 interaction energy decreases. In this case the sandwich- π interaction with Phe330 and Trp84 is also affected due to the displacement. The simultaneous presence of bulky groups at R₃ and R₉, leads to more restrained displacements and less affected cation- π interaction. The high contribution of Glu199 to the IE seems difficult to explain based on its non-classical C-H...O hydrogen bond with the inhibitor. However, it is involved in a hydrogen bond network mediated by water molecules with residues of the catalytic triad in the vicinity of the charged aromatic nitrogen of (-)-huprines.

The effect that a chlorine atom at R₃ position has on the inhibitory efficiency cannot be explained by means of the contribution of the surrounding residues to the interaction energy. The same observation is valid for the effect of the ethyl group at R₉. Thus, the stabilization due to short-range interactions can be ruled out. Actually, it seems that a cooperative effect of the residues of the gorge determine the affinity of the enzyme for these inhibitors. Asp72, Glu199 and His440 contribute significantly to the network of interactions between active site residues, which stabilize the inhibitors in the gorge.

(+)-Huprines are the less active enantiomers, whose arrangement in the active site differs in some aspects from that of (-)-huprines. However, Asp72, Glu199 and His440 are also the residues that most contribute to the IE, even though the relationship between these IE tendencies and the geometrical parameters is not always evident. Emphasis is given to the low contribution of Ser122 to the IE, although this residue forms a hydrogen bond with the aromatic nitrogen of (+)-huprines mediated by a water molecule. This also points out that the affinity for

the inhibitor is due to a cooperative effect of the whole enzyme, where Asp72, Glu199 and His440 have a prominent contribution.

AUTHOR INFORMATION

* Tel.: +34 964 728067. Fax. +34 964 728066.

* E-mail: oliva@uji.es (M.O.)

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Supporting Information

Binding Free Energy Calculations to Rationalize the Interactions of Huprines with Acetylcholinesterase

Érica C. M. Nascimento, Mónica Oliva and Juan Andrés*

Departament de Química Física i Analítica, Universitat Jaume I, 12071 Castelló, Spain

Figure S1. Potential energy evolution (a) and root mean square deviation (RMSD) evolution of the AChE backbone (b) for the AChE-huprine systems as function of the AM1/MM MD simulation time. Variation coefficient (VC) for the potential energy is also included.

ACh*: $X=1$, $Y=1$: ACh, (-)-HUX, (-)- HUY, (-)- HUE, (-)- HUM, (+)-HUE, (+)- HUX,

(+)- HUM: $X=1$, $Y=2$ and (+)- HUY: $X=2$, $Y=1$

	a (Å)	b (Å)	c (Å)	a (°)
ACh*	15.03 ± 0.30	11.44 ± 0.27	3.67 ± 0.13	167.05 ± 4.52
ACh	12.11 ± 0.40	8.84 ± 0.34	4.02 ± 0.30	137.80 ± 7.11
(-)-HUX	13.50 ± 0.27	8.04 ± 0.17	5.99 ± 0.33	148.46 ± 3.05
(-)-HUY	14.08 ± 0.30	8.72 ± 0.21	6.30 ± 0.24	138.67 ± 3.28
(-)-HUE	14.32 ± 0.48	9.04 ± 0.47	5.61 ± 0.22	155.04 ± 3.23
(-)-HUM	14.36 ± 0.37	9.00 ± 0.26	6.20 ± 0.35	141.09 ± 23
(+)-HUE	13.90 ± 0.56	7.27 ± 0.54	7.01 ± 0.12	153.90 ± 2.67
(+)-HUX	16.20 ± 0.27	8.44 ± 0.29	8.11 ± 0.22	156.26 ± 2.38
(+)-HUM	15.01 ± 0.23	7.16 ± 0.26	7.87 ± 0.24	175.38 ± 2.67
(+)-HUY	14.23 ± 0.26	7.95 ± 0.26	6.81 ± 0.13	149.38 ± 3.42

Figure S2. Geometrical parameters in the relationship between residue Asp72, residue Glu199, and N^+ atom of the ligand in AChE-ligand complex interactions. Averaged values from the last 500ps of the QM/MM MD simulation. ACh* represents the acylated AChE-ACh complex.

Figure S3. Average IE of some aromatic residues in the (-)-huprine-AChE systems.

Table S1. Selected averaged distances, in Å, involving the chlorine atom and the surrounding residues for (-)-HUX and (-)-HUY systems.

Distance (Å)	(-)-HUX	(-)-HUY
CG2(Ile 439) - Cl Huprine	3.87 ± 0.18	3.94 ± 0.17
CE (Met436) - Cl Huprine	3.90 ± 0.18	3.79 ± 0.21
NE1(Trp432) – Cl Huprine	3.87 ± 0.22	4.01 ± 0.22
CE2(Trp432) – Cl Huprine	3.65 ± 0.16	3.70 ± 0.16
CA(Phe330) – Cl Huprine	4.30 ± 0.17	4.18 ± 0.21
CB(Phe330) – Cl Huprine	4.09 ± 0.16	4.10 ± 0.20
CE1(Tyr334) – C2 Huprine	3.85 ± 0.19	4.18 ± 0.25
HH(Tyr334) – OD1(Asp72)	1.70 ± 0.13	1.70 ± 0.13
OH(Tyr442) – NE1(Trp432)	2.88*	2.89*
OH(Tyr442) – NE1(Trp84)	2.88*	2.88*
CE1(Tyr442) – Cl Huprine	4.67*	4.72*
CE1(Tyr334) – Cl Huprine	4.66*	4.67*

*values in the last structure of the QM/MM MD.

Figure S4. Average values of the addition of interaction energies (in $\text{kcal} \cdot \text{mol}^{-1}$) of (a) Trp432 + Met436 + Ile 439 and, (b) Phe 330 + Trp432 + Met436 + Ile 439 in the (-)-huprine-AChE systems.

Article 3

6. Systematic study of several Acetylcholinesterase Inhibitors through computational means: A molecular docking study.

*"How can I leave you alone there? You are already alone
such a star on the sky without its moon." (Ibrahim Berksoy)*

Acetylcholinesterase Inhibitors through Molecular Docking Simulations: A critical Overview.

Érica Cristina Moreno Nascimento,^a Nuno M. F. S. A. Cerqueira,^b Mónica Oliva,^a Juan Andrés,^a Pedro A. Fernandes^{b,*}

^a*Departament de Química Física i Analítica, Universitat Jaume I, 12071 Castelló, Spain*

^b*UCIBIO@REQUIMTE, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, 4169-007, Porto, Portugal*

ABSTRACT: The binding interactions of 44 molecules belonging to 8 families of molecules, which have shown promising capabilities as AChE inhibitors, have been explored theoretically employing molecular docking simulations. The results have shown that the best AChE inhibitors should be able to interact with both CAS and PAS sites of the gorge. The main residues of CAS involved in these interactions are Ser200, Glu199 and His440 through the formation of hydrogen bonds. The Trp84, Phe330, and Tyr130 are the most active PAS residues and interact with all molecules through π - π , cation- π and π -stacking interactions in the process of the inhibition of AChE. The docking range scores and experimental IC₅₀ values were compared and a good agreement between theoretical and experimental inhibition was obtained among the AChE all inhibitors that were studied.

Keywords: Acetylcholinesterase, inhibitors, MAOIs, AutoDock4, VsLab.

1. INTRODUCTION

Acetylcholinesterase (E.C. 3.1.1.7), also known as AChE or acetylhydrolase, is a serine protease that hydrolyzes the neurotransmitter acetylcholine (ACh) to acetate and choline.¹

AChE is an ultra-rapid acting enzyme being characterized by high rates of ligand binding and high catalytic activity a condition that is rarely found in biology.²⁻⁴ Each molecule of AChE can degrade about 25000 molecules of ACh (see Scheme 1) per second, approaching the limit allowed by diffusion of the substrate (see Scheme 1).²⁻⁴ ACh is one of many neurotransmitters that are available in the nervous system and acts on both peripheral nervous system and central nervous system. Yet the role played by this neurotransmitter is unique as it is specific in the motor division of the somatic nervous system.⁵⁻⁷

The AChE is anchored in the postsynaptic membrane between nerve cells and muscle cells (post-synaptic membrane) and in other tissues e.g., red blood cells.^{1,5-7}

Scheme 1. Acetylcholine hydrolysis.

During neurotransmission, ACh is released from the nerve cell (axon terminal) into the synaptic cleft and binds to ACh receptors that are located on the post-synaptic membrane^{6,8} (Figure 1). However, for a cholinergic neuron to receive another impulse ACh must be released from the ACh receptor and this only takes place when the concentration of ACh in the synaptic cleft is very low. Here is where the role of AChE becomes crucial, hydrolyzing ACh shortly after it dissociates from the receptor.⁸⁻¹⁰

Figure 1. Role of AChE in the nerves cells (ACh: Acetylcholine; AChE: Acetylcholinesterase)

The formation of choline is subsequently taken up again by the pre-synaptic nerve and ACh is synthesized by combining it with acethyl-CoA through the action of choline acethyltransferase. This effectively stops the nerve signal, allowing the pieces to be recycled and rebuilt into new neurotransmitters that will be used to trigger the next message.^{11,12}

1.1 Structure of the enzyme

AChE is the one of the most important molecular target for therapeutic intervention in the treatment of various kinds of disorders such as Alzheimer's disease (AD), myasthenia gravis, and glaucoma. For treatment of AD, drugs that inhibit ACh hydrolysis aiming to keep the gradient of concentration of the neurotransmitter in the synapses are used.

This enzyme is an allosteric enzyme from the class of hydrolases that is composed by 537 amino acid residues and with high dipole moment (800 - 1800 Debye) that is oriented along its active site (Figure 2).¹³

The structure of AChE presents low flexibility despite having a large number of amino acids residues (more than 500). However, some important structural changes were observed in some residues of the active site pocket when the enzyme interacts with some inhibitor, such as, rivastigmine, bis(7)-tacrine, and huperzine A and B. These conformational changes are particularly relevant with the residues His440, Trp279 and the residues of aromatic peripheral site (Figure 3).²

Figure 2: Superposed 3D structures of *Torpedo californica* –TcAChE, PDB code: 2ACE (ACh shown in cpk model), 2CKM, 1DX6, 1GQR, 1E66, 1EVE, 1SOM (shown in ribbons). SASA of the active site, gorge (colored in gold).

New studies have shown that AChE plays an important role in the formation of β -amyloid senile plaques^{10,14}. A conformational change in the structure of the amyloid precursor protein (APP), takes place in the peripheral site gorge. AChE causes a breakdown of this protein and onset the appearance of amyloid fibers that generates aggregation of beta-amyloid peptides during the initial phase of the AD.¹⁵ Thus, drugs that inhibit the action of AChE, not only in the region of SEH but also in the peripheral region of its active site, have great importance in the treatment of this disease. AChE rules in two directions: promotes the maintenance of cholinergic functions and avoids the appearance of senile plaques that produce neuronal apoptosis.

1.2 Tunnel of the active site and the two regions

The active site of AChE is a deep canyon with approximately 20 Å depths, named “gorge”.^{2,16-19} The active site is characterized by the presence of multiple negative charges around the catalytic triad which would explain the attraction of ACh to SEH.^{2,4,16-21} The gorge, shown in Figure 3, has four sub-sites. The first sub-site is the peripheral site located at the entrance of the protein. The second one is the binding quaternary ammonium site located in the central region between the residues Trp84 and Phe330. These two sub-sites are built from several aromatic residues and hence the whole area covered is termed peripheral aromatic site (PAS) of AChE.^{2,20} Others AChE sub-sites are the peripheral anionic site and the catalytic site located at the bottom of the acylation pocket where the deactivation of ACh occurs, these two subsites are known as the catalytic acylation site (CAS).

Figure 3: Main residues of the active site of AChE, gorge.

1.3 Reaction Catalyzed by the enzyme

There are three types of AChE inhibitors, the irreversible, pseudo-irreversible and the reversible ones. Irreversible AChE inhibitors have been used as insecticides (e.g., malathion) and nerve gases for chemical warfare (e.g., sarin and soman).^{22,23}

Carbamates, esters of N-methyl carbamic acid, are considered pseudo-irreversible AChE inhibitors, once they hydrolysis take some hours or days. The carbamates have been used for medical purposes, e.g., physostigmine in the treatment of glaucoma.^{5,16,24} The main difference between both classes of inhibitors is the stabilization in AChE-ligand complexes formed.

Figure 4. Natural substrate (acetylcholine), reversible (tacrine, galantamine, huaine, donepezil, rasagiline), pseudo-irreversible (phenserine) and irreversible (soman) AChEIs.

In contrast to the irreversible inhibitors, the reversible AChE inhibitors are effective in transiently increasing the ACh level in the synapse cleft. They are used in treatment of diseases and conditions where an increased ACh level is desired. These inhibitors occupy the active site of AChE for short periods of time (seconds to minutes) and are used to treat a range of central nervous system diseases. Some

of the reversible inhibitors of AChE are among the key drugs approved by the FDA in AD treatment.^{1,9,10,25}

People suffering with Alzheimer's disease misplace many nerve cells as the disease progresses. By taking a drug that partially blocks acetylcholinesterase, the levels of the ACh neurotransmitter can be raised, thus strengthening the nerve signals of the remaining operational nerve cells. Some of the drugs have been approved by FDA to improve cognitive function in Alzheimer's disease are galantamine and rivastigmine (Table 1).

Table 1. AChE inhibitors approved by FDA for the AD treatment.^{5,26-29}

Drug	Commercial trade name ®
Tacrine	Cognex (Withdrawn)
Donepezil	Aricept
Rivastigmine	Exelon
Galantamine	Nivalin

There are several other drugs currently in clinical trials. Some of them are in phase II and phase III such as rasagiline and huperzine A,²⁶ along with various derivatives of the first mentioned drugs. In addition, there are several studies and research focused on new drugs that may act on the cholinergic neurons to ensure the maintenance of the levels of acetylcholine in the synapse cleft, ameliorating the cognitive function of AD patients in the best possible level.

The high complexity of AD calls for huge effort to develop molecules able to modulate multiple CNS targets to promote a real improvement of AD therapy. The multi-factorial nature of the disease rules out the use of consolidated mono-therapy and put researches on the way of multipotent drugs hitting cooperatively different targets underlying the onset and/or progression of the disease.³⁰⁻³² This approach is grounded on the idea that an AChEI could exhibit a more efficient therapeutic effect in AD when able to express an additional MAOI activity, and/or inhibit other enzymes implicated in neurodegenerative diseases as BuChE, and also limiting the sources of reactive oxidative species neurotoxicity.³³ Drugs which are selective

against isoforms of particular biological target but nonselective against two or more targets are very useful in multifactorial diseases as AD and Parkinson's disease.³⁴ The development of multifunctional molecules is crucial for treating different diseases that share some particularities as neurodegenerative diseases.

AChE and MAO are currently two drug targets used to the development of new drugs to treat Alzheimer's disease, Parkinson's disease, depression and glaucoma. A polypharmacologic approach is desirable and aiming to treat and modify activities of these targets simultaneously, particularly those associated with the progression of the diseases.²⁶ Currently, many experts discuss which one of these targets is the best way to treat neurodegenerative diseases. There is an agreement for many experts in several areas in which such diseases require the use of multidrug therapy approach in their treatment. The drugs ladostigil (LAD), rasagiline (RAS), selegiline (SEL) and rivastigmine fulfill the role of drug with multiple neuroprotective activities.³⁵⁻³⁷ Thus, drugs that inhibit the action of AChE not only in the region of SEH but also in the peripheral region of the gorge have great importance in the AD treatment; not only to promote the maintenance of cholinergic functions as well as to avoid the appearance of senile plaques that produce neuronal apoptosis. New studies have demonstrated that AChE plays an important role in the formation of β -amyloid senile plaques.¹⁴

1.4 The Inhibitors

1.4.1 Neurotransmitters

The concentration of neurotransmitters in the synaptic clefts rules an important function in the states of movement, mood, emotion, cognition, memory and learning, as they are the regulators of these functions. The increase and/or decrease in the quantity of neurotransmitters in synaptic clefts can produce deleterious effects on the brain.^{11,38,39} The communication between neurons is led

by neurotransmitters, which can carry information of electrical and chemical nature in order to make possible the process of communication between cells of the CNS. The main function of synapse is chemical transmission of information from the presynaptic neuron to the postsynaptic cell.

