SYNTHESIS, CHOLINESTERASE INHIBITORY ACTIVITY AND MOLECULAR DOCKING STUDY OF PIPERIDONE-GRAFTED PYRIMIDINE AND THIAZOLOPYRIMIDINE DERIVATIVES

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2014

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By

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Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

JANUARY 2014

to GOD who created the world,
human, wisdom, knowledge and
love,

to my spiritual Master, who respired
the love in me,

to my parents and parents in-law,
who donate the love to me,

and to Yalda, who was the gift of
love.

ACKNOWLEGEMENTS

My deepest appreciation goes to my main supervisor, Dr. Vikneswaran Murugaiyah, for his kindness, encouragements, great support and enormous patience from the first day my study to finishing this thesis.

I am deeply grateful to my co-supervisor, Prof. Madya Dr. Hasnah Osman, for her myriad helps, continues support and valuable knowledge.

I owe a very important debt to Dr. Raju Suresh Kumar that without his gracious heart, finishing this thesis was formidable.

I would like to thank Mr. Chow, our expert lab assistant, for his great supports and Mr. Zahari, our knowledgeable NMR technician, for his kindness.

I am thankful to government of Malaysia, Institute of Post Graduate studies and the School of Pharmaceutical Sciences, USM for providing me the financial support and all the facilities necessary for the completion of my research project.

I would like to show my appreciation to my uncles Adel, Ebrahim and my lovely aunty Farideh.

I would like to offer my special thanks to my family, my father Mehdi, my father-in-law Firooz, My mother Esmat, My mother-in-law Khatoon and my sisters and brothers: Sara, Nima MU, Atbin, Anopa, Saba and Mohadesse for their great love, persistent encouragements, heartily supports.

Alireza Basiri

January 2014

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

µg	Microgram
µM	Micromolar
^{13}C NMR	Carbon 13 Nuclear Magnetic Resonance
1D	One dimensional
^{1}H NMR	Proton Nuclear Magnetic Resonance
2D	Two-dimensional
AChE	Acetylcholinesterase
AChI	Acetylthiocholine iodide
Ala	Alanine
Asp	Asparagine
BChE	Butyrylcholinesterase
BChI	S-butyrylthiocholine iodide
br.s	broad singlet
$CDCl_3$-d_1	Deuterated chloroform
ChEI	Cholinesterase inhibitors
COSY	Correlated Spectroscopy
d	doublet
DCM	Dichloromethane
dd	Doublet of doublets
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated dimethyl sulfoxide
DTNB	Dithiobisnitrobenzoic acid
Glu	Glutamic acid
Gly	Glycine
*h*AChE	Human acetylcholinesterase
*h*BChE	Human butyrylcholinesterase
His	Histidine
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
IC_{50}	Half maximal inhibitory concentration
J	Coupling constant
Leu	Leucine
m	Multiplet
MeOD-d_4	Deuterated methanol
MHz	Megahertz
mp	Melting point
Phe	Phenylalanine

ppm	Part per million
s	Singlet
Ser	Serine
t	Triplet
*Tc*AChE	*Torpedo californa* acetylcholinesterase
TLC	Thin Layer Chromatography
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine
α	Alpha
β	Beta
δ	Delta
μL	Microliter
σ	Sigma

SINTESIS, PERENCATAN KOLINESTERASE DAN KAJIAN PENDOKAN MOLEKUL TERBITAN PIPERIDON-TERCANTUM PIRIMIDINA DAN TIAZOLOPIRIMIDINA

ABSTRAK

Penyakit Alzheimer (AD) adalah demensia yang paling biasa dalam kalangan orang tua. Berdasarkan hipotesis kolinergik, kehilangan neuron kolinergik dalam otak pesakit AD menyebabkan kemerosotan tahap neurotransmiter asetilkolina (ACh) dan akhirnya menyebabkan disfungsi teruk dalam sistem neuropenghantaran kolinergik. Oleh itu, meningkatkan tahap ACh adalah kaedah terapeutik yang berpotensi untuk memulihkan sebahagian besar kemerosotan ingatan dan disfungsi kognitif pada pesakit AD. Pencarian perencat kolinesterase yang baru sedang berjalan di seluruh dunia. Diinspirasikan oleh kepentingan biologi terbitan tercantum pirimidina, terutamanya dalam perencatan kolinesterase, tujuh puluh empat terbitan tercantum pirimidina yang baru, iaitu piridopirimidina **6(a-l),** piridopirimidition **7(a-l)**, *N*-etilpiridopirimidition **8(a-j)**, *N*-etilmorfolinopiridopirimidition **9(a-j)**, pirimidintiol **11(a-l)** dan thiazolopirimidina **13(a-r)** telah disintesiskan dan dinilai bagi perencatan kolinesterase mereka terhadap asetilkolinesterase (AChE) dan butirilkolinesterase (BChE).

Menariknya, enam puluh lima daripada tujuh puluh empat sebatian yang dihasilkan mempunyai aktiviti perencatan BChE yang poten dengan nilai IC_{50} yang lebih rendah daripada galantamina (IC_{50}=19.34 μM) manakala sepuluh sebatian mempunyai aktiviti

perencatan AChE yang lebih tinggi atau setanding dengan galantamina (IC_{50}=2.19 μM). Sebatian **13d** dan **7e** adalah perencat AChE dan BChE yang paling poten dengan nilai IC_{50} 0.53 dan 1.18 μM, masing-masing.

Secara umumnya, penggantian moieti karbonil (C=O) dalam siri **6** dengan moieti tiokarbonil (C=S) dalam siri **7** telah menambahkan aktiviti perencatan bagi kedua-dua AChE dan BChE. Oleh itu, kajian seterusnya telah ditumpukan kepada pengubahsuaian terbitan siri **7** yang berpotensi. Sebatian dalam siri **8(a-j)** dan **9(a-j)** yang dihasilkan oleh pencantuman moieti etil atau etilmorfolino kepade NH bebas daripade gelang piperidon dalam siri **7** telah memaparkan potensi perencatan AChE dan BChE yang lebih baik. Tambahan pula, sebatian dalam siri **11**, yang dihasilkan oleh penukaran moieti tiokarbonil (C=S) dalam siri **7** dengan entiti tiol (C-SH) telah menghasilkan peningkatan kecil dalam aktiviti perencatan bagi kedua-dua AChE dan BChE. Walaupun pengubahsuaian piridopirimidotion yang tersebut dalam siri **7**, kebanyakan terbitan dalam siri **8**, **9** dan **11** lebih cenderung kepada BChE dan secara amnya lebih lemah dalam aktiviti perencatan AChE. Oleh itu, satu siri thiazolopirimidina (siri **13**) telah disintesis. Sebatian dalam siri **13** bukan sahaja menunjukkan potensi perencatan kolinesterase yang lebih tinggi tetapi juga menunjukkan pemilihan kepada AChE yang lebih baik berbanding dengan siri yang lain. Tambahan pula, sebatian **13(a-d)** juga menunjukkan sifat-sifat perencatan dual terhadap kedua-dua enzim AChE dan BChE.

