AURONES OF AMINE AND CARBAMATE FUNCTIONALITIES AS NEUROPROTECTIVE AGENTS WITH MULTITARGETING POTENTIAL: SYNTHESIS, STRUCTURE-ACTIVITY RELATIONSHIPS AND MODE OF ACTION STUDIES

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by

LIEW KOK FUI

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I dedicate this thesis to the memory of my father,

To my mother,

&

To my dearest wife for always being there for me through thick and thin

ACKNOWLEDGEMENT

TABLE OF CONTENTS

CHAPTER FIVE: *IN VITRO* PHARMACOKINETIC STUDIES OF SELECTED AURONES AND HPLC METHOD DEVELOPMENT FOR THEIR QUANTIFICATION

CHAPTER SIX: NEUROPROTECTIVE POTENTIAL OF A SELECTED AURONE IN *CAENORHABDITIS ELEGANS* (*C. ELEGANS*) MODELS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

μCi	Microcurie
^{13}C	Carbon-13
^{1}H	Proton
6-OHDA	6-Hydroxydopamine
Å	Angstrom
ABL	Aqueous boundary layer
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AChEIs	Acetylcholinesterase inhibitors
AD	Alzheimer's disease
ADE	Anterior deirid
ADMET	Absorption, distribution, metabolism, elimination and toxicity
ADT	AutoDockTools
ANOVA	Analysis of variance
Aβ	Amyloid-beta
$A\beta_{1-40}$	Amyloid-beta 1-40 peptide
$A\beta_{1-42}$	Amyloid-beta 1-42 peptide
BBB	Blood-brain barrier
BSA	Bovine serum albumin
BuChE	Butyrylcholinesterase
C.elegans	*Caenorhabditis elegans*
CE	Capillary electrophoresis
CEP	Cephalic
ChE	Cholinesterase
CNS	Central nervous system
CNS –	Low BBB permeation
CNS +	High BBB permeation

CNS +/–	Uncertain BBB permeation
COSY	Correlation spectroscopy
CYP450	Cytochrome P450
Cβ	Exocylic olefinic carbon
Da	Dalton
DAergic	Dopaminergic
DAT-1	Dopamine transporter 1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DRW	Dynamic range window
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
ER	Efflux ratios
em	Emission
ESI-MS	Electrospray Ionization Mass Spectra
ex	Excitation
FDA	Food and Drug Administration
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GFP	Green fluorescent protein
H_2O_2	Hydrogen peroxide
HBSS	Hanks' balanced salt solution
HCl	Hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFIP	Hexafluoroisopropanol
*h*MAO-A	Human recombinant monoamine oxidase A
*h*MAO-B	Human recombinant monoamine oxidase B
HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry

HSQC	Heteronuclear single quantum coherence spectroscopy
Hz	Hertz
IC_{50}	Half maximal inhibitory concentration
K_2CO_3	Potassium carbonate
K_i	Inhibition constant
K_m	Michaelis-Menten constant
KOH	Potassium hydroxide
L1	Larvae stage 1
LC-MS	Liquid chromatography-mass spectrometer
LOD	Limit of detection
$\log D_{7.4}$	Distribution coefficient at pH 7.4
Log$P$	Logarithm of the octanol/water partition coefficient
LOQ	Limit of quantification
M	Molarity
M.p.	Melting point
MAO	Monoamine oxidase
MAO-A	Monoamine oxidase A
MAO-B	Monoamine oxidase B
MAOIs	Monoamine oxidase inhibitors
MeOH	Methanol
MTDL	Multitarget-directed ligand
MTDLs	Multitarget-directed ligands
Na_2SO_4	Sodium sulphate
NADP	Beta-nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NFT	Neurofibrillary tangles
NGM	Nematode growth medium
NMDA	*N*-methyl-*D*-aspartate
NMR	Nuclear magnetic resonance

NQO1	NAD(P)H: quinine oxidoreductase 1
OAT	Organic cationic transporter
℃	Celcius
P_0	Intrinsic transcellular passive permeation
PAMPA	Parallel artificial membrane permeability assay
P_{app}	Apparent permeability rate
P_{ABL}	Aqueous boundary layer restriction
P_c	Transcellular restriction
P_{para}	Paracellular restriction
P_{filter}	Filter restriction
PAS	Peripheral anionic site
PBEC	Porcine brain endothelial cells
PBL	Porcine brain lipid
PBS	Phosphate buffer saline
PDB	Protein data bank
PDE	Posterior deirid
P_e	Effective permeability
PEG-400	Polyethylene glycol 400
P-gp	Permeability glycoprotein
ppm	Parts per million
PT_{50}	Mean duration at which 50% worms were paralyzed
r^2	Correlation coefficients
RLM	Rat liver microsomes
RMSD	Root-mean-square deviation
RO5	Rule of five
RPM	Revolutions per minute
rt	Room temperature
SEM	Standard error mean
$t_{1/2}$	Half-life

TEER	Transendothelial electrical resistance
ThT	Thioflavin T
t_R	Retention time
US$	United States dollar
UV	Ultraviolet
V_{max}	Maximum velocity
$ZnCl_2$	Zinc chloride
α	Alpha
β	Beta
δ	Delta
μM	Micromolar
μm	Micrometre
π	Pi
Ω	Resistance

LIST OF APPENDICES

AURON DENGAN KEFUNGSIAN AMINA DAN KARBAMAT SEBAGAI AGEN NEUROPROTEKTIF DENGAN POTENSI PELBAGAI SASARAN: SINTESIS DAN KAJIAN HUBUNGAN STRUKTUR-AKTIVITI SERTA MOD TINDAKAN

ABSTRAK

Penyakit Alzheimer (AD) ialah penyakit kompleks pelbagai faktor melibatkan pelbagai mekanisme yang menyumbang kepada pencetusan penyakit ini. Dalam pencarian entiti kimia baru untuk menangani faktor-faktor penyebab penyakit ini, strategi rekaan ligan diarah pelbagai sasaran (MTDL) telah digunakan dalam kajian ini dengan menggabungkan motif struktur yang terpilih (pelbagai amina dan karbamat) daripada dua ubat Alzheimer yang mantap (donepezil dan rivastigmina) ke dalam perancah auron. Auron-auron ini direka berdasarkan premis bahawa perancah tersebut berpotensi digunakan untuk membangunkan suatu sebatian pelbagai sasaran yang bersaiz munasabah kecil untuk neuroprotektif sementara memelihara ciri-ciri menyerupai ubat yang baik. Dalam kajian ini, suatu siri terbitan auron yang membawa kefungsian amina dan karbamat di pelbagai kedudukan (gelang A dan/atau B) perancah telah disintesiskan dan dicirikan dengan menggunakan teknik-teknik spektroskopi. Auron-auron tersebut pada mulanya dinilai dalam aktiviti *in vitro* perencatan asetilkolinesterase (AChE) dan butirilkolinesterase (BuChE). Untuk mengesahkan ciri pelbagai sasaran mereka, auron tersebut dinilai dalam dua aktiviti berkaitan dengan penyakit Alzheimer (AD), iaitu perencatan monoamina oksidase

