Bioanalytical Approach to the Interaction of Novel sym-Triazine Derivatives with Alzheimer’s disease biomarkers

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

Alzheimer’s Disease (AD) is a complex neurological disorder affecting an increasing number of people worldwide. The amyloid hypothesis is the most widely accepted theory for the cause of AD and states that the main origin of the disease stems from the mis-folding and accumulation of the amyloid-beta peptide (Aβ). There is currently no cure for the disease, however, drugs are available to mitigate the symptoms. Multi-target directed ligands (MTDLs) have been implemented as a novel drug design strategy for treatment of AD. sym-Triazine derivatives have been previously reported as effective inhibitors of Aβ aggregation and acetylcholinesterase (AChE). Two novel sym-triazine derivatives (AM-1 and AM-2) have been synthesized, that improve on previous design and were tested for their inhibition through various analytical techniques. The results indicate that the novel compounds were more effective in Aβ aggregation but not as potent in their AChE inhibition in comparison to previous derivatives.
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<tr>
<td>αS</td>
<td>α-synuclein</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-beta</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor proteins</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AChEI</td>
<td>Acetylcholinesterase Inhibitor</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyl transferase</td>
</tr>
<tr>
<td>CC</td>
<td>Cyanuric chloride</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LA</td>
<td>Lactic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectra</td>
</tr>
<tr>
<td>MTDL</td>
<td>Multi-target-directed ligand</td>
</tr>
<tr>
<td>NFTs</td>
<td>Neurofibrillar tangles</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D aspartate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAS</td>
<td>Peripheral anionic site</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin-T</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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Chapter 1
Alzheimer’s Disease

1 Introduction

Alzheimer’s Disease (AD) is an irreversible, progressive neurodegenerative disorder that is having a significant effect on the world’s aging population, affecting 5.1 million people in the United States alone in 2001. The number is expected to increase to 42 million people worldwide by 2020 with the absence of an effective treatment and with the aging population.

This complex disorder is one of many diseases that is associated with aberrant folding of normally soluble proteins into insoluble deposits that accumulate in the extracellular space in various tissues. These insoluble aggregates tend to adopt similar properties that are independent of their sequence suggesting that aggregation into β-rich sheets is a generic property of polypeptides.

Common properties amongst these mis-folded peptides include spontaneous self-aggregation to form morphologically similar β-sheet rich structures termed amyloids. X-ray fiber diffraction also revealed that a common structural motif for all amyloid peptides are cross β-sheets oriented perpendicular to the long axis of the fibril. While other neurological disorders such as Parkinson’s and Creutzfeldt-Jakob disease may be accredited to the mis-folding of α-synuclein (αS) and prion protein respectively, AD is histopathologically characterized by the overproduction and accumulation of amyloid-beta (Aβ) peptides in the hippocampus and cerebral cortex of the brain.

AD is the most prevalent form of age-related dementia characterized by considerable memory loss, changes in perception and emotional behaviour, decline in cognitive abilities and these symptoms only worsen as time progresses. Downstream effects of AD in the brain may also include oxidative stress through formation of reactive oxygen species (ROS), unregulated Ca2+ overproduction in the brain and activation of microglia.

Although medication is available for the treatment of AD to delay or impede symptoms, no cure is currently available. Treatments are insufficient to stop the disease completely mainly because research into the actual mechanism of disease onset and progression are still underway.
Understanding the kinetics of Aβ fibril formation in AD is critical in finding novel compounds to inhibit this process, and ultimately this disease.

1.1 Amyloid-beta (Aβ) Peptide

While there are two distinctive hallmarks for Alzheimer’s disease, the mis-folding and aggregation of Aβ is believed to be the primary suspect for the onset of the disease as opposed to hyperphosphorylation of the tau protein leading to the accumulation of neurofibrillary tangles (NFTs). Aβ spontaneously aggregates to form mature fibrils (Figure 1.1). In many cases, NFTs are actually viewed as a downstream symptom of Aβ. This can be attributed largely to the fact that inherited mutations in the amyloid protein can lead to early manifestation of AD while inherited mutations in the tau protein however, does not lead to AD but rather a less common but equally destructive form of the disease. In addition, studies on Aβ aggregation have been shown to cause phosphorylation of tau leading to its associated downstream symptoms with neurotoxicity. Genetic mutations associated with the precursor protein to Aβ have also been linked to early onset of the disease and production of Aβ.

Figure 1.1: Schematic of amyloid-beta (Aβ) peptide fibrillogenesis in AD. Aβ monomers spontaneously self-aggregate to form oligomers, which in turn aggregate into protofibrils, and finally into mature fibrils. Fibrils deposit in extracellular tissues as insoluble senile plaques. Recent studies have indicated that the toxic species in the sequence of Aβ fibrillogenesis are the soluble oligomers and together with fibrils, cause downstream symptoms in the brain that eventually lead to neuronal death.
Aβ is composed of 39-43 amino acid residues and is the major constituent of insoluble plaques that accumulate in the extracellular space of the AD brain. Electron microscopy (EM) studies have revealed the structure of amyloid fibrils to be rigid and unbranched with a diameter between 5-13 µm. X-ray diffraction has displayed a cross β-sheet structure within the fibrils where the β-strands are oriented perpendicular to the long axis of the fibril. Progressive accumulation of the peptide into plaques, have been associated with altered axons and dendrites, activated microglia, and mitochondrial dysfunction.

1.1.1 Cleavage from APP

Amyloid-beta peptides (Aβ) are ~4 kDa peptides resulting from specific proteolysis of a larger precursor protein known as amyloid precursor protein (APP). This transmembrane protein may be cleaved by a variety of enzymes, but most notable are α-, β-, and γ-secretase. Cleaving the peptide by α-secretase generates a non-toxic soluble molecule termed sAPPα through the non-amyloidogenic pathway. However, when β-site APP cleaving enzyme, or BACE1, performs the initial cleavage of APP at the N-terminal, it generates two products; a small fragment from the N-terminal termed sAPPβ that gets released into the extracellular compartment, and the C-terminal transmembrane protein termed C99. C99 then becomes cleaved by γ-secretase which produces either a 40- or 42- amino acid long peptide, termed Aβ40 and Aβ42. Production of these two isoforms is termed the amyloidogenic pathway due to the capability of the peptides to spontaneously form amyloid fibrils.
Figure 1.2: Formation of Aβ monomer is through sequential cleavage of the amyloid precursor protein (APP). APP may be cleaved by a variety of enzymes, producing different peptides of varying lengths and toxicity. Initial cleavage by α-secretase results in sAPPα and a non-toxic soluble molecule through the non-amyloidogenic pathway. The amyloidogenic pathway occurs through β-secretase cleavage followed by γ-secretase produces the 40- and 42- amino acid residue long peptide, termed Aβ\textsubscript{40} and Aβ\textsubscript{42} respectively, that is the crucial in AD.\textsuperscript{24, 32}

1.1.2  Aβ\textsubscript{40} and Aβ\textsubscript{42}

Aβ that is mainly found in the neuronal deposits is composed of 40 or 42 amino acid long peptide, termed Aβ\textsubscript{40} and Aβ\textsubscript{42}.\textsuperscript{7, 33} They are both amphipathic with the C-terminal being hydrophobic and N-terminal hydrophilic. Aβ\textsubscript{42} constitutes only about 10% of secreted Aβ compared to Aβ\textsubscript{40} but is the major component found in neuritic plaques in the brain.\textsuperscript{34, 35} In view of the amino acid sequence, Aβ\textsubscript{42} contains an additional 2 hydrophobic amino acids at its carboxy terminus compared
to Aβ40 that allows it to aggregate at a much faster rate; thereby making it the more toxic form.\(^{22}\) Increased ratio between Aβ42/Aβ40 leads to copious amounts of Aβ plaques being produced and accelerates Aβ deposition.\(^{37, 38}\) In addition to the C-terminal of the peptide being of influence to the aggregation rate, residues 16-23 contains a hydrophobic core that plays an important role in initiating the aggregation process of the peptide and its ability to polymerize.\(^{39}\)

**Figure 1.3:** (a) Amino acid sequence of monomeric Aβ\(_{40/42}\) peptide. Aβ42 contains two additional hydrophobic amino acids (in red) than Aβ40 that allow it to aggregate at a much faster rate and thereby deeming it the more toxic form. (b) Structure of the monomeric form of Aβ\(_{40/42}\) in a mature fibril. In the Aβ40 strand, residues 1-10 are unstructured (grey) and 11-40 form a β-turn-β fold formation. Side-chain packing (blue) is observed between residues His13 and Val40, between Gln15 and Val36 and Phe19 with Val36, Leu34 and Ile32. In Aβ42, 1-17 may be unstructured and 18-42 form the β-turn-β fold structure. Molecular contacts may be present between Phe19 and Gly38 (red) and also may occur between Met35 and Ala42 (orange). In both forms, the β-turn-β fold formation is stabilized by a salt bridge present between Asp23 and Lys28 (black) and through hydrophobic interactions between certain residues (green) (adapted from Ahmed *et al.*).\(^{40}\)
1.1.3 Amyloid Cascade Hypothesis

The amyloid cascade hypothesis is the most widely accepted theory for the underlying cause of AD. It suggests that the mis-folding of Aβ initiates a cascade of pathogenic events that ultimately lead to synapse malfunction and neuronal cell death.\textsuperscript{41-44} This hypothesis is largely influential not only in academic research but also in the pharmaceutical industry. Aβ fibrillogenesis occurs through a nucleation-dependent aggregation mechanism.\textsuperscript{45, 46} A nucleus or seed is formed after a “lag phase” after which fibril growth is accelerated and becomes rapid. The protein has to attain a certain concentration before fibrillization can occur making the nucleation phase of the process the limiting step.\textsuperscript{47} Aβ spontaneously self-assembles to form soluble oligomeric aggregates. These aggregates gradually become larger until they form protofibrils. Protofibrils elongate through the accumulation of monomers until eventually forming the mature fibril that is found in abundance in neuronal deposits.\textsuperscript{48-50} The aggregation process is speculated to be driven by β-sheet formation due to the spontaneous reaction of the peptides converting from random coil structure to β-sheet rich conformation.\textsuperscript{51} A molecular dynamic simulation study supported this by showing that by stabilizing the β-sheet prone state of the peptide through hydrogen bonds, amyloid fibrillization was promoted with the presence of oligomeric intermediates.\textsuperscript{52}
Figure 1.4: Schematic of the nucleation-dependent process of Aβ fibrillogenesis occurring through a “lag-phase” mechanism depicted as a sigmoidal curve. During the lag phase, monomeric Aβ self-aggregate, assembling into dimers, trimers and oligomers, forming a “seed” or “nucleus” onto which additional assemblies may join to form larger oligomers. Through a fast elongation phase, the nucleus is further elongated through assembly of monomers and oligomers until a mature fibril is formed. Mature fibrils contain highly ordered β-sheets and form insoluble senile plaques that are present in large amounts in the AD brain.