Perencat AChE dan BChE yang paling aktif yang menunjukkan potensi lebih baik atau setanding dengan galantamina telah didokkan ke dalam tapak aktif AChE dan BChE

untuk tujuan penyiasatan orientasi dan ciri-ciri interaksi pengikatan mereka. Perencat ini lebih cenderung menunjukkan interaksi hidrofobik dan π, π-menyusun dengan sisa-sisa asid amino aromatik yang membarisi tapak anionik periferal (contohnya Tyr70, Tyr121, Trp279 dan Tyr334 dalam *Tc*AChE) dan tapak pengikatan kolina (contohnya Phe330 dan Trp84 dalam *Tc*AChE) pada enzim yang mungkin mencegah penyisipan dan hidrolisis substrat di tapak mangkin enzim yang menyebabkan aktiviti perencat kolinesterase yang kuat. Ramalan ciri-ciri fizikokimia bagi kebanyakan perencat yang paling aktif telah mendedahkan bahawa semua sebatian ini adalah molekul organik yang kecil dan sangat lipofilik, oleh itu mempunyai keupayaan yang baik untuk melalui halangan darah otak dan aktiviti sistem saraf pusat serta penyerapan oral yang baik dalam saluran gastrousus. Antaranya, sebatian **7e**, **7i** dan **8a** menunjukkan sifat-sifat fizikokimia yang paling memuaskan dan boleh dipertimbangkan sebagai calon drug yang berpotensi.

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia among the elderly people. Based on the cholinergic hypothesis, loss of cholinergic neurons in AD patients' brain leads to the decline of acetylcholine (ACh) neurotransmitter level and eventually causes severe dysfunctions in the cholinergic neurotransmission. Thus, increasing the ACh levels is a promising therapeutic approach to restore the substantial impairment of memory and cognitive dysfunctions in AD patients. The search for new cholinesterase inhibitors is still ongoing worldwide. Inspired by the biological significance of pyrimidine-grafted derivatives, especially in cholinesterase inhibition, seventy-four novel pyrimidine embedded derivatives, namely pyridopyrimidines **6**(**a-l**), pyridopyrimidothiones **7**(**a-l**), *N*-ethyl-pyridopyrimidothiones **8**(**a-j**), *N*-ethylmorpholino pyridopyrimidothiones **9**(**a-j**), pyrimidinethiols **11**(**a-l**) and thiazolopyrimidines **13**(**a-r**) were synthesized and evaluated for their cholinesterases inhibitory potential against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).

Interestingly, sixty-five out of seventy-four synthesized compounds had potent BChE inhibitory activities with IC_{50} values lower than galanthamine (IC_{50}=19.34 μM) while ten compounds had higher or comparable AChE inhibitory activity to that of

galanthamine (IC_{50}=2.19 μM). Compounds **13d** and **7e** were the most potent AChE and BChE inhibitors with IC_{50} values of 0.53 and 1.18 μM, respectively.

Generally, replacement of carbonyl (C=O) moiety in series **6** with thiocarbonyl (C=S) moiety in series **7**, improved both AChE and BChE inhibitory activities. Therefore, further studies were focused on the modifications of potentially active derivatives of series **7**. The compounds in series **8**(**a-j**) and **9**(**a-j**), afforded by attachments of ethyl or ethylmorpholino moieties to the free NH of piperidone ring in series **7** displayed better AChE and BChE inhibition potencies. Furthermore, compounds in series **11,** prepared by the conversion of the thiocarbonyl (C=S) moiety in series **7** with thiol (C-SH) entity also resulted in slight improvements in both AChE and BChE inhibitory activities. Despite the aforementioned modifications on pyridopyrimidothiones in series **7**, most of derivatives in series **8**, **9** and **11** had more selectivity toward BChE and generally weaker AChE inhibitory activity. Therefore, a series of thiazolopyrimidines (series **13**) were synthesized. The compounds in series **13** not only showed higher cholinesterases inhibition potencies but also displayed better selectivity towards AChE compared to the other series. Moreover, compounds **13**(**a-d**) also displayed dual inhibitory properties, against both AChE and BChE.

The most active AChE and BChE inhibitors displaying more or comparable potency to that of galanthamine were docked into the active sites of AChE and BChE to investigate their orientations and binding interactions characteristics. These inhibitors preferentially displayed hydrophobic and π,π-stacking interactions with aromatic amino acid residues

composing peripheral anionic site (e.g. Tyr70, Tyr121, Trp279 and Tyr334 in *Tc*AChE) and choline binding site (e.g. Phe330 and Trp84 in *Tc*AChE) of the enzymes that plausibly prohibit insertion and hydrolysis of substrate at catalytic site of the enzymes and ensue strong cholinesterase inhibitory activities. Physicochemical properties predictions for the most active inhibitors revealed that all these compounds are small organic molecules, highly lipophilic, thus having good ability to pass through blood brain barrier and central nervous system activity as well as good oral absorption in the gastrointestinal tract. Among them, ccompounds **7e**, **7i** and **8a** displayed the most favourable physicochemical properties and may be considered as promising drug candidates.

CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD) is a prevalent, irreversible neurodegenerative disorder that according to the World Alzheimer's Report, till now affected more than 35 million people worldwide (Prince *et al.*, 2011). The etiology of AD is not completely understood. The appearance of extracellular β-amyloid plaques and formation of intracellular neurofibrillary tangles are considered as main pathological hallmarks of this disease (Pimplikar, 2009). AD is clinically characterized by progressive cognitive impairments, loss of memory and learning disabilities along with a diverse range of neuropsychiatric symptoms (Ferri *et al.*, 2004).

Based on the so-called cholinergic hypothesis, loss of cholinergic neurons in the forebrain, cortex and hippocampus of AD patients' brain lead to decline in acetylcholine (ACh) neurotransmitter level, which eventually causes memory and cognitive impairments due to severe dysfunctions in cholinergic neurotransmission system (Bartus, 2000). Two cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are responsible for degradation and regulation of acetylcholine in human body, however they differ in kinetics and substrate selectivity (Giacobini, 2004). Acetylcholinesterase (AChE) plays a pivotal role in central and peripheral nervous systems to terminate the nerve impulse transmissions from the nerve cell to postsynaptic membrane or from the nerve cell to skeletal muscles. On the other hand, the role of butyrylcholinesterase (BChE) is not clearly known, although it is

proposed that this non-specific cholinesterase, protects AChE by hydrolyzing harmful toxins that may damage or deactivate AChE (Giacobini, 2003).