Neurodegenerative diseases involve multifactorial situations.³⁶ In order to explain this diseases many hypotheses and biochemical evidences have been provided to support a cascade of events that include an accentuated decreasing of the graduate of concentration of the neurotransmitters acetylcholine, dopamine, epinephrine, norepinephrine, serotonin, melatonin and phenethylamine in the synapses cleft; and factors as the oxidative stress, the inflammatory process, the apoptosis. Many neuropathologies are associated with the decrease in the amount of neurotransmitter in the synapse, among them Alzheimer's disease where the drastic reduction of the neurotransmitter acetylcholine at cholinergic synapses causes cognitive impairment leading to the dementia. Hence the study of the mechanism that trigger this imbalance as well as the development of the effective drugs that promote the concentration gradient of neurotransmitters, at synapses are fundamental actions to mitigate and even, why not, heal the neuropathology on medium and long term.

1.4.2 Acridines Analogues

The family of acridines is based in the 1,2,3,4-Tetrahydro-9-acridinamine, THA, the first drug approved by the FDA for AD treatment.^{16,40} Although tacrine is not first drug of choice in AD treatment, it is taken as reference in search of more potent AChEIs.⁴¹ THA exhibits a dual inhibitory activity: inhibiting AChE and free radical scavenging activity.⁴² THA analogues are widely investigated and tested for inhibition of AChE. One of most studied family of AChEIs derived from THA is constituted by carbon.^{30,43} The compounds formed when $n=5$ and 7, the Bis(5)-tacrine (F11) and Bis(7)-tacrine (DMT) show the greater selectivity and higher activity in the inhibition of AChE.⁴³ DMT act as a dual inhibitor, inhibiting AChE

and MNDA activity in synapse.^{30,42,44} There are several compounds from acridines derivatives proposed as AChEI candidate. Two of them are the photosensitizer methylene blue (MTB)⁴⁵ a strong reversible AChEI, and the edrophonium (EDR) a competitive inhibitor of AChE used to treat myasthenia gravis. Acridines derivatives from THA and DMT are largely studied in the pursuit of best AChEI to treat AD.³⁰

Some acridines are well known AChE inhibitors; one of the most relevant interactions of the family is the strong electrostatic interaction between its piperidinium ring and the backbone oxygen atom of His440 residue. Other important interactions observed in this family of inhibitors is the sandwich π -stacking between Trp84, and Phe330 residues forming a kind of complexed π - π and cation- π arrangement.^{18,43,46}

1.4.3 Alkaloids Analogues

The alkaloids are an important and promising class of AChEI for the AD treatment. The GNT was the first alkaloid approved in 2001 to treat AD, especially for promoting significant improvements in cognitive state and inhibit the formation of tangles of beta-amyloid protein.⁴⁷⁻⁴⁹ This alkaloid is largely studied due its dual biological activity. Several research groups are developing year by year heterodimeric derivatives based in this AChEI, aiming the association of tolerability of GNT and potency of dual-target inhibitors in AD treatment.⁵⁰ The GNT derivatives with indole moiety⁵⁰ bivalent derivative as (GL8), and bis-functional derivatives⁵⁰ as the N-Piperidinopropyl-galantamine (G3X) are some of new alkaloids proposed as potent AChE inhibitors.

Other important alkaloid AChEI is the HUP. This inhibitor is a natural alkaloid found in the *Huperzia serrata* herbs and a potent reversible inhibitor of AChE. This alkaloid is a potential drug candidate for AD treatment, considering its prolonged action on the CNS, low toxicity, and protective action on neurons. The

HUP is an efficient drug against glutamate excess that can lead to death of nerve cells.^{10,40} This inhibitor also have higher oral bioavailability and longer AChE inhibition in comparison with others alkaloids molecules.⁴⁷ With synthetic route not yet defined to be produced on large scale, the research to develop HUP derivative analogues has been intensified. Some of its analogs as the huprines are proved to be 200 times more potent than HUP in the AChE inhibition.⁵¹

1.4.4 Carbamates analogues

The molecules of this class of carbamates are derivated from compounds carbamic acids (NH_2COOH). These compounds are used in different diseases as AD, myasthenia gravis, glaucoma, and Parkinson's disease. Some carbamates as physostigmine (PHY) and rivastigmine (RIV) are reversible AChEIs.⁴⁷ The natural carbamate PHY was the first drug clinically studied to be applied in the AD treatment.³² But, due its low efficiency in the long-term clinical phases II and III was rejected by the FDA.^{16,24,47} This carbamate is a molecular reference object in the development of new analogues for the treatment of AD, even no longer being used as a therapeutic drug. In mid-1998 was approved in Europe the rivastigmine (RIV), a drug derived from PHY by molecular modifications. RIV is a selective and pseudo-irreversible carbamate⁷ but exhibits superior pharmacological profile than its precursor, with good selectivity and bioactivity in vivo. It is well tolerated by patients, moreover presents neuroprotective properties and improves in cognitive function of AD patients.⁵²

The phenserine (PHS) a new drug for AD treatment, is a phenyl carbamate analog of physostigmine. It is a highly selective and a pseudo-irreversible AChEI.¹ Nowadays is in II clinical trials phase, PHS is a new promise considering its relative wide therapeutic window compared to other carbamate used for AD treatment.¹⁷

1.4.5 Huprines Analogues

In the beginning of 2000s Camps and coworkers⁵¹ designed a new class of AChEIs provided from combination of two well-known inhibitors the THA and HUP. By combining the carbobicyclic substructure of HUP with 4-aminoquinoline group of THA, the hybrid molecule called huprine was designed. This new class of AChEI provides the development of various derivatives by the substitution of the position 9 (ethyl group e.g. huprine E: HUE, Figure 4) and position 3 (chlorine e.g. huprine X: HUX; the huprine Y: HUY with R9= Methyl, Figure 4).^{30,51}

Some huprines as HUX presented more affinity by AChE than others earlier AChEIs reported till 2000s. The huprines interact with AChE as THA and HUP, in addition present some others important interactions with conservative residues of gorge as Asp72 and Glu199.^{30,51} Following the tendency of others classes of AChEIs a huge amount of multi-target heterodimeric derivatives of huprines were developed.

1.4.6 MAO inhibitors

The monoamine oxidase (MAO, EC 1.4.3.4, amino-oxygen reductase) is a flavoenzyme localized at the inner of the outer mitochondrial membrane, which is tightly bonded through its C-terminal sequence. The biochemical function of MAO is the oxidative deamination of monoamine neurotransmitters as serotonin (5-HT), noradrenalin or norepinephrine (NOR), and DOP.^{33,35} The enzyme presents two distinct isoforms, MAO-A and MAO-B. The MAO-A isoform preferentially deaminates 5-HT, EPI, and NOR, and selective inhibitors as clorgyline. The MAO-B isoform metabolizes neurotransmitters as dopamine (DOP) and benzylamine (PHE), and the inhibitors as rasagiline (RAS) and selegiline (SEL).

The increasing level of MAO-B in brain may contribute to onset of neurodegenerative diseases as AD and Parkinson, through the formation of free radicals via MAO-B action. In this way, the inhibitors of MAO isoforms, the

MAOIs, play a beneficial neuroprotective action in neurodegenerative diseases by reducing oxidative stress conditions, preventing neuronal damage and decrease apoptosis in neural cells caused by MAO isoforms actions in synaptic clefts.^{37, 53, 54} Therefore SEL, RAS and its derivative were also studied as a potential AChEI for AD treatment and as multi-target drugs. The propargyl moiety in SEL and RAS may have intrinsic neuroprotective activity not related to its ability to bind covalently to MAO-B and inhibit it.³¹

Association between AChEIs and MAOIs molecules has been studied aiming to search multi-target drugs for neurodegenerative diseases. A more efficient molecule able to inhibit in the same concentration AChE and MAO isoforms is a special target in biochemistry and computational chemistry field. A new class of multi-functional drugs - the AChEI-MAOI drugs is emerging.³⁰ Youdim et al. studied a large series of dual MAO-AChE inhibitors with propargyl moiety (present in SEL and RAS). This study was based in the molecular design of molecules combining in same molecule propargylaminic fragment characterizing some well-known MAOIs as SEL and RAS, with carbamoyl moiety, which could add the AChE inhibitory property.^{33,36}

The AChEI-MAOI, ladostigil (LAD) emerged from the Youdim's studies. This dual inhibitor is synthesized by linking directly the carbamoyl moiety from RIV (AChEI) at RAS (MAOI) molecule.^{35,36} Ladostigil show anti-depressant activity while inhibiting both MAO isoforms, and also the ability to increase EPI and 5-HT levels on respective synaptic clefts. In addition LAD possesses multiple therapeutic activities as reduces apoptosis and stimulates the processing of APP protein.⁴² The LAD nowadays is in II phase of clinic test for AD treatment.

1.4.7 N-benzylpiperidines Analogues

The class of N-benzylpiperidine is other important group of AChEI focus in AD treatment. In 1996 donepezil (DNP) was the second AChEI approved for AD treatment. This drug was developed by using studies from molecular recognition

QSAR.^{40,55} It was developed to overcome the disadvantages of the side effects produced by the THA. When donepezil is used in the treatment of mild and moderate AD significant improvements in compared treatment with THA were observed.^{56,57}

This AChEI is a dual site inhibitor being able to interact with CAS and PAS subsites of AChE through hydrophobic interactions and hydrogen bonds with gorge residues.³⁰ In addition DNP is a potent AChEI acting with inhibitory power in the β -secretase, BACE-1, thus offering neuroprotective effect to brain cells. AD patients treated with DNP shown ameliorated delay in cognitive and functional decline during the treatment.³¹

Other N-benzylpiperidine studied as AChEI is the 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin (CP0), an anticancer prodrug approved for the treatment of colon cancer, with known AChE inhibitory action. CP0 has a dipiperidino moiety, making similar interactions as the AChE-DNP complex.⁵⁸ Some others N-benzylpiperidines derivatives in which indanone moiety of DNP is replaced by different others groups were also proposed as AChEI. The bioisosteric N-benzylpiperidinebenzoxazoles presented higher selectivity and potency than DNP.³²

1.4.8 Organophosphates Analogues

The organophosphorus compounds (OPs) are esters or thiols derived from phosphoric, phosphonic and phosphinic acid. This class of compound has been used as chemical weapon agent, pesticides, plasticizers, pharmaceuticals and parasiticide in veterinary medicine.^{47,59} OPs are extreme toxic molecule acting directly in CNS and muscles cells of mammals and some others animals. The acute toxic effect of OPs is related to irreversible inactivation of AChE. This occurs through the phosphorylation of the enzyme to form a stable OP-AChE adduct by the covalent bond formed between phosphonyl group of OP and the –OH group of Ser200 residue.⁵⁹

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Some OPs were studied from the 1930s as potent irreversible inhibitors of AChE in very low concentrations.^{47,59-61} Sarin (SAR) gas was the first OP molecule studied as AChE inhibitor.⁶² This AChEI is a very potent nervous agent and acts on AChE producing a strong phosphonylated complex via effective covalent bond with the residue Ser200 of gorge.⁶³⁻⁶⁵

Other organophosphorous compound studied as AChEI, the 2-(3,3-dimethylbutyl) methylphosphonofluoridate, soman (SOM) is a non-selective and irreversible cholinesterase inhibitor. These compounds inhibit potently both AChE and BuChE enzymes. The SOM, as SAR also form an effective phosphonylated AChE complex.⁶⁵⁻⁶⁷

Metrifonate (MTR) is a prodrug, and unique AChEI of OP family studied as potential drug for the treatment of patients with AD. When metabolized, MTR produces the active metabolite 2,2-dichlorovinyl dimethyl phosphate (DCV) that acts as selective and pseudo irreversible inhibitor on AChE with very slow rate of dephosphonylation.^{59,62} MTR was rejected on its FDA-approval because presented severe side effects in muscles and respiratory system for some patients in phase III of clinical trials.³²

The AChE-OP adducts formed with SAR, SOM, DCV, and other OPs have a very slow dephosphonylation reaction. In case of DVC, it takes some days for completely dephosphonylation of AChE, but in other OPs as SAR the inhibition is considered irreversible. AChE-ACh adduct shows very fast deacylation step, regenerating the enzyme in its free form. On the other hand the dephosphorylation is very slow (or definitive) and the phosphorylated enzyme is unable to hydrolyze ACh.⁴⁷ As a consequence of the irreversible inhibition of AChE by OPs, ACh is accumulated in synaptic cleft and an over-stimulation of cholinergic receptors is produced, blocking the neurotransmission.

In this work, we present a detailed theoretical study, performed by molecular docking simulation, on the binding interactions of a total of 44 molecules that were divided in 8 families, depending if they are reversible, irreversible, classical and non-classical inhibitors of AChE.

The first family of molecule studied are the acridines:^{41,68,69} Tacrine (THA),^{24,25,70} followed by bis(5)-Tacrine (F11),⁴³ bis(7)-Tacrine (DMT),^{43,70} (RS)-Tacrine(10)-Huperdone (A2E),⁷¹ methylene blue (MTB)⁴⁵ and edrophonium ion (EDR),⁷² all reversible AChEI molecules. The second family of molecules is composed by alkaloids,^{73,74} as galantamine (GNT),^{28,55,75,76} huperzine A (HUP),^{3,25,77} N-piperidinopropyl-galantamine (G3X)⁷⁶ and bis-acting galantamine (GL8).⁷⁸ The pseudo-irreversible AChEI family of the carbamates composed by rivastigmine (RIV),^{24,25,29,34} physerine (PHS)^{1,28,79} and physostigmine (PHY)^{24,28,69,80} are the third class of inhibitors studied. Huprines molecules (-)-huprine X (HUX) and (-)-huprine Y (HUY) and its derivatives (HUR), (HUE) and their (+)-huprines stereoisomers⁸¹ (RUX, RUY, RUE, and RUR), see Figure 4 in a total of 8 molecules was also studied. The fifth family belongs to the monoamino oxidase inhibitors,^{34,53,82,83} such as the isocarboxazid (ISO),³³ iproniazid (IPR),³³ nialamide(NIA),^{82,84-86} moclobemide (MOC),³³ phenelzine (PHZ),³³ tolloxatone (TOL),³³ tranlylcyclopropamine (TRA),³³ rasagiline (RAS),^{82,84-86} selegiline (SEL)^{82,84-86} and the dual target MAO-AChE ladostigil (LAD)^{34,54,87} inhibitor. The family of neurotransmitters is compound by acetylcholine (ACh),^{6,88,89} and the monoamines dopamine (DOP),^{88,90,91} epinephrine (EPI),^{88,90} norepinephrine (NOR),^{88,90} serotonin (5-HT),^{88,90,91} melatonin (MEL)⁹⁰ and phenethylamine (PHE).⁹⁰ The N-benzylpiperidine family of molecules is formed by donepezil (DNP)^{24,28,55,92-94} and the prodrug 7-ethyl-10-[4-(1-piperidino)-1(piperidino)]-carbonyloxycamptothecin (CP0).⁵⁸ Finally, the irreversible inhibitors dichlorvos (DCV),^{59,95} metrifonate (MTR),^{28,96} soman (SOM)⁶⁷ and sarin (SAR)^{67,97} form the family of organophosphates of our study.

2. METHODOLOGY

2.1 Molecular Docking

Molecular docking techniques are many times used to predict the binding pose and the affinity between a receptor and its biological ligand. The interactions they establish are fundamental for the functionality of a given receptor.

The molecular interaction between AChE and 44 ligands were studied using the molecular docking software AutoDock4⁹⁸ and the vsLab⁹⁹ plug-in. Due the diversity of nature and size of inhibitors, the structure of the receptor was built from the PDB crystallographic structure specifically selected to bind each family of molecules. As the gorge suffered rearrangements in the rotamers of crucial residues, such as Trp84, Phe330, His440 and Gly118 when AChE interacts with inhibitors such as DMT⁴³ and HUP,⁷⁷ the use of a unique protein structure to carry out molecular docking calculations would not be recommended. Thus, 8 different structures were selected in present work: 2CKM⁴³ to acridines; 1DX6⁵⁵ to alkaloids; 1GQR⁷⁴ to carbamates; 1E66¹⁰⁰ to huprines; 1GQR⁷⁴ to MAOIs; 2ACE¹⁰¹ to neurotransmitters; 1SOM⁶⁷ to organophosphates and 1EVE⁹³ to N-benzylpiperidine. All crystallographic structures contain the *TcAChE* monomeric form and present resolution below to 2.5 Å.

