Rational Design of \textit{sym}-Triazines For Multitarget-Directed Modulation of Cholinesterases and Amyloid-Beta in Alzheimer’s Disease

By

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

Department of Chemistry
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Abstract
Alzheimer’s disease (AD), a progressive age-related neurodegenerative disorder is characterized by impairments in memory and cognitive functions. The two main pathogenic hallmarks associated with the progression of this multifactorial disease include accumulation of amyloid-beta (Aβ) plaques and the deterioration of the cholinergic system in the brain. Using cost-effective synthetic procedures, mono-, di-, and tri- substituted *sym*-triazine derivatives incorporating acetylcholine substrate analogues and aromatic phenyl moieties were synthesized for the targeted modulation of Aβ aggregation and acetylcholinesterase (AChE) activity. A subset of these *sym*-triazines demonstrated dual inhibition of Aβ fibrillization and AChE hydrolytic activity *in vitro studies*. These highly effective compounds were also shown to be well tolerated by differentiated human neuronal cells in cell viability tests. These novel compounds have the potential to undergo future *in vivo* pharmaceutical analysis and have a positive impact on the quality of life of the people living with this devastating disease and their caretakers.
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<tr>
<td>Aβ</td>
<td>Amyloid-beta</td>
</tr>
<tr>
<td>APP</td>
<td>Aβ precursor proteins</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>BuChE</td>
<td>Butyrylcholinesterase</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CC</td>
<td>Cyanuric chloride</td>
</tr>
<tr>
<td>ChEIs</td>
<td>Cholinesterase inhibitors</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LA</td>
<td>Lipoic acid</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectra</td>
</tr>
<tr>
<td>MTDL</td>
<td>Multi-target-directed ligand</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NFTs</td>
<td>Neurofibrillary 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>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>SOCCs</td>
<td>Store-operated calcium channels</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>VDCCs</td>
<td>Voltage-dependent calcium channels</td>
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Chapter 1
Alzheimer’s Disease and Its Pathologies

1 Introduction

Alzheimer’s disease (AD), a complex neurodegenerative disorder prevalent in the aging population (1), is characterized by progressive deterioration in memory (2, 3) and cognitive functions (4-6). 25 million cases of AD were reported worldwide in the year 2000 and it is expected to increase to 114 million by 2050 if new therapies do not emerge in the near future (7).

Cross-sectional (8-10) and longitudinal (11-16) neuroimaging data in AD research have indicated that AD patients experience a decrease in total brain volume and expansion in ventricular system compared to individuals without AD. A typical magnetic resonance imaging (MRI) of the brain of a control (normal volunteer) and a patient suffering from AD can demonstrate the atrophy in certain regions of the brain, mainly the hippocampus and cerebral cortex in AD patients along with an increase in size of the AD brain (8, 9, 15-17).

Pathological deterioration of AD patients has been strongly associated with the overproduction and disregulation of the amyloid-beta (Aβ) peptide (6, 18). Aβ has been implicated in a number of neurotoxic pathways related to the formation of reactive oxygen species (ROS) (19), the disruption of calcium (Ca^{2+}) homeostasis (20), formation of neurofibrillary tangles (NFTs) (21) and chronic activation of microglia (22). One prominent strategy to reduce neurodegeneration has thus emphasized the removal of neurotoxic Aβ oligomers by implementing small-molecule aggregation modulators that disrupt π-π stacking between β-sheets in order to impede Aβ self-association (23, 24). Although the development and commercialization of Aβ-modulating agents are underway (25, 26), currently available pharmacological treatments have targeted more distal pathways of neurodegeneration, which are limited to two classes of compounds: N-methyl-D aspartate (NMDA) receptor antagonists and cholinesterase inhibitors (ChEIs) (27). The aim of such drug therapies is not to impede the proposed aetiopathologies, but rather to ameliorate behavioral and cognitive dysfunctions, which have significantly delayed - and in some cases avoided – the need for institutionalization (6, 28).¹ In order to develop drugs for the efficient

therapy of AD, it is vital to understand the multiple pathologies that contribute to the etiology of this complex disease (29).