The active sites of these two cholinesterase are located at the bottom of a 20 Å long, narrow gorge comprising five important regions to accommodate and hydrolyze the substrate, namely the catalytic triad, oxyanion hole, choline binding site, acyl binding pocket and peripheral anionic site. While the overall structure of human BChE is similar to that of human AChE, the active site of BChE has many of the channel-lining aromatic residues replaced by residues with aliphatic side chains, such as leucine (Leu) and valine (Val), making BChE more proper to accommodate bulkier substrates and inhibitors (Nicolet *et al.*, 2003). Acetylcholine or inhibitors guidance inside the gorge is facilitated by hydrophobic interactions with aromatic amino acid residues lining the gorge wall (Koellner *et al.*, 2002).

Hitherto, clinically approved treatments for AD are limited to cholinesterase inhibitors (e.g. donepezil, galanthamine, rivastigmine, huperzine A), which act by inhibiting cholinesterases and also *N*-methyl d-aspartate receptor antagonists (e.g. memantine), which act at the glutaminergic pathway (Farlow *et al.*, 2009). Organic synthesis is a valuable tool to prepare a library of drug candidate molecules and their bioactivity guided modifications to amplify the desired activities and to minimize or eliminate the unwanted properties for extensive biological, pharmacological and animal studies (Lednicer, 2009). Among the biologically active lead compounds, pyrimidine grafted entities were found to possess a wide range of biological properties such as

antibacterial, anti-inflammatory, antiviral, anti HIV (Lednicer, 2009) as well as cholinesterase inhibitory activity (Mohamed *et al.*, 2010).

Problem statement

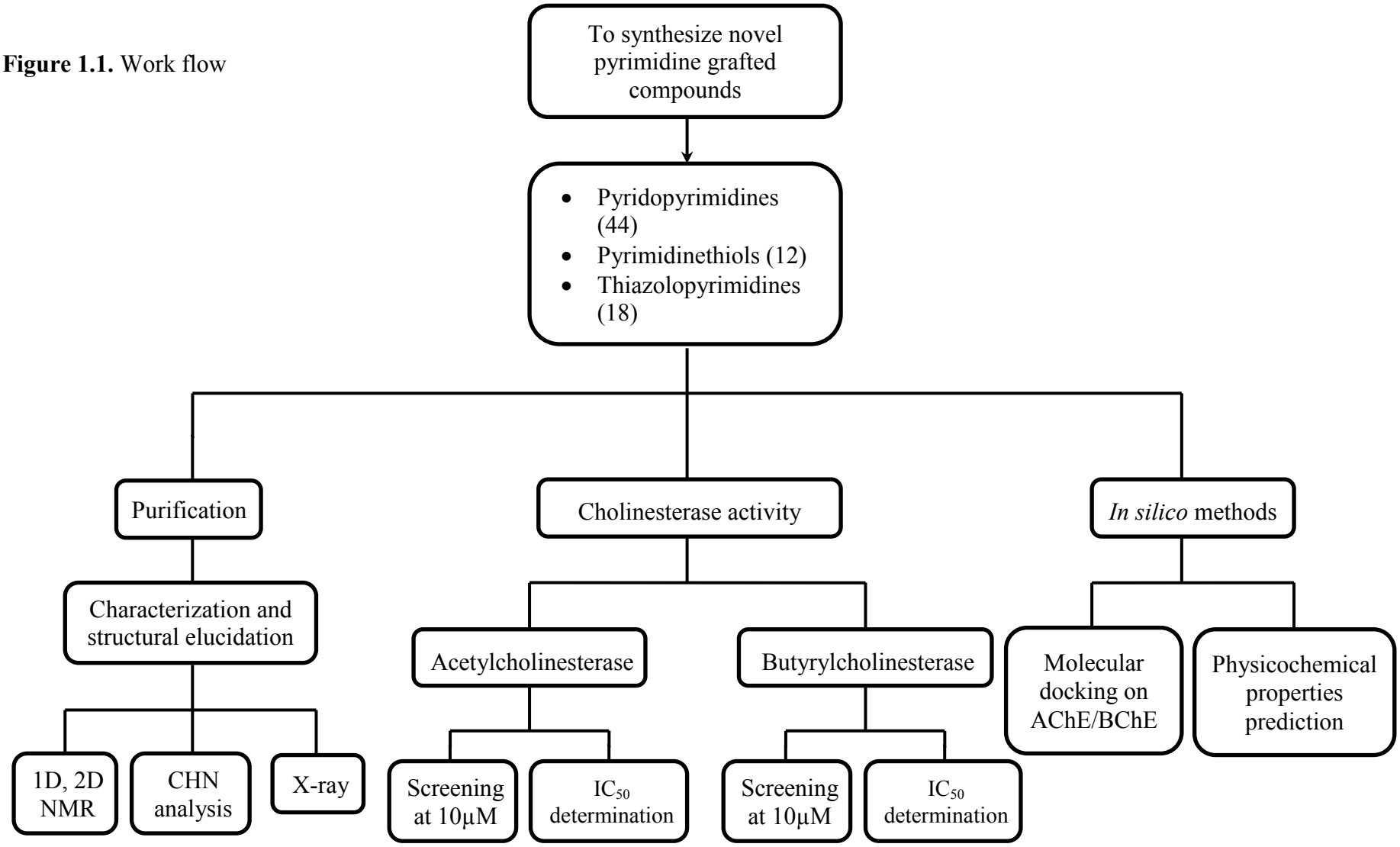
Currently, the most widely used approach for symptomatic treatment and/or alleviation of severe cognitive impairments in the individuals suffering from AD is limited to cholinesterase inhibitor drugs such as donepezil or galanthamine. Despite the tremendous efforts in search of novel disease modifying agents working *via* β-amyloid or tau pathways, none is clinically available due to their adverse effects. Moreover, limitation of potent cholinesterase inhibitor drugs, keeps the search for new inhibitors going worldwide. On this note, in the present study, novel piperidone grafted pyrimidine and thiazolopyrimidine derivatives were synthesized and evaluated for their cholinesterases inhibitory activities, in search for new cholinesterase inhibitors. Furthermore, their molecular interactions and orientation with cholinesterase were studied.