(MAO) dan pengagregatan amiloid beta (Aβ). Pada masa yang sama, kestabilan metabolik dan ketelapan rintangan darah-otak (BBB) auron terpilih yang poten diperiksa untuk mengenal pasti suatu sebatian optimum dengan kombinasi pelbagai potensi dan profil farmakokinetik yang baik. Kesan neuroprotektif aurone **4-3** yang paling baik kemudian diperiksa dengan dua model neurodegenerasi *Caenorhabditis elegans* (*C.elegans*), iaitu kelumpuhan diaruh Aβ dan neurodegenerasi diaruh 6-hidroksidopamina (6-OHDA). Kajian hubungan struktur-aktiviti mendedahkan beberapa perencat kuat AChE selektif yang membawa moieti piperidina dan pirolidina di gelang A atau B, dengan nilai-nilai IC_{50} submikromolar. Sebagai tambahan kepada aktiviti perencatan AChE mereka, potensi pelbagai sasaran telah diperhatikan dalam dua auron, iaitu auron **2-2** (perencat MAO-B) dan **4-3** (perencat pengagregatan Aβ). Pengeraman mikrosom hati tikus dengan auron mengenalpasti **4-3** sebagai paling stabil secara metabolik berbanding dengan auron-auron lain. Penilaian ketelapan BBB menggunakan cerakinan ketelapan membran tiruan selari (PAMPA) dan ketelapan dwiarah sel endothelium otak khinzir (PBEC) mendedahkan kesemua auron yang diuji sangat telap secara pasif merentasi BBB serta penerapan aurone **4-3** adalah melibatkan mekanisme pengambilan aktif merentasi BBB. Tambahan, auron **4-3** yang menjanjikan juga menunjukkan perlindungan kepada nematod daripada ketoksikan diaruh Aβ dan 6-OHDA dalam model neurodegenerasi *C.elegans*. Maka, auron **4-3** yang ditemui dalam kajian ini mewakili suatu sebatian petunjuk menyerupai ubat yang menjanjikan kepada perkembangan lanjut perancah auron sebagai agen pelbagai poten yang berpotensi untuk penyakit neurodegeneratif.

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ABSTRACT

Alzheimer's disease (AD) is a complex multifactorial disease involving diverse mechanisms contributing to the onset of the disease. In the search for novel chemical entities to address the causative factors of the disease, the multitarget-directed ligand (MTDL) design strategy has been applied in the present study by incorporating selected structural motifs (various amines and carbamate) from two established Alzheimer drugs (donepezil and rivastigmine) into the aurone scaffold. These aurones were designed on the premise that the scaffold could be utilised to develop a reasonably small sized multitargeting compound (targeting cholinesterase, monoamine oxidase, and amyloid-beta aggregation) for neuroprotection while maintaining good drug-like properties. In this study, a series of aurone derivatives carrying amine and carbamate functionalities at various positions (ring A and/or B) of the scaffold was synthesized and characterized using spectroscopic techniques. These aurones were initially evaluated for their *in vitro* acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities. To further substantiate their multi-targeting properties, the aurones were evaluated on two Alzheimer's disease (AD)-related activities, namely monoamine oxidase (MAO) and amyloid-beta (Aβ)

aggregation inhibitions. In parallel, the metabolic stability and blood-brain barrier (BBB) permeability of selected potent aurones were examined to identify an optimal compound with a combination of multipotency and favourable pharmacokinetic profile. The neuroprotective effect of the most promising aurone **4-3** was then examined on two *Caenorhabditis elegans* (*C. elegans*) neurodegeneration models, namely Aβ-induced paralysis and 6-hydroxydopamine (6-OHDA)-induced neurodegeneration. Structure-activity relationship study revealed several potent selective AChE inhibitors carrying piperidine and pyrrolidine moieties at ring A or B, with submicromolar IC_{50} values. In addition to their AChE inhibitory activity, multi-targeting potential was observed in two aurones, namely aurone **2-2** (MAO-B inhibitor) and **4-3** (Aβ-aggregation inhibitor). Rat liver microsomal incubation of the aurones identified aurone **4-3** to be the most metabolically stable compared to the other aurones. BBB permeability evaluation using the parallel artificial membrane permeability assay (PAMPA) and porcine brain endothelial cells (PBEC) bidirectional permeability revealed all the tested aurones to be highly passive permeable across the BBB and the permeation of aurone **4-3** to involve active uptake mechanism across the BBB. In addition, the most promising aurone **4-3** also showed protection on the nematodes against both Aβ- and 6-OHDA-induced toxicities in the *C.elegans* neurodegeneration models. Hence, aurone **4-3** discovered in the present study represents a promising, drug-like lead for further development of the aurone scaffold as potential multi-potent agents for neurodegenerative diseases.

CHAPTER 1

INTRODUCTION

1.1   Neurodegenerative diseases

Neurodegenerative diseases are debilitating disorders characterized by progressive loss of nerve structure and function in the central nervous system (CNS). They include Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis. Although there are a variety of risk factors (aging, genetics, environment, and lifestyle) leading to the onset of these diseases (Nieoullon, 2011), the outcome is the same: loss of cognitive function and motor control progressing to brain atrophy and death (Brettschneider *et al*., 2015). Despite their enormous diversity in clinical manifestations and pathogenesis, most neurodegenerative diseases share some common key features: dramatic loss of synapses and neurons and cerebral deposits of insoluble misfolded protein aggregates (Forman *et al*., 2004). These deposits can be considered trademarks for the different neurodegenerative disorders because the main protein component involved is different in each disease (Ramanan and Saykin, 2013). In the particular case of AD, two types of protein deposits are considered to be the pathological hallmarks of the disease, namely senile plaques (also known as amyloid plaques) that are associated with extracellular amyloid beta (Aβ) protein (Glenner and Wong, 1984) and intracellular neurofibrillary tangles (NFT) that are composed of hyperphosphorylated tau protein (Huang and Mucke, 2012; Longo and Massa, 2004).

1.1.1 Prevalence and pathogenesis of AD

Among the various neurodegenerative diseases that have been diagnosed, AD stood out to be the most prominent in terms of prevalence and socioeconomic burden (Maslow, 2008). The disease is named after Alois Alzheimer, the pathologist who in 1907 first observed the plaques and tangles in the brains of AD patients (Forman *et al*., 2004). It is age-related and affects approximately 5 - 8 % of people over the age of 65, 15 - 20 % of those over the age of 75, and 25 - 50 % of those over the age of 85 (Shah *et al*., 2008). Epidemiologic studies show that around 35 million in the world are suffering from AD and this will grow to more than 100 million cases by 2050 (Aggarwal *et al*., 2012; Thies and Bleiler, 2013). In parallel with the increase in the number of people affected with AD, the annual financial cost to society due to AD in 2010 was estimated approximately US$ 604 billion worldwide (Wimo *et al*., 2013) and expected to increase by the year of 2030 (Abbott, 2011), highlighting the enormous socioeconomic impact of AD.