1.1 Amyloid-Beta (Aβ)

Aβ, the peptide responsible for the formation of the senile plaques in the brain of AD patients, is one of main pathological factors of AD (29). The peptide was first sequenced twenty years ago from the meningeal blood vessels of patients suffering from AD as well as individuals with Down syndrome (30, 31). The following year, Aβ peptide was recognized as the main component of senile plaques of brain tissues in AD patients (32). The amyloid hypothesis, which states that the Aβ accumulation in the brain is the primary driving force of AD pathology, has become a significant focus in AD research in the past 10 years since the discovery of amyloidogenic Aβ derived from mutations in Aβ precursor proteins (APP) (33). Although this hypothesis has its strengths and weakness, Aβ pathology has been a strong focus for AD therapy (34). Aβ aggregation has been considered as the triggering event for further neurotoxic effects such as, mitochondrial dysfunction (35), induction of apoptotic genes (36), activation of protein-kinases that may form toxic neurofibrillary tangles (NFTs) (37), stimulation of microglia cells, oxidative stress, and ultimately neuronal damage and cell death (29, 38).

1.1.1 β- and γ- Secretase Enzymes

Aβ peptide is generated by endoproteolytic cleavages of the APP, a cell surface glycoprotein consisting of 770 amino acids and involved in neural signaling. The APP gene is abundantly expressed in several tissues to form APP, a fraction of which undergoes endoproteolysis by three proteases called α-, β-, and γ- secretases. This generates the Aβ peptide that can deposit and aggregate (or vice versa) in extracellular insoluble plaques (39).

The β-secretase pathway involves the formation of the amyloidogenic form of Aβ peptide and is initiated with the first cleavage of APP, which is carried out by the β-site APP cleaving enzyme

or BACE (40, 41). BACE generates the N-terminal of the APP (βAPPs) and the C-terminal fragment (C99) by cleaving APP at the N-terminal of the Aβ sequence (39, 42). The C99 protein intermediate is then subsequently cleaved by the γ-secretase to produce the amyloidogenic peptide, which is a heterogenous event that leads to the generation of Aβ with different C termini (39, 42). The most common forms of the Aβ peptide are the Aβ1-40 and Aβ1-42, which are generated when γ-secretase cleaves the C99 fragment at Val at position 40 and Ala at position 42, respectively (39). Alternatively, the α-secretase causes the release of the non-amyloidogenic form of Aβ, a soluble N-terminal portion of APP (αAPPs) and a C-terminal fragment (C83), by cleaving the APP inside the Aβ sequence (39).

### 1.1.2 Aβ1-40 and Aβ1-42 Peptides

Aβ1-42 is the most dominant form of Aβ peptide in senile plaques and more neurotoxic than Aβ1-40 (39). Aβ1-42 differs in its amino acid sequence from Aβ1-40 by the addition of two amino acids-Ile in position 41 and Ala in position 42-in the C-terminus (43) (amino acid sequence shown in Fig. 1.1). Recent studies have indicated that these two predominant Aβ isoforms demonstrate different oligomeric pathways (44, 45) during fibrillization of the peptide with kinetic studies indicating that Aβ1-42 is more fibrillogenic than Aβ1-40 (46, 47). Soluble Aβ can be converted to high β-sheet content following a conformational change; thus, making it prone to aggregation into soluble oligomers and larger insoluble fibrils in senile plaques. This can direct the fibrillogenetic Aβ1-42 isoform to stimulate the misfolding of other Aβ species (1). Aβ1-42 deposition can increase the accumulation of Aβ1-40 and trigger the amyloid cascade processes (43).

![Amino acid sequences of Aβ1-40 and Aβ1-42. Aβ1-42 incorporates two hydrophobic amino acid residues, Ile and Ala in the C-terminal that are absent in Aβ1-40 and make it more fibrillogenic than Aβ1-40 (adapted from Jarrett et al, 1993 (43)).](image)

**Fig. 1.1:** Amino acid sequences of Aβ1-40 and Aβ1-42. Aβ1-42 incorporates two hydrophobic amino acid residues, Ile and Ala in the C-terminal that are absent in Aβ1-40 and make it more fibrillogenic than Aβ1-40 (adapted from Jarrett et al, 1993 (43)).
1.1.3 Aβ Fibrillogenesis Pathway

Both *in vitro* (48, 49) and *in vivo* (50, 51) studies have strongly indicated that soluble oligomeric species of Aβ incorporating a β-sheet secondary structure are the main factors causing toxicity in neuronal cells (51, 52). Haas has argued that these soluble oligomers provide a larger surface area for interaction with neuronal cells compared to Aβ aggregates; thus, allowing it to rapidly diffuse into the synaptic cleft and cause dysfunction in neuronal transmission (53, 54).