For a time, fibrils were thought to be the causal species associated with neurodegeneration. Recently however, there is strong evidence that proposes that the β-sheet rich soluble oligomeric intermediates, formed prior to the mature fibrils, are the cause of cytotoxicity in the brain. These soluble oligomers displayed neurotoxicity both in vitro and in vivo. When soluble oligomers were implemented with brain slices, disruption of hippocampal long-term potentiation (LTP) was observed. LTP is important for signal transmission between neurons and is a key aspect in memory mechanisms. These toxic oligomers have also been linked to synapse degeneration and memory loss. Gong et al. have proposed that the oligomers have high affinity and specificity for cell-surface attachment to some membrane proteins, confirming the fact that AD is a synapse disease. All these studies suggest that the soluble intermediates are part of, in addition to the insoluble plaques, the main culprits for neurodegeneration in AD. Both isoforms of Aβ can self-aggregate forming intermediate assemblies that subsequently lead to fibril formation and understanding this mechanism of oligomerization may lead to the discovery of potential inhibitors.

1.2 Other pathologies of AD

Other pathologies exist in addition to the toxic neuritic plaques found in the AD brain. Aβ is seen as the major causal pathway towards the disease but what makes AD such as heterogeneous and complex disease to treat is the involvement of several different pathologies. These diverse pathologies are what give AD its multifactorial nature allowing multiple symptoms and pathways to manifest in one very complicated disease to cure.
1.2.1 Neurofibrillary Tangles (NFTs)

Neurofibrillary tangles (NFTs) are another important identifying hallmark of AD and is composed of twisted fibers of a protein named tau.\textsuperscript{63} Tau is natively found to be associated with microtubules and is responsible for its stabilization. The state of protein phosphorylation is important for the modulation of neurons and synapse and mitochondrial function.\textsuperscript{64, 65} When the protein becomes hyperphosphorylated, it dissociates and aggregates into NFTs and plaques causing a downstream cascade that ultimately leads to neurotoxicity in the brain.\textsuperscript{52} Efforts have been made to mediate this by studying molecules that can inhibit the kinases responsible for phosphorylation of tau\textsuperscript{66, 67}, or similarly by activating the dephosphorylation of tau\textsuperscript{68, 69}, by inhibiting tau aggregation,\textsuperscript{70} or by stabilizing the microtubule.\textsuperscript{71} Many of these approaches encounter problems associated with specificity and selectivity which can lead to limited efficacy and unwanted side effects with other targets. Some have reached clinical trials but most have not yet undergone extensive evaluations.\textsuperscript{1} Research is still being conducted for tau modulation and inhibition.

**Figure 1.5:** Schematic depiction of the formation of neurofibrillary tangles (NFTs) from tau protein. Tau is natively found to be responsible for stabilization of microtubules. Hyperphosphorylation of the protein causes it to dissociate from the microtubule and aggregation leads to assemblies that deposit as NFTs.
1.2.2 Oxidative Stress

Oxidative stress plays a major role in neurodegeneration that results in cell death in AD.\textsuperscript{72} Increased concentrations of reactive oxygen species (ROS) are a common result and major species include superoxide anion (O$_2^-$), which are prone to convert to hydrogen peroxide (H$_2$O$_2$).\textsuperscript{73} These ROS are formed normally under regular metabolic conditions in low concentrations, however, in AD, the balance in the production and clearance of these ROS is disrupted leading to abnormal elevated levels. Aβ has the potential to enter mitochondria and in disrupting their regular function, increase the production of ROS and therefore induce oxidative stress.\textsuperscript{74, 75}

Studies have found that Aβ upsets regular function by blocking protein transport and the electron transport chain which can in turn create free radicals.\textsuperscript{76} ROS are highly reactive and will lead to lipid, protein, DNA, and RNA peroxidation depending on which substrate it comes into contact to.\textsuperscript{77} Products of these will result in membrane impairment, reduced protein synthesis, and loss of functions of certain activities within the neuron that can ultimately lead to neuronal dysfunction.\textsuperscript{78} Mitochondrial dysfunction, and in parallel oxidative stress, occurs as an early onset in AD and contributes a large part to many of the downstream symptoms associated with the disease. In order to address this pathology, antioxidants have been and still being researched extensively as possible therapeutic agents to counteract the production of free radical in oxidative stress.

1.2.3 Calcium Homeostasis

Aβ causes neuronal membranes to lose their ability to regulate ion concentration. One important ion in particular is calcium and its increased concentration causes major neurological problems in AD. Calcium is a key component in signal messaging in the brain, for synapse plasticity and apoptosis and an imbalance of Ca$^{2+}$ has been linked to neurodegeneration as well as deficits in cognitive functions.\textsuperscript{79, 80} Aβ is speculated to effect Ca$^{2+}$ dysregulation by a number of hypothesis. One is through the membrane pore hypothesis which states that Aβ oligomeric assemblies fuse to and form a channel on the lipid membrane through which Ca$^{2+}$ may flow through thereby increasing the concentration of the ion in the membrane. This has been seen through in vitro study of direct binding of Aβ to lipid membranes.\textsuperscript{81-83} Another hypothesis suggests that Aβ binds to and activates ion channels, specifically L-type voltage-gated Ca$^{2+}$ channels. This increases the influx
of Ca\textsuperscript{2+} leading to neurons being more susceptible to glutamate excitotoxicity in the brain that ultimately lead to cell death.\textsuperscript{84}

One of the therapeutic drugs currently available on the market is Memantine, an N-methyl D-aspartate (NMDA) receptor antagonist.\textsuperscript{85} An increased influx of Ca\textsuperscript{2+} occurs when this receptor is activated by glutamate. This drug inhibits this receptor and in turn has restored the balance of Ca\textsuperscript{2+} within the cell membranes and delayed AD associated symptoms.\textsuperscript{86} Inhibiting Ca\textsuperscript{2+} influx can be seen as a therapeutic option for novel drug design.

1.3 Conclusions

AD is considered a multifaceted disease in that it contains numerous downstream symptoms that is speculated to stem from the mis-folding of Aβ.\textsuperscript{6} Spontaneous aggregation of Aβ leads to neuronal deposits in the brain that can cause oxidative stress\textsuperscript{87}, calcium imbalance\textsuperscript{14} and neurofibrillary tangles (NFTs)\textsuperscript{47} that ultimately lead to neurotoxicity and cell death. Defining symptoms of AD include cognitive decline, impaired judgement, abnormal behaviour and memory loss that can be a result of lack of synaptic function and loss of neurons in the brain.\textsuperscript{4} These symptoms are irreversible and only worsen as time progresses. No drugs are presently available on the market that targets the underlying cause for the disease and research is still being conducted to find novel drugs to address this problem.
Chapter 2
Current therapeutic approaches to Alzheimer’s disease

2 Introduction

Reduction of Aβ and NFTs in the brain has been the subject of discussion for the treatment of AD for the past 25 years due to their significance in the disease etiology. Roughly 100 molecules have been developed to target these hallmarks from 1998 to 2011 but with many being unsuccessful.¹ The imbalance between production and clearance of Aβ has been widely accepted as the primary cause of symptoms in AD.⁸⁸ Drugs designed to target this pathology have included β- and γ-secretase inhibitors⁸⁹ and Aβ plaque removal using vaccines and antibodies.⁸⁸ Reasons behind why drugs have been ineffective in this area have been attributed to the underlying unknown mechanism associated with Aβ aggregation and production.⁹⁰ Therefore, understanding this complex progression is of utmost importance for future drug discovery research.

There is currently no cure for AD and current FDA approved drugs being prescribed to patients are only aimed at delaying the symptomatic effects of the disease.⁹¹, ⁹² The need for novel therapeutic treatments for AD is still progressing. Previous drugs and drug design have been based on a “one-compound, one-target” paradigm which was shown to be highly successful in the past.⁹³ However, with the multifactorial complexity of many neurodegenerative disease, such as AD which does not necessarily depend on a single entity, this method of drug design might not be sufficient. In lieu of this, multi-target directed ligands (MTDLs) have emerged as a novel technique to combat this type of situation.

2.1 Current Treatments for AD

There are currently two classes of drugs available to mitigate the symptoms of AD; acetylcholinesterase inhibitors (AChEI) and N-methyl-D-aspartate (NMDA) receptor antagonists. Three FDA approved AChEI, donepezil (Pfizer)⁹⁴, rivastigmine (Novartis) ⁹⁵, and galantamine (Janssen)⁹⁶, and one NMDA receptor antagonist, memantine, are available and widely used for the treatment of AD.⁹⁷, ⁹⁸ A fourth AChEI and actually the first drug approved for AD in
1993, tacrine, was dropped out and is now discontinued for use due to its hepatotoxicity and toxic side effects. Structure of all five drugs are depicted in Figure 2.1.