Notwithstanding the many efforts to understand the AD pathogenesis, the precise aetiology of AD remains incomplete (Leon *et al*., 2013). Advancements in molecular biology and immunology over the past two decades have enabled further understanding of the disease and the identification of molecular targets that mediate the pathogenesis of AD. Several theories have come into prominence to explain the aetiology of AD (Klafki *et al*., 2006; Suh *et al*., 2005): the cholinergic deficit hypothesis (Bartus *et al*., 1982), the amyloid cascade hypothesis (Hardy and Allsop, 1991; Hardy and Selkoe, 2002; Karran *et al.*, 2011), oxidative stress and mitochondrial dysfunction (Garcia-Escudero *et al*., 2013; Marcus *et al*., 1998; Moreira *et al*., 2010), excitotoxic and neuroinflammatory processes (Mishizen-

Eberz*et al*., 2004) and monoaminergic abnormalities (Baker and Reynolds, 1989, Trillo *et al*., 2013), altogether depicting a complex picture of the disease.

The cholinergic hypothesis is particularly important as it has been the cornerstone to the discovery of the present treatments for AD. This hypothesis postulates that the cognitive impairment and symptoms (dementia, memory loss) experienced by AD patients are due to the extensive loss of cholinergic neurons in certain regions of the brain such as the hypothalamus, the amygdala, and the neocortex. Drugs that can restore this cholinergic deficit in the CNS would therefore be able to slow the cognitive decline associated with the disease.

Also of prominence is the amyloid cascade hypothesis which originates from the observation of Aβ plaques in the AD brains. The deposition of insoluble Aβ fibrils amidst the neurons and the generation and aggregation of Aβ monomers into protofibrils and oligomers have been shown in studies *in vitro* and *in vivo* to be toxic to the neurons (Klein, 2013). The mechanisms by which Aβ exerts neuronal toxicity and the identification and dynamics of the different Aβ forms (for examples, protofibrils, soluble oligomers) that are toxic are among the most extensively studied subjects in AD research with the aim of finding a potential therapeutic targeting Aβ in mind (Benilova *et al*., 2012).

1.1.2   Current treatment modalities for AD

To date, there is no efficacious treatment available that allows the recovery and reversal of the inevitable degenerative process of AD (Simoes *et al*., 2014). Current drugs available for AD treatment in the clinic are mainly acetylcholinesterase

inhibitors (AChEIs) (Leon *et al.*, 2013) based on the cholinergic deficit hypothesis. This hypothesis arised from evidence that AD patients suffered from cognitive dysfunction because of the loss of cholinergic neurons and activity in certain parts of the brain. Thus, a drug that inhibits the hydrolysis of the cholinergic neurotransmitter acetylcholine by presynaptic acetylcholinesterase would augment cholinergic activity and relieve the symptoms of AD. Only a handful of AChEIs have been approved by the U.S. Food and Drug Administration (FDA) and launched in the market for the treatment of mild and moderate stages of AD. They are tacrine (1993), donepezil (1996), rivastigmine (2000) and galanthamine (2001) (Hong-Qi *et al.*, 2012). Tacrine was the first acetylcholinesterase inhibitor approved for the treatment of AD (Tumiatti *et al*, 2010). However, tacrine was eventually abandoned due to its limited therapeutic use because of its poor oral bioavailability and severe adverse effects such as hepatotoxicity and gastrointestinal reactions (Alfirevic *et al.*, 2007; Mehta *et al.*, 2012).

In 2003, another drug called memantine has been approved by FDA that acts as a non-competitive antagonist of glutamate receptors (Mehta *et al.*, 2012; Rodda and Carter, 2012). This drug marks a departure from the previous AChEIs in that it has a different mechanism of action; it binds to the *N*-methyl-*D*-aspartate (NMDA) receptor to block the excitotoxic effects of elevated glutamate levels that may lead to neuronal dysfunction (Rodda and Carter, 2012).
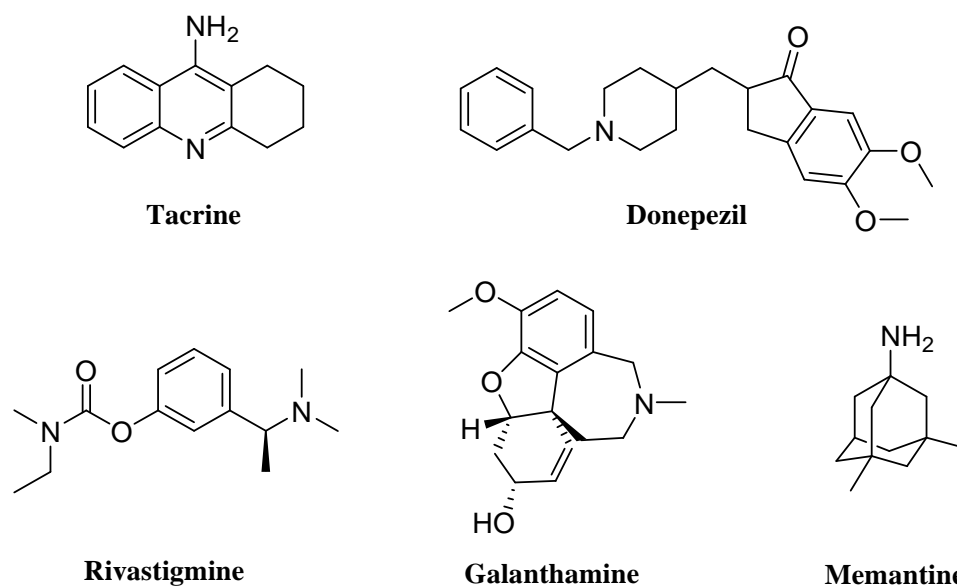
Figure 1.1: Chemical structures of clinically used drugs for the treatment of AD.

1.1.3 Emerging paradigm: Multitarget-directed ligand (MTDL)

The current drugs available for AD are considered as short-term treatments and appear to be palliative as they temporarily slow down the progressive loss of cognitive function and do not address AD's causative factors (Hong-Qi *et al*., 2012; van Marum, 2008). The molecular basis of AD can be considered as a complex network of protein targets with multiple pathological pathways inter-relating with each other (Minarini *et al*., 2012). Due to the multifactorial nature of AD and the diverse molecular and cellular mechanisms implicated in the disease, these "one-drug-one-target" drugs are only capable of symptomatic relief and are unable to prevent the neurodegenerative process hence the limited efficacy of the current clinical therapy for AD (Pisani *et al*., 2011). The lack of effective treatment of the disease in spite of the complexity of AD prompted many research efforts to search for potential disease-modifying agents that target the other factors associated with AD (Citron, 2010; Galimberti and Scarpini, 2011; Salomone *et al*., 2012). Examples of AD causative factors are amyloid-beta (Aβ) aggregation and generation of toxic