Bitan *et al.*, 2003 demonstrated that oligomerization of Aβ monomers are initiated with the formation of pentamer/hexamer units called paranuclei. These minimal units can oligomerize to form larger oligomers, protofibrils and fibrils (39, 45, 54). These monomeric, paranuclei and large oligomeric species are mainly unstructured and consist of short β-sheet/β-turn and helical elements (Fig. 1.2) (39).

![Fibrillogenesis pathway of Aβ in AD](image)

**Fig. 1.2:** Fibrillogenesis pathway of Aβ in AD. The Aβ monomers can form amorphous aggregates and small oligomers, which are in equilibrium with each other. The large oligomers formed from the small ones can form the metastable protofibril that eventually form the fibril structure responsible for the development of senile plaques. The oligomeric species have been shown to be toxic to neuronal cells (adapted from Stains *et al.*, 2007 (54)).

1.2 The Cholinergic System

The cholinergic system has received considerable attention in AD disease research due to the early and selective dysfunction in cholinergic transmission associated with this neurodegenerative disease (55). The cholinergic hypothesis that stems from pathological, biochemical, and pharmacological observations, suggests that the cognitive and behaviour
deficits experienced in AD patients arise from the impairment in cholinergic neurotransmission (56-58). The pathological changes such as, depletion of cholinergic neurons due to decrease in neurotransmission usually have implications in biochemical functions. These biochemical disruptions in cholinergic functions are associated with severe dementia (7). Pharmacological studies done in animal models such as, rats and rodents further supported the hypothesis indicating the close relation between cognition and cholinergic neurotransmission (7, 59, 60). Cholinergic antagonists such as, antimuscarinic agents, have shown to impair performances in memory during behavioural experiments including spatial learning tests, operant tasks, and avoidance procedures (60).

Because cholinergic function plays a major role in neuronal plasticity in the adult brain, impairment in the cholinergic function in AD patients not only affects neuronal transmission but also deteriorates the brain’s ability to compensate for progressive neurodegeneration (55). Cognitive dysfunction associated with deterioration of cholinergic neurons of the basal forebrain and neocortex leads to profound deficits in the production of the neurotransmitter, acetylcholine (ACh) (Fig. 1.3) (61). The gradual loss of ACh can become lethal as in AD and not only cause impairment in cognitive function but also in autonomic and neuromuscular functions (62, 63).

![Chemical structure of acetylcholine (ACh)](image_url)

**Fig. 1.3:** Chemical structure of acetylcholine (ACh) (adapted from Milkani et al, 2011 (64)).

### 1.2.1 Acetylcholine (ACh) Production

As a result of all the research studies and findings, the primary approach for AD therapy to date involves the cholinergic replacement strategy that includes the use of acetylcholinesterase inhibitors (ChEIs) (65). ChEIs act upon the catabolic enzyme, acetylcholinesterase (AChE) in order to increase the availability of acetylcholine in the synaptic cleft (7).

Being the most biologically potent neurotransmitter in the brain, ACh is generally strictly regulated in the synaptic cleft (55). It is synthesized in the cholinergic neurons from acetyl CoA and choline with the aid of the enzyme, choline acetyltransferase (7, 55). Once produced, it is
released from the presynaptic cell into the synaptic cleft following depolarization of the cell, which allows for its binding to muscarinic and nicotinic cholinergic receptors in the pre- and post-synaptic cells. In the case of presynaptic cells, this interaction leads to the further release of Ach in the synaptic cleft. However, when Ach acts upon the postsynaptic receptors, it leads to the activation of the cell’s biochemical pathways regulating neurotransmission. For the controlled regulation of ACh in the synaptic cleft, AChE can inactivate the neurotransmitter in the synaptic cleft by hydrolyzing it into choline and acetate. Choline is taken up into the presynaptic cell for further production of ACh (Fig. 1.4) (7). Due to decreased levels of ACh in patients suffering from AD, inhibiting AChE to prolong the time ACh remains in the synaptic cleft for postsynaptic neurotransmission, can be a useful therapy in AD (7, 55).
Fig. 1.4: Schematic representation of the biosynthesis and action of ACh. ACh can be produced from acetyl CoA and choline by the action of choline acetyltransferase. It is released to the synaptic cleft upon cell depolarization upon which it can either bind to presynaptic receptors for its further release or bind to postsynaptic receptors for synaptic transmission. Acetylcholinesterase (AChE) breaks down ACh into choline and acetate, which are then taken up by the presynaptic cell (adapted from Lleo et al, 2006 (7)).