![Chemical structures of drugs]

**Figure 2.1:** Drugs for the treatment of AD. Rivastigmine, galanthamine and donepezil are acetylcholinesterase inhibitors (AChEIs) and memantine is an NMDA receptor antagonist. These four drugs are currently available and FDA approved for the palliative symptoms of AD. Tacrine, the first drug approved, was dropped out and not currently used for its toxicity.

### 2.1.1 Acetylcholinesterase Inhibitors (AChEI) – Cholinergic Hypothesis

A widely used approach for recent drug therapy for the past 20 years in AD is based on the cholinergic hypothesis. A defining symptom of AD is the deterioration in cognitive function in conjunction with abnormal behaviour which can be attributed to a result of cholinergic neuron loss in the brain. A distinct region in the cerebral cortex that contains a large population of cholinergic neurons was found to be reduced in the post-mortem study of AD brain. Patients that showed early onset for AD also showed decreased activity in choline acetyl transferase
(ChAT), the enzyme that produces Ach.\textsuperscript{105} Decline in cognitive abilities has been closely linked to the decrease of ACh and in the cholinergic system.\textsuperscript{106}

A class of drugs known as acetylcholinesterase (AChE) inhibitors aim to increase or restore the amount of available neurotransmitter in the brain in hopes to ameliorate this symptom, but reliefs tend to be short-term.\textsuperscript{107} Acetylcholinesterase inhibitors have shown to decrease the amount of time patients need to spend in full-time institutionalization compared to no treatment at all. A review published by Takeda \textit{et al.} stated that AChEIs were able to delay cognitive impairments for at least 6 months.\textsuperscript{108} However, the clinical significance of this is minor and use of these drugs tend to contain adverse side effects associated with overstimulation of cholinergic systems resulting in confusion, hallucinations, abnormal behavioural shifts, nausea and stomach pains.\textsuperscript{99,109} In addition, AChEIs are not designed to resolve the underlying problem associated with AD, which is the imbalance in the production and clearance of Aβ.\textsuperscript{110}

2.1.2 Dual Activity of AChEIs

Sussman \textit{et al.} have revealed the structure of \textit{Torpedo californica} AChE through X-ray crystallography and determined for the first time the binding pocket of the active site of the enzyme.\textsuperscript{111} He determined that the active site consisted of a catalytic triad (Ser200-His440-Glu327) that is located at the bottom of a deep and narrow gorge, about 20 Å long and lined with 14 aromatic amino acids. ACh binds to this active site by contact of its quaternary group to the indole ring of Trp84. By studying the tacrine-AChE interaction, they also determined that the inhibitor mainly functions through $\pi-\pi$ stacking interactions with Trp84 and hydrogen bonding with His440.

The classical activity of AChEIs involves inhibiting AChE, the enzyme that breaks down ACh. It has been accepted that the decrease in cognitive abilities in AD is due to lack of ACh concentration in the synaptic cleft. To compensate this shortage of neurotransmitter in the brain, AChEIs were designed to increase ACh concentration so cognitive function may be restored and used for neurotransmission. This has been the basis for majority of the current drugs available on the market to treat AD. And most recently studies have indicated that in addition to restoring the amount of acetylcholine, AChEIs may have another function that has spurred its interest in the past years as a therapeutic route in modulating AD.
Inestrosa et al. have proved that AChE increases aggregation of Aβ and its toxicity. Along with the active gorge site of AChE which hydrolyzes acetylcholine (ACh), the enzyme also possesses a peripheral anionic site (PAS) that is located spatially away from the active site. This peripheral binding site is speculated to be involved with accelerating Aβ aggregation to form fibrils. The PAS of the enzyme complexes with Aβ, catalyzing the peptide to form β-sheet structures and thereby accelerating its aggregation. Inhibitors of the enzyme can have the potential of complexing with and consequently inhibiting the acyl-enzyme intermediate that is formed during hydrolysis, but can also block the PAS from associating with Aβ. Examples such as propidium, is able to block Aβ aggregation through interaction with Trp279 of the peripheral site. This premise generated the high interest surrounding drug discovery based on AChE inhibition and their potential for having multiple target sites as well as combining the cholinergic hypothesis and amyloid hypothesis in one drug.

Figure 2.2: Structural schematic of acetylcholinesterase (AChE). X-ray crystallography has revealed an active site lined with hydrophobic residues at the bottom of a narrow gorge of the enzyme. The active site consists of a catalytic triad comprising serine, histidine and glutamate. In addition to the traditional activity of AChE to break down ACh, a peripheral anionic binding site (PAS) was discovered that allow the enzyme to have dual activity that is important in AD. This
PAS is located spatially away from the binding site and may have an effect in facilitating the aggregation of Aβ.

Early efforts in combining these two properties together include constructing heterodimers and homodimers of already existing AChEIs and Aβ aggregating units together with a linker. Donepezil, an AChEI currently in the market, was shown to have anti-aggregation properties along with its inhibitory activity towards AChE. Tacrine has been shown to bind to the PAS of AChE leading to a family of heterodimer compounds that combines tacrine and donepezil to achieve anti-aggregating properties as well as AChE inhibitory activities. Another novel compound based on donepezil-tacrine hybrid is Figure 2.3. C. In hopes to increase its potency Camps et al. preserved only active sites of each compound so that the novel hybrid compound can maintain the same contact sites as the parent compounds during its interaction. This family of compounds displayed potent inhibitory activities towards AChE and Aβ aggregation with low concentrations. Many other compounds were also synthesized based crystal structure and molecular modelling programs. One compound in particular termed NP-61, is one of the first of these dual AChEIs to hit phase II of clinical trials and many more are in development demonstrating increased interest in this area.

Figure 2.3: Dual binding site AChE inhibitors. (a) and (b) depicts heterodimer molecules that aim to combines tacrine and donepezil to achieve AChE inhibitory activity as well as anti-aggregating properties associated with Aβ.
2.2 β- and γ-secretase Inhibitors

One of the main reasons why targeting β- and γ-secretase inhibition has been difficult is mainly due to the multiple cleavage sites as well as the numerous different substrates the enzyme may possess. Inhibiting the protease may affect its ability and significance in other pathways. For example, one of the main substrates of γ-secretase is Notch, which makes inhibiting this enzyme problematic. Notch signalling pathway is important in cell communication and inhibiting this process may have serious consequences in cell proliferation in the adult and embryonic life.

2.3 Multi-target Directed Ligands (MTDLs)

Majority of the strategies for the development of novel inhibitor molecules have been based on having the compound target one specific site. However, in light of the multifaceted nature of the disease, single-target therapeutic methods may be inadequate in treating AD. AD contains several pathological events as a result of the complex network of biological and pathogenic responses of the disease. To combat this disease efficiently, having a compound that can target multiple sites in parallel would be beneficial and advantageous.

Multi-target directed ligands (MTDLs) are emerging as a novel approach by designing compounds that can target multiple sites to achieve maximum efficacy in treating the disease. Initial efforts include combining different pharmacophores each with their own distinct function onto a core structure to create a hybrid molecule that can have multiple targets. Developing molecules that contain AChE inhibitory activity may prove to be an attractive route for MTDL research, as this class of compounds have been suggested to have the potential to inhibit Aβ aggregation, in addition to its native function. A very promising drug currently being explored is Memoquin, a compound synthesized by Cavalli et al. This compound has exhibited exemplary properties that have beneficial effects in AD. It is shown to have anti-oxidant properties to counteract the production of reactive oxygen species (ROS) produced in AD, an effective inhibitor of AChE, inhibition of Aβ aggregation, prevent tau phosphorylation, BACE-1 inhibitor, and was also shown to rescue AD induced neurodegeneration in all stages of development in
mice.\textsuperscript{124} This drug is recognized as a breakthrough in the current development for the discovery of MTDLs for AD.

Discovering innovative drugs with the ability to target multiple sites is advantageous and simpler than administering multiple drugs which can have unwanted pharmacokinetic and pharmacodynamics consequences. To target this complex disease, MTDLs provide a solid starting point.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.4.png}
\caption{Structure of memoquin, a multi-target directed ligand capable of targeting the multifactorial nature of AD.\textsuperscript{124}}
\end{figure}

2.4 \textit{Aβ} Modulating Compounds

Small molecules and chaperones have been identified to modulate amyloid fibrillogensis.\textsuperscript{50, 125, 126} Instead of producing Aβ fibrils, molecular chaperones Hsp70 and Hsp40 have been shown to form soluble, amorphous protein aggregates from polyglutamine (polyQ), the protein responsible for Huntington’s disease.\textsuperscript{127} They redirected the protein from amyloid fibrillization to an off-pathway mechanism that produced non-amyloidogenic assemblies. In another study, the chaperonin, TRiC, along with Hsp70, were shown to work synergistically in inhibiting fibril formation in polyQ into soluble non-toxic oligomers.\textsuperscript{128}

Naturally occurring flavonoids have been seen as potential modulators for therapy of neurodegenerative diseases such as AD for their antioxidant and anti-inflammatory properties.\textsuperscript{65} They have been shown to have beneficial effects on neurological functions in the brain in both human and animal models. Flavonoids are speculated to improve neuronal health by promoting
learning, memory and cognitive functions in the brain through their antioxidant properties by protecting neurons and stimulating neuronal regeneration. A natural polyphenol found abundantly in green tea, epigallocatechin-3-gallate (EGCG), was found to be a potent modulator of polyQ, α-synuclein (αS) (the protein responsible for Parkinson’s Disease), as well as Aβ. EGCG was found to bind to the natively unfolded polypeptide, disrupting the aggregation process early on avoiding the on-pathway mechanism towards fibrillogenesis. The molecule redirects the peptide by inhibiting the conversion into β-sheet structure and into an off-pathway alternative route creating highly stable non-toxic oligomers.