Objectives

The aims of the present study are as follow:

1. To synthesize, isolate, characterize and elucidate the structure of novel piperidone grafted pyrimidines, pyrimidinethiols and thiazolopyrimidine derivatives.
2. To evaluate the cholinesterases inhibitory activities of synthesized compound against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).
3. To investigate the structure-activity relationship (SAR) studies of the synthesized compounds.
4. To investigate the orientations and binding interaction types of the most active inhibitors inside the active site gorge of AChE and BChE by molecular docking analysis and predict their relevant physicochemical properties.

Figure 1.1. Work flow



CHAPTER 2

LITERATURE REVIEW

2.1. Alzheimer's disease

2.1.1. Prevalence, incidence and impact of Alzheimer's disease

According to the 2012 World Alzheimer report, there are an estimated of 35.6 million people with dementia worldwide (Prince *et al.*, 2011). Alzheimer's disease (AD) is the most common form of dementia, which accounts for 60 to 80 % of dementia cases in the elderly (Beckett *et al.*, 2010). This number doubles every 20 years, estimated to reach 65.7 million by 2030 and 115.4 million by 2050. Approximately 58% of people with dementia live in developing countries, but by 2050 this will rise to 71%. The fastest growth in the elderly population is taking place in China, India, and their south Asian and western Pacific neighbours (Alzheimer's Disease International, 2013).

As the population ages, the prevalence of AD and related dementias increase. In United States, one in eight people age 65 and older (13 %) show symptoms of AD. Almost half of people age of 85 and older (45%) are suffering from AD (Hebert *et al.*, 2003). Researches also revealed that, women are more affected by AD and other dementias than men. Based on estimations, 16% of women at age of 71 and older have AD or other dementias compared to 11% of men (Plassman *et al.*, 2007). This larger proportion of older women having AD or other dementias is basically due to the fact that women live longer than men (Seshadri *et al.*, 1997).

Prevalence and incidence studies showed that people with higher years of education seem to be at lower risk for AD and other dementias than those with less years of education (Gurland *et al.*, 1999). Researchers believed that higher level of education provides a "cognitive reserve" that enables individuals to tolerate the changes in the brain which could ensue AD or another dementia (Roe *et al.*, 2007).

Despite affecting a comparatively lesser number of people that other chronic diseases such as diabetes mellitus and heart related diseases, the impact of AD on social and economic burden, due to its huge financial resource consumption (e.g. Medicare and caregivers costs) is tremendous. In 2012, in United States more than 15 million family members and other caregivers provided an estimated 17.5 billion hours of care to people with AD with a value of more than USD 216 billion. Medicare payments for services to beneficiaries age 65 years and older with AD are three times more than the payments for beneficiaries without these conditions. Total payments in 2013 for health care, long-term care, and hospice services for people age 65 years and older with dementia are expected to be USD 203 billion (Mebane-Sims, 2009).

2.1.2. Clinical symptoms of Alzheimer's disease

AD is manifested by a progressive impairment of cognitive functions including memory loss that disrupts daily life, challenges in planning and solving the problems, confusion with time or places and withdrawal from work or social activities. At advanced stages of the disease, AD patients also exhibit behavioural disturbances including agitation,

irritability, anxiety, delusions and depression resulting in morbidity and eventual mortality (Ferri *et al.*, 2006).

2.1.3. Pathogenesis of Alzheimer's disease

The main and dominant pathological changes of the brain believed to contribute in the development of AD are the accumulation of β-amyloid (Aβ) plaques outside the neurons and neurofibrillary tangles, composed of hyper-phosphorylated tau proteins, inside the neurons.

The β-amyloid plaques first appear in the frontal cortex, and then spread over the entire cortical region, while hyper-phosphorylated tau and insoluble tangles initially appear in the limbic system and then progresses to the cortical region (Pimplikar, 2009). The pathogenesis of AD is explained by various hypotheses such as amyloid, tau and cholinergic hypotheses.

2.1.3.1. Amyloid hypothesis

Although the exact cause of AD has been the subject of considerable debate, the amyloid hypothesis remains one of the best defined and most studied conceptual framework for AD (Pimplikar, 2009). As originally proposed in the early 90s, the accumulation of hydrophobic amyloid-β peptides outside the neurons in basal forebrain due to amyloid precursor protein (APP) over expressed cleavage results in aggregation and formation of insoluble plaques (senile plaques), which trigger a cascade of

deleterious changes, resulting in neuronal death and thus causing AD (Hardy *et al.*, 1992).

It is also proposed that basal forebrain cholinergic neurotransmission functions (e.g. ACh regulation and release as well as receptor signalling) are impaired by these neurotoxic plaques (Yankner *et al.*, 1990) which is a causal factor for the symptoms of AD (Auld *et al.*, 2002). However, further studies showed that plaque load does not correlate well with the degree of dementia in humans. Furthermore, many AD patients with severely impaired memory showed no plaques at post-mortem analysis (Terry *et al.*, 1991). On the other hand, recent advances in neuroimaging techniques have shown the presence of huge plaques in cognitively normal people (Nordberg, 2008).

2.1.3.2. Tau hypothesis

The pioneering findings suggested that tau hyper-phosphorylation could constitute a common pathogenic pathway in different neurodegenerative diseases such as AD (Iqbal *et al.*, 1986; Wood *et al.*, 1986). Physiologically, high level of abnormal tau proteins inside the neurons, form insoluble neurofibrillary tangles that inhibit the transportation of nutrients and other essential molecules throughout the cell (Weiner *et al.*, 2012). In healthy subjects, tau protein is a component of microtubules representing the internal support structures for the transport of nutrients, vesicles, mitochondria and chromosomes within the cell. Microtubules also stabilize growing axons, which are necessary for the development and growth of neurons (Griffin, 2006).

In AD, tau protein is abnormally hyper-phosphorylated and forms insoluble fibrils, originating deposits within the cell. This process also contributes to cell death. The brain of patients in advanced stages of AD shows dramatic shrinkage from cell loss and widespread debris from dead and dying neurons. These pathological hallmarks begin to appear decades before symptoms onset and long before the dementia stage is reached (Toledo *et al.*, 2012).

2.1.3.3. Cholinergic hypothesis

Cholinergic hypothesis argues that the activity of choline acetyltransferase, the enzyme responsible for the synthesis of ACh remarkably decreases in the cortex and hippocampus of AD patient's brain (Bowen *et al.*, 1976; Davies *et al.*, 1976). Diminished ACh synthesis and two other specific function of cholinergic synapses *viz.* depolarization-induced ACh release as well as choline uptake in nerve terminals to replenish the acetylcholine synthetic machine (Nilsson *et al.*, 1986) result in loss of acetylcholine neurotransmitter levels in these brain regions that eventually leads to memory loss and other cognitive symptoms of AD (Bartus, 2000).