1.2.2 Acetylcholinesterase (AChE) Structure and its Mechanism of Action

Crystal structures of AChE from mouse (66), Drosophila (67), and human beings (68) were derived and found to be very similar to one another. The two sites of AChE include the active site and the peripheral anionic site (PAS) (Fig. 1.5). The active site of AChE has been shown to be located in a deep narrow gorge of about 20 Å long and located more than halfway into the enzyme (63, 69). Early kinetic studies have demonstrated that the active site contains two subsites, the ‘esteratic’ and ‘anionic’ subsites that correspond to the catalytic site and choline-binding site, respectively (70). 40% of the surface of the active-site gorge consists of 14 aromatic
amino acid residues. The gorge contains only few acidic residues including aspartic acid, glutamic acid, and tyrosine (69). Ser 203, His 447 and Glu 334 compose the catalytic triad in the ‘esteratic’ site of mammalian AChE (71, 72). AChE can be classified as a serine hydrolase due to the presence of this catalytic triad that is similar to that of other catalytic sites in serine proteases such as, trypsin and blood clot factors. These serine proteases usually have Asp in the catalytic triad; however, in AChE, Glu is substituted for Asp as the acidic residue (63). The anionic site consists of the amino acid residues Trp 86, Tyr 133, Tyr 337 and Phe 338, involved in binding to the quaternary trimethylammonium group of ACh while positioning the ester of ACh optimally at the catalytic or acylation site (72, 73).

**Fig. 1.5:** Structural features of the AchE enzyme. X-ray crystallography has identified the active site found in the deep narrow gorge of AChE and the peripheral anionic site (PAS) found near the lip of the active site (adapted from Soreq et al, 2001 (63)).

While the anionic active site is responsible for binding the quartenary trimethylammonium moiety, the esteratic active site consists of the nucleophilic Ser residue responsible for the hydrolysis of the ester bond in ACh (74, 75). The imidazole proton from a His residue causes a concerted protonation of the carbonyl oxygen and the hydroxyl group of Ser attacks the partial positive carbon of the ester carbonyl moiety of ACh. This results in the formation of a tetrahedral intermediate that collapses due to its instability, releasing choline and acetylated AChE. This
acetylated complex undergoes subsequent hydrolysis with water to release the active form of AChE and acetic acid (74, 75).

Site-directed mutagenesis studies have also indicated the presence of the PAS on the surface of AChE, which is about 20 Å distant from the active site (69, 71). It is involved in the allostERIC modulation of the protein and binds ACh during the first step of the catalytic pathway (76-78). It also has non-cholinergic functions such as, cell adhesion and neurite outgrowth in neuronal cells (77-80). Tyr 72, Asp 74, Tyr 124, Trp 286 and Tyr 341 include the five residues of the mammalian PAS found near the entrance of the active site gorge (81-83).

1.2.3 Cholinesterase Inhibitors (ChEIs)

ChEIs have been implemented effectively to restore pathologically reduced neurotransmitter levels by modulating native hydrolytic degradation catalyzed by the enzyme, AChE (6, 84). Currently, four ChEIs have been approved by the FDA for clinical treatment of mild to severe stage AD. These include: Galanthamine (REMINYL ®), Rivastigmine (EXELON ®), Tacrine (COGNEX ®) and Donepezil (ARICEPT ®) (Fig. 1.6) (85, 86). Tacrine is rarely used due to its hepatotoxicity effects (86, 87). In view of the limited number of commercially available drug therapies for AD, continued progression towards more biologically compatible ChEI is imperative (6).

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Fig. 1.6: FDA approved AChE inhibitors and the first generation of AD drugs - Tacrine (Cognex), Donepezil (Aricept), Rivastigmine (Exelon), and Galanthamine (Reminyl) - used for the symptomatic treatment of AD (adapted from Palmer et al, 2002 (87)).