Aβ oligomers, the formation of tau protein fibrils within the neurons, neuroinflammation, excitotoxic insults, and oxidative stress (Figure 1.2). This has led to the idea of multitarget-directed ligand (MTDL) in medicinal chemistry, molecules designed to combine functionalities or moieties needed in one molecule to hit multiple targets simultaneously (Nepovimova *et al.*, 2014; Rosini *et al.*, 2005; Zhang, 2005). In principle, each of the functionalities combined in a hybrid molecule should retain their ability to interact with their corresponding targets and consequently, to produce pharmacological responses which as a whole modulate the neurodegenerative process (Cavalli *et al.*, 2008). Such an approach may hold the promise for treating a multifactorial disease such as AD.
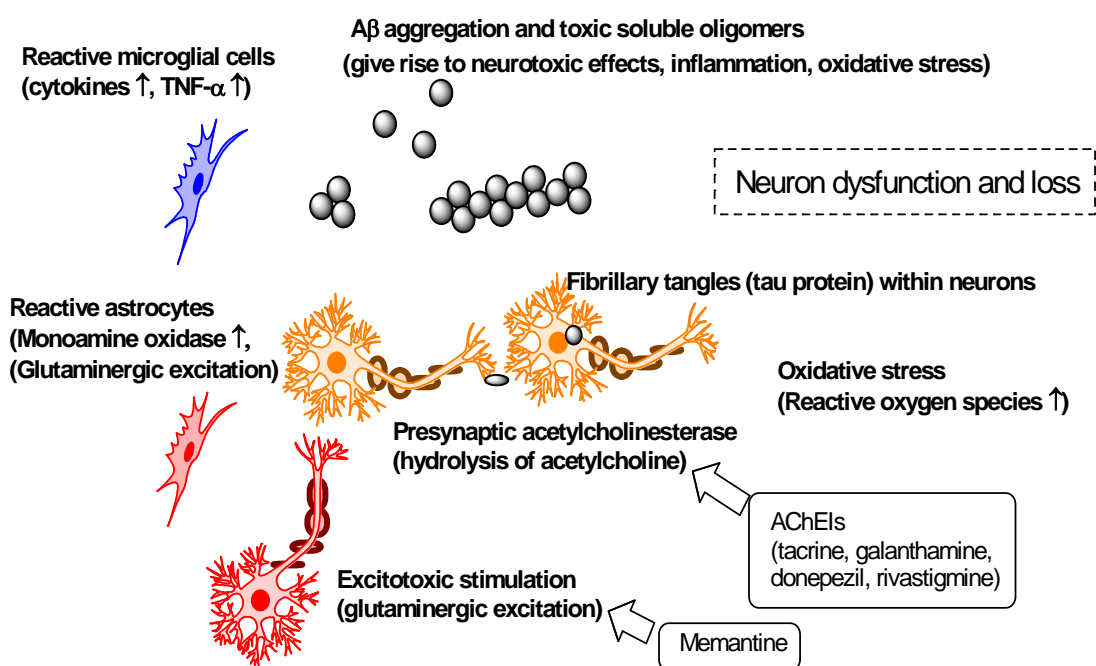
Figure 1.2: A simplified diagram showing the multiple factors implicated in the pathogenesis of AD and the present drugs available to address them.

Over the past decade, many potential multifunctional agents have been developed for AD treatment. The majority of these focused on the modifications of existing drugs with specific biological activities (Guzior *et al.*, 2015; Nepovimova *et*

al., 2014). One of the most widely adopted designs in this approach was a dual-binding AChEI in which modification was made on the existing AChEIs with additional biological properties such as Aβ anti-aggregating activity (Bajda *et al*., 2011; Giacobini, 2004; Pepeu and Giovannini, 2009). Previous studies showed that the peripheral anionic site (PAS) of the enzyme acetylcholinesterase (AChE) can induce the formation of Aβ fibrils (Bartolini *et al*., 2003; Inestrosa *et al*., 1996); therefore, dual-binding AChEI that is able to bind to both the catalytic and peripheral sites of the enzyme could simultaneously inhibit the hydrolysis of acetylcholine and block the Aβ-aggregating action of the enzyme (Dinamarca *et al*., 2010; Johnson and Moore, 2006). In an attempt to examine new dual-binding AChEIs, Tang and co-workers (2011) synthesized a series of novel compounds bearing tacrine and oxoisoaporphine moieties linked by an aminoalkyl tether (Figure 1.3). In this approach, tacrine acts as catalytic-site inhibitor and oxoisoaporphine as a PAS inhibitor of AChE. All the synthesized compounds were found to be AChEIs, with IC_{50} values in the nanomolar range (3.4 to 910 nM) and showed inhibitory activities (35.5 - 85.8 %) on self-induced Aβ aggregation at 10 μM (Tang *et al*., 2011).
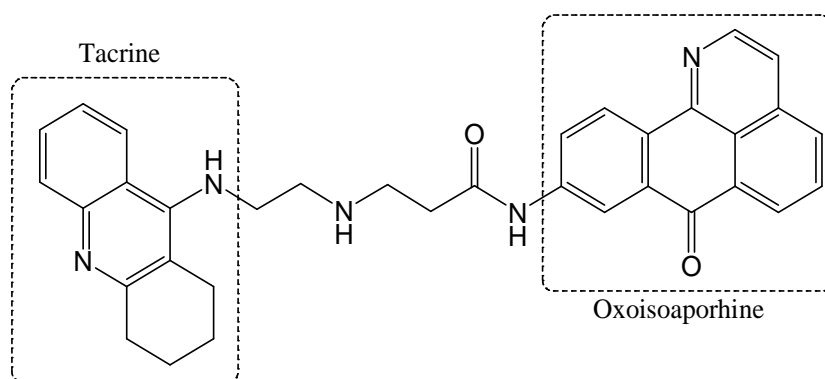
Figure 1.3: Structure of tacrine heterodimers with promising AChE and Aβ aggregation inhibitory activity (Tang *et al*., 2011).

Another successful approach of multifunctional agents based on dual-binding AChEI is the donepezil-tacrine hybrid compound. In 2010, a series of AChE and Aβ aggregation inhibitors was synthesized by Camps and co-workers by combining both donepezil and tacrine molecules, linked by a variety of spacer subunits (Figure 1.4). All compounds of this series were able to inhibit the human AChE and butyrylcholinesterase (BuChE) in the nanomolar range together with several compounds showing multipotencies by significantly inhibiting the AChE-induced Aβ aggregation at the concentration of 100 μM (Camps *et al*., 2010).
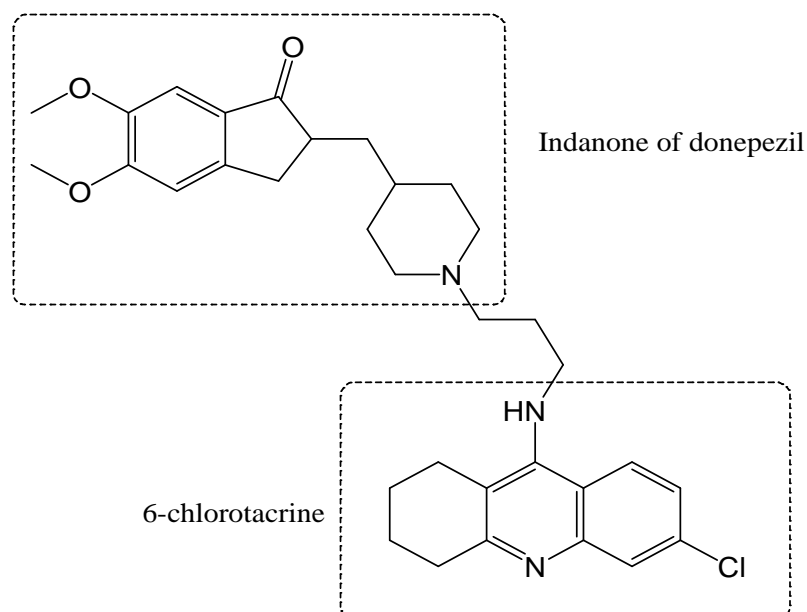
Figure 1.4: Structure of tacrine-donepezil hybrid compound with selective AChE and AChE-induced Aβ aggregation inhibitory activities (Camps *et al*., 2010).