In this way, since there is at least one PDB structure AChE-Ligand complex for each family (excepting for MAOIs).

To begin with, we took the crystallographic ligands out of the X-ray structures and redock them again with AutoDock4. The selection of the structure to be used with each family was done on the basis of the RMSD values between the predicted docking solutions and the corresponding crystallographic structures, for each inhibitor (Table 2). In addition, we also performed RMSF study for the active site residues shoot for the best PDB structure elections (see Table S1 in supplementary information).

Table 2. Comparison of RMSD values for each inhibitor with all PDB structures by family.

Family	Inhibitor						
<i>Acridine</i>		2CKM	1ACJ	1AX9	2CMF	1ZGC	2W9I
	DMT	4,412	5,188	5,297	5,114	5,276	4,886
	THA	0,105	0,112	0,108	0,119	0,105	0,111
	EDR	2,104	2,246	2,108	2,221	2,254	2,191
	F11	3,587	3,973	3,907	3,73	3,527	4,341
	A2E	2,998	3,617	3,24	3,889	3,093	4,045
	MBT	0,817	1,103	0,857	1,031	0,837	0,809
<i>Alkaloid</i>		1DX6	1W4L	3I6M	1VOT		
	GNT	3,016	3,077	3,132	3,139		
	GL8	4,637	5,044	5,009	4,981		
	G3X	2,671	3,309	2,676	3,071		
	HUP	1,389	1,389	1,389	1,391		
<i>N-Benzylpiperidine</i>		1EVE	1U65				
	DNP	2,308	2,453				
	CP0	8,711	8,085				
<i>Organophosphate</i>		1SOM	1CFJ				
	SOM	0,843	1,078				
	SAR	0,189	0,437				
	DCV	0,13	0,455				
	MTR	1,103	0,939				

The ligands were built with GaussView,¹⁰² neutral and protonated at physiological pH and optimized with Gaussian09¹⁰³ (B3LYP/6-31G (d,p)). The docking experiments show that there is no significant difference in scores tendency (range) and poses between neutral and charged inhibitors. For this reason, since there are molecules that have just the neutral form at physiological pH, e.g. organophosphates family, in this study we will present MD results considering all inhibitors molecules in neutral form.

In the docking process, the Lamarckian genetic algorithm (LGA) was used. The number of generations, energy evaluations, and docking runs were set to

2500000, 27000, and 150, respectively. The atomic charges types were Kollman-all-atom for the receptor and Gasteiger for the compounds. The final solutions were retrieved based on the score and interacting energy.

The dimensions of the active site box were chosen specifically for each family of inhibitors, in order to consider all residues of the gorge. The dimensions of the box in this MD was set to be 24.0 Å x 22.0 Å x 24.0 Å to acridines; 22.0 Å x 19.0 Å x 21.0 Å to alkaloids; 18.0 Å x 20.0 Å x 21.0 Å to carbamates; 22.0 Å x 24.0 Å x 21.0 Å to family of the huprines; 19.0 Å x 17.0 Å x 21.0 Å to MAOIs; 16.0 Å x 21.0 Å x 20.0 Å for N-benzylpiperidine; 19.0 Å x 17.0 Å x 21.0 Å to neurotransmitter family; and 19.0 Å x 17.0 Å x 20.0 Å to organophosphorous compounds. Here we considered the value of the point space equal to 0.250 for all families of compounds.

3. RESULTS AND DISCUSSION

Although AChEIs present common inhibition properties, the nature of their chemical structure and interaction are different, as shown in Figure 4. The selection of the molecules studied in this work was taken based on the importance of them in AD treatment, as well the relevant function of these molecules while interacting with the CNS, as the neurotransmitters which play a role in brain functions.

In the next sections, we follow a simple schedule to identify our results and original crystallographic structure of molecules deposited in PDB data bank. In our results the compounds are drawn in licorice representation in red and pink color; the inhibitors in the original PDB structures are drawn in licorice representation in blue color.

3.1 Docking of NTs

Here, we studied the interaction between the neurotransmitters ACh, DOP, EPI, NOR, 5-HT, MEL and PHE with AChE, to understand how all these inhibitors interact with the enzyme. Using the structure of the complex AChE-ACh deposited in PDB data bank under the code 2ACE, we run a series of molecular docking simulations to understand the binding pose and recognition mechanism of these NTs at molecular level.

Best docking results for NTs and AChE are show in Figure 5. The lowest docking score was observed with ACh (-5.11) and the highest with NOR (-8.79) ligands. This seems to be reasonable since ACh shouldn't bind better than the inhibitors (otherwise the inhibitors would not really inhibit the enzyme).

Figure 5. Best poses for inhibitors of the neurotransmitters family in AChE using 3D structure under PDB code 2ACE (ACh, from PDB X-ray structure is represented in blue color). (a) The natural substrate ACh (red color, molecular docking pose) is docked well in to the active site, and with highest dock score. (b) Best poses found on the flexible ligands docking of DOP, EPI, NOR, and 5-HT in AChE.

As seen in Figure 5 (a), the best pose of ACh fitted with high accuracy the PDB conformation of the molecule (RMSD deviation for the X-ray structure of 2.892). The ACh quaternary ammonium cation of ACh is directed at the Trp84 residue, making a cation- π interaction. The methyl groups of ACh are making π -

stacking interactions with Trp84 and Phe330 residues. The ester group of ACh is near of Ser200 residue (red color: 2.41 Å), compared to 1.48 Å at the 2ACE crystallographic structure complex. This result is in conformity with the expected AChE mechanism for ACh hydrolysis.

For others NTs, all poses shown binding interactions that are similar to the natural substrate (Figure 5 b). The NTs DOP, EPI, NOR and 5-HT have similar characteristic to fit in the gorge of AChE, through cation- π interactions with its NH_3^+ groups and π -stacking interactions between its aromatic rings and indole ring of Trp84, and hydrogen bond interactions between OH group of these NTs and OH group of Ser200.

3.2 Docking of Acridines

The R-L interaction of the acridines THA, DMT, F11, A2E, MTB and EDR was studied. In Figure 6 are shown the best poses of acridines. They are ranked by the docking score ranking, intermolecular energy and theoretical K_i (calculated through molecular docking).

In general, the docking results are in agreement with site directed set up of acridines. Monomeric acridines interacting with CAS residues, and dimeric compounds interacting with both CAS and PAS residues, Figure 6. The DMT presents the highest docking score (-14.52) and EDR the lowest (-6.62) for all acridines studied. Most of the poses shown π -steking interactions between the AChEIs aromatic rings and Trp84, Phe330, Trp279, and Tyr70 (not showed).

The good agreement between our results superposed with DMT PDB structure is shown in Figure 6 (a). This AChEI is the most potent inhibitor studied in the family of acridines. All interactions between DMT^{43,46} and AChE reported in literature also were observed in our results, specially the interaction between the N atom of the piperidine ring of DMT and the backbone oxygen atom of His440.

For other acridines this distance is reported in table 2, as for THA,¹⁸ EDR,⁷² F11,⁴³ and A2E⁷¹ inhibitors.

Figure 6. Best Docking poses of acridines (docked ligands represented in red color) using the TcAChE (PDB code 2CKM) structure. (a) The ligand DMT (PDB 2CKM); (b) A2E (PDB A2E); (c) THA (PDB 1ACJ) are docked well into the gorge, and with the highest dock score. Molecules represented in blue color are extracted from original PDB structure of each AChEI. (d) Superposition of all ligands of acridines family in the active site of AChE (2CKM).

Table 3. Main electrostatic interaction in acridines compounds based in THA. The hydrogen bounding AChEI:N....O:His440. All values are represented in Å.

<i>N....O:His440</i>	DMT	F11	A2E	THA	EDR
<i>PDB structure</i>	3.02	2.95	3.13	3.24	5.37
<i>Docking</i>	2.63	2.64	3.42	2.53	4.72

In our flexible docking ligand study for THA was observed a divergence in the conformation of this rigid molecule while comparing with its original

crystallographic structure (1ACJ). In this study, the -NH₂ group of tacrine is interacting by hydrogen bond with the backbone oxygen atom of His440, distinguishing this acridine of others as DMT, F11 and A2E, where this interactions take a place in the N atom of the piperidinium group. This acridine is a condensed rigid three member rings molecule, which presents high symmetry in its structure. Thus, in attempt to get proper pose as indicated in 1ACJ structure, we performed a short study considering as flexible, not only the ligand, but also the residues Phe330 and Trp84. Surprisingly the poses generated keep the same orientation of THA. In this study the -NH₂ group of tacrine is hydrogen bonded to the backbone oxygen atom of His440, distinguishing this acridine of other as DMT, F11 and A2E where the interaction involving the tacrine NH₂ group is replaced by the interaction involving the N atom of the piperidinium group.

3.3 Docking of Alkaloids

The interactions between AChE and the alkaloids GNT, GL8, G3X and HUP were studied by means of the molecular docking simulations. The best poses for this alkaloid, as well as their docking score are shown in Figure 7. All poses shown similar binding interactions once the compounds GNT, GL8 and G3X have similar characteristic to fit in CAS subsite of gorge, and a similar sub-unit of GNT (in G3X and GL8 dimers). Most of the poses reveled hydrogen bonding interactions between GNT oxygen atoms and the CAS residues Asp72, Ser200, Glu199, His440. Since GNT act as a hydrogen bond donor⁵⁰ is reasonable find these kind of interactions.

The GNT and its derivatives also exhibit π - π interactions with Trp84 and Phe330 residues. The Trp84 promotes a π -stacking interaction with the indole ring of GNT monomeric structure.^{46,80} The main interactions between residue Phe330 and GNT unit take a place by cation- π interactions.^{55,80} Besides this function, since the Phe330 residue is quite flexible and able to adopt different side-chain

conformation in AChE active site, this residue acts, during the inhibition of AChE, as a swinging gate in order to interact through π - π and cation- π interactions with the bound ligands.⁷⁶

The results of HUP alkaloid docking study confirm predictions of previous studies that suggest interactions between the amino group of the inhibitor and indole ring of Trp84, and aromatic ring of Phe330 through cation- π interactions.^{46,101} Other important hydrogen bonds interactions were observed with HUP and residues the Tyr130 and Glu199.

Figure 7. Best poses for flexible ligand docking of alkaloids (docked ligands molecules represented in red color) using the TcAChE (PDB code 1DX6) structure. Molecules represented in blue color are extracted from original PDB structure of each AChEI. (a) GNT (PDB 1DX6); (b) G3X (PDB 3I6M)

The lowest docking score in the family of alkaloids studied here correspond to GNT (-9.45) and the highest score to GL8 (-15.35), are in agreement to the experimental IC_{50} values.

3.4 Docking of Carbamates

Since the crystallographic structure of RIV with entry PDB ID code 1GQR was crystallized in adduct form AChE-RIV, the desirable superposition between it and the pose of docking studies is quite difficult, whereby request special considerations. The RIV inhibitor is fragmented in two groups identified as EMM (carbamoyl moiety bonded with OG:Ser200) and SAF [1-(dimethylamino)ethyl]phenol group. In this way, best poses for RIV (-7.33), PHS (-11.15) and PHY (-8.68) carbamates along their docking scores (numbers in parenthesis) are shown in Figure 8. Most of the poses revealed hydrogen bond and π -stacking interactions between AChE and the carbamates.

The superposition of the best pose for RIV docked and the EMM, and SAF (come from crystallographic structure) fragments is shown in Figure 8 (a). The carbamoyl moiety of RIV form strong hydrogen bond with residues Ser200 (2.41 Å), Gly118 (2.28 Å), and Gly119 (2.19 Å) of the CAS site of the gorge. These interactions were previously related for Bar-on et. al.⁷⁴ The position of the SAF fragment and the similar correspondent in RIV docked structure (our pose) reveals a possible conformation for this carbamate before the catalytic reaction with AChE. And the 1-(dimethylamino)ethyl]phenol group of RIV is stacked against Trp84 and Phe330 residues.

For PHS inhibitor all generated poses shown hydrogen bond interactions between its carbamoyl moiety and the (not usually relevant residues) Gln69 (2.15 Å) and Asn85 (2.77 Å). The PHY carbamoyl moiety shown different pattern of interactions than RIV and PHS is hydrogen bonded to Tyr130 (2.72 Å), Gly117 (2.22 Å) and Ser122 (2.71 Å) residues. These observations indicate that the PHS and PHY carbamates probably are not able to acylate AChE in its catalytic site.

Figure 8. Best poses for ligand docking of carbamates, (a) ligand represented in red (RIV), and (b) ligands represented in pink color (PHS), light red color (PHY) using the TcAChE (PDB code 1GQR) structure. The SAF and EMM fragments are extracted from rivastigmine ligand from original PDB structure code 1GQR (are represented in blue color).

All poses of RIV, PHS and PHY show π -stacking interactions with residues Trp84 and Phe330 residues (Figure 8b). These results highlight the importance of this kind of interactions between carbamates and aromatic PAS residues.

3.5 Docking of Huprines

The R-L interaction of the (7S,11S) huprines HUX, HUY, HUE, HUR, and its (7R, 11R) stereoisomers RUX, RUY, RUE and RUR with AChE was studied through molecular docking.

The docking scores for huprines family range from HUX (-11.42) to HUR (-9.4), Figure 9 shown the best poses and the superposition of huprines with its analogue that come from PDB ID 1E66.

The docking results point out the presence of hydrogen bond interactions between huprines and the residues Asp72, Glu199, His440 and Ser122. The hydrogen bonds typical in acridines (AChEI:N.... O:His440) also observed in the huprines. This interaction is an important electrostatic interaction for this class of molecules.⁵¹ As in the derivatives compounds of the acridine THA, the huprines studied presents some van der Waals interactions between the ligand and Trp84 and Phe330 residues. Both cation- π and sandwiched π -stacking binding interactions were shown in the pose of the best docking results (see Figure 9).

Figure 9. The ligands of the huprine family, are docked well into, the active site of AChE, and with the highest dock score. Best pose of huprines, (a) Ligands represented in red color are the (7S,11S) huprines. (b) Compounds represented in pink color are the (7R,11R) huprines. The HUX coordinates (represented in blue color) come from the original PDB structure 1E66

In addition, some complementary interactions appear along the profile of binding interactions of the huprines. In the studied huprines, a T-stacking interaction takes places between their aromatic ring and the indole ring of Trp432

residue of AChE. As for HUX and HUY a π -stacking interaction between Cl atom and the methyl groups of the Met436 and Ile439 residues of the AChE, and different van der Waals long range interactions between the bridge double bounded carbon atoms and residues Tyr442, Ile444 can be sensed (see supplementary information Table S2).

3.6 Docking of MAOIs

The R-L interaction of a set of the MAOIs as ISO, IPR, NIA, MOC, PHZ, TOL, TRA, RAS, SEL and LAD was exhaustively studied. In order to perform this MD study, the AChE structure 1GQR was taken, since at the present day there isn't a crystallographic structure of AChE-MAOI complex. Thus, our MD study, comparison of poses, and superposing between docking solutions and original position were performed using the PDB ID 1GQR, the crystal structure of the AChE-RIV. This structure was chosen since the hybrid AChEI-MAOI inhibitor ladostigil (LAD) is a drug designed using the RIV and RAS molecules as model.

The best poses for LAD and MOC MAOIs inhibitors along their docking score are shown in Figure 10. All MAOIs presented the AChE binding interaction profile quite similar to LAD and MOC. π - π interactions between aromatic benzene ring of compounds and Trp84 residue were observed. In the same way, π -stacking interactions between MAOIs and Phe330 were observed. Some others van der Waals interactions were observed between LAD and Tyr130 residue was also observed.

Figure 10. Best poses for flexible ligand docking of LAD (pink color) and MOC (red color) in AChE. The SAF and EMM fragments are extracted from rivastigmine ligand from original PDB structure code 1GQR (represented in blue color).

Some electrostatic binding interactions were observed during docking performance of the MAOIs with AChE. The carbamoyl moiety of LAD interacts by hydrogen bonds with Ser200, and Glu199 residues. The same kind of interactions was previously observed for RIV inhibitor. The best pose of the TRA involves a hydrogen bond interaction between the ligand and Glu199 residue (not shown).

The interaction profile of the MAOIs family can be divided in two main groups. A set formed by LAD, RAS, SEL, ISO, TRA and PHZ ligands, which present their main interactions with the residues of the pocket and Trp84 residue. And, other set of ligands formed by MOC, TOL, IPR and NIA that interact mainly with the residues of the PAS site of the AChE.