1.3 AChE induced Aβ aggregation

Further motivation for the synthesis of novel ChEI has stemmed from the recent identification of AChE as an accelerant of Aβ aggregation via interaction with the PAS of the enzyme (6). This has been further confirmed by studies with butyrylcholinesterase (BuChE), a non-specific cholinesterase variant that effectively lacks a PAS, which is unable to induce such accelerated growth (88). Rivastigmine is a ‘psuedo-irreversible’ inhibitor that binds to the catalytic active site region forming a carbamylated enzyme intermediate that dissociates slowly. Conversely, Donepezil, Tacrine and Galanthamine are rapidly reversible inhibitors capable of only short-term interaction with the AChE PAS.(89, 90) Notably, certain PAS-binding ChEI, such as Donepezil, have been shown to impede AChE-induced Aβ aggregation, thereby prompting the development of a novel class of ChEI capable of mediating long-term beneficial changes (91).³

1.4 Other Pathologies in AD

Although AChE plays a pivotal role in neuronal dysfunction in AD and research findings have suggested that Aβ is the triggering event in AD pathology, there are other secondary events involved that make this multifaceted disease very challenging to treat (29). Some of these factors include oxidative stress (19), imbalance in calcium (Ca\textsuperscript{2+}) homeostasis (20), formation of NFTs (21), and activation of the microglia cells (22, 29).

1.4.1 Oxidative Stress

Many scientists have debated for years whether oxidative stress was a cause or a consequence of neurodegenerative disorders but have come to a consensus of intracellular oxidative imbalance being an early event in the neurodegenerative cascade (92). It is therefore considered to play a significant role in the pathology of neurodegenerative diseases (93). Imbalance in the homeostasis in pro-oxidant versus antioxidant in the central nervous system (CNS) can lead to the generation of toxic radical and non-radical ROS that are involved in initiating and/or propagating radical chain reactions. Damage caused by ROS is commonly observed within the brain of AD patients as neuronal tissues are extremely sensitive to oxidative stress (29). It is seen within every class of biological macromolecules: nucleic acids, proteins, lipids, and carbohydrates (94, 95). Aβ-induced free radicals can also cause secondary to excessive oxidative stress (29, 96). All the multifactorial biological pathways involved in AD seem to share oxidative stress as a common factor; hence, suggesting it to play a causative role in AD pathogenesis (29).

1.4.2 Calcium (Ca\textsuperscript{2+}) Dyshomeostasis

Khachaturian (97) was the first to propose the Ca\textsuperscript{2+} hypothesis in AD, which basically states that Ca\textsuperscript{2+} dyshomeostasis is a proximal cause in the pathogenesis of AD. This hypothesis was initially not supported by experimental evidence; however, evidence for its role in AD has now emerged (98). Studies have shown that disregulation of Ca\textsuperscript{2+} have implications on Aβ accumulation and tau hyperphosphorylation in the brain. Experimental evidence from human subjects have suggested that disruptions in the signaling of Ca\textsuperscript{2+} occur during the early phases of AD (99). However, there has been an ongoing debate as to whether this phenomena is correct or does Ca\textsuperscript{2+} dyshomeostasis precede Aβ in the cascade because it has been suggested that disregulation in
Ca\(^{2+}\) signaling is one of the ways through which A\(\beta\) toxicity is manifested (\(100, 101\)). Many researchers have argued that A\(\beta\) can exert a toxic effect on Ca\(^{2+}\) regulation (\(102, 103\)).

Ca\(^{2+}\) entry, including ligand-gated channels, voltage-dependent calcium channels (VDCCs) that permit calcium entry after membrane depolarization, and store-operated calcium channels (SOCCs), are regulated in many ways by neurons (\(104\)). An influx of extracellular Ca\(^{2+}\) into the cytosol through SOCCs on the plasma membrane triggers the decrease of calcium stores in the endoplasmic reticulum (ER). The ER membrane then pumps Ca\(^{2+}\) through the sarco-/endoplasmic reticulum Ca\(^{2+}\) ATPase located in the ER membrane to replenished the Ca\(^{2+}\) load in the ER. This mechanism of store-dependent entry of Ca\(^{2+}\) is known as capacitative calcium entry, and has been shown to contribute in the pathogenesis of Alzheimer’s disease (\(103, 105-108\)).