In another example, Bolea and co-workers (2011) reported a series of donepezil-based hybrid compounds capable of interacting simultaneously with cholinesterases (ChE) and monoamine oxidase (MAO), two targets related to AD and other neurodegenerative diseases. The structural design was based on the combination of the benzylpiperidine moiety of donepezil and indolylpropargyl amine

subunit present in the structure of monoamine oxidase inhibitors (MAOIs) connected by methylene chains of various lengths. Among the compounds tested, a compound (Figure 1.5) was identified as a promising compound which showed selective inhibition on monoamine oxidase A (MAO-A) with an IC_{50} of 5.2 nM and non-selective inhibition towards the cholinesterase enzymes in submicromolar range. In conjunction, this compound was also shown to be able to inhibit Aβ self-induced aggregation as well as AChE-induced Aβ aggregation. These results suggested that this compound might be a promising multitargeting drug candidate to address the multifactorial nature of AD (Bolea *et al.*, 2011).
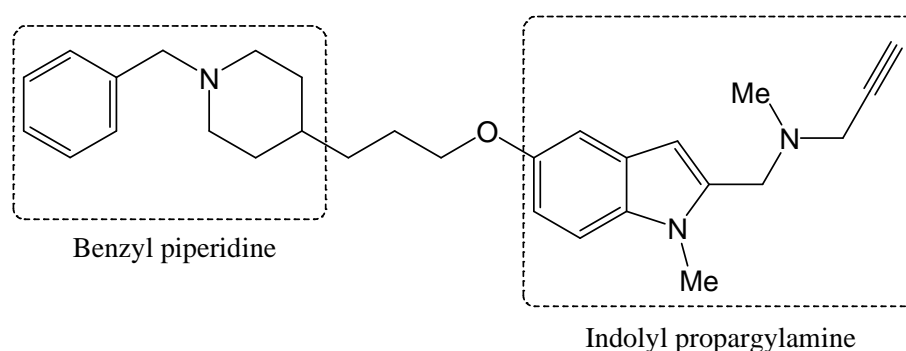
Figure 1.5: Structure of donepezil-propargylamine hybrid compound with dual ChE/MAO inhibitory activity (Bolea *et al.*, 2011).

Aside from this, more interesting multitarget-directed ligands (MTDLs) designed utilising this hybrid approach include the cholinesterase inhibitors with neuroprotective and antioxidant activity (Fernandez-Bachiller *et al.*, 2009; Samadi *et al.*, 2011), monoamine oxidase B (MAO-B) inhibitors with metal-chelating property (Fernandez-Bachiller *et al.* 2010), and metal chelators with antioxidant activity (Avramovich-Tirosh *et al.*, 2007; Zheng *et al.*, 2009). Most of these ligands have been shown to display promising biological activity *in vitro* and proved to be superior to those of one-target specific drugs. Interestingly, several studies also

suggested that MTDLs may give rise to neuroprotective effect (Bajda *et al*., 2011; Bolea *et al*., 2013; Minarini *et al*., 2012). Such a desirable effect has been attributed to the combination of two or more functionalities in the hybrid molecules enabling them to intervene concertedly on several biological pathways.

1.1.4   The importance of drug-like properties

Drug discovery is a complex and demanding process, involving many disciplines and investigations into various aspects, often with complications on the road to realize a successful drug. In the earlier history of drug discovery, the trend in industry practice was to optimize the biological target activity or potency of the drug candidate first. Little attention was given to the bioavailability and pharmacokinetic properties of the potent compounds. These aspects were only addressed in the later stages of development. This was one of the reasons many compounds with high potencies failed in clinical trials due to poor pharmacokinetic profiles and bioavailability (Lipinski, 2000). In the case of drugs developed to treat CNS diseases such as AD, of the many potential compounds in development, only 2 % are able to enter the brain in sufficient concentrations to produce the desired therapeutic effect (Pardridge, 2001). These clinical failures marked a tremendous loss in terms of money, research effort, and time.

In recent years, considerable attention has been placed on assessing drug-like properties during the early phase of discovery. These drug-like properties include absorption, distribution, metabolism, elimination and toxicity (ADMET) in addition to the other physicochemical aspects to ensure the pharmacokinetic feasibility of compounds brought forth during development (Di and Kerns., 2003; Liu *et al*.,

2004). The term "drug-like" is a qualitative concept used in drug design to describe certain intrinsic properties (structural, physicochemical, biochemical and pharmacokinetic) of a compound that would contribute to its bioavailability (availability in the system) and its availability in the CNS for drugs aimed for treating CNS diseases (Kerns and Di, 2008; Li, 2005). Looking for and optimizing drug-like features in the molecules synthesized during the early part of discovery would be beneficial in ensuring quality, pharmacokinetically sound (and not any) compounds are being studied throughout the various stages of development.

Many high-throughput *in vitro* assays have been developed over the past decade for evaluating drug-like properties in order to avoid unnecessary complexities associated with animal models and to minimize the time and cost needed to screen large numbers of target compounds (Di *et al.*, 2004; Liu *et al.*, 2004). These assays measure the fundamental physicochemical and biochemical properties which determine the higher level properties such as the pharmacokinetics of a drug (Li, 2004). They include blood-brain barrier permeability assays, gastrointestinal permeability assays, plasma stability assay, solubility assay, and liver microsomal stability assay that are able to sort out candidates with acceptable drug-like properties in the earlier drug design phase with fewer resources and in a shorter amount of time (Di *et al.*, 2003; Kerns and Di, 2008; Li, 2005). Moreover, these methods also showed good correlation with *in vivo* findings and could reflect the *in vivo* pharmacokinetic condition (Li, 2005). Table 1.1 lists several commonly assessed drug-like properties and the *in vitro* assays used to evaluate them.

Table 1.1: Selected drug-like properties and their *in vitro* methods of evaluation (Di and Kerns, 2003).

Drug-like Properties	Methods/Assays
Oral/gastrointestinal permeability	Parallel Artificial Membrane Permeability Assay (PAMPA), Caco-2 cell monolayer, immobilized artificial membrane high performance liquid chromatography (HPLC), everted gut sac.
Lipophilicity	Shake flask method, reversed phase HPLC, capillary electrophoresis.
Blood-brain barrier permeability	PAMPA-BBB, cell-based method.
Metabolic stability	Liver microsomes, S9 fraction, cytosol, hepatocytes.
Toxicity	Cell toxicity, hERG block assays, zebrafish.
CYP450 inhibition and induction	Liver microsomes, hepatocytes.

In order to design a compound with drug-like properties, the Lipinski's rule of five or simply Rule of five (RO5) plays a guiding role, whereby it states that a drug-like molecule in general should have less than 5 hydrogen bond donors and 10 hydrogen bond acceptors, a molecular mass less than 500 Da, and an octanol-water partition coefficient (log P) value less than 5 (Lipinski, 1997). Early evaluation of a chemical structure as suggested by the RO5 would allow one to select the possible drug candidates that would be more drug-like for further pharmacological testing (Lipinski, 2004). This rule of thumb together with the assays for evaluating drug-like properties are used to drive the design, synthesis, selection, and optimization of compounds in conjunction with the main biological activity evaluation.