3.7 Docking of N-benzylpiperidines

The R-L interaction of the N-benzylpiperidine DNP and CP0 was studied. The results reveal a good reproducibility of the docking algorithm used to perform

this study. As shown in Figure 11, the superposition of the best docking score pose of the DNP and CP0 piperidine derivatives are in total agreement with the crystal structure pose.

Figure 11. Best position for N-benzilpiperidine ligands. (a) The ligand DNP (PDB 1EVE), is docked in to gorge of AChE with the highest dock score. (b) The CP0 ligand docked well in to the active site of AChE. The compounds represented in blue color is extracted from original PDB structures DNP:1EVE, CP0:1U65.

Both DNP and CP0 presented π -stacking interaction with Trp84 residue and cation- π interactions with Phe330 residue. Other π - π interaction observed in these piperidine derivatives poses was the π -stacking between its piperidine moiety and Trp279 PAS residue.

An important hydrogen bond interaction between the piperidine moiety of these inhibitors and Asp72 ligand recognition residue was observed.

3.8 Docking of Organophosphates

In the docking study of organophosphorous (OPs) derivatives SAR, SOM, MTR and DCV the best poses of all OPs are shown in Figure 12. It was observed a good agreement in the superposition between the docked pose of these compounds and its experimental conformation that come from the crystallographic structure.

The OPs present the lowest docking score values in comparison with the other seven ligands families, DCV (-4.96), SOM (-4.90), MTR (-4.37), and SAR (-3.75). It is well known that OPs are very fast competitive inhibitors of AChE by producing a stable adduct with the enzyme.

Generally, the interactions between the studied OPs and AChE are similar to the AChE-ACh complex. Several hydrogen bonds were observed between SAR, SOM, MTR and DCV and catalytic Ser200 residue; also hydrogen bonds were observed between OPs and Gly117, Gly118, Gly119 (the oxyanion hole of AChE) residues of the gorge.

Some π - π interactions between methyl groups of OPs and aromatic residues of AChE as Trp84, Trp233, Phe290 were also observed.

Figure 12. The ligands SOM, SAR, DCV and MTR are well docked into, the gorge of the AChE. The fragments of the compounds represented in blue color is extracted from original PDB structures SOM:1SOM and SAR:1CFJ

3.9 Qualitative analysis of binding interaction per residue

An analysis of the results renders that all poses represented in previous Figures 5-12 shown similar binding interactions while comparing the 44 molecules studied here. All compounds have some similar characteristic to fit in active site, in CAS and/or PAS sites of the gorge. The small size of the molecules (inhibitors) allows a favorable interaction with the residues of the pocket area of the gorge. The main residues involved are Ser200, Glu199 and His440 through the formation of hydrogen bonds. The Trp84, Phe330, and Tyr130 are the PAS site residues with more favorable interaction in the inhibition of AChE for small size molecules. Molecules as ACh, OPs ligands, THA, NTs ligands, the MAOIs ISO, SEL and PHZ showed similar binding interactions. These compounds have similar characteristics to fit in active site in several conformations due its small molecular size.

As can be observed in Figure 13, the most potent AChEIs present important interactions with main residues of the active site of AChE. The largest inhibitors as GL8, DMT; F11, A2E, DNP, and CP0, interacts with majority residues of PAS site (hachured area in blue), and CAS site residues as well the acylation pocket (hachured area in gray), the oxyanion hole (hachured area in green) and catalytic subsite residues (hachured area in red). This kind of inhibitors, presenting aromatic rings, are capable to block AChE through several van der Waals interactions with Tyr70, Trp279, Trp84, Phe330, Phe331 residues.^{43,46} In addition, these inhibitors present some heteroatoms acting as donor and receptor of hydrogen bonds, forming strong hydrogen bond interactions with Asp72, His440, Tyr130, Glu199 and Tyr121 residues; in some cases these interactions are intermediated by water molecules of the gorge.^{46,104}

Without any exception, all 44 molecules studied here share the same profile of interaction with the residue Trp84, see Figure 13. This residue has as main function to trap the $(\text{CH}_3)_3\text{N}^+$ group of ACh during the process of its hydrolysis. In

similar way, this residue seems to be involved in the π -stacking, π - π and cation- π , interactions between AChE and ligands, to trap and orientate the molecules during the process of inhibition.^{46,105}

The ligands of families of neurotransmitters, organophosphates, carbamates, alkaloids, and the MAOIs as LAD, RAS and ISO have interactions with all residues of the oxyanion hole and catalytic subsite of AChE. The relation between the inhibitors that interacts with the enzyme making covalent bond with Ser200 residues, in general the neurotransmitter ACh, the organophosphates compounds (irreversible interaction), and the carbamates (pseudo-reversible inhibition) is well known.^{46,105} For a ligand that perform such kind of interaction, the inhibition through covalent bond, is notorious the presence of electrophilic atom (e.g. carbonyl carbon and phosphoryl phosphorus) able to attack by the nucleophilic OH:Ser200 group.

Some interactions were observed between ligands and other residues of the gorge of the AChE, and some others not localized in the active site of the enzyme. As the huprines family compounds that interacts with Met436 and Ile439 residues, as well with Ser81 and Gly441 residues. A full table with all less significative interactions is available in supplementary materials (see Table S2).

Figure 13. Families of molecules and its main interactions observed between ligands and residues of the gorge at distance of 5 Å from the ligand. NTs family is hachured in gray color, OPs is represented in ochre color, the carbamates family is hachured in orange, the alkaloids in dark blue, the MAOIs are shown in pink, the N-benzylpiperidine family is hachured in dark green, the acridines are in light blue, and huprines in light green color.

The Figure 14 show the dramatic rearrangement of residues Trp279, Phe330 and His440 produced when some inhibitors are binding with AChE.¹⁰⁶ With a simple superposition of between the seven PDB crystallographic structures of AChE with the ligands ACh, DMT, DNP, GNT, RIV, HUX and SOM, it is quite easy to understand how large size molecules as DMT change the arrangement of gorge. DMT displaces in the XY plane of conformational of the Phe330 and Trp279 residues arrangement.¹⁰⁶

Figure 14. Comparison of the active site residues superposition (His440, Phe330 and Trp279 residues) and the dramatic rearranged of gorge conformation with seven families of AChEIs. For DMT (acridines) residues colored in light blue; for GNT (alkaloids) residues painted in blue; for RIV (carbamates and MAOIs) residues traced in orange; and for others families studied here the structure are perfect superposed.

As shown in Figure 15, at least 36 of a set of 44 ligands present interactions with top 10 most accessed residues of gorge during the receptor-ligand interaction. In general, considering a distance of 5 Å, any AChEI presents the profile through short and long-range interactions with PAS residues Trp84, Phe330, Phe331,

Tyr121 and Tyr130, and with CAS residues Glu199, Gly117, Gly118, Ser200 and His440. Thus, the fundamental profile of interaction to any ligand characterized as AChEI should promote these interactions between the ligand and the active site residues of AChE.

Figure 15. Top 10 residues most accessed by AChEIs during the AChE-ligand interaction.

3.10 Quantitative analysis of binding interaction

The score rates obtained from molecular docking study of 44 molecules considered as AChE inhibitors reveals the most potent inhibitors are composed by a set of molecules including: acridines (DMT, A2E, F11, MTB and THA), alkaloids (GL8, G3X, HUP and GNT), carbamates (PHS, PHY and RIV), huprines (HUX, HUY, HUE, RUX and RUE), MAOIs (LAD, NIA, and TOL), the N-benzilpiperidine (DNP and CP0) and the neurotransmitters (NOR, 5-HT and EPI).

Figure 16. Decreasing order of the power of inhibition through score range tendency.

As shown in Figures 16 and 17 the organophosphorous compounds and majority of MAOIs, and NTs are not potent AChE inhibitors comparing its theoretical docking scores, and experimental IC_{50} values. The ligands that belong of the family of alkaloid, acridines, huprines and carbamates are the best and most potent AChEIs.

Figure 17. Nonlinear logarithmic regression of experimental pIC_{50} (nM) values versus theoretical Score of AChEIs.

As can be observed in Figure 17 the best compounds elected as AChEIs according the correlation between experimental data pIC_{50} and theoretical score values are primarily not related with the chemical family. The most potent AChEI with good correlation is the acridine derivative DMT, followed by the (7S,11S) HUX and HUY, HUE and HUR huprines stereoisomers, in the sequence the N-piperidine DNP an approved AChEI for AD treatment; the alkaloids HUP and GNT, the carbamates PHS, PHY and RIV; and the hybrid MAOI-AChEI LAD.

The best inhibitors have a set of properties and characteristics that lead it as good AChEI. The pharmacophoric profile of a good AChEI is clearly related with presence of some chemical groups in its molecular constitution. The presence of aromatic rings, pyridine, piperidine, indole groups, carbamoyl and propargyl moiety, as well groups that promote electrostatic interactions between the ligand and AChE.^{46,107}

4. CONCLUSIONS

In this work, we have investigated the binding interactions of 44 molecules belonging to 8 families of molecules, which have shown promising capabilities as AChE inhibitors. The investigation has been carried out by using molecular docking (MD) simulations and employing a computational protocol previously and successfully used in a series of systems. The conclusions of the present work which highlight interactions similarities within a set of different class of inhibitors of AChE are mainly:

- i) A good inhibitor for AChE should be able to interact with both CAS and PAS sites of the gorge.
- ii) In the development of more potent AChEIs several factors play a key role: the molecular size; presence of aliphatic chain as linker of the two subunits in the dimer inhibitors; as well the presence of aromatic rings and heteroatoms able to act as hydrogen bond donor in the compounds.

- iii) Considering the approved available drugs, the donepezil is the best posed AChEI, followed by the galantamine, and rivastigmine; these inhibitors are used as AD treatment medicines; others well evaluated AChEIs are ladostigil, huperzine A, and phenserine that are in clinical trials phases as promising anti-AD drugs.
- iv) The interactions between a ligand and residues of the CAS and/or PAS sites as Trp84, Phe330, His440, Ser200, Glu199, Gly118 and Gly119 are strongly desirable in drug design of molecules with potential inhibition of AChE.
- v) The design of dimer and/or hybrids molecules composed by combination of two monomer unit or fragment of two or more ligands coming from inhibitors of these four families of AChEIs: alkaloids, acridines, huprines and carbamates, in order to act as dual bind or dual target drugs seems a promising proposal in the development of more potent AChEIs able to being apply in multifactorial diseases treatment. Since observing that a better inhibitor is achieved by those compounds which interact with both the active site at the bottom of gorge (CAS) and the PAS.⁷⁶

We hope the results reported here can stimulate further experimental investigations on these promising systems for their potential as AChE inhibitors, and their possible application in AD treatment.

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Supporting Information

Acetylcholinesterase Inhibitors through Molecular Docking Simulations: A critical Overview.

Érica Cristina Moreno Nascimento,^a Nuno M. F. S. A. Cerqueira,^b Mónica Oliva,^a
Juan Andrés,^a Pedro A. Fernandes^{b,*}

^a*Departament de Química Física i Analítica, Universitat Jaume I, 12071 Castelló, Spain*

^b*UCIBIO@REQUIMTE, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, 4169-007, Porto, Portugal*

Table S1. Comparison of RMSF values for each inhibitor with all PDB structures by family.**Family:PDBcode****Acridine:2CKM**

Resolution (Å)	2CKM 2.15	1ACJ 2.8	1AX9 2.8	2CMF 2.5	1ZGC 2.1	2W9I 2.4	
Asp72	0	0,464	0,351	0,471	0,354	0,328	<<<
Trp84	0	0,392	0,082	0,217	0,689	0,06	
Tyr130	0	0,473	0,258	0,307	0,178	0,226	
S200	0	0,162	0,398	0,419	0,134	0,149	
Trp279	0	0,343	0,249	0,455	0,212	0,318	
Phe330	0	0,194	0,175	0,909	0,301	0,267	<<<
Phe331	0	0,194	0,131	0,986	0,267	0,281	<<<
Glu199	0	0,148	0,166	0,19	0,143	0,123	
Glu327	0	0,344	0,352	0,366	0,201	0,124	
His440	0	0,21	0,339	0,279	0,932	0,155	<<<

Alkaloid:IDX6

Resolution (Å)	IDX6 2,1	1W4L 2.16	3I6M 2.26	1VOT 2.5	1GPK 2.1	
Asp72	0	0,21	0,232	0,365	0,192	<<<
Trp84	0	0,051	0,098	0,389	0,294	
Tyr130	0	0,065	0,084	0,278	0,16	
S200	0	0,07	0,065	0,253	0,195	
Trp279	0	0,138	0,149	0,345	0,246	<<<
Phe330	0	0,262	0,083	0,252	0,258	<<<
Phe331	0	0,254	0,117	0,26	0,239	<<<
Glu199	0	0,041	0,117	0,135	0,153	
Glu327	0	0,191	0,126	0,265	0,28	
His440	0	0,091	0,102	0,198	0,203	

N-benzylpiperidine: IEVE

Resolution (Å)	IEVE (2.5)	1U65 2.6	
Asp72	0	0,636	<<<
Trp84	0	0,228	
Tyr130	0	0,299	
S200	0	0,098	
Trp279	0	0,245	
Phe330	0	0,63	<<<
Phe331	0	0,598	<<<
Glu199	0	0,118	
Glu327	0	0,271	
His440	0	0,275	

Organophosphate: ISOM

Resolution (Å)	ISOM (2.2)	1CFJ 2.6	
Asp72	0	0,132	<<<
Trp84	0	0,081	
Tyr130	0	0,09	
S200	0	0,132	
Trp279	0	0,07	

Phe330	0	0,141	<<<
Phe331	0	0,144	<<<
Glu199	0	0,072	
Glu327	0	0,101	
His440	0	0,088	

Table S2. Families of molecules and it main interactions observed between ligands and residues of the AChE at distance of 5 Å from the ligand.

NTs family is hachured in gray color, OPs is represented in ochre color, the carbamates family is hachured in orange, the alkaloids in dark blue, the MAOIs are show in pink, the N-benzylpiperidine family is hachured in dark green, the acridines are in light blue, and huprines in light green color.

Article 4

7. Computational Modeling and Simulations on Acetylcholinesterase Inhibitors: Towards a Relationship among Ligands Properties, Pharmacophoric Profile, and Inhibition Mechanism.

*“... a bloomed white bud: which is my one side.
My other side feels cold
my mountains: my white snow colored mountains.” (Ibrahim Berksoy)*

Computational Modeling and Simulation on Acetylcholinesterase Inhibitors: Towards a Relationship among Ligands Properties, Pharmacophoric Profile, and Inhibitor Mechanism.

Érica Cristina Moreno Nascimento^a, Laura Cleofas-Sánchez^{b,*}, Mónica Oliva^a, Juan Andrés^a

^a*Departament of Physical Chemistry and Analytical, Universitat Jaume I, 12071 Castelló, Spain*

^b*National Institute of Genomic Medicine Genómica, Ciudad de México, D.F, México.*

Abstract

In this work, computational modeling and simulation, based on principal component analysis, pattern recognition and data mining tools (clustering, classification and missing values methods), were performed to find a relationship between 29 molecules (20 AChEIs, 4 MAOIs, and the 5NTs), and their inhibitory action against AChE. The molecules were studied considering the state of protonation in physiological pH. PCA studies revealed that properties as molar mass, number of chiral carbon in “S” stereoisomer, molecular orbital energy of the LUMO, molecule size and number of aromatic rings are the most relevant properties in the study of AChE inhibitors. The missing data values, i.e., the distance of the nitrogen atom and the oxygen atoms in the molecules, are important variable in the study of recognition pattern and correlation in our data set. Hierarchical clustering method is the best predictor with cross-validation coefficient equal to 0.931.

Keywords: Acetylcholinesterase inhibitors, Alzheimer’s Disease, Clustering, Missing Values Methods, Classification, PCA, Multivariate Analysis.

1. INTRODUCTION

Alzheimer's disease (AD) was described at the first time by psychiatrist Alois Alzheimer in 1907. After performed several autopsies in the brains of patients with the same kind of dementia, doctor Alzheimer observed similarities in the cells of the cortex structure in the brain samples. The majority of the samples presented a drastic reduction of brain mass, in addition to dark tarnish and fibrillar tangles.¹⁻³ This is the main physiological characteristic of the AD disorder into the brain cells. Nowadays it is estimated that 60 million people worldwide have some form of dementia, and 20 million of them have symptoms characteristic of AD.