1.4.3 Formation of Neurofibrillary Tangles (NFTs)

NFTs consist of paired helical filaments that are composed of tau proteins in its abnormal hyperphosphorylated form (Fig. 1.12). Tau proteins are generally involved in the stability of cytoskeletal microtubule assembly, which gets hindered during phosphorylation within the microtubule binding domain of the protein (\(21\)). The tau and tangle hypothesis is AD states that the normal activity of tau proteins is impaired in AD and the microtubules are replaced with NFTs in the neurons (\(109\)). This can cause disruption in the neuronal transmission and ultimately lead to cell death. Isolating tau protein from NFTs and its subsequent dephosphorylation can restore its activity in stabilizing microtubules, which suggests that phosphorylation/dephosphorylation kinetics greatly contributes to the neurodegeneration in AD (\(21\), (29). Recent studies have shown that inhibition of the neurofibrillary degeneration in AD can be achieved by phosphoseryl/phosphothreonyl protein phosphatase-2A activation or glycogen synthase kinase-3\(\beta\) and cyclin-dependent protein kinase inhibition (\(21\), (29).

1.4.4 Chronic Activation of Microglia

Microglia cells, macrophages found in the brain and spinal cord, have received considerable attention in AD because they have been recognized in contributing to the neuroinflammation in the CNS (\(29\)). They produce cytokines, chemokines, and neurotoxins when activated by A\(\beta\) proteins. These are potentially toxic to neurons and ultimately lead to neuronal degeneration
Therefore, modulating the neuroinflammation of CNS is considered a therapeutic target for AD (29, 110).

1.5 Conclusion

AD, a multifactorial syndrome, is triggered by a cascade of molecular events such as, Aβ aggregation (18), depression of the cholinergic system, formation of toxic NFTs in neurons (21), oxidative stress (19), Ca^{2+} dyshomeostasis (20) and activation of the microglial cells (22). Although this disease is involved in multiple pathologies, ChEIs are the only drugs marketed for AD therapy. Hence, current therapeutic approaches should focus on a multi-targeted approach for drug development for the efficient treatment of such a complex disorder (29, 111). The objective of the research conducted in our laboratory involved the synthesis of potential drugs that could act as multi-target-directed ligands (MTDLs) for AD.
Chapter 2
Multi-Target-Directed Approach in Alzheimer’s Disease Therapy

2 Introduction

Combination targeting of distinct AD pathologies using multiple, independently acting drug therapies has emerged as a highly potent strategy to address the complex, degenerative nature of AD (Fig. 2.1) (6). Combination studies of ChEI have a synergistic enhancement of therapeutic effects compared to single drug therapies (112). However, the implementation of multiple single-drug entities also raises a potential for conflicting bioavailabilities, pharmacokinetics and metabolism between compounds (113, 114). More recent innovations have sought to address these concerns through incorporation of multiple pharmacophores into single drug entities, which still retain the ability of each individual component to interact with its intended target (29). Multi-targeted therapies are also expected to simplify therapeutic regiments for patients leading to improved applicability for AD care. An approach in multi-target drug therapy development for AD is to integrate a ChEI pharmacophore with a second unit capable of targeting a separate AD pathology (6, 111, 115-117).4

Fig. 2.1: Possible molecular factors involved in the pathology of AD and the potential inhibitors to target these etiologies. The multifactorial nature of the disease make it challenging to treat and requires a multi-target-directed approach for therapy (adapted from Cavalli et al., 2008 (29)).

2.1 Dual Binding ChEIs

ChEIs that are able to simultaneously interact with both the active and peripheral sites of AChE can not only help restore the cholinergic system in AD patients but also target Aβ aggregation (29). Such a compound with the ability to have dual inhibition properties was demonstrated in Melchiorre et al., 2003. This compound named caproctamine (2.1) was synthesized from a tetraamine disulfide benextramine compound, which was previously shown to act as a reversible inhibitor of AChE (Fig. 2.2) (118). Docking studies to analyze the binding mode of compound

2.1 indicated that it can bind to both AChE sites; however, this property was not verified through experimental studies (29). Structure-activity relationship (SAR) studies of 2.1 were elaborated by replacing the octamethylene spacer separating the two amide moieties with dianiline moieties to form compound 2.2 (Fig. 2.2). Compound 2.2 demonstrated the most potent inhibition of AChE (pIC$_{50} = 8.48 \pm 0.02$) in vitro. (29, 119).

**Fig. 2.2:** Design strategy for development of compounds with AChE inhibition and AChE-induced Aβ aggregation inhibition activities. Compounds 2.2 was derived through the octamethylene linker from the diamine diamide compound 2.1 (adapted from Cavalli et al, 2008 (29)).