2.5 sym-Triazine based Multi-target Directed Ligands (MTDLs)

Our group has previously synthesized a library of 12 novel sym-triazine derivatives that have exhibited multiple functions, mainly AChE inhibition and β-sheet breaking activity. sym-Triazines, also known as 1,3,5-triazines, have a number of uses and applications in a variety of fields; including those in the pharmaceutical, textile, plastic and rubber industries. It has been shown to contain anti-cancer, anti-bacterial, and anti-viral properties, and therefore displays immense potential for therapeutic purposes. Most sym-triazines are derived from cyanuric chloride (CC), a popular initial reagent used for the ease of susceptibility of its chlorine atoms to nucleophilic attack in the presence of various hydrochloride accepters.

3 different substituent moieties were implemented onto the sym-triazine core to enhance their inhibitory activity; R₁) 1-dimethylamino-2-propanol, R₂) 3-dimethylamino-1-propanol, R₃) 2-dimethylaminoethanol, all being various analogs of ACh to target AChE. R₁ contained an additional methyl subunit onto the ACh analogue, R₂ consists of an elongated alkyl chain, and R₃ represented the exact analogue of ACh. Overall, they found the effectiveness of the inhibitors to be R₃ > R₁ > R₂. Mono-, di-, and tri-substituted sym-triazines were synthesized and tested for their activity with the result of tri-substituted sym-triazines (3d and 3e) being the most potent inhibitors. The tri-substituted compounds were improved on by converting them into quaternary amine salts (3f and 3g) to increase their positive charges for the purpose of enhancing their affinity towards the negatively charged PAS of the enzyme. This proved to be an effective method in that these compounds displayed a >50% drop in IC₅₀ values for inhibiting the enzyme compared to their non-derivatized counterparts. 3f and 3g demonstrated to be effective inhibitors in both Aβ
aggregation and AChE activity. Through transmission electron microscopy (TEM) studies, the compounds were shown to modulate Aβ from progressing into amyloidogenic fibrils into soluble oligomeric assemblies, much like how EGCG was shown to disrupt aggregation by redirecting the peptide towards an off pathway course to generate non-toxic soluble oligomers. Our compounds may have the same effect on Aβ as the flavonoid in redirecting the peptide to forming these non-toxic oligomers.

Figure 2.5: Structure of acetylcholine (ACh) and the three varying substituents (R₁, R₂, and R₃) implemented in the synthesis of sym-triazine derivatives to target acetylcholinesterase (AChE) inhibition. The groups were chosen for their comparable structure to the native substrate. R₁ contains an additional methyl subunit onto the ACh analogue, R₂ consists of an elongated alkyl chain, and R₃ represents the exact analogue of ACh.

3f and 3g, also termed TAE-1 and TAE-2, went on to perform in vitro studies on differentiated human SH-SY5Y neuronal cells to test for their neurotoxicity. The results indicated that the compounds promoted cell growth through neurite extension and cell differentiation through an increase in the neuronal marker MAP2. In addition to these beneficial biological activities, the compounds also showed greater AChE inhibition than donepezil, which was used as a control, as well as Aβ-aggregation inhibitor. These results confirmed the multi-target activities of sym-triazine compounds and verified them as potential drug candidates for future AD related therapies.
Figure 2.6: Twelve novel multi-target sym-triazine derivatives synthesized and designed to target Aβ aggregation and acetylcholinesterase (AChE) in AD. These compounds were tested to have anti-aggregation properties as well as inhibitory activity towards AChE. 3f and 3g in particular showed the highest activity amongst the compounds.\textsuperscript{134}

2.6 Conclusions

Two classes of FDA approved drugs are currently available on the market for the treatment of AD. They include acetylcholinesterase inhibitors (AChEIs) and N-methyl-D-aspartate (NMDA) receptor antagonists. The first drugs developed for AD therapy were AChEIs in 1993 with 3 compounds now available for patients; donepezil (Pfizer) \textsuperscript{94}, rivastigmine (Novartis) \textsuperscript{95}, and galantamine (Janssen).\textsuperscript{96} The aim of this class of drugs is based on the cholinergic hypothesis and
its goal is to increase the amount of acetylcholine (AChE) in the brain by inhibiting the enzyme that normally breaks it down. The second class of drugs termed NMDA receptor antagonists is believed to restore the concentration of Ca\(^{2+}\) in the brain and protect neurons from excitotoxicity.\(^{145}\) Clinical trials have verified that administration of these drugs can delay symptoms associated with AD, but toxic side effects may prove the drugs to have limited use in long-term treatment for severe cases of AD.

Multi-target directed ligands (MTDLs) are viewed as a more efficient and advantageous technique for AD drug design. Because AD does not solely possess one symptom, but multiple differing effects, having a compound that can target multiple sites would be more effective. *sym*-Triazine compounds have been proven to be successful in targeting both Aβ aggregation and AChE inhibition, combining both the amyloid cascade hypothesis and the cholinergic hypothesis.\(^{134}\) This drug design strategy can be viewed as a potential therapeutic route towards AD.
Chapter 3
Synthesis of \textit{sym}-Triazine Compounds

3 Introduction

\textit{sym}-Triazines have been implemented and used in a number of different purposes and contains properties such as anti-microbial\textsuperscript{140}, anti-cancer\textsuperscript{138}, and anti-viral\textsuperscript{135}. Our group has previously synthesized and tested novel \textit{sym}-triazine compounds that could act as potential multi-target inhibitors for AD.

In the present study, we aim to expand on the library of \textit{sym}-triazine compounds and improve on this work by optimizing and increasing the number of hydrophobic aromatic groups onto the \textit{sym}-triazine core. It was suggested that the addition of aromatic subunits will aid in disrupting the β-sheet structure that is crucial for Aβ aggregation. Docking studies reported by Xing \textit{et al.}\textsuperscript{146} also revealed that phenyl subunits might function as hydrophobic pharmacophores was significant in AChE inhibitory activity. The two novel compounds AM-1 (1d) and AM-2 (2d) were synthesized following \textbf{Scheme 1}. AM-1 utilizes the advantage of increased activity of tri-substituted \textit{sym}-triazines, in comparison to mono- or di-substituted compounds, with a diphenyl subunit as one of the substituents. AM-2 is similar in being tri-substituted but containing a phenoxy moiety instead of the diphenyl.
Figure 3.1: Schematic for the synthesis of 1d (AM-1) and 2d (AM-2).
3.1 Reaction Scheme

The degree of substitution of the chloride atoms on the sym-triazine ring is highly dependent on temperature, hence careful monitoring of temperature can yield mono-, di-, or tri-substituted sym-triazine compounds. Through pragmatic experimentation, mono-substitution of CC can be attained by performing the reaction at 0 °C, di-substitution at room temperature and tri-substitution at 60 °C.

3.2 Experimental Procedure

3.2.1 Procedure for sym-Triazine Based Methoxy Compounds

\[ \text{2,4,6-trichloro-1,3,5-triazine} + \text{diphenylamine} \rightarrow \text{4,6-dichloro-N,N-diphenyl-1,3,5-triazin-2-amine (1a)} + \text{HCl} \]

\text{Figure 3.2:} Synthesis of sym-triazine based derivative 1a from cyanuric chloride and diphenylamine at equal ratios. Reaction was kept at 0 °C to ensure mono-substitution.

\text{4,6-dichloro-N,N-diphenyl-1,3,5-triazin-2-amine (1a):} Cyanuric chloride was dissolved in acetone (20 mL) and cooled to 5 °C in an ice-salt bath. Diphenylamine and sodium carbonate was added in small portions to the cyanuric chloride solution and stirred for two hours in an ice-salt bath while maintaining temperature at 5 °C. Completion of reaction was monitored using TLC (acetone:hexane, 1:1) and the yellow solution was poured into a water/ice mixture and the precipitate was filtered and washed with cold water. Compound was recrystallized using isopropanol to form light blue crystals with a yield of 65%.

M.p. 170-173°C; \text{\textsuperscript{1}H NMR (500 MHz, [D₆]DMSO, 25°C, TMS):} δ = 7.52-7.28 ppm (m, 10 H); \text{FTIR (ATR):} 3074.93-2962.30 (w, CH stretch aromatic), 1487.19, 1442.73 (C[dbond]C, C[dbond]N stretch aromatic), 1173.53 (m, CN stretch), 668.57 cm\textsuperscript{-1} (s, CCl stretch).
Figure 3.3: Synthesis of sym-triazine based derivative 1b from cyanuric chloride and phenol at equal ratios. Reaction was kept at 0 °C to ensure mono-substitution.

2,4-dichloro-6-phenoxy-1,3,5-triazine (2a): Cyanuric chloride (9.22 g, 0.05 mol) was dissolved in minimum amounts of chloroform (40 mL) and cooled to 5 °C in an ice-salt bath. Phenol (5.2 g, 0.05 mol) and sodium hydroxide (2.25 g, 0.05 mol) were added to the cyanuric chloride solution while maintaining temperature of solution at 5 °C. Solution was stirred for 30 minutes at room temperature and then for another two hours at 50 °C. Chloroform layer was collected through separatory funnel and air dried to produce a white precipitate. White precipitate was recrystallized with petroleum ether and toluene mix (3:1). Final product was a peach coloured crystal with 76% yield.