2.1.4. Pharmacological management of Alzheimer's disease

Presently, there are two classes of drugs being used for the treatment of AD, namely the cholinesterase inhibitors and glutamate receptor antagonist. These agents are mainly for symptomatic treatment of AD and are widely prescribed to ameliorate cognitive impairments in these patients (Weinera *et al.*, 2010). Despite the tremendous efforts in

search of novel disease modifying agents working *via* β-amyloid or tau pathways, none are clinically available due to their adverse effects.

2.1.4.1. Acetylcholinesterase inhibitors

The dominant effect of acetylcholinesterase inhibitors (e.g. galanthamine, donepezil, rivastigmine and huperzine A) is to restore the cognitive loss as the prevailing symptom in AD. These cognitive improvement makes it possible for the patients to respond more appropriately and promptly to the environment and facilitates daily living, thus increases their quality of life (Pepeu *et al.*, 2012). Acetylcholinesterase inhibitors display consistent but modest clinical efficacy against cognitive decline (Lanctôt *et al.*, 2003). The inhibition of AChE is currently the most promising and widely used approach for treating AD. Researchers also revealed that AChE inhibition could play a pivotal role in alleviating amyloid β-peptide (Aβ) plaques deposition inside the brain (Inestrosa *et al.*, 1996).

2.1.4.2. *N*-methyl-d-aspartate (NMDA) receptor antagonists

To date there is only one *N*-methyl-d-aspartate (NMDA) receptor antagonist, namely memantine, which acts at glutaminergic pathway and being clinically used to treat AD. However, its highly undesirable side effects at therapeutic doses, makes cholinesterase inhibitors a better choice for symptomatic improvement of AD (Parsons *et al.*, 1999).

2.2. Cholinesterase enzymes

Cholinesterases (ChE's) are a family of enzymes that catalyze the hydrolysis of ACh into choline and acetic acid, an essential process to restore the cholinergic transmission inside the brain. There are two types of ChE in a mammalian body differing by their functions and substrate specificity: acetylcholinesterase (AChE; EC 3.1.1.7) (Figure 2.1A) and butyrylcholinesterase (BChE; EC 3.1.1.8) (Figure 2.1B) (Pohanka, 2011).

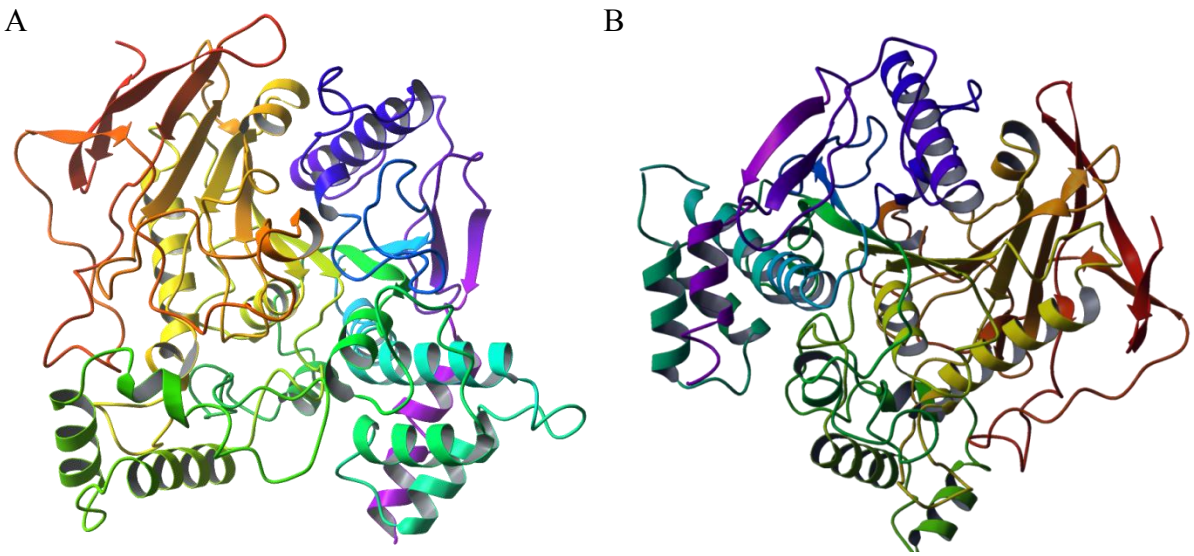


Figure 2.1. Representation of *Torpedo californica* AChE (A) and human BChE (B) (Protein Data Bank, 2013)

2.2.1. Physiological functions

As shown in Figure 2.2, acetylcholinesterase (AChE) plays a pivotal role in central and peripheral nervous systems. The synaptic AChE terminates the nerve impulse transmissions from the nerve cell presynaptic membrane to postsynaptic membrane through the synaptic gap or from the nerve cell membrane to skeletal muscles through fast hydrolysis of acetylcholine (Silman *et al.*, 2005).

Butyrylcholinesterase (BChE), which is also known as pseudo-cholinesterase or plasma cholinesterase is a non-specific enzyme that hydrolyzes many different choline esters. BChE physiological function is still unclear, but due to the wide distribution of ChE agents in plants, it has been proposed that BChE might be a naturally developed protecting enzyme against these toxicants (Antokhin *et al.*, 2010). Apart from that, there has been some evidence that neuronal BChE may play a role in certain non-cholinergic pathways such as cell differentiation (Giacobini, 2001).