### 2.2 Dual Inhibition of AChE and Other Neurotransmitter Systems

Behavioural disturbances and changes experienced in AD patients are not only related to severity in loss of ACh but also deterioration in serotonergic and noradrenergic systems that have been linked to depression (29, 120, 121). Neurotransmitters including serotonin, noradrenalin, and dopamine undergo deamination during the catalytic activity of the enzyme, monoamine oxidase (MAO). This results in the production of hydrogen peroxide that is a potential source of oxidative stress to neurons in the brains of AD patients (122). Hence, MAO inhibition property
incorporated in a hybrid molecule designed to be a multi-target-directed ligand (MTDL), can be a very useful property in AD therapy (29). Research studies to develop compounds with both MAO and AChE inhibition activities resulted in a series of compounds shown in Fig. 2.3. These compounds retained the propargylamine pharmacophore that have shown to exhibit irreversible MAO inhibition properties (123). Rasagiline (2.4) has previously demonstrated MAO inhibition along with neuroprotective effects in vitro and in vivo studies (29). The potential MAO/AChE inhibitor 2.5 with an indane scaffold was rationally designed by incorporating a carbamate group seen in rivastigmine (2.3) (an FDA approved ChEI) in order to exhibit AChE inhibition properties (124). Compound 2.5, named ladostigil is currently under phase II clinical trials for treatment in dementia (29, 125).

Fig. 2.3: Design strategy for the development of compounds with AChE inhibition and monoamine oxidase (MAO) inhibition activities. Compounds 2.5 incorporates the proparylgamine moiety residing the MAO inhibition property and a carbamate moiety shown to have AChE inhibition property in the FDA approved AChE inhibitor rivastagmine (2.3) (adapted from Cavalli et al, 2008 (29)).
2.3 Multi-Target Inhibition of AChE, AChE-Induced Aβ Aggregation and Oxidative Stress

Oxidative stress is considered a significant factor in AD pathology due to the neuronal damage caused by ROS (29). Lipoic acid (LA) (2.6), an antioxidant has previously shown to exert neuroprotective effects (126). Combining this antioxidant feature with a pharmacophore exhibiting a biological property such as, AChE inhibition can be an optimal design strategy for a MTDL (127). The disulfide cyclic moiety of LA was also argued to have the potential to bind to the PAS of AChE and inhibit AChE-induced Aβ aggregation. Based on these properties of LA, it was combined with the tetrahydroacridine compound, tacrine (2.7) (an FDA approved AChE inhibitor) in order to develop the compound lipocrine (2.8) for restoring cholinergic transmission, inhibit Aβ aggregation, and decrease damage caused by oxidative stress (Fig. 2.4). 2.8 demonstrated nanomolar affinity against AChE and kinetic studies confirmed its interaction with PAS of AChE. It was highly efficient in inhibiting AChE-induced Aβ aggregation and protecting cells against ROS formation compared to its parent compound, 2.6 (29, 128).

Fig. 2.4: Design strategy for the formation of 2.8 derived from the organosulphur compound 2.6 residing the antioxidant properties and the tetrahydroacridine compound 2.7 possessing the inhibition properties of AChE and AChE-induced Aβ aggregation (adapted from Cavalli et al, 2008 (29)).

2.4 Multi-Target Inhibition of AChE, BuChE and Ca^{2+} Channels

Ca^{2+} dyshomeostasis is another factor contributing to the pathology of AD as it can ultimately cause neuronal cell injury and death (29). This may occur due to overactivation of NMDA receptors that leads to the influx of excessive Ca^{2+} through the receptor’s ion channel (111).
Compounds that can possess Ca\(^{2+}\) channel blockage activity by inhibiting VDCC in dorsal root ganglionic cells \((129)\) along with AChE inhibition property would have a pivotal impact in AD research \((130, 131)\). A series of tacrine derivatives were explored for their properties as AChE/BuChE inhibitors and VDCC modulators \((132-137)\). Modifications of the structure of tacrine was achieved by replacement of the benzene ring with various substituted heterocyclic moieties and by changing the size of the cyclohexane ring to generate two series of compounds labeled \(2.15\) and \(2.16\) in Fig. 2.5. Compound \(2.17\), a 1-dihydropyridine hybrid from compound \(2.15\) series termed tacripyrine demonstrated a 4-fold higher AChE inhibition \((IC_{50} = 45 \text{ nM})\) compared to tacrine \((2.11)\) and exhibited a high selectivity towards BuChE in vitro studies. Furthermore, it displayed efficient Ca\(^{2+}\) blockage activity and provided neuroprotection in cells with Ca\(^{2+}\) overload. It also acted as a neuroprotectant to cells exposed to hydrogen peroxide with an activity 1.5 fold higher than that of its prototype \(2.14\) \((29, 137)\).