1.2 Overview of aurones and their AD-related activities

Aurones (2-benzylidenebenzofuran-3-(2H)-ones) are small molecules belonging to the flavonoid family that are naturally present as bright yellow pigments in fruits and flowers of certain terrestrial and marine plants (Harborne *et al*., 1988). Naturally occurring aurones derivatives are often found as their hydroxylated, methoxylated or glycosylated forms (Beney *et al*., 2001). Although aurones are structurally related to the other subclasses of flavonoids (flavones, isoflavones and chalcones), aurones hold a unique place in nature due to the remarkably low quantities present in plants (Haudecoeur and Boumendjel, 2012).
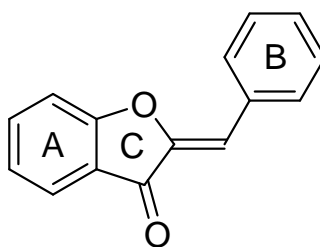
Figure 1.6: The aurone scaffold.

Ever since the first review by Boumendjel (2003), aurones have gained considerable attention as a "privileged structure" due to its promising utility in many medicinal chemistry projects (Boumendjel, 2003; Haudecour and Boumendjel, 2012; Zwergel *et al*., 2012). Aurones have been found to possess antiproliferative effect in cancer cells (Lawrence *et al*., 2003), anti-tyrosinase activity (Okombi *et al*., 2006), anti-viral activity (Liu *et al*., 2008), anti-microbial activity (Bandgar and Patil, 2010), antimalarial activity (Souard *et al*, 2010; Carrasco *et al*., 2014), and are potential chemopreventive agents via the induction of the cytoprotective NADP(H) Quinone Oxidoreductase 1 (NQO1) (Lee *et al*., 2010). More recent studies revealed that aurones possess anti-inflammatory activity by reducing the production of nitric oxide

and prostaglandin E_2 (Shin *et al*., 2011), and were potent inhibitors of the breast cancer resistance protein ABCG2 (Sim *et al*., 2011) and Hepatitis C Virus RNA Polymerase (Haudecoeur *et al*., 2012; Meguellati *et al*., 2014).

Sheng and co-workers (2009) were the first to report AD related activities of synthetic aminomethylaurones through AChE inhibition. Most of the synthesized aurones demonstrated a high inhibition towards AChE with IC_{50} in micromolar range (Sheng *et al*., 2009). The authors also proposed that the π-π stacking interaction of an aurone planar ring system could be a reason for the enhanced AChE inhibitory activity of the aurones (Sheng *et al*., 2009). In another investigation, a series of 6-alkoxy aurone derivatives all possessing pyridinium as the nitrogen-bearing motif showed moderate to high AChE inhibition activities (Nadri *et al*., 2010). However, these aurones were focused mainly on the cholinesterase inhibitory activity and were not designed for multitargeting purposes. In addition, aurones have also been reported to exhibit high binding affinity for Aβ aggregates *in vitro* (Maya *et al*., 2009) and more recently showing MAO inhibitory activity (Morales-Camilo *et al*., 2015). These reported activities provide interesting leads to exploit the scaffold in the development of novel multitargeting anti-Alzheimer's agents.

Sheng et al. (2009)   Nadri et al. (2010)

Figure 1.7: Aurones with reported acetylcholinesterase inhibitory activity.

1.3 Problem statement and hypotheses

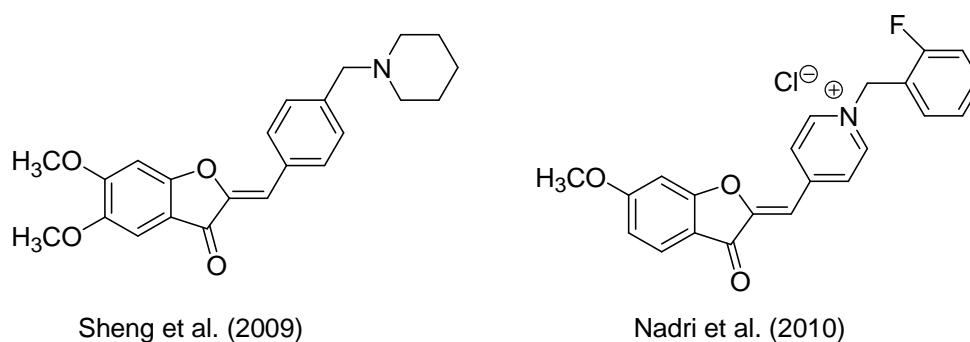The purpose of this thesis is to investigate the potential of aurones as neuroprotective agents via a multi-target directed ligand design while placing equal emphasis to maintain a balanced drug-like profile particularly their metabolic stability and ability to permeate the blood-brain barrier. Numerous MTDLs have been developed and were shown to be have potent multi-faceted activities (Bajda *et al*., 2011; Guzior *et al*., 2015; Pisani *et al*., 2011). However, despite having high potencies and strong binding affinities for their target proteins (many in the nanomolar range), these ligands were generally bulky, had high molecular weights (> 500 Da), and were tethered hybrids of two drug molecules as can be observed from several MTDL examples cited in Section 1.1.3. Such structural designs cast doubts about their drug-like properties. Large molecular weights in these compounds could hinder their ability to permeate the blood-brain barrier which is necessary for a CNS-active compound while high lipophilicity which came with their large size may attract high liver metabolism and clearance particularly by the phase 1 cytochrome P450 (CYP450) enzymes. It can be argued that in the design of these hybrid MTDLs, drug-like considerations (which are necessary for the development of CNS drugs) have been overlooked in achieving high target protein affinities and multitarget potencies.

To develop a novel multipotent anti-Alzheimer drug that retains good drug-like properties (metabolic stability and blood-brain barrier permeability), the aurone platform was utilised in the present investigation. The aurone scaffold with a tenable molecular weight of 222.24 Da and a topological polar surface area (26.3 Å^2) would provide a suitable foundation for which to introduce additional key functionalities

15

while maintaining reasonable molecular weight and polar surface area in compliance with the RO5 (Lipinski, 1997; 2000). Over the past decade, the aurone scaffold has gained importance as a "privileged structure" due to its prevalence in many pharmacologically active compounds (Boumendjel, 2003; Zwergel *et al.*, 2012). It is proposed that by incorporating selected structural motifs (various amines and carbamate) from two established Alzheimer drugs (rivastigmine and donepezil) into the scaffold, a novel multitargeting AChEI that has additional mechanism(s) of action may be uncovered. It would also seem likely that the proposed multitargeting aurones may exhibit neuroprotective effect, as has been observed in studies on other MTDLs (Bajda *et al.*, 2011; Bolea *et al.*, 2013; Minarini *et al.*, 2012). A promising aurone obtained from this study would provide the tool to investigate this premise.
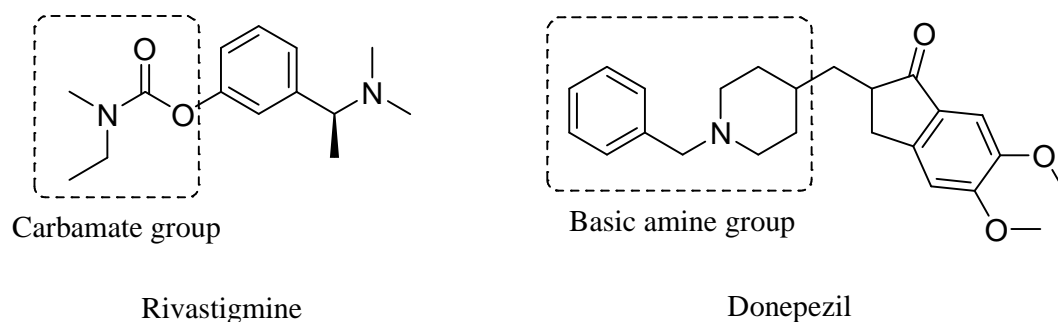

Figure 1.8: Selected structural motifs (basic amine and carbamate) from donepezil and rivastigmine used in the present study.