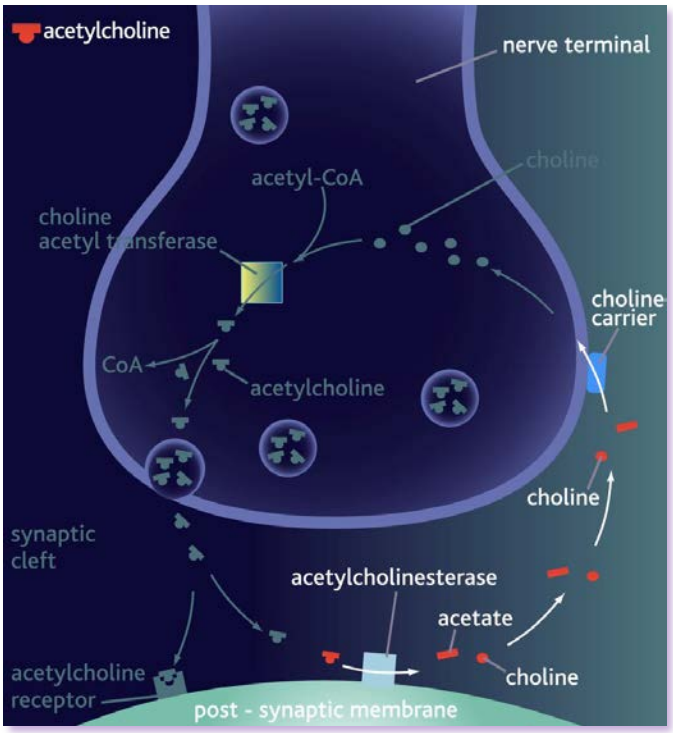


Figure 2.2. Schematic representation of AChE action (Katzung *et al.*, 2004)

Previous studies revealed that as the AD progresses, the activity of AChE decreases whereas the activity of BChE remains unaffected or even increases (Giacobini, 2004). In the brain of advanced staged AD patients, BChE can compensate for AChE when the

activity of AChE is inhibited. Thus, BChE hydrolyzes the already depleted levels of ACh in these patients (Xie *et al.*, 2000; Greig *et al.*, 2005). It has been also proposed that individuals with low-activity of BChE can sustain cognitive functions better comparing to individuals with normal BChE activity (Holmes *et al.*, 2005). Therefore, inhibitors with good balance between AChE and BChE inhibitory activity are valuable therapeutic targets in AD therapy.

2.2.2. Structural specifications

The overall architecture of the AChE and BChE enzymes is quite similar. The active site is located at the bottom of a 20 Å deep cavity named as "aromatic gorge". Substrate and inhibitor guidance down the aromatic gorge is facilitated by hydrophobic interactions with aromatic residues lining the gorge wall such as phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr) (Koellner *et al.*, 2002). The overall structure of human AChE (*h*AChE) is very similar to that of *Torpedo californica* (*Tc*AChE), whereby only the residues numbering are different. On the other hand, in the active site of BChE, aromatic residues such Trp and Phe, are replaced with hydrophobic ones including leucine (Leu) and valine (Val), making BChE more appropriate to accommodate bulkier substrates and inhibitors (Nicolet *et al.*, 2003).

2.2.3. Residue compositions of the active site gorge

Active site gorge of AChE and BChE can be classified into five regions, namely the catalytic triad, oxyanion hole, acyl pocket, choline binding site, and peripheral anionic site. The active site is located at the bottom of the gorge, lying 20 Å below the surface.

The catalytic triad is the most important site of the enzymes, where the hydrolysis of acetylcholine takes place. Both AChE and BChE possess the catalytic triad (CT), which is composed of Ser200, His440 and Glu327 residues in *Tc*AChE and His438, Ser198 and Glu325 in *h*BChE (Sussman *et al.*, 1991). The mechanism of substrate complexation to CT is plausibly *via* nucleophilic attack of serine hydroxyl moiety to carbonyl group of acetylcholine to give an acyl-enzyme intermediate. In the next step, water molecule deacylates serine by hydrolyzing its ester linkage of the substrate and converts acetylcholine to acetic acid and choline (Figure 2.3).

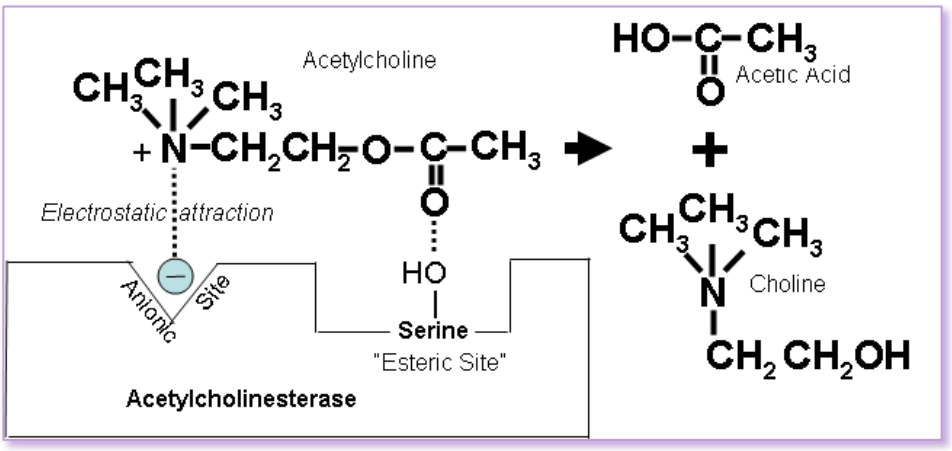


Figure 2.3. Acetylcholine hydrolysis in AChE active site (ATSDR, 2013)

Four other binding sub-sites play important role for guiding, orientation and insertion of the choline substrate in the catalytic cavity (Figure 2.4). Using *Tc*AChE residue numbering, the functions of these sub-sites are described as follows:

(i) A three-pronged “oxyanion hole” formed by the amides backbone of Gly118, Gly119, and Ala201, stabilizes the negative charge developed at the C=O moiety of the substrate in the acylation/de-acylation process (Harel *et al.*, 1996).

(ii) A concave hydrophobic pocket (acyl-binding pocket) consisting of residues Phe288 and Phe290 is located in the so-called acyl loop, in which the acetyl or propanoyl moiety of the substrate is bound (Pezzementi *et al.*, 2011).

(iii) While the catalytic triad hydrolyzes the ester bond, the anionic site interacts with the acetylcholine quaternary ammonium atom and is responsible for its correct orientation. The aromatic rings of Trp84 and Phe330 at “choline binding site or α-anionic site” of the enzyme stabilize the quaternary ammonium function of the choline moiety through cation-π interactions (Harel *et al.*, 1993).

(iv) A peripheral anionic site (β anionic site), composed of aromatic Tyr 70, Asp72, Tyr 121, Trp279 and Tyr 334 residues guides the substrate to catalytic triad (Çokuğraş, 2003). The peripheral anionic site is located 15Å above the active site, close to the mouth of the gorge.