AD is the most common dementia, and it mainly affects people over the age of 65. The disease is characterized by the drastic reduction of the neurotransmitters (NTs) acetylcholine (ACh), serotonin (SER) and dopamine (DOP) in the synapses of cholinergic, serotonergic and dopaminergic neurons. The decrease of NTs in synaptic cleft causes cognitive deficit and altered mood behavior leading to dementia as Alzheimer's disease and Parkinson's disease.¹⁻³ In AD patients, the gradient of the concentration of ACh in the transmission of neuronal impulses is markedly reduced, due its low production within the neurons, thus causing the cholinergic deficit and the serious compromise of the cognitive functions.

Since AD does not have known cause, its cure has not yet been determined. Therefore some strategies are employed in an attempt to remedy the problem.⁴ In order to treat the disease the hypothesis of cholinergic deficit was suggested. This hypothesis targets the use of drugs capable of maintaining or increasing the gradient of concentration of ACh in the synapses of cholinergic neurons by inhibiting or blocking the hydrolysis of this NT.¹ The use of the acetylcholinesterase (AChE) inhibitors is the main strategy used in the treatment of AD. This strategy is known as cholinergic therapy. This treatment derived from the cholinergic hypothesis, and consists in the improvement of the transmission of nerve impulses in the cholinergic synapses of AD patients. The use of correct medicine, which hand increasing the concentration of ACh in the synapse has, over

the years, effectively retarded the progression of the disease in AD patients.^{1,5}

The neurotransmitter ACh has as precursors the choline and acetyl-coenzyme A, which under the action of the enzyme acetylase react by producing it within nerve cells. ACh (Figure 1) is the most abundant neurotransmitter in the brain and the main responsible for the propagation of the nerve impulse in the synaptic clefts of the cholinergic neurons.

Hitherto four inhibitors of AChE (the AChEIs) have been approved by FDA for treatment of the AD. Tacrine (THA)⁶ was the first approved drug, nowadays no longer recommended because its use caused severe side effects in AD patients. The second drug approved to treat AD patients was donepezil (DNP),⁷ followed by galantamine (GNT)⁸, and the rivastigmine (RIV).^{9,10}

Some AChEIs, in addition to the AChE inhibition also present neuroprotective function. Drugs as DNP and RIV act more efficiently in the AD treatment. These drugs act in a way to prevent hyperphosphorylation of the β -amyloid protein, inhibiting the breakdown of this protein and preventing the formation of amyloid plaques.¹¹⁻¹⁵

Several studies were devoted developing new drugs able to act on cholinergic synapses in order to guarantee the maintenance of the levels of ACh in the synaptic clefts and thus to guarantee a better efficiency in the performance of the cognitive activities of AD patients. Therefore, efforts in the research and development of drugs that act more effectively in the treatment of AD are the target of several fields of knowledge.

Figure 1. Structure of some classical and non classical AChEIs.

Molecular modeling is one of the strands of theoretical and computational chemistry methods with huge development in last three decades. Rational drug design is one of the fields of application in molecular modeling with high relevance in the treatment and cure of diseases. The use of computational approaches in the prediction of molecules with well-defined pharmacological activities brings the expectation of reducing the time in researches and the expenses with synthesis and preclinical tests. In this way, the calculation of molecular properties which are relevant to determining how interactions between biological receptor (R) and a ligand (L) take place.

Through modeling the system receptor-ligand (R-L) is possible to obtain important information about the interactions occurring between them. The molecular interactions R-L established the functionality of a given molecule. However, the studies of interactions by itself do not provide enough parameters to

the development of new ligands. In the prediction and rational design of new drugs, it is fundamental to use robust computational techniques that provide mathematical and statistical relationships that consider the structural, electronic and chemical properties obtained both theoretically and experimentally of ligands and its correlated biological activity.

It is well known that the biological activity of a given molecule on a proteomic target is a result of the contribution of different properties of this molecule. These properties are intrinsic factors to each molecule which in turn can be described by means of two-dimensional parameters such as physical-chemical, electronic, geometric and topological, three-dimensional and four-dimensional properties. It is important to note that a set of properties, taken in isolation, do not explicitly reveal, in a simplified way, the biological activity of a molecule. The way to define how the properties of a given ligand are correlated with its biological activity is using techniques able to describe the molecular recognition relationships and the properties involved in this process.

The pattern recognition is a tool used to analyze a lot of real world problems, which is applied in problems involving classifications, clustering among others. In this way, the pattern recognition can be used to treat biochemical problems, as the correlation between properties of a set of molecules and its activity in a given protein.

Pattern recognition is based in how the human being recognizes objects of the real world such as faces, heart diseases, type of wines, among others. By means of the natural process of the human brain to assimilate the information to recognize real world objects, a lot of information can be achieved. In this context, pattern recognition is defined as the smart reasoning of computational processes made by means of a supervised or unsupervised learning. The first of them considers a prior knowledge; the label of object is determined by an expert in the area. Comparing to supervise learning, the second one unsupervised learning does not need a prior knowledge to make its learning model. In this work, an unsupervised learning is used to performance data clusters analysis from data set. And an unsupervised

learning is carried out to evaluate clusters by means of classification strategies. In addition, data mining methods are used to obtain missing values present in data set.

Computational modeling and simulation methods such as the principal component analysis, and pattern recognition tools (clustering, classification and missing values methods) were carried out to elucidate the correlation profile between 29 molecules and their inhibitory activity against AChE. The molecules were studied considering the state of protonation in physiological pH. We investigated exhaustively 25 determinant properties to find a relationship between these 29 molecules and their performance in the acetylcholinesterase inhibitions. Among these, 20 AChEIs, 4 MAOIs and the 5 NTs involved in the development of dementias as AD, Parkinson's disease and depression.

2. METHODOLOGY

In order to elucidate the correlation between the principal properties of 29 molecules and their inhibitory activity against AChE, quantum theoretical studies associated with principal component analysis, clusters analysis, classification and missing values methods were carried out. The molecules were studied considering the state of protonation in physiological pH that means in protonated chemical state. We investigated exhaustively 25 determinant properties that correlate these 29 molecules and their performance in the central nervous system. Among these, 20 AChEIs, 4 MAOIs and the 5 NTs involved in the development of dementias as AD, Parkinson's disease and depression.

Table 1. AChEIs, MAOIs and NTs molecules studied.

Class	AChEI	MAOI	NT
<i>AChEI/MAOI</i>	Ladostigil (LAD)	Ladostigil (LAD)	Acetylcholine (ACh)
		Rasagiline (RAS)	Dopamine (DOP)
<i>Acridine</i>	Tacrine (THA)	Selegiline (SEL)	Epinephrine (EPI)
	Bis(5)-tacrine (F11)	Tranylcipromine(TRA)	Norepinephrine (NOR)
	Bis(7)-tacrine (DMT)		Serotonin (SER)
	Edrophonium (EDR)		
	Methylene blue (MTB)		
<i>Alkaloid</i>	Galantamine (GNT)		
	Bis-acting galantamine (GL8)		
	N-piperidinopropyl-galantamine (G3X)		
	Huperzine A (HUP)		
<i>Carbamate</i>	Rivastigmine (RIV)		
<i>Huprine</i>	(-)-Huprine (HUR)		
	(-)-Huprine E (HUE)		
	(-)-HuprineX (HUX)		
	(-)-Huprine Y (HUY)		
	(+)-Huprine (RUR)		
	(+)-Huprine E (RUE)		
	(+)-HuprineX (RUX)		
	(+)-Huprine Y (RUY)		
<i>N-benzyl piperidine</i>	Donepezil (DNP)		
	Camptothecin (CP0)		

2.1 Principal Component Analysis (PCA) of AChEIs

To analyze the properties of AChEIs molecules we have computed the electronic and structural descriptors of those molecules. 2D parameters (constitutional, geometric, electronic and lipophilicity), and 3D (spatial conformation, conformers, enantiomers, atomic charges, dipole moment, and

energy of the frontier molecular orbitals) are calculated by performing *ab initio* calculations *in vacuum*. The 4D parameter the docking score and the theoretical K_i (constant of inhibition) were taken for our previous work performed by means of molecular docking simulations with AutoDock4.2 algorithm tools.

The theoretical studies of the inhibitors were performed using the Gaussian 09¹⁶ in order to determine the best electronic and geometrical parameters of AChEIs molecules, see Table 2. Calculations based in the density functional theory (DFT) at the B3LYP hybrid functional level with 6-31+G(d,p) basis set, were performed in order to obtain the molecular properties. The geometries of the target drugs were first optimized using internal coordinates to minimize all geometric data. The molecular structures were taken from Cambridge Structural database (CSD), DrugBank¹⁷ and PubChem.¹⁸

In this work, all the structural and electronic properties of the molecules obtained from the calculations and optimization of geometry were exhaustively studied by exploring the properties listed in Table 2. These properties can be visualized as coordinates of points in a dimension space equal to 29 (20 AChEIs + 4 MAOIs + 5 NTs), where each axis is associated with a property (variable). The PCA analysis was applied to plot the correlation profile of the described molecules. From the values of the calculated properties listed in Table 2, the properties of significative weight for the activity of the compounds were evaluated.

The PCA was used to correlate 25 properties of 29 molecules to reduce the initial parameter set and determine the most relevant data for the AChEIs. PCA was conducted using the auto-scale method once the structural and electronic properties have different dimensions. Since the properties studied here have different dimensions, autoscaling method is required. This method consists of translate and stretches axes, centering the data on average and divide them by their standard deviation. Thus, each variable presents zero mean and variance equal 1, giving the same significance for all variables.^{19,20} At first five principal components (PCs) were generated for the data set analyzed, consequently based in the results of the preliminary analysis, finally three PCs were generated.

Table 2. 2D, 3D and 4D descriptors, and 25 studied properties.

Descriptor Type		Property
2D	Constitutional	Number of aromatic rings (NAR), molar mass (MM).
	Geometric	Molecular volume (VOL), molecule size (SIZE), distance between the most acid hydrogens (H-H), polar surface area (PSA), degree of freedom (DGF), distance of nearest N and O [d(O-N)] atoms, and the distance of nearest N and O atom of the carbonyl group [d(O=N)].
	Electronic	Number of H-bond acceptors (H-acc), number of H-bond donors (H-don), polarizability (DEP).
	Lipophylicity	Partition coefficient (LogP)
	Conformer	Number of chirality centers (NCC), number of chiral carbon in -S form (NCS), number of chiral carbon in -R form (NCR).
3D	Electronic	Dipolar moment (DIP), energies of the frontier molecular orbitals: HOMO, HOMO-1, LUMO, LUMO+1, GAP (LUMO-HOMO energy), charge* of heteroatoms (Chrg-), charge* of most acid hydrogens (Chrg+).
4D	Docking	Docking score rate (SCO), Constant of inhibition (<i>K_i</i>)

*The charge used was obtained from the Chelpg population analysis (charge from electrostatic potentials using a grid-based method).

The theoretical studies to determine the structural and electronic parameters were carried out through the Gaussian 09¹⁶, AlogPS2²¹ and OSIRIS²² programs. The PCA studies were performed with the MATLAB[®] program.²³

During the molecular recognition of ACh by AChE, some important interactions are observed between the ligand and the active site of the enzyme. The quaternary nitrogen atom of the substrate forms a strong electrostatic interaction with the carboxyl group of the Asp72 residue (3.67 ± 0.13 Å).²⁴ In addition, strong

hydrogen bound interactions are observed between the oxygen atom of the ester group of ACh and the residues Gly118, Ala201 and Tyr130.^{24,25} In this way, the distance between the nitrogen and the oxygen atoms in a molecule which will be recognized by AChE should be at least similar as the ACh molecule (3.5 - 6.5Å).

Due the diversity of the molecular structure of some ligands, the properties distance of nearest N and O atoms [d(O-N)] atoms, and the distance of nearest N and O atom of the carbonyl group [d(O=N)] are discarded, i.e., the absence of oxygen and/or nitrogen atom in the molecule is observed in 22 of the 29 inhibitors studied. The PCA method is not possible to be performed using as variable a set of values with some missed data. For this reason, we performed the PCA analysis without consider the properties d(O-N) and d(O=N) distances.

On the other hand, considering the importance of the properties d(O-N) and d(O=N) distances in the molecular recognition of the AChE, and in attempt to mark the load of these two properties in this biochemical process we took on board potent pattern recognition and data mining tools to treating all data set including the missed data.

2.2 Clusters and missing data methods applied in AChEIs data

In order to enrich and validate PCA results, clustering studies were performed with pattern recognition algorithms. Also, missing values problem was treated to avoid missing data may to affect the clustering analyses. In this way, missing values were obtained with K-Nearest Neighbor Imputation and K-Means Imputation methods. After trying that problem, data sets without the presence of missing values were used to make clustering models. In addition, clustering analysis also was carried out discarding the presence of missing values in data sets. Those clustering models were performed with called Farthest First, Hierarchical and K-means algorithms. Finally, the clusters validation is based in classification methods. For the experiments, KEEL and Weka repositories of pattern recognition methods were used.^{26,27}

2.2.1 Missing values methods

The K-Nearest Neighbor Imputation (KNNI) algorithm is used to replace missing data by admissible data from a data set, this process is called imputation method, in this case the k-nearest prediction algorithm is considered as imputation method,²⁸ which is independent of the learning algorithm. This situation allows applying a suitable imputation in the data sets, replacing missing values without skewing results. For this, qualitative or quantitative attributes (characteristics of objects of the life world) should not be highly correlated between them, because the best prediction models could not be obtained. For qualitative attributes, the k-nearest algorithm searches the most frequent values among its nearest neighbors to determine the missing values. When quantitative attributes were considered, the k-nearest algorithm obtains the mean value among nearest neighbors. In addition, it is important to mention that the k-nearest is a good prediction algorithm to treat patterns with a lot missing values.

In the literature, it is described that KNNI gets better results than mean, mode, C4.5 and CN2 imputation methods, despite that these last has been widely used to get missing data. The first two methods employ the mean metric to obtain missing values from known data; the mean method is used when the values are quantitative and the mode method is performed when the data are qualitative. The last two methods obtain the missing values with CN2 and C4.5 prediction algorithms known in this case as imputation methods. The first of them uses a simple method to try missing data, considering the most represented value before to get the entropy measure. The second of them is based on a probabilistic approach.

The fuzzy k-means clustering algorithm (KMI) is used as imputation method to obtain missing values from known data, this method may tolerate imprecision and uncertainty implicit in study problems, just because in some cases patterns attributes may be assigned to more of a cluster, due to that they are not well separated by the k-means method, in this case the fuzzy function is included within

k-means method with the aim of to resolve this problem.²⁹ In this sense, the KMI algorithm determines the first well-distributed centroids to avoid that clusters are built when a local minimum is present. Then the fuzzy function and centroids are updated. Finally, the missing values are obtained with respect to clusters information. In addition, according some authors the KMI shows better results than k-means clustering method.

2.2.2 Clustering methods

On the other hand, in this section it will be briefly described Farthest First, Hierarchical and K-means clustering methods that were used to perform data sets clustering analyses based in unsupervised learning. The concept of cluster is defined as the disjoint division of the data set.

The k-means algorithm is well-known by the scientific community of pattern recognition to obtain clustering by means of some parameters that define the number of partitions over data sets, taking into account that the first centroids (patterns) that are randomly choose to each group, after that, centroids are updated calculated a mean value from each cluster, and then new partitions are got. This process is iteratively performing until that the distances sum of each group is less than a specified threshold.

The Farthest-First clustering was created in 1985 by Dorit S. Hochbaum and David B. Shmoys, which performs clustering process, taking into account a simple approximation algorithm based in requisite triangle inequality. This algorithm works as the k-means algorithm; but centroids are calculated as follows: a new centroid is remotely calculated of clusters already obtained.

The Hierarchical clustering process seeks to build groups take into account hierarchies to get partitions from data sets. This method considers strategies such as agglomerative and divisive. The first of them builds an initial group for each pattern, after that the clusters are merged with respect to its hierarchy level. In the second case, clusters are recursively formed from each pattern belongs to data sets

until the partitions are hierarchically replaced.²⁷

2.3 Clusters Validation

In order to validate clusters, classification methods consider k-fold cross validation strategies was used in this work. In this sense, the classification in pattern recognition is known as the division of the space of study in disjointed classes. The supervised classification process is performed through learning and test phases. In the learning phase is built the learning model from data sets, in the test phase the new patterns are presented to the learning model to validate this model.