In addition to examining the pharmacological potential of the aurones, it is also important to gain accurate data on their drug-like properties as such information is needed to identify an optimal compound with a combination of good activity/potency and favourable pharmokinetic profile (metabolic stability, blood-brain barrier permeability). Little is known about the drug-like properties of aurones, particularly their stability in the face of phase 1 CYP450 liver clearance and their

ability to permeate the blood-brain barrier, important features in developing a CNS-active compound. It is hypothesized that the close resemblance between the aurone's benzofuranone and the pharmacokinetically optimized donepezil's indanone might give rise to favourable drug-like properties in the target aurones in addition to their "streamlined" (small molecular weights and polar surface area) design. Furthermore, the proposed introduction of amines and carbamate moieties into the scaffold may influence the physicochemical properties of the scaffold and hence their drug-like potential.

1.4   Objectives of study

To verify the aforementioned hypotheses, the specific objectives of the study are as follow:

(i)   To design and synthesize a series of aurone derivatives incorporating the selected structural motifs from the known AChEIs donepezil and rivastigmine.

(ii) To elucidate the structure-activity relationship of the aurones with respect to their cholinesterase inhibitory activity as well as their mode of binding.

(iii) To investigate the multitargeting potential of the aurones in two AD-related targets (monoamine oxidase and Aβ aggregation) and their respective binding mechanisms.

(iv) To evaluate the drug-like properties of selected aurones using *in vitro* pharmacokinetic assays (metabolic stability, blood-brain barrier permeability).

(v)   To investigate the neuroprotective effects of the most promising aurone in two *Caenorhabditis elegans* (*C.elegans*) neurodegeneration models.

CHAPTER 2

DESIGN AND SYNTHESIS OF TARGET COMPOUNDS

2.1   Introduction

This chapter describes the design and synthesis of target compounds for the evaluation of potential neuroprotective properties based on their anti-cholinesterase activity. These compounds possess the aurone scaffold with modifications at rings A and B with various basic amines and carbamate functionalities. The rationale underlying the design, chemical considerations of their synthesis and the experimental methods are presented. The structures of the synthesized compounds were identified by proton-1 (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectroscopy, and their mass spectra. Spectroscopic data, melting points, yields and purities of each compound are listed in Appendix 1.

2.2   Rationale of target compound design

The aurone scaffold was chosen in this investigation owing to the close resemblance of its benzofuranone core with the indanone of the clinically used Alzheimer drug donepezil (Figure 2.1). Donepezil is a well-tolerated, orally bioavailable acetylcholinesterase inhibitor for the treatment of AD (Sugimoto *et al*., 2002; Wilkinson, 1999). It serves as a lead for further modifications on the aurone scaffold to uncover novel compounds with a similar pharmacokinetic profile. Furthermore, studies on a series of pyridinium aurone derivatives synthesized as acetylcholinesterase inhibitors revealed that some of these compounds exhibited high anti-cholinesterase activity (Nadri *et al*., 2013; Nadri *et al*., 2010). In addition,

aurones have been developed as probes for imaging Aβ plaques using single photon emission computed tomography (SPECT) that showed high affinity for Aβ aggregates (Ono *et al*., 2007) suggesting a potential multi-targeting property in the scaffold.
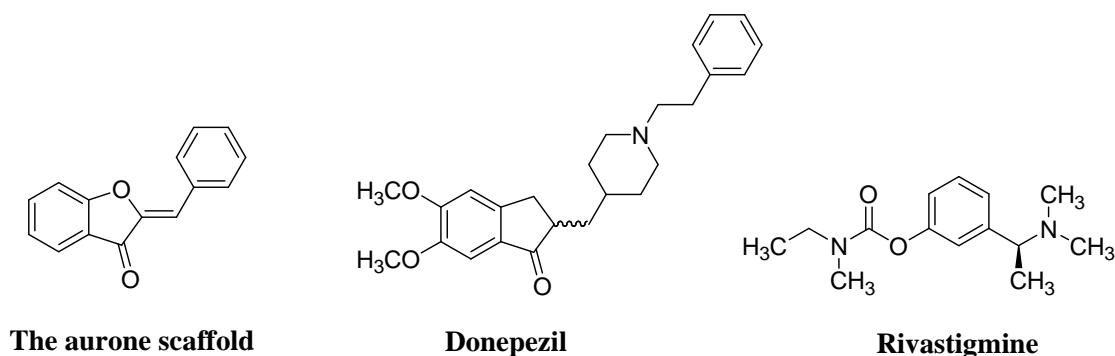

The aurone scaffold　　　　**Donepezil**　　　　**Rivastigmine**

Figure 2.1: The aurone scaffold and the structures of AChEIs donepezil and rivastigmine.

In this study, a total of forty-one target compounds were synthesized and grouped into six series based on the modifications at rings A and B of the aurone scaffold. Series 1 and 2 consist of 6-hydroxyaurones and 6-methoxyaurones respectively with different substituents at the 4'-position of the ring B (Table 2.1 and 2.2). They were introduced because such oxygenated aurones have been reported to exhibit multiple bioactivities including anti-inflammatory properties (Shin *et al*., 2011), a desirable feature in developing a multi-targeting agent. Moreover, hydroxyl and methoxyl groups would provide a good comparison between the two series with regard to their lipophilicities and hydrogen bond donor and acceptor properties. Series 3 comprises of aurones with "reversed" functionalized equivalents of Series 2 in which the various substituents are placed at the 6-position of ring A while the methoxyl group is at the 4'-position of ring B (Table 2.3). They provide suitable comparisons with Series 2 and Series 5 by which to explore the placement of the

amine or carbamate moieties at the other end of the scaffold. Series 4 aurones are akin to Series 3 with the functionalization at ring A but with a 3', 4', 5'-trimethoxy motif at ring B (Table 2.4). This modification was inspired by a report on a series of multipotent AChEIs in which the use of this motif gave rise to potent inhibitors (Belluti *et al.*, 2005). Series 5 are 6-diethylcarbamoylaurones with the amine functionalities at ring B to examine the effect of having the carbamate moiety at ring A (Table 2.5). Series 6 aurones are chlorinated equivalents of Series 2 where one chlorine is placed at positions *ortho* and *meta* to the amine functionality at ring B (Table 2.6). This series serves to assess whether the placement of an electron-withdrawing group (such as chlorine) near a potentially labile site can increase the metabolic stability of the compounds.