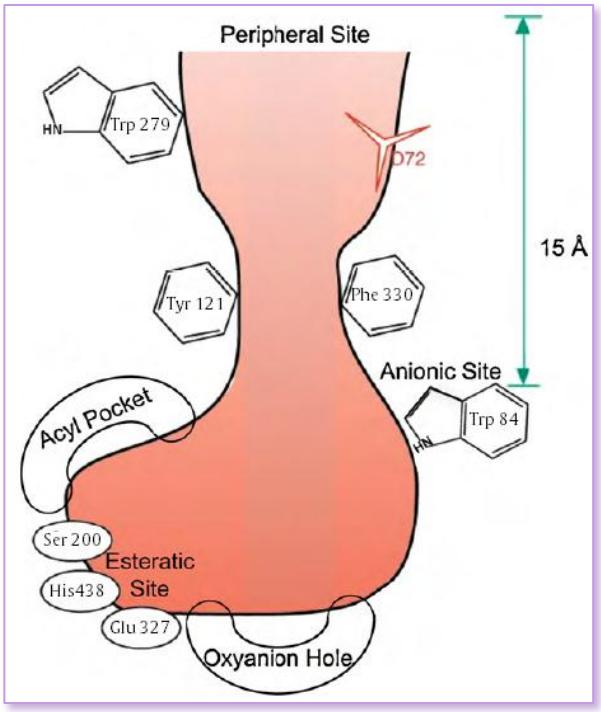


Figure 2.4. Active site residue compositions in *Torpedo californica* AChE (Dvir *et al.*, 2010)

The enzyme active site gorge residue compositions of *Tc*AChE, *h*AChE and *h*BChE are summarized in Table 2.1. The overall structure of human AChE (*h*AChE) and *Torpedo californica* (*Tc*AChE) are similar, they only differ in the residues numbering. On the other hand, in the active site of BChE, aromatic residues such Trp and Phe, are replaced with hydrophobic ones (Cheung *et al.*, 2012).

Table 2.1: Residue compositions of the enzyme active sites in *Tc*AChE, *h*AChE and *h*BChE

Site name	Residue composition in *Tc*AChE (Cheung et al., 2012)	Residue composition in *h*AChE (Cheung et al., 2012)	Residue composition in human BChE (Nicolet *et al.*, 2003)
Catalytic triad	Ser200, His440 &Glu327	Ser203, His447 & Glu201	His438, Ser198 & Glu325
Choline binding site (α-anionic site)	Trp84 & Phe330	Trp86 & Phe338	Trp82 & Phe329
Acyl-binding pocket	Phe288 & Phe290	Phe295 & Phe297	Leu286 & Val288
Oxyanion hole	Gly118, Gly119 & Ala201	Gly121, Gly122 & Ala204	Gly116, Gly117& Ala199
Peripheral anionic site (β-anionic site)	Tyr70, Asp72, Tyr121, Trp279 & Tyr334	Tyr72, Asp74, Tyr124, Trp286 & Tyr341	Trp231 & Phe398

2.2.4. Cholinesterase inhibitors

Cognitive impairment is the most prominent and clinically relevant feature of AD. Cholinesterase inhibitors act by inhibiting cholinesterase enzymes from hydrolyzing acetylcholine, thus restoring the cognitive abilities in AD patients (Posner *et al.*, 2013). To date, there are few prominent cholinesterase inhibitors available, namely tacrine, physostigmine, rivastigmine, donepezil, galanthamine and huperzine A, which structures are depicted in Figure 2.5.

Tacrine or tetrahydroaminoacridine was the first FDA approved drug for the treatment of mild or moderate AD. Tacrine is a centrally acting, reversible AChE inhibitor, which exhibit a variety of pharmacological properties including interacting with nicotinic

receptors (Svensson, 2000). Tacrine is not prescribed clinically after multiple reports on its severe hepatotoxicity.

Physostigmine is a competitive inhibitor of the AChE with the ability to diffuse through the blood brain barrier. It has been suggested as an alternative for protection against organophosphate brain toxicity as well as symptomatic treatment of AD. The therapeutic use of physostigmine is limited due to its biological constraints such as short elimination half-life, narrow effective dose range, low *in vivo* stability and low oral bioavailability (Walter *et al.*, 1995).

Rivastigmine (Exelon®) is a novel carbamate type, reversible dual cholinesterase inhibitor used for symptomatic treatment of mild to moderate dementia in AD and idiopathic Parkinson's disease. This inhibitor displays specific activity for central AChE over peripheral AChE (Kumar *et al.*, 2009; Thomas *et al.*, 2012). Adverse effects of rivastigmine are generally those associated with a second generation AChE inhibitor, in particular gastrointestinal effects, such as nausea and vomiting (Blesa González *et al.*, 2011). A new rivastigmine formulation has recently been developed in the form of transdermal patches, which provide a stable release of drug over time (Wentrup *et al.*, 2009). It is a safe, well tolerated system, which at the same time allows good adherence and effective drug penetration via the skin, with good local tolerability, ensuring ease of use together with optimized pharmacokinetics (Farlow *et al.*, 2009).

Galanthamine, a tertiary alkaloid extracted from *Galanthus* and *Narcissus* species (Amaryllidaceae), is a competitive and reversible AChE inhibitor. In addition to inhibition of AChE, galanthamine interacts allosterically with nicotinic ACh receptors to potentiate the action of agonists of these receptors and amplifies the ACh response by increasing ACh release (Samochocki *et al.*, 2003; Nakao *et al.*, 2008). Galanthamine has more than a 10-fold selectivity for AChE in contrast to BChE. The inhibition of AChE ceases 24 hours after the discontinuing galanthamine, therefore anesthetic agents and muscle relaxants, can be safely administered within a short period of stopping galanthamine (Coyle *et al.*, 2001).

Donepezil is a reversible and non-competitive cholinesterase inhibitor for the treatment of AD. It is a selective inhibitor toward AChE rather than BChE. This drug was demonstrated to be potent and selective toward brain AChE with lower adverse effects in comparison to tacrine (Yu *et al.*, 2005). Several studies have demonstrated its efficacy in slowing the deterioration of cognitive function and its safety in long-term treatment. This drug has been shown to be effective for mild-to-moderate AD, and the start of treatment at earlier stage has been recommended (Zhang *et al.*, 2007).

Huperzine A, a novel Lycopodium alkaloid discovered from the Chinese folk medicine *Huperzia serrata,* has been found to inhibit AChE selectively and possess well tolerated properties that may be especially suitable for AD treatment (Zhao *et al.*, 2002). Huperzine A is approved by the United States Food and Drug Administration as a dietary supplement for memory improvement and has been employed for centuries to

treat swelling, fever and blood disorders in China. Studies had showed its effectiveness to improve cognitive abilities in AD patients (Wang *et al.*, 2009).