The classification is made before gets training model with the 70 % of the patterns from original data set, after that test phase considers 20 % of the patterns to prove the classifier performance. In addition, 5-fold cross validation (FCV) method was considered to do those process five times taking into account five subsets for each phase. The aim FCV method is to divide the original data set in disjointed partitions. From each training subset is determined a classification model, which is proved with its respective test subset. On this way, the FCV prove classifier performance.

The k-Nearest Neighbor can be used as classification algorithm, which built from data set a classification model. Its classification process consists in to obtain the distance between each test pattern and its k-Nearest Neighbor from training set, after that the test pattern label is assigned with respect to the most frequent label of the patterns.

3. RESULTS AND DISCUSSION

In the analysis of a huge number of variables, it is very difficult find empirically the relationship among the data set. In practice, many variables are strongly correlated, and it is desirable that a small subset of variables may explain a full picture of the described phenomenon.

The PCA was the first method used in this work to analyze our data set. In the sequence, we performed a series of experiments on AChEIs data sets, which are characterized by containing missing data values and the size of data sets is very small. The experiments were performed in two forms:

I). -the problem of missing data was treated with K-Nearest Neighbor Imputation and the fuzzy k-means clustering methods, for this, missing data algorithms consider a k parameter equal to 4 (value in order to obtain the new data),

II). -missing values was removed from original data sets, this is not the best form to obtain new subsets, because we can lose valuable information for our study problem.

After that missing values were calculated or removed from the original data set, the clusters were carried out through three pattern recognition algorithms, such as Farthest-First, k-means, and Hierarchical. Of each one of them, several groups were determined based on missing values algorithms.

3.1 Principal Component Analysis (PCA) of AChEIs

For the analysis of principal components the geometry optimizations of each molecule were performed at the level B3LYP/6-31+G(d,p). The data of the ligands properties are shown in Table S1 (see supplementary material). The ACh was the molecule chosen to compare the other molecules in this PCA analysis once it is the substrate of AChE. This choice was made with the aim of searching, by similarity,

the properties that allow to the process of inhibition of AChE, by different classes of ligands.

Two experiments were performed using the PCA method aiming an evaluation of which properties, of molecules are the most relevant and correlate these molecules with the inhibitory action of the AChE. At first, an analysis was carried out with the 23 properties studied here. Since PCA analysis calculations cannot be performed using missing values, those variables that contain missing data were not taken in account. At the first 5 principal components were generated to find out which are the most relevant properties within the set of variables and the degree of correlation between all these properties.

The first PCA (Box 1) shows that 76% of the information forming the set of 29 ligands and 23 properties can be represented by 5 PCs. Thus, it is worth to say that the properties observed represent up to 76% the main properties that characterize these inhibitors and correlate them with their function: molecules with molecular recognition by AChE.

CV PC1	CV PC2	CV PC3	CV PC4	CV PC5
31%	48%	59%	68%	76%
PC1= 0.128 _{MM} + 0.125 _{VOL} + 0.109 _{SIZE} + 0.107 _{NAR} + 0.09 _{HOMO} (1)				
PC2= 0.123 _{DEP} + 0.119 _{NCC} + 0.108 _{DGF} + 0.090 _{LogP} + 0.007 _{LUMO+1} (2)				
PC3= 0.208 _{H-don} - 0.193 _{Chrg+} - 0.30 _{Chrg-} + 0.095 _{H-H} - 0.083 _{H-acc} (3)				
PC4= 0.258 _{LUMO} + 0.209 _{LUMO+1} + 0.066 _{DIP} - 0.065 _{Chrg-} - 0.056 _{PSA} (4)				
PC5= 0.278 _{NCS} + 0.145 _{PSA} + 0.133 _{NCC} + 0.065 _{Chrg-} - 0.070 _{GAP} (5)				

Box 1. Cumulated Variance (CV) of 29 ligands and 23 properties.

In a first analysis, the properties that presented the highest score value for each PC are MM (0.128), DEP (0.123), H-acc (0.208), the energy of the orbital LUMO (0.258), and NCS (0.278) respectively, (See equations 1-5 in Box 1, values

in bold). It was observed in both PC1 vs. PC2 and PC1 vs. PC5 graphs (see supporting information) that the molecules with the highest values of MM and VOL is approached through the first component PC1 (CP0, DMT, F11) and they are separated through PC2 and PC5, which the properties with higher score are DEP (0.123) and NCC (0.278) consecutively. Another prominent observation of this first analysis is the generation of clusters with the molecules of the classes of the huprines (22-29).

In PC1 vs. PC2 is observed the exact separation of some ligands by classes such as the cluster formed by huprines, the NTs (DOP, EPI, NOR, SER) and group between MAOIS (TRA, RAS). The group formed by (RIV, LAD) is quite interesting at once the inhibitor LAD is a hybrid molecule formed by half part of the RIV ligand and half part of RAS molecule.

In the graphic of scores PC1 vs. PC5 were observed clusters where the molecules of the family of huprines are separated in two different subset, one with the S stereoisomers molecules (22 - 25) and other with the R stereoisomers (26 - 29).

The generation of clusters described above is a good qualitative indicator that such properties represent the role set and reflect its biochemical function by up to 76%. The properties MM, VOL, DEP, H-don, LUMO, are important parameters to design pharmacophoric profile of the molecules studied here considering their classification as inhibitors of AChE.

With the objective of eliminating the correlation between the 23 properties studied here, the second experiment was carried out by combining the most relevant properties obtained in the first step of the first experiment (with 5 principal components). That is, selecting the properties with direct or indirect correlation between them, eliminating those of less weight. Therefore, increasing the accuracy, listing properly the properties that independently represent the totality of the sample space in the experiment.

To increase the accuracy and to determine in a systematic way the principal components of this analysis were realized the reduction of the dimensional space

for 3 PCs, and using 5 variables (properties). In this way, we obtain the higher dispersion (covariance value) with the lowest number of possible variables (1:5), see Box2.

Thus, reducing the number of variables to 5, but keeping the sample space of 29 objects (ligands), was observed up to 94% of the information that characterizes these molecules as being recognized by AChE.

CV PC1	CV PC2	CV PC3
52%	74%	94%
PC1= 0.355 _{MM} + 0.346 _{SIZE} + 0.289 _{NAR} + 0.005 _{NCS} - 0.004 _{LUMO} (6)		
PC2= 0.010 _{MM} + 0.001 _{SIZE} - 0.054 _{NAR} + 0.800 _{NCS} - 0.135 _{LUMO} (7)		
PC3= 0.005 _{MM} + 0.016 _{SIZE} - 0.023 _{NAR} + 0.112 _{NCS} + 0.843 _{LUMO} (8)		

Box 2. Cumulated Variance (CV) of 29 ligands and 5 properties.

The first PC represents 52% of all significance, followed by PC2 and PC3 which representing 22% and 20% respectively, where using only 3 major components and 5 variables its possible get up to 94% the covariance dispersion.

The variables MM, NCS, LUMO, SIZE and NAR are the properties that correlate all the 29 molecules studied in the most dispersive way possible. Although the dispersion is higher than 90%, is observed the composition of clusters aggregating some molecules in the same group.

The graph of scores PC1 vs. PC2 (see Figure 2) is representing the new experiment, keeping the 29 objects and reducing the number of variables to 5 properties. We observe two clusters formed by subgroups with the molecules [(HUP, HUY, HUE, HUR), (LAD, HUX, GNT, G3X)] and [(DOP, EDR), (SER, SEL), (RUX, RUY, RUE, RUR)] and the clusters (RAS, TRA, NOR, EPI, RIVA), and (DMT, F11). Groups are approached by PC1 and PC2, the MM and NCS properties are the variables with the highest score in these PCs and represent 74% of all covariance

Figure 2. PC1 x PC2 scores from B3LYP/6-31G(d,p) data set.

Figure 6 depicts the scores of PC1 versus PC3. In this case there are 5 standards in PC3, this is reasonable since PC3 represents the orbital energy of LUMO (+0.843) see Equation 8, these molecules have values close to the LUMO and VOL (see Table S1).

Figure 3. PC1 x PC3 scores from B3LYP/6-31G(d,p) data set.

Figures 2 and 3, equations 6-8 generated in the PC indicates that the constitutional parameters Molar Mass and NAR, and conformer parameter NCS, and the electronic parameter the value of the orbital energy LUMO and the geometrical parameter SIZE, are the most significant properties of the ligands which can be considered AChE inhibitors.

The PC2 has the property NCS as the most significant with the highest score (0.800). The chirality of molecules is vital for life conditions and existence. It is well known that the preference of the life organism cells with the levorotatory form of molecules to express biochemical specificity and functions. In Figure 4 is represented the molecule LAD and its electrostatic potential map. In this representation, the regions hachured in blue indicate the low density of the electrons in the molecular space (in other words, positive charged part of the molecule), while the red color indicates the region rich in electronic density. LAD has a chiral carbon atom expressing its stereoisomer S.

Figure 4. Electrostatic potential map of LAD.

The distribution of frontier orbital in the molecule is important to determine which atoms are most relevant for the interaction between the ligand and the receptor.

Figure 5 shows the regions of the frontier orbital LUMO of some ligands studied in this work. In general, this orbital is located in the region of the most positive charged nitrogen atom (blue sphere) and over the aromatic rings of the well-known inhibitors of AChE, as RIV, DNP, THA and GNT. In other hand is possible observe that in the case of the MAOI TRA and the NT NOR the LUMO orbital is placed only in the H_3N^+ region of such molecules, mimetizing the substrate of AChE.

Figure 5. LUMO of some ligands calculated at B·LYP/6-31+G(d,) level.

3.2 Missing values analysis

In this section, we show results values obtained with K-Nearest Neighbor Imputation (Table S2, see supporting information) and the fuzzy k-means (Table

S3, see supporting information) algorithms. In this way, missing values results can be observed in Atr18 and Atr19 attributes (in columns of Table S2 and Table S3, all that in bold), which were calculated from the original data, and, the molecules are represented in rows.

It is possible to observe that KMI algorithm indicate similar/equal values in Atr18 and Atr19 attributes of the molecules, which may mean that KMI centroids were not suitably updated, due to low-samples size problem.

On the other hand, the KNNI algorithm calculate suitable missing values from original data, due to the fact of have more variety in results, and because the algorithm works best with low-samples

3.3 Clustering analysis

In this section, we observe experiments obtained with clusters methods to get molecules groups that may be related to each other. Results each cluster algorithms are indicated in columns, considering a value 4 as parameter to build the groups. Also, the Farthest-First algorithm is represented by “F”, the k-means is indicated by “M”, and Hierarchical method is showed by “H”. The identifications of each molecule that belong to the groups are found in rows. In addition, in the Table 3 are shown the results of clusters built when the Atr18 and Atr19 attributes (see Table S2 and S3 in supplementary information) are removed from the original data set. Other molecules groups are obtained when missing values methods are applied (Tables 3).

Table 3. Clusters results with missing and no missing values to K-means and k-NN algorithm.

	missing values with k-means			missing values with k-NN			no missing values based in k-means			no missing values based in k-NN		
	F4	M4	H4	F4	M4	H4	F4	M4	H4	F4	M4	H4
ACh	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0
RIV	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster0	cluster2	cluster0	cluster2	cluster2	cluster0
GNT	cluster0	cluster0	cluster0	cluster0	cluster0	cluster0	cluster0	cluster3	cluster0	cluster0	cluster0	cluster0
GL8	cluster3	cluster3	cluster0	cluster3	cluster3	cluster0	cluster1	cluster3	cluster0	cluster3	cluster3	cluster0
G3X	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0
HUP	cluster0	cluster2	cluster0	cluster0	cluster0	cluster0	cluster0	cluster3	cluster0	cluster0	cluster0	cluster0
DMT	cluster1	cluster3	cluster1	cluster1	cluster3	cluster1	cluster1	cluster2	cluster1	cluster1	cluster3	cluster1
F11	cluster1	cluster3	cluster1	cluster1	cluster3	cluster1	cluster1	cluster2	cluster1	cluster1	cluster3	cluster1
THA	cluster0	cluster1	cluster2	cluster0	cluster1	cluster2	cluster0	cluster1	cluster2	cluster0	cluster1	cluster2
MTB	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0	cluster0	cluster2	cluster0	cluster0	cluster3	cluster0
EDR	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0
DNP	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0
CP0	cluster3	cluster3	cluster3	cluster3	cluster3	cluster3	cluster1	cluster3	cluster3	cluster3	cluster3	cluster3
SER	cluster2	cluster2	cluster0	cluster0	cluster2	cluster0	cluster2	cluster2	cluster0	cluster0	cluster2	cluster0
DOP	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0
EPI	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0
NOR	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0
SEL	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0
TRA	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0	cluster2	cluster2	cluster0
RAS	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0
LAD	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0
HUX	cluster0	cluster0	cluster0	cluster0	cluster0	cluster0	cluster0	cluster2	cluster0	cluster0	cluster2	cluster0
HUY	cluster0	cluster0	cluster0	cluster0	cluster0	cluster0	cluster0	cluster3	cluster0	cluster0	cluster0	cluster0
HUR	cluster0	cluster0	cluster0	cluster0	cluster0	cluster0	cluster0	cluster3	cluster0	cluster0	cluster0	cluster0
HUE	cluster0	cluster0	cluster0	cluster0	cluster0	cluster0	cluster0	cluster3	cluster0	cluster0	cluster0	cluster0
RUX	cluster0	cluster0	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0
RUY	cluster0	cluster0	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0
RUR	cluster0	cluster0	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0
RUE	cluster0	cluster0	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0	cluster0	cluster3	cluster0

In general, the number molecules in each cluster depend of parameters established to make the partition of the data set. If the parameter number is large then the molecules number in each group is less. To the low-sample size problem is recommended that the number of groups is not very large.

In this work, we also search which molecules group is the best, this aspect will be solved when we have the subsets classification were obtained. These experiments show us which molecules are suitably grouped with each cluster method.

Same pattern in the formation of the clusters were observed when calculations were carried out with Farthest-First algorithm and when missing values were obtained in both k-means and K-NN algorithms results. The groups composed by (DMT, F11), (GL8, CP0), (ACh, RIV, EDR, DOP, SEL, TRA) (GNT, G3X, HUP, Huprines, THA, MTB, DNP, NOR, EPI, SER, RAS) were formed. In the case of the calculations discarding the missing values, it was observed same pattern when the cluster K-NN is applied. In other hand, there was a disruption in the group aggregation when k-means algorithm is performed used with no missing values (graphic not shown).

(a)

(b)

Figure 6. Clusters results using Farthest-First algorithm. (a) Calculations with k-means missing values method; (b) Calculations with k-NN missing values method.

Comparing the pattern with the groups observed in the graphic PC1 vs. PC2 (Figure 2) the clusters formed with Farthest-First (F4) algorithm show similarities with the clusters (DMT, F11), (GL8, CP0) (ACh, DOP, EDR, SEL) generated with the PCA analysis. Some divergences were observed due the numbers of parameters used to carry out the calculations, equal 4, may not the best suitable one to represent properly the groups of our data set.

Clusters study performed with k-means algorithm revealed the overlapping of some clusters previously observed in F4 algorithm. This can be generates dubious interpretation in the chemical pattern recognition of our data set. Since some molecules are part of the same class, it is not reasonable expect the huprines at the first moment is separated, see Figure 7 (a).

When the calculations are carried out eliminating the missing data the effect of overlapping is remarked, Figure 7 (b), and some clusters seem be suffering cross-contamination. These results shown that missing data are important values and should be considered in the analysis of our data set.

(a)

(b)

Figure 7. Clusters results using k-means algorithm. (a) Calculations with missing values; (b) Calculations without missing values.

In general, using hierarchical method (H) it was observed the same pattern (Figure 8) in clusters aggregation formed when calculations are performing with or without missing values. In all cases, for all calculations it was observed 4 groups as (DMT, F11), (THA), (CP0) and all other molecules grouped at the same cluster.

Figure 8. Clusters results with K-means values using hierarchical methods with missing values.

Comparing this pattern with the graphic PC1 vs. PC2 (Figure 2) it is possible correlate both methods while grouping molecules with similarities.