M.p. 103-105°C; $^1$H NMR (500 MHz, [D$_6$]DMSO, 25°C, TMS): $\delta$=7.63-7.47 (m, 2 H), 7.47-7.23 ppm (m, 3 H); FTIR (ATR): 3055.86-3015.75 (CH stretch aromatic), 1532.54, 1483.06 (C[dbond]C, C[dbond]N stretch aromatic), 1292.67 (CN stretch), 691.81 cm$^{-1}$ (CCl stretch); HRMS (TOF MS ES$^+$): $m/z$ calcld for C$_9$H$_5$Cl$_2$N$_3$O: 240.98; found: 240.9 [M+H]$^+$.
3.2.2 Procedure for sym-Triazine Based Acids

Figure 3.4: Synthesis of sym-triazine based acid 1b from reaction with its methoxy derivative 1a and 4-hydroxybenzoic acid. Substitution of the remaining chlorine atoms require the reaction to be performed at 40°C.

4,4’-((6-(diphenylamino)-1,3,5-triazine-2,4-diyl)bis(oxy))dibenzoic acid (1b): Sodium hydroxide (2.02 g, 0.05 mol) and 4-hydroxybenzoic acid (3.48 g, 0.025 mol) was dissolved in water (20 mL) and added to 1a (4.00 g, 0.0126 mol) dissolved in acetone (20 mL) at a temperature of 0°C. After addition, solution was stirred at 40°C for two hours and left stirring at overnight at room temperature. Completion of reaction was monitored using TLC (acetone:hexane, 1:1). Solution was acidified to pH 3.0 where white precipitate formed. Compound was filtered and dried in oven. Final product was a white powder with a yield of 77%.

M.p. 290-294°C; \(^1\)H NMR (500 MHz, [D\(_6\)]DMSO, 25°C, TMS): δ = 12.95 (s, 2 H), 7.92-7.85 (m, 4 H), 7.44-7.25 (m, 12 H), 7.24-7.17 ppm (m, 2 H); FTIR (ATR): 3068.23 (OH stretch), 2889.76-2807.62 (CH stretch), 1685.42 (C\{dbond\}O stretch), 1536.14, 1491.70 (C\{dbond\}C, C\{dbond\}N, stretch aromatic), 1370.25 (CN stretch), 1217.56 cm\(^{-1}\) (CO stretch).
**Figure 3.5:** Synthesis of *sym*-triazine based acid 2b from reaction with its methoxy derivative 2a and 4-hydroxybenzoic acid. Substitution of the remaining chlorine atoms require the reaction to be performed at 40ºC.

4,4'-(6-phenoxy-1,3,5-triazine-2,4-diyl)bis(oxy)dibenzoic acid (2b): NaOH (2.42 g, 0.066 mol) and 4-hydroxybenzoic acid (4.56 g, 0.033 mol) was dissolved in water (20 mL) to make a solution and added dropwise to compound 2a (4.00 g, 0.015 mol) dissolved in minimum amounts of acetone (20 mL). After addition, solution was heated to 50 ºC for 30 minutes and allowed to stir at room temperature overnight. The milky white solution was poured into 50 mL of cold water and acidified to pH 3.0. White precipitate was collected and washed with cold water. Product was then oven dried and recrystallized using water and small amounts of DMF. Resulting product was a white powder with a yield of 92%.

M.p. 267-269ºC; 1H NMR (500 MHz, [D6]DMSO, 25ºC, TMS): δ = 13.09 (s, 2 H), 8.01-7.96 (dq, J = 2.2 Hz, 4 H), 7.47-7.34 (m, 6 H), 7.31-7.18 ppm (m, 3 H); FTIR (ATR): 3056.83 (OH stretch), 2548.19-2830.21 (CH stretch), 1682.92 (C[dbond]O stretch), 1596.92, 1489.17 (C[dbond]C, C[dbond]N stretch aromatic), 1361.76 (CN stretch), 1202.12, 1162.30 (CO stretch); HRMS (TOF MS ES-): m/z calcd for C23H15N3O: 444.09; found: 444.06 [M-H]⁻.

**3.2.3 Procedure for *sym*-Triazine Based Acyl Chlorides**

![Image of the reaction](image)

**Figure 3.6:** Synthesis of *sym*-triazine based acyl chloride derivative 1c. Excess thionyl chloride (SOCl₂) was used as the chlorinating agent in this reaction with 1b, with pyridine as a base and dry chloroform (CHCl₃) as the solvent.

4,4'-(6-(diphenylamino)-1,3,5-triazine-2,4-diyl)bis(oxy)dibenzoyl chloride (1c): Thionyl chloride (5.57 mL, 0.0768 mol) and 1b (2.00 g, 0.0038 mol) and a drop of pyridine (catalyst) was
dissolved in dry chloroform (30 mL). Reaction was refluxed and boiled at 60 °C for 3 hours until solution turned clear. Progress of reaction was monitored by TLC for completion. Chloroform was distilled off at 60 °C where the solution turned a light yellow colour. 40 mL of hexane was poured into solution and allowed to precipitate for an hour. White precipitate was filtered and washed with petroleum ether. Final product had a yield of 50%.

M.p. 165-170°C; \(^1\)H NMR (500 MHz, [D\(_6\)]DMSO, 25°C, TMS): \(\delta = 8.00-7.86\) (m, 4 H), 7.51-7.25 (m, 8 H), 7.22-7.17 ppm (m, 6 H); FTIR (ATR): 3128.31 (C-H stretch, aromatic), 1735.04-1765.32 (C[dbond]O stretch), 1535.2, 1487.66 (C[dbond]C, C[dbond]N stretch aromatic), 1361.72 (CN stretch), 1198.83-1162.28 (CO stretch), 640.811 cm\(^{-1}\) (CCl stretch).

**Figure 3.7:** Synthesis of *sym*-triazine based acyl chloride derivative 2c. Excess thionyl chloride (SOCl\(_2\)) was used as the chlorinating agent in this reaction with 2b along with pyridine as a base and dry chloroform (CHCl\(_3\)) as the solvent.

**4,4′-((6-phenoxy-1,3,5-triazine-2,4-diyl)bis(oxy))dibenzoyl chloride (2c):** A solution of thionyl chloride (2.2 mL, 0.03 mol), compound 2b (2.0 g, 0.005 mol) and a drop of pyridine (catalyst) was dissolved in dry chloroform (30 mL). Reaction mixture was stirred and refluxed at 60 °C for 3 hours. Completion of reaction was monitored using TLC. Excess chloroform as eliminated using simple distillation until solution turned a pale yellow colour. Hexane (40 mL) was poured into resulting mixture to produce white precipitate that was collected. Compound was a white powder collected at 74% yield.

M.p. 140-141°C; \(^1\)H NMR (500 MHz, [D\(_6\)]DMSO, 25°C, TMS): \(\delta = 8.08-7.95\) (m, 4 H), 7.65-7.43 (m, 2 H), 7.41-7.34 (m, 4 H), 7.29-7.21 ppm (m, 3 H); FTIR (ATR): 3067.41 (CH stretch,
3.2.4 Procedure for *sym*-Triazine Based Esters

**Figure 3.8**: Synthesis of *sym*-triazine based ester 1d. Reaction proceeded using its acyl chloride counterpart 1c and 1-dimethylamino-2-propanol as reagents in dry tetrohydrafuran (THF) in the presence of trimethylamine as a base.

**Bis(1-(dimethylamino)propan-2-yl)4,4’-((6-(diphenylamino)-1,3,5-triazine-2,4-diyl)bis(oxy))dibenzoate (1d)**: A solution containing 1-dimethylamino-2-propanol (0.25 mL, 0.002 mol) and triethylamine (0.28 mL, 0.002 mol) in dry THF (5 mL) was added dropwise to a solution of compound 1c (0.56 g, 0.001 mol) in dry THF (5 mL). Solution was stirred and refluxed for 3 hours at 40 °C. Completion of reaction was monitored by TLC. Yellow filtrate was gravity filtered off and washed with dry THF. Filtrate was evaporated under vacuum conditions. Resulting residue was washed with petroleum ether which formed white precipitate. Resulting compound was a white powder with a yield of 55%.

M.p. 145°C; ¹H NMR (500 MHz, [D₆]DMSO, 25°C, TMS): δ = 7.91-7.89 (d, 4 H), 7.36-7.29 (m, 8 H), 7.23-7.20 (m, 6 H), 2.09 ppm (s, 12 H); FTIR (ATR): 2962.31 (CH stretch), 1683.10 (C=O stretch), 1605.61, 1498.98 (C=O stretch), 1364.95 (CN stretch), 1201, 1159.29 cm⁻¹ (CO stretch); HRMS (TOF MS ES⁺): *m/z* calcd for C₃⁹H₄₂N₈O₆: 690.32; found: 691.32 [M+H]⁺.
**Figure 3.9:** Synthesis of *sym*-triazine based ester 2d. Reaction proceeded through reacting its acyl chloride counterpart 2c with 1-dimethylamino-2-propanol in dry tetrahydrofuran (THF) in the presence of trimethylamine as a base.

**bis(1-(dimethylamino)propan-2-yl) 4,4'bis(1-(dimethylamino)propan-2-yl) 4,4''-(6-phenoxy-1,3,5-triazine-2,4-diyl)bis(oxy))dibenzoate (2d):** Compound 2c (1.20 g, 0.0025 mol) was dissolved in dry THF (7 mL). A solution of 1-dimethylamino-2-propanol (0.66 mL, 0.005 mol) and triethylamine (0.75 mL, 0.005 mol) in dry THF (5 mL) was added dropwise to the above solution. Reaction mixture was stirred and heated at 40 °C for 3 hours. Filtrate was collected through gravity filter, washed with dry THF, and dried under vacuum conditions. The resulting residue was washed with petroleum ether to form a white precipitate. Compound was a white powder with a yield of 34%.