As for the type of substituents, they were limited to tertiary amines such as dimethylamino-, diethylamino-, pyrrolidine, and piperidine. The rationale underlying this choice was because most centrally active AChEIs such as donepezil, galanthamine and recently synthesized derivatives (Pan *et al.*, 2014; Yan *et al.*, 2012) hold basic, ionisable nitrogen-containing moieties which contribute to their activities and pharmacokinetic properties. Carbamate functionality (diethylcarbamate) borrowed from the known AChEI rivastigmine (Figure 2.1) and xanthostigmine derivatives (Rampa *et al.*, 2001) is another group introduced into the design of the present aurones. The incorporation of the aforementioned amines and carbamate to the aurone scaffold is expected to influence the anti-cholinesterase activity of the target compounds.

Table 2.1: Structures of compounds in Series 1
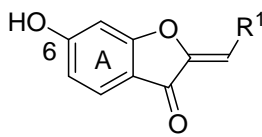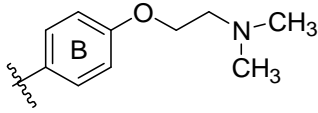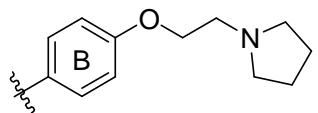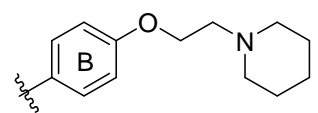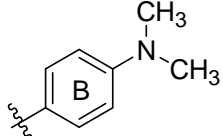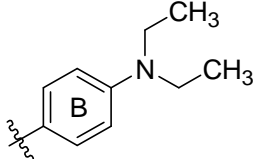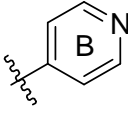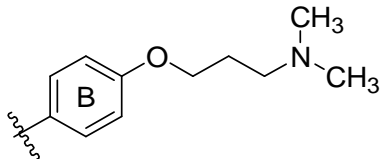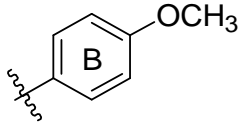
Compound	R^1	% Yield
Series 1		
1-1		63
1-2		64
1-3		59
1-4		99
1-5		81
1-6		53
1-7		84
1-8		68

Table 2.2: Structures of compounds in Series 2
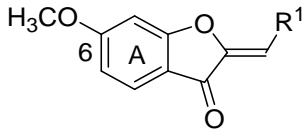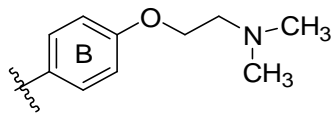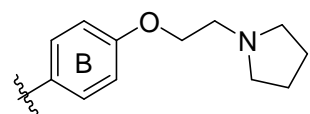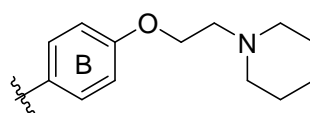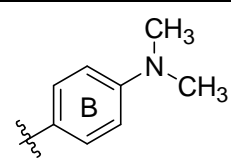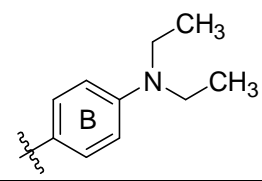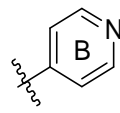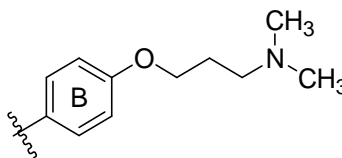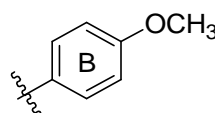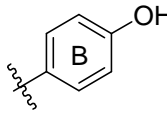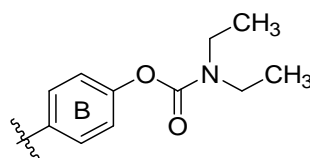
Compound	R^1	% Yield
Series 2		
2-1		53
2-2		26
2-3		19
2-4		42
2-5		47
2-6		38
2-7		33
2-8		36
2-9		98
2-11		20

Table 2.3: Structures of compounds in Series 3
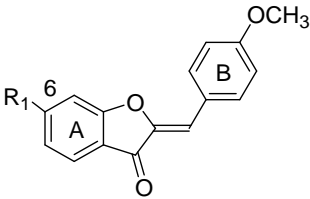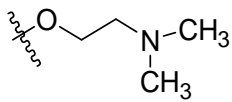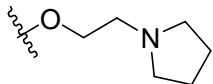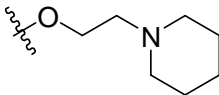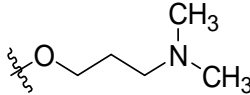
Compound	R^1	% Yield
Series 3		
3-1		46
3-2		40
3-3		46
3-7		35

Table 2.4: Structures of compounds in Series 4
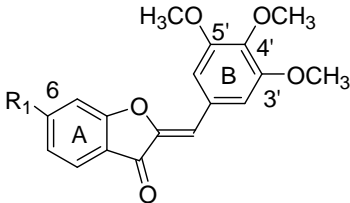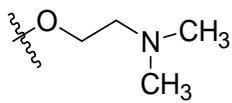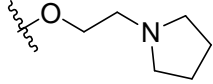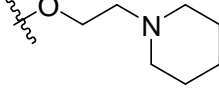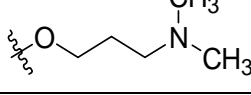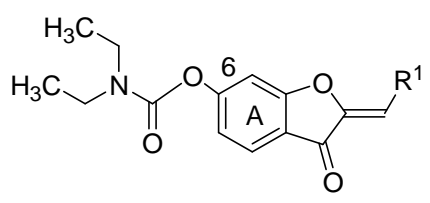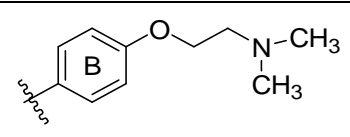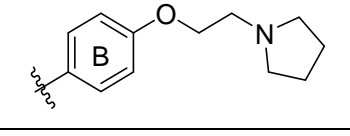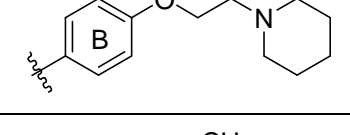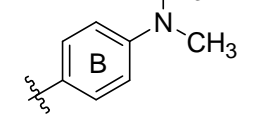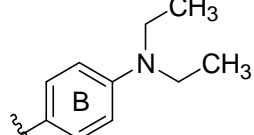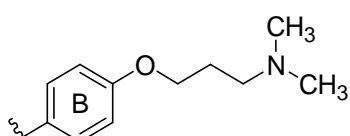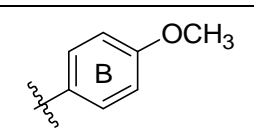
Compound	R^1	% Yield
Series 4		
4-1		42
4-2		59
4-3		70
4-7		39

Table 2.5: Structures of compounds in Series 5

Compound	R^1	% Yield
Series 5		
5-1		58
5-2		68
5-3		47
5-4		76
5-5		75
5-7		55
5-8		63