Performing the validation of our experiments using the cluster validation classification with cross-validation we found the best predictor cluster method is the hierarchical clustering (H), with classification $R=0.9310$ while considering missing data values (Figure 8). For no-missing data set the best ranked predictor is the k-means cluster method (M), with classification cross-validation equal to 0.9310 (Figure 7b).

4. CONCLUSIONS

Principal component analysis method and pattern recognition algorithms were used based in DFT calculations within B3LYP functional has been performed to find a relationship between 29 molecules (20 AChEIs, 4 MAOIs, and the 5NTs) and their inhibitory action against AChE.

Preliminary studies with PCA method shown the most relevant properties which correlate the molecules with AChE inhibition are Molar Mass, Number of Chiral Carbon in “S” stereoisomers, energy of the orbital LUMO, Number of Aromatic Rings and Molecule Size.

With pattern recognition tools were generated clusters using Farthest-First, k-means and hierarchical algorithms revealed similarities with clusters generated by PCA. This convergence was strongly observed when the calculations were performed with missing data values. New calculations considering 5 and 10 parameters are strongly recommended in order to identify precisely the profile of our data set.

To indicate the most potent inhibitors of AChE, the cluster validation is mandatory. To validate the clusters is necessary to consider the subsets classification base in cross-validation methods. Taking in account the theoretical K_i calculated by molecular docking studies are being carry out to determinate precisely the profile of the pharmacophoric profile of AChE inhibitors. The best predictor using missing data is the hierarchical method using k-means algorithm. In other hand, the best predictor using no-missing data is the K-means clustering method using the k-means algorithm.

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Supporting Information

Computational Modeling and Simulation on Acetylcholinesterase Inhibitors: Towards a Relationship among Ligands Properties, Pharmacophoric Profile, and Inhibitor Mechanism.

Érica Cristina Moreno Nascimento^a, Laura Cleofas-Sánchez^{b,*}, Mónica Oliva^a, Juan Andrés^a

^a*Departament of Physical Chemistry and Analytical, Universitat Jaume I, 12071 Castelló, Spain*

^b*National Institute of Genomic Medicine Genómica, Ciudad de México, D.F, México.*

Table S1. Values of 2D, 3D and 4D descriptors properties at B3LYP/6-31+G(d,p).

		HOMO	LUMO	GAP	VOL	SIZE	H-H	DIP	Chrg+	Chrg-	HOMO-1	LUMO-1	PSA	LogP	H-acc	H-don	NAR	DGF	d(O-N)	d(O=N)	MM	DEP	NCS	NCR	NCC	pKi	SCO
1	ACH	-10.9	-4.1	6.9	190	9.0	1.79	17.9	0.16	-0.57	-12.0	-3.4	26	-1.4	1	0	0	4	3.74	5.14	146.2	0.27	0	0	0	3.9	-5.1
2	RIV	-9.4	-4.1	5.4	325	10.9	2.23	4.5	0.30	-0.63	-9.9	-3.8	33	2.9	2	1	1	9	2.26	4.27	251.3	0.59	1	0	1	5.7	-7.8
3	GNT	-8.7	-4.0	4.7	338	10.3	9.79	11.4	0.37	-0.64	-9.5	-3.7	42	1.4	0	2	1	1	5.21	missd	288.4	0.34	2	1	0	6.8	-9.6
4	GL8	-9.0	-4.1	2.2	627	19.9	2.93	8.0	0.40	-0.51	-9.2	-3.6	81	1.4	3	1	2	9	5.21	7.26	530.7	0.35	2	1	0	10.7	-15.0
5	G3X	-8.6	-3.9	4.7	493	15.3	3.25	4.3	0.44	-0.74	-8.9	-3.6	45	2.8	2	1	1	4	5.24	6.86	399.5	0.31	2	1	0	9.4	-13.1
6	HUP	-9.4	-4.9	4.5	295	9.3	1.66	10.0	0.35	-0.58	-10.1	-4.7	59	2.6	1	2	1	1	missd	6.28	243.3	0.31	2	0	2	7.0	-9.8
7	DMT	-3.9	-3.9	0.2	614	20.8	5.61	1.9	0.39	-0.70	-8.2	-2.8	59	3.9	0	4	4	9	missd	missd	494.7	0.52	0	0	0	10.7	-14.6
8	F11	-5.6	-5.2	0.4	581	17.0	5.65	4.5	0.39	-0.81	-8.5	-3.6	50	7.6	0	4	3	8	missd	missd	466.6	0.63	0	0	0	10.1	-13.7
9	THA	-12.3	-2.3	10.0	243	9.5	5.68	4.6	0.42	-0.80	-13.0	-1.4	39	3.1	1	2	2	0	missd	missd	199.3	0.15	0	0	0	6.0	-8.2
10	MTB	-7.9	-4.7	1.5	337	13.5	3.41	2.2	0.16	-0.64	-8.9	-4.5	19	1.0	4	1	3	2	missd	missd	285.4	0.23	0	0	0	6.2	-8.4
11	EDR	-9.6	-4.2	2.4	175	8.9	3.47	5.9	0.44	-0.58	-10.7	-3.5	20	-2.1	0	1	1	3	4.89	missd	167.2	0.63	0	0	0	4.9	-6.6
12	DNP	-8.0	-4.2	3.8	457	16.7	2.43	14.7	0.20	-0.55	-8.7	-3.9	39	4.1	3	1	3	2	10.41	6.68	280.5	0.24	1	1	2	8.7	-12.8
13	CP0	-7.7	-6.5	1.2	653	22.6	5.78	10.2	0.43	-0.76	-9.3	-5.1	114	4.1	8	2	4	5	5.53	5.85	588.7	0.16	1	0	1	9.8	-12.7
14	SER	-8.4	-5.3	3.1	216	8.9	6.88	16.7	0.42	-0.60	-8.7	-3.7	62	-1.0	0	2	2	2	6.95	missd	177.2	0.31	0	0	0	6.3	-9.7
15	DOP	-8.9	-5.4	3.5	188	8.8	1.66	17.5	0.31	-0.64	-9.7	-3.8	67	0.2	0	3	1	2	7.86	missd	154.2	0.70	0	0	0	6.1	-8.4
16	EPI	-8.9	-4.5	4.5	215	10.2	2.91	11.2	0.43	-0.60	-9.9	-3.6	73	-0.7	0	4	1	3	7.86	missd	184.2	0.65	1	0	1	5.5	-7.5
17	NOR	-9.4	-4.8	4.6	195	8.3	3.11	10.2	0.47	-0.63	-10.3	-4.0	87	-1.0	0	4	1	4	6.73	missd	170.2	0.67	1	0	1	6.6	-9.0
18	SEL	-9.8	-4.4	5.5	259	11.4	4.31	7.9	0.28	-0.27	-9.9	-3.8	32	2.8	0	1	1	5	missd	missd	188.3	0.11	0	1	0	5.5	-7.4
19	TRA	-9.9	-5.3	4.6	179	8.3	1.65	12.7	0.33	-0.29	-10.0	-4.0	26	1.4	0	1	1	2	missd	missd	134.2	0.05	1	1	2	5.0	-6.8
20	RAS	-10.4	-6.5	2.4	224	10.2	4.37	5.3	0.28	-0.23	-11.1	-4.7	12	1.8	1	1	1	1	missd	missd	172.0	0.10	1	0	1	5.5	-7.5
21	LAD	-9.1	-4.6	4.5	330	12.3	1.64	12.9	0.31	-0.57	-9.7	-4.0	42	2.1	1	2	1	3	5.60	7.10	273.3	0.37	1	0	1	7.1	-9.6
22	HUX	-9.5	-5.8	3.7	334	11.3	5.72	3.1	0.42	-0.87	-10.2	-4.6	39	5.8	2	2	2	1	missd	missd	299.3	0.27	2	0	2	8.7	-11.9
23	HUY	-9.6	-5.8	3.8	314	10.8	5.72	2.3	0.42	-0.84	-10.2	-4.6	39	5.5	2	2	2	1	missd	missd	285.8	0.20	2	0	2	7.7	-10.5
24	HUR	-9.5	-5.7	3.8	300	10.2	5.72	3.6	0.42	-0.86	-10.1	-4.5	38	5.0	2	2	2	1	missd	missd	251.4	0.18	2	0	2	7.3	-9.9
25	HUE	-9.5	-5.6	3.8	322	10.9	5.72	4.7	0.43	-0.86	-10.0	-4.5	38	4.9	2	2	2	1	missd	missd	265.4	0.18	2	0	2	7.6	-10.3
26	RUX	-9.5	-5.8	3.7	343	10.7	5.72	2.6	0.42	-0.84	-10.2	-4.6	39	5.8	2	2	2	1	missd	missd	299.3	0.24	0	2	2	7.4	-10.6
27	RUY	-9.6	-5.8	3.8	321	10.8	5.72	2.3	0.42	-0.86	-10.2	-4.6	39	5.5	2	2	2	1	missd	missd	285.8	0.29	0	2	2	7.0	-9.9
28	RUR	-9.5	-5.7	3.8	310	10.2	5.72	3.6	0.42	-0.84	-10.1	-4.5	38	5.0	2	2	2	1	missd	missd	251.4	0.29	0	2	2	7.1	-9.9
29	RUE	-9.5	-5.7	3.8	327	10.1	5.72	4.4	0.42	-0.85	-10.1	-4.5	38	4.9	2	2	2	1	missd	missd	265.4	0.24	0	2	22	7.4	-10.7

Figure S1. PC1 x PC2 scores from B3LYP/6-31G(d,p) data set, generated in the experiment using 5 PCs.

Figure S2. PC1 x PC5 scores from B3LYP/6-31G(d,p) data set, generated in the experiment using 5 PCs.

Table S2. Calculating missing values with K-Means.

	Atr1	Atr2	Atr3	Atr4	Atr5	Atr6	Atr7	Atr8	Atr9	Atr10	Atr11	Atr12	Atr13	Atr14	Atr15	Atr16	Atr17	Atr18	Atr19	Atr20	Atr21	Atr22	Atr23	Atr24
ACh	-10.94	-4.09	6.85	190	9.033	1.786	17.88	0.163	-0.572	-11.98	-3.44	26.4	-1.35	1	0	0	4	3.741	5.142	146.21	0.271	0	0	0
RIV	-9.41	-4.05	5.36	325	10.928	2.228	4.47	0.299	-0.632	-9.94	-3.75	32.8	2.86	2	1	1	9	2.257	4.272	251.337	0.586	1	0	1
GNT	-8.69	-4.03	4.66	338	10.327	9.788	11.36	0.373	-0.642	-9.53	-3.7	41.9	1.39	0	2	1	1	5.207	5.885	288.35	0.337	2	1	0
GL8	-9.01	-4.09	2.22	627	19.89	2.93	7.95	0.395	-0.513	-9.21	-3.59	80.8	1.37	3	1	2	9	5.214	7.26	530.65	0.348	2	1	0
G3X	-8.56	-3.87	4.68	493	15.278	3.248	4.25	0.437	-0.738	-8.87	-3.62	45.2	2.75	2	1	1	4	5.237	6.86	399.54	0.313	2	1	0
HUP	-9.42	-4.92	4.5	295	9.318	1.658	9.96	0.354	-0.576	-10.12	-4.7	58.9	2.6	1	2	1	1	4.355	6.281	243.32	0.305	2	0	2
DMT	-3.93	-3.92	0.2	614	20.752	5.607	1.89	0.385	-0.696	-8.2	-2.76	58.5	3.89	0	4	4	9	5.214	7.26	494.71	0.515	0	0	0
F11	-5.56	-5.18	0.39	581	17.009	5.646	4.54	0.388	-0.813	-8.46	-3.63	49.8	7.58	0	4	3	8	5.214	7.26	466.64	0.632	0	0	0
THA	-12.3	-2.29	10.01	243	9.52	5.676	4.62	0.417	-0.804	-12.96	-1.37	38.9	3.13	1	2	2	0	6.337	5.142	199.26	0.148	0	0	0
MTB	-7.85	-4.67	1.47	337	13.521	3.412	2.2	0.164	-0.64	-8.85	-4.48	19.4	1.04	4	1	3	2	4.355	5.885	285.4	0.227	0	0	0
EDR	-9.55	-4.18	2.38	175	8.878	3.47	5.91	0.438	-0.577	-10.68	-3.53	20.2	-2.07	0	1	1	3	4.887	5.142	167.24	0.826	0	0	0
DNP	-7.97	-4.17	3.79	457	16.748	2.433	14.71	0.203	-0.548	-8.73	-3.88	38.8	4.14	3	1	3	2	10.406	6.883	280.5	0.239	1	1	2
CP0	-7.68	-6.53	1.16	653	22.63	5.776	10.2	0.434	-0.757	-9.3	-5.06	114	4.1	8	2	4	5	5.532	5.847	588.68	0.163	1	0	1
SER	-8.39	-5.32	3.07	216	8.928	6.878	16.66	0.42	-0.6	-8.7	-3.67	62	-1.01	0	2	2	2	6.945	5.142	177.22	0.308	0	0	0
DOP	-8.86	-5.39	3.46	188	8.835	1.657	17.52	0.305	-0.639	-9.74	-3.78	66.5	0.17	0	3	1	2	7.857	5.142	154.18	0.699	0	0	0
EPI	-8.9	-4.5	4.49	215	10.188	2.911	11.22	0.431	-0.598	-9.89	-3.55	72.7	-0.68	0	4	1	3	7.86	5.142	184.2	0.645	1	0	1
NOR	-9.43	-4.77	4.64	195	8.293	3.111	10.19	0.471	-0.626	-10.3	-4.03	86.7	-0.98	0	4	1	4	6.734	5.142	170.18	0.665	1	0	1
SEL	-9.81	-4.39	5.51	259	11.439	4.309	7.85	0.278	-0.269	-9.86	-3.78	32.4	2.8	0	1	1	5	6.337	5.142	188.28	0.109	0	1	0
TRA	-9.87	-5.3	4.58	179	8.34	1.654	12.73	0.328	-0.29	-10.02	-3.98	26	1.4	0	1	1	2	6.337	5.142	134.19	0.054	1	1	2
RAS	-10.39	-6.52	2.35	224	10.186	4.365	5.27	0.283	-0.227	-11.11	-4.67	12	1.8	1	1	1	1	6.337	5.142	172	0.099	1	0	1
LAD	-9.12	-4.6	4.53	330	12.268	1.641	12.94	0.31	-0.574	-9.72	-3.95	41.6	2.08	1	2	1	3	5.601	7.102	273.34	0.372	1	0	1
HUX	-9.51	-5.78	3.74	334	11.295	5.722	3.1	0.424	-0.866	-10.17	-4.62	38.92	5.77	2	2	2	1	4.355	5.885	299.32	0.266	2	0	2
HUY	-9.56	-5.79	3.76	314	10.813	5.723	2.25	0.42	-0.843	-10.19	-4.64	38.91	5.5	2	2	2	1	4.355	5.885	285.79	0.198	2	0	2
HUR	-9.5	-5.66	3.84	300	10.23	5.72	3.58	0.424	-0.857	-10.07	-4.47	38	5.01	2	2	2	1	4.355	5.885	251.35	0.177	2	0	2
HUE	-9.46	-5.64	3.82	322	10.88	5.722	4.68	0.425	-0.857	-10.04	-4.45	38	4.85	2	2	2	1	4.355	5.885	265.37	0.177	2	0	2
RUX	-9.52	-5.78	3.74	343	10.726	5.723	2.6	0.417	-0.837	-10.17	-4.62	38.92	5.77	2	2	2	1	4.355	5.885	299.32	0.236	0	2	2
RUY	-9.56	-5.79	3.76	321	10.813	5.723	2.3	0.422	-0.859	-10.19	-4.62	38.91	5.5	2	2	2	1	4.355	5.885	285.79	0.289	0	2	2
RUR	-9.5	-5.66	3.84	310	10.23	5.72	3.62	0.416	-0.842	-10.08	-4.47	38	5.01	2	2	2	1	4.355	5.885	251.35	0.292	0	2	2
RUE	-9.47	-5.65	3.82	327	10.141	5.723	4.4	0.422	-0.846	-10.05	-4.46	38	4.85	2	2	2	1	4.355	5.885	265.37	0.238	0	2	22

Table S3. Calculating missing values with KNNI.