M.p. 155°C; $^1$H NMR (500 MHz, [D$_6$]DMSO, 25°C, TMS): δ = 8.30-8.16 (m, 4 H), 7.49-7.39 (m, 2H), 7.26-2.24 (m, 4 H), 6.97-6.93 (m, 1 H), 6.83-6.82 (m, 2 H), 4.25 (m, 2 H), 3.71 (m, 2H), 3.34 (s, 12 H), 1.36 ppm (s, 6 H); FTIR (ATR): 2989.76-2829.45 (CH stretch), 1690.33 (C[dbond]O stretch), 1595.61, 1490.17 (C[dbond]C, C[dbond]N, stretch aromatic), 1358.47 (CN stretch), 1195.75, 1158.27 cm$^{-1}$ (CO stretch); HRMS (TOF MS ES$^+$): $m/z$ calcd for C$_{33}$H$_{37}$N$_5$O$_7$: 615.27; found: 616.37 [M+H]$^+$. 
3.3 Discussion

Procedures for the synthesis of *sym*-triazine derivatives were adapted from Veloso *et al.*, and Dudley and comprise of multiple subsequent nucleophilic aromatic substitutions. The first of which includes a reaction involving reacting the commercially available starting reagent CC, with either phenol (1.0 equivalent) or diphenylamine (1.0 equivalent) in 0 °C temperature to yield compounds 1a and 2a, respectively. Reaction at this temperature ensured only one of the chlorine atoms is to be displaced to generate mono-substituted *sym*-triazine intermediates leaving the other two chlorine atoms free to react in subsequent reactions. Addition of the phenoxy and diphenyl moiety groups were specifically chosen to increase hydrophobicity of the compound and to target Aβ aggregation. It was previously suggested that aromatic hydrophobic groups, such as the ones employed in the reaction, could disrupt π-π stacking that is critical in the formation of β-sheets in Aβ aggregation. Having these specific substituents would allow the compounds to have anti-aggregation effects in AD therapy. Compounds 1a and 2a continue through a second nucleophilic aromatic substitution with 4-hydroxybenzoic acid (2.0 equivalent) at 60 °C to displace the remaining two chlorine atoms to form the stable tri-substituted carboxylic *sym*-triazines 1b and 2b. This second reaction required the temperature to be increased to 60 °C, in comparison to 0 °C in the initial substitution where only one of the chlorine atoms needed reacting.

Acyl chlorides are the more reactive species compared to its carboxylic analogues and often used as an intermediate for the reaction with carboxylic acids. To form the corresponding acyl chlorides from the tri-substituted carboxylic derivatives 1b and 2b, excess thionyl chloride (SOCl₂) was used as the chlorinating agent in the subsequent step. The reaction proceeded under dry conditions in chloroform at 60 °C. It was essential to maintain dry conditions during this step as the resulting hygroscopic compounds were highly sensitive to water and care was taken to prevent exposure to air. Due to the strong reactivity of the acyl chlorides, water can quickly react with the compounds to revert them back to their carboxylic equivalents.

As a consequence, esterification of these two compounds were conducted immediately in the following step with 1-dimethylamino-2-propanol (2.0 equivalent) to form the more stable, corresponding tri-substituted ester compounds, 1d and 2d, also known as AM-1 and AM-2. The compounds were synthesized under dry conditions in THF with the use of triethylamine as a base.
1-dimethylamino-2-propanol was used as a substituent group attached to the _sym_-triazine core to allow the compound to have AChE inhibitory activity. This moiety resembles acetylcholine, the native substrate for the enzyme, and previous studies conducted on compounds that contain this analogue have shown to impede AChE activity. Conversion into the ester derivative were 55 and 34% for AM-1 and AM-2, respectively.

All reactions were monitored using thin layer chromatography (TLC) with a 1:1 hexane:acetone system. New compounds were characterized with IR, ESI-MS and _1^H_ NMR spectroscopy (Provided in the Supporting Information).

### 3.4 Conclusions

Two novel _sym_-triazine based compounds, AM-1 and AM-2, were successfully synthesized with 55 and 34% respectively. By controlling the temperature of the reaction with cyanuric chloride (CC), different degrees of substitution can be achieved. To achieve the multi-functions of the compounds and for its use as a potential MTDL, different substituent moieties were added onto the _sym_-triazine core. To target AChE, 1-dimethylamino-2-propanol was used as a moiety group due to its resemblance to the native substrate of AChE and comprises two of the substituent arms. The third substituent consisted of either a diphenylamine group for AM-1 or a phenoxy moiety for AM-2. These hydrophobic aromatic groups were added to the _sym_-triazine core to increase the inhibition activity for Aβ fibrillogenesis. Together, the compounds should possess AChE inhibition activity as well as disruption for B-sheet during Aβ aggregation. The activity of the compounds are tested through various analytical techniques in the next chapter.
Chapter 4
Bioanalytical Techniques for the interaction of small molecules with Alzheimer's disease biomarkers

4 Introduction

After successfully synthesizing the two \textit{sym}-triazine based compounds, their inhibition activity towards Aβ aggregation and AChE were tested through various inhibition tests. These results were then tested against and compared to the two most effective inhibitors previously synthesized in our group, 3f and 3g, also termed TAE-1 and TAE-2.

We compare the strength of AChE inhibition and Aβ aggregation of the novel compounds, AM-1 and AM-2, with previously tested compounds TAE-1 and TAE-2 through Thioflavin T (ThT) fluorescence analysis and Ellman’s colourimetric assay. In addition, circular dichroism (CD) spectroscopy and transmission electron microscopy (TEM) was implemented to provide a visual representation of the aggregation process of Aβ peptides over time with and without the presence of our molecules.

4.1 Ellman’s Colourimetric Assay

Figure 4.1: Reaction scheme of Ellman’s colourimetric assay. Acetylthiocholine (ATCh) is hydrolyzed by the enzyme acetylcholinesterase (AChE) to form thiocholine and acetate.
Thiocholine is then able to react with Ellman’s reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form the yellow anion of DTNB, TNB⁻, which can be monitored at 410 nm.

In order to assess the AChE inhibition properties of the compounds, Ellman’s colourimetric assay was implemented. In this spectrophotometric assay, acetylcholinesterase was incubated with various concentrations of inhibitor before initiation of the reaction with acetylthiocholine iodide (ATCh), the used substrate for AChE. This hydrolysis reaction results in the production of a yellow anion (TNB⁻) that is formed when reacted with Ellman’s reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Figure 4.1). The product can be monitored at 410 nm and the intensity of the signal is proportional to the amount of uninhibited enzyme available. Signal for AChE should have the highest intensity signal while those with inhibitors should be attenuated. These signals are then compared and will give an idea of the compounds effectiveness towards AChE inhibition.

Figure 4.2: Non-linear inhibition plots of AM-1 (●) and AM-2 (▲) incubated with acetylcholinesterase (AChE) with varying inhibitor concentrations. Enzymes were incubated with inhibitors at 37°C with 200 rpm shaking prior to initiation of reaction with acetylthiocholine iodide (AChI). Optical density values at 410 nm were obtained for the first 5 minutes of reaction and used to acquire the initial velocity ($V_0$) for each inhibitor concentration. Reaction was performed in 0.1 M PBS pH 8.0.

In addition to the active gorge site of AChE which hydrolyzes acetylcholine (ACh) and similarly ATCh, the enzyme also possesses a peripheral anionic site (PAS) that is located spatially away
from the active site. This peripheral binding site is speculated to be involved with accelerating Aβ aggregation to form fibrils. Inhibitors of the enzyme can have the potential of complexing with and consequently inhibiting the acyl-enzyme intermediate that is formed during hydrolysis, but can also block the PAS from associating with Aβ. This premise generated the high interest surrounding drug discovery based on AChE inhibition and their potential for having multiple target sites.

We examined the hydrolytic activity of AChE towards ATCh after incubation with AM-1 and AM-2. Results showed a 50% inhibition of the enzyme with 109 µM of AM-1 and 124 µM with AM-2 (Table 1). Compared to the IC₅₀ values for previous synthesized TAE-1 and TAE-2, the concentration required for the novel molecules are much higher. TAE-1 and TAE-2 are both tri-substituted compounds with all 3 substituent groups being of acetyl-choline-like analogue. AM-1 and AM-2 only have 2 side chains with the potential for enzyme interaction as we replaced the last group with aromatic functional groups aimed at increasing Aβ aggregation inhibition. A higher concentration of AM-1 and AM-2 might be needed for inhibition because of result of the molecules not being of the quaternary amine salt analogues of the compounds and therefore not contain the positive charges associated with it. Positive charges may increase the attraction of the molecules towards the negatively charged PAS region of the enzyme and therefore increase inhibition. The modified analogues were shown to have a >50% drop in IC₅₀ values when compared to their counterparts and could be one of the reasons why AM-1 and AM-2 may have such high IC₅₀ values.