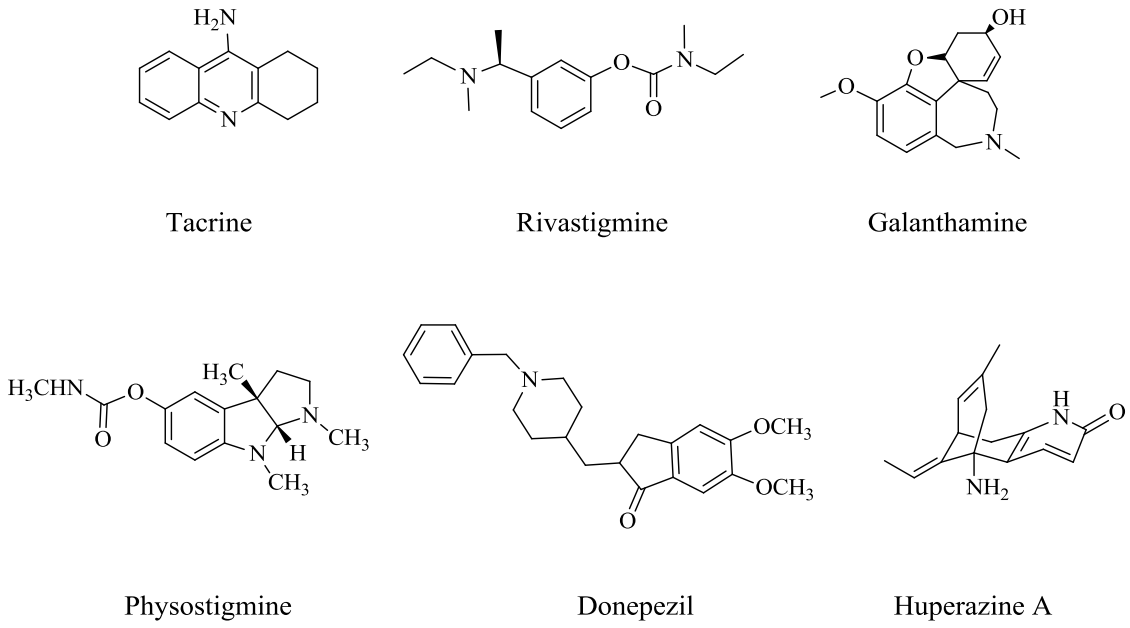


Figure 2.5. Cholinesterase inhibitors used in the treatment of Alzheimer's disease

2.3. Molecular docking

Molecular docking is a process that predicts the conformation of a ligand within the active site of a receptor or enzyme and finds the lowest energy binding modes between them. It has become a useful tool in drug discovery efforts and is a primary component in many drug discovery programs (Kitchen *et al.*, 2004). Pioneering research works in the area of molecular docking date back into the early 1980s. However, it took at least a decade for this technology to become popular among computational chemists and pharmaceutical researchers (Kuntz *et al.*, 1982). Molecular docking procedure typically consists of two interrelated task: (i) to sample possible lowest energy conformational

states of the protein-ligand complex and (ii) to calculate of the free energy of these complex to produce a score, which can be further correlated to biological activities or other functions, the so-called scoring (Novikov *et al.*, 2009). Docking simulation or *in silico* methodology is a valuable tool in investigation of the binding orientation of probable active ligands inside the active site of a specified protein from a particular library of compounds (McInnes, 2007).

2.3.1. The role of molecular docking in cholinesterase inhibitors design

The active site of AChE and BChE enzymes are composed of 5 major sub-sites, namely, peripheral anionic site (PAS), acyl binding pocket, choline binding site, oxyanion hole and catalytic triad. Compounds inhibiting ChE can be divided into three particular groups, which are

(i) Compounds binding at catalytic triad of the enzyme (e.g. nerve agents).

(ii) Compounds interacting with the choline binding site of the enzyme (e.g. galanthamine).

(iii) compounds binding at the peripheral anionic site of the enzyme (e.g. huperzine A).

The catalytic triad binding inhibitors are the compounds with the chemical structure of organophosphorus or carbamate derivatives mainly including toxins, chemical warfare agents or pesticides. These compounds interact with serine residue in the catalytic triad of the enzyme, providing stable, irreversible esters (Bajgar, 2004). On the other hand, inhibitors binding to the choline binding site of the enzyme, typically contain condensed aromatic cores (e.g. galanthamine). In comparison to the catalytic triad inhibitors, the

compounds interact with the choline binding site are reversible inhibitors (Pohanka, 2011).

The peripheral anionic site is the main target of newly synthesized drugs for AD treatment. Studies showed that the deposition of amyloid plaque in AD might be accelerated or even triggered by interaction of β-amyloid with the peripheral anionic site of the enzyme. Thus, inhibition of the peripheral anionic site not only improve the symptomatic effects of AD due to the enhancement of acetylcholine availability but also slows down the deposition of β-amyloid plaques, as one of the major pathological hallmarks of AD (Berson *et al.*, 2008; Arce *et al.*, 2009). Moreover, drugs binding to the peripheral anionic site as well as choline binding site (e.g. donepezil, galanthamine) inhibit AChE more effectively.

Thus, the therapeutic features of newly synthesized inhibitors can be demonstrated by *in silico* interaction analysis with the amino acid residues composing active site of cholinesterase enzymes prior to employing expensive and time-consuming *in vivo* techniques.

2.4. Medicinal chemistry

Medicinal chemistry is the science that deals with discovery and design of new therapeutic chemicals and their development into useful medicines (Lednicer, 2009). It may involve isolation of compounds from nature or the synthesis of new molecules, investigation of the relationships between the structure of molecules and their activity,

and elucidation of the interactions between this molecules and the receptors of various kind (Bauer, 1969).

Medicinal chemistry has been practiced for several thousand years. The earliest written records of Chinese, Indians, South Americans and Mediterranean civilizations described the therapeutic effect of different plants (Sneader *et al.*, 1985). However, the modern therapeutic is considered to have begun with an extract of foxglove plant for the treatment of dropsy (congestive heart failure) in 1785 by Withering (Withering, 1785). As a result of advances made in synthesis and separation methods as well as biochemical techniques since the late 1940s, a rational approach to design, synthesis and chemical modification to improve or change their medicinal properties was established.

2.4.1. The role of organic synthesis in drug discovery

Generally, drugs are not discovered. What is more likely to be discovered is known as a lead compound. The lead is a prototype compound that has a number of attractive pharmacological activities and pharmaceutical applications, but may have other undesirable properties including high toxicity, absorption difficulties, insolubility or metabolism difficulties. The structure of the lead compound is modified by organic synthesis to amplify the desired activities and to minimize or eliminate the unwanted properties to prepare a drug candidate, which is a compound appropriate for extensive biological, pharmacological and animal studies (Lednicer, 2009).