	Air1	Air2	Air3	Air4	Air5	Air6	Air7	Air8	Air9	Air10	Air11	Air12	Air13	Air14	Air15	Air16	Air17	Air18	Air19	Air20	Air21	Air22	Air23	Air24
ACh	-10.94	-4.09	6.85	190	9.033	1.786	17.88	0.163	-0.572	-11.98	-3.44	26.4	-1.35	1	0	0	4	3.741	5.142	146.21	0.271	0	0	0
RIV	-9.41	-4.05	5.36	325	10.928	2.228	4.47	0.299	-0.632	-9.94	-3.75	32.8	2.86	2	1	1	9	2.257	4.272	251.337	0.586	1	0	1
GNT	-8.69	-4.03	4.66	338	10.327	9.788	11.36	0.373	-0.642	-9.53	-3.7	41.9	1.39	0	2	1	1	5.207	5.687	288.35	0.337	2	1	0
GL8	-9.01	-4.09	2.22	627	19.89	2.93	7.95	0.395	-0.513	-9.21	-3.59	80.8	1.37	3	1	2	9	5.214	7.26	530.65	0.348	2	1	0
G3X	-8.56	-3.87	4.68	493	15.278	3.248	4.25	0.437	-0.738	-8.87	-3.62	45.2	2.75	2	1	1	4	5.237	6.86	399.54	0.313	2	1	0
HUP	-9.42	-4.92	4.5	295	9.318	1.658	9.96	0.354	-0.576	-10.12	-4.7	58.9	2.6	1	2	1	1	4.355	6.281	243.32	0.305	2	0	2
DMT	-3.93	-3.92	0.2	614	20.752	5.607	1.89	0.385	-0.696	-8.2	-2.76	58.5	3.89	0	4	4	9	6.028	6.329	494.71	0.515	0	0	0
F11	-5.56	-5.18	0.39	581	17.009	5.646	4.54	0.388	-0.813	-8.46	-3.63	49.8	7.58	0	4	3	8	6.028	6.329	486.64	0.632	0	0	0
THA	-12.3	-2.29	10.01	243	9.52	5.676	4.62	0.417	-0.804	-12.96	-1.37	38.9	3.13	1	2	2	0	5.676	5.699	199.26	0.148	0	0	0
MTB	-7.85	-4.67	1.47	337	13.521	3.412	2.2	0.164	-0.64	-8.85	-4.48	19.4	1.04	4	1	3	2	6.126	6.239	285.4	0.227	0	0	0
EDR	-9.55	-4.18	2.38	175	8.878	3.47	5.91	0.438	-0.577	-10.68	-3.53	20.2	-2.07	0	1	1	3	4.987	5.231	167.24	0.626	0	0	0
DNP	-7.97	-4.17	3.79	457	16.748	2.433	14.71	0.203	-0.548	-8.73	-3.88	38.8	4.14	3	1	3	2	10.406	6.683	280.5	0.239	1	1	2
CP0	-7.68	-6.53	1.16	653	22.63	5.776	10.2	0.434	-0.757	-9.3	-5.06	114	4.1	8	2	4	5	5.532	5.847	588.68	0.163	1	0	1
SER	-8.39	-5.32	3.07	216	8.928	6.878	16.66	0.42	-0.6	-8.7	-3.67	62	-1.01	0	2	2	2	6.945	5.231	177.22	0.308	0	0	0
DOP	-8.86	-5.39	3.46	188	8.835	1.657	17.52	0.305	-0.639	-9.74	-3.78	66.5	0.17	0	3	1	2	7.857	5.231	154.18	0.699	0	0	0
EPI	-8.9	-4.5	4.49	215	10.188	2.911	11.22	0.431	-0.598	-9.89	-3.55	72.7	-0.68	0	4	1	3	7.86	5.231	184.2	0.645	1	0	1
NOR	-9.43	-4.77	4.64	195	8.293	3.111	10.19	0.471	-0.626	-10.3	-4.03	86.7	-0.98	0	4	1	4	6.734	5.231	170.18	0.665	1	0	1
SEL	-9.81	-4.39	5.51	259	11.439	4.309	7.85	0.278	-0.269	-9.86	-3.78	32.4	2.8	0	1	1	5	5.676	5.699	188.28	0.109	0	1	0
TRA	-9.87	-5.3	4.58	179	8.34	1.654	12.73	0.328	-0.29	-10.02	-3.98	26	1.4	0	1	1	2	5.676	5.699	134.19	0.054	1	1	2
RAS	-10.39	-6.52	2.35	224	10.186	4.365	5.27	0.283	-0.227	-11.11	-4.67	12	1.8	1	1	1	1	5.676	5.699	172	0.099	1	0	1
LAD	-9.12	-4.6	4.53	330	12.268	1.641	12.94	0.31	-0.574	-9.72	-3.95	41.6	2.08	1	2	1	3	5.601	7.102	273.34	0.372	1	0	1
HUX	-9.51	-5.78	3.74	334	11.295	5.722	3.1	0.424	-0.866	-10.17	-4.62	38.92	5.77	2	2	2	1	6.126	6.239	299.32	0.266	2	0	2
HUY	-9.56	-5.79	3.76	314	10.813	5.723	2.25	0.42	-0.843	-10.19	-4.64	38.91	5.5	2	2	2	1	6.417	6.084	285.79	0.198	2	0	2
HUR	-9.5	-5.66	3.84	300	10.23	5.72	3.58	0.424	-0.857	-10.07	-4.47	38	5.01	2	2	2	1	5.676	56.992	251.35	0.177	2	0	2
HUE	-9.46	-5.64	3.82	322	10.88	5.722	4.68	0.425	-0.857	-10.04	-4.45	38	4.85	2	2	2	1	6.417	6.084	265.37	0.177	2	0	2
RUX	-9.52	-5.78	3.74	343	10.726	5.723	2.6	0.417	-0.837	-10.17	-4.62	38.92	5.77	2	2	2	1	6.126	6.239	299.32	0.236	0	2	2
RUY	-9.56	-5.79	3.76	321	10.813	5.723	2.3	0.422	-0.859	-10.19	-4.62	38.91	5.5	2	2	2	1	6.417	6.084	285.79	0.289	0	2	2
RUR	-9.5	-5.66	3.84	310	10.23	5.72	3.62	0.416	-0.842	-10.08	-4.47	38	5.01	2	2	2	1	6.417	6.084	251.35	0.292	0	2	2
RUE	-9.47	-5.65	3.82	327	10.141	5.723	4.4	0.422	-0.846	-10.05	-4.46	38	4.85	2	2	2	1	6.417	6.084	265.37	0.238	0	2	22

Conclusiones, Perspectivas y Continuidad

“...One never notices what has been done;
one can only see what remains to be done.”
(*Maria Skłodowska-Curie*)

8.1 CONCLUSIONES

De forma general, la presente Tesis Doctoral constituye un estudio teórico sobre el proceso de inhibición de la acetilcolinesterasa por moléculas que bloquean el sitio activo de la proteína. Se han estudiado un conjunto de 44 inhibidores, dividido en 8 clases distintas, por método QM/MM, análisis estadístico utilizando los métodos de análisis multivariados de datos, reconocimiento de patrones y de algoritmos de agrupamiento. Como se puede comprobar, la presente Tesis Doctoral está constituida por cuatro contribuciones. Las conclusiones más importantes se pueden encontrar en la parte final de cada una de ellas. A continuación, y de forma resumida las enumeramos, siguiendo el mismo orden en que se presentan estas contribuciones

Se ha estudiado la interacción y se ha calculado la energía libre de enlace (ΔG_{bind}) entre la AChE y un subconjunto de 14 moléculas de manera sistemática, utilizando los métodos híbridos QM/MM MD asociados a los métodos de la FEP y del BIE.

Se han utilizado algoritmos de docking molecular para analizar la interacción receptor-ligando de los 44 inhibidores. Este método es una buena herramienta para estudiar un número elevado de moléculas.

Las propiedades más relevantes de los inhibidores que están involucradas en el proceso de inhibición de la AChE han sido estudiadas en detalle y de forma sistemática para determinar cómo moléculas de distintas clases interactúan sobre la misma diana biológica. Para alcanzar este objetivo se han realizado análisis estadísticos, utilizando algoritmos de agrupamiento.

Los métodos empleados nos han permitido comprender los procesos de inhibición de la enzima, y encontrar correlaciones estructurales y electrónicas de los inhibidores. De esta forma, podemos entender la interacción entre los

residuos de la enzima, las moléculas de agua estructurales y los diversos inhibidores, a nivel atómico, que son fundamentales para el diseño de nuevos inhibidores y drogas con aplicaciones directas en la enfermedad de Alzheimer.

8.1.1 Estudios QM/MM MD – FEP y BIE

Los resultados de los cálculos de energía libre de enlace por el método de la FEP reproducen la tendencia de los datos experimentales, tanto en los estudios con inhibidores clásicos pertenecientes a distintos grupos como dentro de una misma familia. La correlación teórico-experimental se puede considerar buena, lo que nos permite validar el método empleado.

El análisis de los distintos términos (electrostáticos y de van der Waals) de la energía libre de interacción ha revelado una característica poco habitual en las interacciones enzima-inhibidor. El término de van der Waals es tan importante como el término electrostático en el proceso de inhibición de la AChE. La menor contribución del término de van der Waals en la energía libre de interacción se obtiene para el inhibidor de la clase de las huprinas, (-)-Huprina Y (38%), sin embargo, para el inhibidor huperzine A (53%) es más importante que el término electrostático. Este comportamiento se justifica por el elevado número de residuos aromáticos que componen su sitio activo, y la estructura química de los inhibidores que en su totalidad presentan sistemas π conjugados.

El análisis de la energía de interacción por residuo ha permitido determinar los residuos clave para la interacción de los diferentes ligandos con la enzima. Se ha observado que algunos residuos del sitio CAS de la AChE como His440, Glu327, Glu199, Gly118, Tyr130 y la Ser200, y los residuos del PAS de la AChE como Trp84, Phe330, Asp72, Phe331, y Tyr121 contribuyen significativamente a la estabilización de todos los sistemas.

Estructuralmente se observó que el mantenimiento del set up de la triada catalítica Ser200-Glu327-His400 de la AChE es fundamental para la eficiencia del inhibidor. Otro factor importante en la eficiencia y el potencial de inhibición está relacionado con el número de interacciones del tipo cation- π que el inhibidor puede establecer.

Las moléculas de agua tienen papel fundamental durante el proceso de inhibición de la AChE. Su función consiste en mediar las interacciones de enlace de hidrógeno entre los residuos polares de la AChE y los inhibidores, como por ejemplo la interacción entre el inhibidor DMT y los residuos Asp72, Glu199 y Glu278.

En los diferentes inhibidores se establecen enlaces de hidrógeno entre la AChE y los ligandos, mediados por moléculas de agua. Estas interacciones son fundamentales para estabilizar a los inhibidores en el gorge. Sin embargo, debido a naturaleza de las interacciones hidrofóbicas y la similar intensidad de las interacciones hidrofílicas de los inhibidores tanto en el agua como en la enzima, no se han observado valores de BIEs significativos en este estudio.

El efecto de los sustituyentes en las posiciones R₃ y R₉ de las huprinas no puede explicarse mediante interacciones de corto alcance, sino mediante el efecto de conjunto de toda la proteína donde los residuos Asp72, Glu199 y His440 tienen una contribución predominante.

8.1.2 Estudios Docking Molecular

Los resultados de los estudios del docking nos permitió reforzar los datos obtenidos con los métodos QM/MM MD tanto cualitativamente cuanto cuantitativamente. La tendencia de inhibición observada con los valores de los scores calculados en el docking está en concordancia con el orden de los valores de inhibición calculados con el método de la FEP.

Los estudios de docking revelaron de qué manera los diferentes inhibidores interaccionan con la AChE en el gorge. Se puede concluir que los inhibidores de tamaño molecular menor a 10 Å tienden a interactuar con los residuos del CAS, mientras que los inhibidores más grandes, en general pueden interaccionar con la mayoría de los residuos a lo largo del sitio activo de la AChE. Este resultado concuerda con los datos obtenidos.

Otra importante conclusión obtenida con el estudio sistemático con docking fue el hecho de que aquellos inhibidores que contienen centro quiral, el estereoisómero (S) presenta mejor perfil de interacción con la AChE y mejor poder inhibitorio que el estereoisómero (R).

8.1.3 Análisis de componente principal y agrupamientos

Los resultados del estudio PCA revelaron que las propiedades Masa Molar, presencia de uno o más centros quirales “S”, el valor de la energía del orbital de frontera LUMO, el volumen molecular y el número de sistemas aromáticos evidencian el perfil farmacofórico que correlaciona todas las moléculas estudiadas como inhibidores de la AChE. Ese perfil es un indicador para el diseño de moléculas con mayor poder inhibitorio.

Se puede concluir que hay una convergencia en cuanto a los resultados obtenidos con los diferentes métodos. De forma general se puede afirmar que un buen inhibidor de la AChE debe presentar, por un lado, una estructura química que permita la presencia de interacciones con los principales residuos del gorge, un tamaño molecular entre 9,0 y 21,0 Å, y anillos aromáticos con sistemas π -conjugados para favorecer las interacciones del tipo π - π , principalmente las del tipo catión- π . Por otro lado, debe tener uno o más centro quirales, preferiblemente los conformeros de la forma “S”, y que el orbital de

frontera LUMO este localizado cerca del heteroatomo más positivo (R_3N^+) y sobre los anillos aromáticos de las moléculas.

8.2 Perspectivas y Continuidad

Una vez concluida la presente Tesis Doctoral, queremos finalizarla con este apartado donde se pondrán de manifiesto las perspectivas de futuro, tanto a nivel metodológico como de aplicación, que constituirían la continuidad del presente trabajo.

8.2.1 Inhibición de la AChE por inhibidores que forman enlace covalente

El estudio del mecanismo de inhibición de la AChE es de gran importancia para el tratamiento de algunas enfermedades como la enfermedad de Alzheimer, Parkinson y otras formas de demencia. Conocer como tiene lugar la inhibición de la AChE tanto con inhibidores que actúan bloqueando la enzima, como con los que forman un enlace covalente con la serina catalítica de la enzima es fundamental para el desarrollo de nuevos medicamentos. La prolongación natural de los estudios de la presente Tesis Doctoral sería estudiar el mecanismo de la reacción de la AChE con el carbamato rivastigmina y el organofosforado diclorvos.

8.2.2 Campo Eléctrico de la AChE

Los resultados obtenidos en la presente Tesis Doctoral ponen de manifiesto la importancia de las distintas interacciones entre el centro activo de la enzima y los diferentes inhibidores. Existen tanto interacciones de tipo π -stacking como enlaces de hidrogeno, generalmente mediados por moléculas de agua. Todas estas interacciones contribuyen a estabilizar a los ligandos en el gorge. Sin embargo, destaca la contribución a la energía de interacción de algunos de los residuos del gorge, como el Asp72 y el Glu199. Se ha observado que las interacciones entre los átomos de oxígeno de los residuos Asp72 y Glu199 con el átomo del inhibidor donde está localizada la carga positiva, en general el nitrógeno amonio cuaternario, están relacionadas con el poder inhibitorio de los ligandos. En el caso particular de las huprinas, se ha determinado que el efecto de los diferentes sustituyentes en la afinidad por la enzima se debe al clúster de interacciones del centro activo donde tanto Asp72 como Glu199 contribuyen decisivamente. Estos datos evidencian la importancia del campo eléctrico generado por la AChE sobre la carga positiva del sustrato natural, ACh, y de los inhibidores estudiados. Se está llevando a cabo el cálculo del potencial y campo eléctrico de los sistemas AChE-ligandos para comprobar esta relación.

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“The end of science is not to prove a theory,
but to improve mankind.” (Manly Hall)

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To: You

Who never forgot something?
How many times we forget anything?
When the forgetness is a memory lapse, everything is fine...
You are still you... You are still there. But...
When the forgetness is a condition imposed by the unknown...
So, What to do? You are disappearing in each active thought...
You are gone through the black door of the unknown things...
So, What to do? Hope...? So, Where are you now?...
Please my friend be know... We will never give up...
We will remember you in each atom linked at this protein.

At 10^{-16} seconds: this is the enough time to lose you...
The eternity: this is the minimun time to search you...
This is my promisse to you:
I will never let you cross the dark line of the forgetness.

With love...

from: Erica

European Joint Doctorate on
Theoretical Chemistry and
Computational Modelling (TCCM)

UNIVERSITAT
JAUME I

Grupo de Química Teórica y Computacional - UJI