Table 1: sym-Triazine based derivatives AM-1, AM-2, TAE-1 and TAE-2 and their inhibition of Aβ fibrils 40/42 and IC₅₀ values. Percent inhibition values were attained with equivalent inhibitor and peptide concentration and IC₅₀ values were obtained through non-linear regression plots with varying inhibitor concentrations. All reactions were incubated at 37 °C with 200 rpm shaking for 72 hours and measured in 50 mM PBS pH 7.4.
<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>% Inhibition of Aβ40 fibrils</th>
<th>% Inhibition of Aβ42 fibrils</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1d</td>
<td>(AM-1)</td>
<td>74.89</td>
<td>80</td>
<td>109.0 ± 0.3</td>
</tr>
<tr>
<td>2d</td>
<td>(AM-2)</td>
<td>71.93</td>
<td>78.73</td>
<td>124.0 ± 0.2</td>
</tr>
<tr>
<td>3f</td>
<td>(TAE-1)</td>
<td>70.2</td>
<td>24.9</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>3g</td>
<td>(TAE-2)</td>
<td>89.9</td>
<td>52.6</td>
<td>2.8 ± 0.1</td>
</tr>
</tbody>
</table>
4.2 Thioflavin-T Aβ aggregation study

The ability of the compounds to inhibit peptide aggregation was evaluated by Thioflavin-T (ThT) fluorescence tests. ThT is a common dye that is used to detect the amount of β-sheet present in protein aggregates. The dye has an affinity for and binds to highly structured β-sheets that is existent in elevated amounts in Aβ. The fluorescence emission at 485 nm is monitored and compared with the uninhibited peptide.152, 153

![Molecular structure of ThT](image)

![Cross β-sheet structure of amyloid fibrils](image)

![Schematic of β-sheet conformation with side chains (R) indicated on each residue](image)

**Figure 4.3:** Interaction between thioflavin-T (ThT) and β-sheets in mature amyloid fibrils. (a) Molecular structure of ThT. (b) Cross β-sheet structure of amyloid fibrils. Fibrils are arranged perpendicular to the long axis of the fibril. (c) Schematic of β-sheet conformation with side chains (R) indicated on each residue. ThT is believed to bind along the surface of the side chain grooves of the fibril. The dye is proposed to bind parallel to the long axis of the fibril. Double headed arrow illustrates one of the channels through which the dye may bind.

Spontaneous self-aggregation of Aβ is widely accepted to be the defining factor in the formation of toxic oligomers in AD and as a result, inhibiting this process is significant in AD drug
therapeutic design. Hydrophobic functional groups are believed to disrupt the π-π stacking in β-sheet structure and therefore incorporation of aromatic analogues into the sym-triazine core may allow the compound to inhibit protein aggregation. This is supported by our previous study that incubated Aβ with donepezil, a current commercially available AChE inhibitor, which did not have any effect on Aβ aggregation.

Previous compounds TAE-1 and TAE-2 have been shown to modulate Aβ aggregation by values indicated in Table 1. Incubation with only 0.5:1 ratio of AM1 to peptide concentration resulted in 52.9% fibril inhibition. At 1:1 concentration of inhibitor to peptide ratio, 70.23% inhibition of fibril formation and 74.9% inhibition at 2X the concentration. AM-2 provides 69.8% and 71.3% inhibition at 1X and 2X compound concentration, respectively. AM-1 shows higher inhibitory activity than AM-2, possibly due to it having a diphenyl moiety versus AM-2 having a benzyoxy substituent which would make the compound more hydrophobic. Comparing the novel compounds synthesized in this study with previously recorded TAE-1 and TAE-2, a major distinction between them are the addition of hydrophobic groups. These aromatic functional groups allow the compound to have a higher inhibitory activity towards Aβ aggregation. Furthermore, the same trend is seen using Aβ42 with AM-1 having the higher % inhibition than AM-2.

**Figure 4.4:** Thioflavin-T fluorescence measurements of inhibitors AM-1 and AM-2 incubated with (a) Aβ40 and (b) Aβ42. Measurements were taken after 72 hours incubation of enzyme with inhibitor at 37 °C with 200 rpm shaking in 50 mM PBS pH 7.4. Control Aβ fibrils were at a 50 µM concentration and inhibitor concentration varied between 25, 50 and 100 µM. Each measurement was performed in triplicates (n=3) with error bar denoting standard deviation.
4.3 Secondary Structure Analysis using Circular dichroism (CD) Spectroscopy and Transmission Electron Microscopy (TEM)

Circular dichroism (CD) results from the uneven absorbance of left handed (counter-clockwise) circularly polarised light (L) and right handed (clockwise) circularly polarised light (R).\textsuperscript{155, 156} If a sample passes through with L or R not being absorbed, or should both components be absorbed to the same extent, the resulting light would be polarised light in the original plane (Figure 4.5). However, if differential absorption is observed between the two components, then the resulting light will be elliptically shaped and that forms the basis of CD spectroscopy. The difference between the absorbance is generally reported in terms of ellipticity ($\theta$) and is measured as a function of wavelength.

Circular dichroism (CD) spectroscopy is a powerful tool used to detect secondary structure of a number of proteins usually in the far UV-regions of the spectrum, 190 – 260 nm.\textsuperscript{157} It is used in support to the dye-binding and kinetic studies conducted in this research to monitor the conformational change of large bio-molecules and their interaction with small molecules. A$\beta$ transitions from unordered random coil structure to $\beta$-sheet rich conformation and the aggregation of the peptide can be directly correlated to the formation of $\beta$-structure.\textsuperscript{158, 159} Although dye-binding methods is used commonly to detect small molecule association with A$\beta$ and to estimate protein aggregation, in some cases dye-binding assays may give false positive results. This can be attributed to the fact that both small molecule and the dye can associate via the same binding site(s) of A$\beta$ aggregates. As CD spectroscopy can detect conformational changes, the data obtained from the incubation of A$\beta$ with small molecules would be unambiguous, thus making it a very advantageous technique for A$\beta$ studies.
Figure 4.5: Basis for circular dichroism effect. (a) If both left handed (counter-clockwise) circularly polarised light (L) and right handed (clockwise) circularly polarised light (R) are absorbed to the same extent, then the resulting light (red) will be in the original plane. (b) If differential absorption is observed during sample absorption, the resulting light will be elliptically polarised.

Inhibitor-free incubating solutions at 0 hours displayed a typical CD spectrum of random coil secondary structure with a characteristic negative peak at 198 nm (Figure 4.6). Transition into a β-sheet rich structure can be seen after 12 hours incubation with the formation of a negative peak at 216 nm and the disappearance at 198 nm. The β-sheet spectrum was maintained throughout the first 72 hours and similar results were seen for Aβ42 with the CD spectra displaying β-structure after incubation for 6 hours. This was consistent with literature that the Aβ42 aggregating at a much faster rate.\textsuperscript{160, 161}
**Figure 4.6:** Circular dichroism (CD) spectra of Aβ incubated with AM-1 and AM-2. (A) Time control study of Aβ$_{40}$ depicting the change in secondary structure observed in the natural aggregation process of the native peptide. At 0 h, the peptide displays a negative peak at 198 nm that disappears and slowly transforms into β a broad negative peak at 216 nm that depicts β-sheet conformation after 72 h. (B) CD spectra of Aβ$_{40}$ incubated with AM-1 and AM-2 after 72 hours. (C) Aβ$_{42}$ incubated with AM-1 and AM-2 with measurement taken after 72 hours. Concentration of both Aβ and inhibitors were 50 µM. All solutions were prepared in 50 mM PBS pH 7.4 and incubated at 37 ºC with 200 rpm shaking. Measurements were taken using 0.1 mm quartz cuvette and measured from 190-260 nm. Each measurement was an average taken from three individual scans with baseline corrected.

Figure 4.6 B and C shows the CD spectra obtained when Aβ$_{40/42}$ peptides were incubated with AM-1 and AM-2. There is a clear difference in the spectra obtained between inhibitor free Aβ$_{40/42}$ and Aβ with AM-1 and AM-2. While both Aβ$_{40/42}$ generated a conformation indicative of β-sheet formation with a broad negative peak at 216 nm, Aβ$_{40}$ incubated with AM-1 displayed negative peaks at 220 and 208 nm and a positive peak at 193 nm and Aβ$_{40}$ with the addition of AM-2 also displayed comparable results with two negatives peaks, at 216 and 208 nm, and a positive peak at 195 nm. Similar results can be said about Aβ$_{42}$ with AM-1 and AM-2 as can be seen in Figure 4.4 C. There is a distinctive dissimilarity between the samples incubated with inhibitors and the one without. The ones with the addition of the small molecules showed two sharp negative peaks at 220 and 210 nm versus the Aβ$_{42}$ strand depicting a broad peak at 218 nm. The conclusion that can be drawn from this data is that with the addition of our small molecules, the formation of β-structure was lessened or not observed.

In the control sample, inhibitor-free Aβ would rapidly aggregate from disordered random coil structure into β-sheets through fibrillogenesis by way of the traditional route.$^{10, 162, 163}$ Aβ transitions from monomers to larger oligomeric aggregates before forming mature fibrils that contain cross β-sheets.$^{42}$ Through solid state NMR and scanning-tunneling electron microscopy studies, Aβ fibrils may take on a β-strand-turn-β-strand (β-turn-β) conformation with the turn stabilized by hydrophobic interactions and a salt bridge between Asp23 and Lys28.$^{40}$ We speculate that with the addition of AM-1 and AM-2, the small molecules interact with the oligomers that are
formed pre-fibril, and disrupt the intermolecular interactions required for Aβ aggregation. The molecules may alter the stabilizing interactions within the peptide and thereby inhibit β-sheet formation. These results correlate with the data obtained from ThT fluorescence assay which showed considerable β-sheet fibril inhibition with the addition of the molecules.

Walsh et al. have observed that the process of fibrillogenesis involves the formation of an oligomeric, α-helix containing intermediate and that stabilization of this transition state intermediate may inhibit Aβ fibrillogenesis and formation of insoluble plaques. Because Aβ fibrillogenesis involves a nucleation-dependent process, the reaction must overcome a certain energy barrier before rapidly aggregating into larger fibrils. By stabilizing the intermediate complex enough to increase the activation energy, the progression into β-rich fibrils would be hindered resulting in lowering the toxicity associated with fibril growth. The CD spectra obtained in this study could be a representation of the secondary structure of the oligomeric intermediate that is stabilized by our molecules and provide insight into the conformation of these stable aggregates.

These stable oligomeric species could be the result of AM-1 and AM-2’s ability to redirect the peptide into non-toxic soluble oligomers similar to the green tea compound, epigallocatechin-3-gallate (EGCG). These soluble oligomers as compared to the toxic species associated with AD, are formed through an off-pathway mechanism and are shown to be non-toxic. EGCG has been reported to inhibit the fibrillogenesis of Aβ through interaction with the aromatic core of the peptide (residues 10-20). The hydrophobic aromatic functional groups attached to the sym-triazine compounds could potentially inhibit aggregation through the same mechanism as EGCG and the reason why Aβ aggregation is disrupted and no β-sheets were observed. The importance of aromatic interactions have been shown to be a significant portion in Aβ aggregation. In addition, the bulkiness of the compound could provide steric hindrance to the conformation of the peptide and also prevent the correct interactions to occur between amino acid residues for β-sheet formation. To support this hypothesis, transmission electron microscopy (TEM) images were taken of Aβ peptides after incubation to obtain a visual representation of the morphologies of the end stage of Aβ (Figure 4.7).
Figure 4.7: Transmission electron microscopy (TEM) of (A) 50 µM Aβ$_{40}$ (control) (B) Aβ$_{42}$ with AM-1 (C) Aβ$_{40}$ with AM-2 (D) 50 µM Aβ$_{42}$ (control) (E) Aβ$_{42}$ with AM-1 (F) Aβ$_{42}$ with AM-2. Grids were stained using 1% uranyl acetate. Images were taken after 72 hours incubation at 37 °C with 200 rpm shaking. All samples were in 50 mM PBS pH 7.4. Scale bars represent 100 nm.

Inhibitor-free Aβ attained fibrils after 72 hours incubation at 37 °C (Figure 4.7 A and D for Aβ$_{40}$ and Aβ$_{42}$ respectively). Peptides that were incubated with AM-1 and AM-2 (Figure 4.5 B and C, respectively) formed amorphous non-fibrillar aggregates. These results are comparable to ones obtained previously published data with TAE-1 and TAE-2.$^{134}$ In the presence of sym-triazine inhibitors, the peptide was redirected in a different route than the common amyloidogenic pathway that would produce the end stage of amyloid fibrils. Our inhibitors were able to modulate the course by diverting from the fibrillogenesis process, consistent with ThT and CD data which demonstrated a decrease in β-sheet structure that is normally present in fibrils. Instead of the highly structured β-sheet structure, amorphous assemblies were seen to have formed. Because EGCG was previously shown to have redirect the peptide into forming non-toxic oligomers, we suggest our sym-triazine compounds also work in the same manner. We propose that the compounds work by stabilizing the peptide, and blocking and inhibiting the inter-chain packing between β-sheets that is necessary for fibrillogenesis. Figures 4.7 E and F display Aβ$_{42}$ fibrils incubated in the presence
of AM-1 and AM-2. The images demonstrate similar results seen with the Aβ_{40} fibrils and are suggested to interact in a related matter.

### 4.4 Conclusions

The inhibition activity of the two novel *sym*-triazine derivatives, AM-1 and AM-2, were tested and compared to the previously reported values of TAE-1 and TAE-2. IC_{50} values were obtained for each compound by evaluating the % inhibition of AChE with increasing inhibitor concentrations. The values obtained were 109 ± 0.3 and 124 ± 0.2 µM respectively for AM-1 and AM-2. These values were higher than previously reported *sym*-triazine values for TAE-1 and TAE-2 indicating a decrease in the potency of the novel compounds. ThT data confirmed β-sheet inhibition with the novel compounds having a higher inhibition rate with equal or lower peptide ratio concentration. Support for a decrease in β-sheet formation was supported by CD and TEM which showed a deviation from the traditional aggregation pathway of Aβ. We hypothesize that our *sym*-triazine derivatives have the potential to modulate the pathway of aggregation from fibrillogenesis to an off-pathway reaction that forms non-toxic amorphous aggregates by stabilizing the intermediate composite. *sym*-Triazine compounds may open a new route for novel AD drug design therapy.
Chapter 5
Future Directions

5 Future Directions and Conclusions

AD is the most common form of dementia, affecting 60-80% of cases in the elderly.\textsuperscript{167} It results in cognitive decline, memory loss, and abnormal behavioural changes.\textsuperscript{168} A distinct hallmark of AD and what is viewed as the origin of the symptoms for AD are abnormal accumulations of Aβ aggregates in the brain.\textsuperscript{169} Other histopathological symptoms include formation of neurofibrillary tangles (NFTs), oxidative stress, mitochondrial dysfunction and calcium imbalance, all ultimately leading to neurotoxicity and cell death. Because of the numerous differing symptoms that are associated with AD, the disease is sometimes referred to as being complex and multifaceted due to the multiple symptomatic pathways.\textsuperscript{122}

Two classes of drugs are available for treatment of AD but may be insufficient to treat and cure the neurodegenerative disorder. Multi-target directed ligands (MTDLs) are shown to be an efficient method to alleviate multiple pathologies at once using one compound that can target multiple sites.\textsuperscript{93} A recent spark in interest for AChEIs as potential therapeutic agents for AD was evidence of a second function of the enzyme.\textsuperscript{170} In addition to the classical method of the compound to inhibit AChE, a peripheral anionic site (PAS) is present on the enzyme located spatially away from the active gorge site that has the ability to accelerate Aβ aggregation. Because of this new discovery, AChEIs can function as multi-target inhibitors.

We have synthesized novel sym-triazine derivatives to act as multi-target ligands that have inhibition activity of Aβ aggregation and AChE.\textsuperscript{134} Through various conducted inhibition studies, our molecules have displayed their potential in inhibiting β-sheet formation and also inhibition of AChE. They have the prospective of acting as promising drug candidates for future drug therapy for AD.

Future directions for the compounds may include conducting additional experiments to test their toxicity through cell viability studies. Additional analytical techniques that can be employed to explore other properties of the compound include using surface plasmon resonance (SPR) to test for the binding of the compound to Aβ. SPR is an upcoming technique that is becoming popular...
amongst pharmaceutical companies to screen for binding of novel compounds to the target protein or peptide. The high-throughput of the machines allow for multiple solutions to be automatically tested with a single experiment.

Isothermal titration calorimetry (ITC) is used to observe quantitative thermodynamic properties of the compounds. Binding affinity of the compounds can be determined as well as enthalpy change. It is frequently used by pharmaceutical companies to test for their affinity towards large biomolecules such as proteins and DNA. It would be beneficial to know the affinity of AM-1 and AM-2 to Aβ so additional modifications to the compounds can be made if necessary.

Because our group has successfully synthesized and verified the validity of inhibition of sym-triazine compounds, further modifications on the sym-triazine compounds can be considered to increase their inhibition properties as well as to incorporate other properties onto the core to increase its functionality in AD therapy. Oxidative stress plays a major role in neurotoxicity in AD that can eventually lead to cell death. Therefore, antioxidants are believed to be a favourable route for drug therapy. Antioxidant properties are regarded as a beneficial quality and may be added onto the sym-triazine core as a moiety group to enrich the number of targets for the MTDL.

Examples of antioxidants that have been incorporated with other compounds to achieve multi-target effects are depicted in Figure 5.1. Lipoic acid (LA) contains protective effects during neurodegeneration and the cyclic moiety in the compound is speculated to interact with the PAS in AChE which is important in Aβ aggregation. Combining LA with tacrine, a known AChE inhibitor, results in a new family of compounds termed lipocrine that have multi-target effects.
Figure 5.1: Drug design strategy combining tacrine, a known AChE inhibitor and lipoic acid (LA), an antioxidant, to form lipocrine, a potential multi-target ligand for AD therapy

A similar design strategy was applied with melatonin, another antioxidant that was implemented with tacrine to produce a compound with multi-target effects. Schematic of the design is shown in Figure 5.2. Melatonin is a powerful antioxidant that plays an important role in aging and contains neuroprotective abilities.¹⁷³
**Figure 5.2:** Drug design strategy combining tacrine, a known AChE inhibitor and melatonin, an antioxidant, to form compound 4, a potential multi-target ligand for AD therapy. \( R_1 = 6,8 \text{ Cl}_2, n = 6. \)

These strategies can be applied to designing novel *sym*-triazine based derivatives that contain anti-oxidant properties to create multi-target ligands that have antioxidant, anti-aggregating and AChE inhibiting activities.

MTDLs can be viewed as a novel upcoming approach for the future drug design of complex neurodegenerative disorders, such as AD. Due to the multiple downstream symptoms the disease may possess, having a compound to target multiple sites may be advantageous and more efficient than administering multiple drugs at once that can have unwanted interactions amongst different drugs. *sym*-Triazine derivatives can provide a solid foundation for novel MTDLs that can exhibit multiple functions, which is central in many neurological diseases, such as AD.
References


Appendices
A.1. IR spectrum of \textbf{1a}. 
A.2. IR spectrum of 2a.
A.3. IR spectrum of 1b.
A.4. IR spectrum of 2b.
A.5. IR spectrum of 1c.
A.6. IR spectrum of 2c.

A.1. IR spectrum of 1a.
A.7. IR spectrum of 1d.
A.8. IR spectrum of **2d**.
Appendix B: Mass Spectra (MS) of \textit{sym}-Triazine Derivatives

B.1. MS of 2a.
B.2. MS of 2b.
B.3. MS of 1d.
B.4. MS of 2d.
Appendix C: $^1$H-Nuclear Magnetic Resonance (NMR) Spectra of $sym$-Triazine Derivatives

C.1. $^1$H-NMR spectrum of 1a.
C.2. $^1$H-NMR spectrum of 2a.
C.3. $^1$H-NMR spectrum of 1b.
C.4. $^1$H-NMR spectrum of 2b.
C.5. $^1$H-NMR spectrum of 1c.
C.6. $^1$H-NMR spectrum of 2c.
C.7. $^1$H-NMR spectrum of 1d.
C.8. $^1$H-NMR spectrum of 2d.