Fig. 2.5: Design strategy for the development of compounds with AChE, and butyrylcholinesterase (BuChE) inhibition properties and voltage-gated calcium channel (VDCC) blockade activity. Compound \(2.10\) was derived from \(2.7\) by altering the cyclohexane ring size and replacing the benzene ring with a heterocyclic moiety. Compound \(2.15\) includes the
dihydropyridine ring from compound 2.9 that resides the Ca$^{2+}$ channel inhibition property (adapted from Cavalli et al, 2008 (29)).

2.5 Multi-Target Inhibition of AChE, AChE-induced Aβ aggregation, Oxidative stress and N-Methyl-D-Aspartate (NMDA) Receptor

Because blocking the NMDA receptor can also modulate Ca$^{2+}$ dyshomeostasis, incorporating a pharmacophore with NMDA receptor antagonist activity in a hybrid molecule with other inhibition properties can generate a significantly potent AD drug (111). Rosini et al, 2008 reported the rational development of a potential AD drug incorporating AChE inhibition, vasodilating beta-blocker, antioxidant, and low-affinity NMDA receptor antagonist activities (111). They chose the carbazole moiety of carvedilol (2.11) as the building block for the design of their MTDL because it has been previously shown to possess antioxidant properties (I38) and inhibition of Aβ aggregation (139). In order for the carbazole compound to act as an effective AChE inhibitor, they selected the chloro-substituted tetrahydroacridine moiety of 6-chlorotacrine, which was previously used as a starting point for the formation of lipocrine (5-[1,2] dithiolan-3-ylpentanoic acid [3-(6-chloro-1,2,3,4-tetrahydroacridin-9-ylamino)-propyl] amide) (2.12), a promising lead as a MTDL for AD (127). Lipocrine’s non-cytotoxicity and bis(7)-tacrine’s (N,N’-bis-1,2,3,4-tetrahydroacridin-9-yl)heptanes-1, 7-diamine) neuroprotective property through the moderate NMDA receptor blockade (140, 141) supported the selection of the tetrahydroacridine core for the synthesis of the series of compounds 2.13, 2.14, 2.15, and 2.16, shown in Fig. 2.6 (111).

Compounds 2.13, 2.14, 2.15, and 2.16 showed AChE and BuChE inhibitory activities on human recombinant AchE and Lineweaver-Burk plots derived for AChE inhibition indicated that all the compounds demonstrated a mixed-type inhibition suggesting that they can interact with the catalytic site and PAS of AChE. These compounds also exhibited a good inhibitory potency on AChE-induced Aβ aggregation that was assessed through a Thioflavin T (ThT)-based fluorometric assay (111, 142). Activity profiles of 2.13, 2.14, 2.15, and 2.16 tested on Ca$^{2+}$ -permeable NR1/NR2A NMDA receptors expressed in Xenopus laevis revealed that all the compounds had NMDA receptor antagonist effects comparable to that of memantine, a popular NMDA receptor blocker. Compound 2.13 presented greater NMDA receptor blocking effect than
memantine and was able to counteract the production of ROS in human neuronal SH-SY5Y cells when treated with the oxidative damage inducer tert-butyl hydroperoxide (I11).

![Chemical structures](image)

**Fig. 2.6:** Design strategy for the development of compounds with AChE, AChE-induced Aβ aggregation, oxidative stress and N-methyl-D-aspartate (NMDA) inhibition activities. Compounds 2.13, 2.14, 2.15, and 2.16 possessing these inhibition properties incorporate the carbazole moiety residing the Aβ inhibiton and antioxidant properites from 2.11. It also contains the tetrahydroacridine moiety from 2.7 which has shown to possess AChE inhibition and low affinity NMDA antagonist properties (adapted from Rosini et al, 2008 (111)).

### 2.6 Conclusion

In summary, MTDL are better candidates for AD therapy due to their ability to hit multiple targets at once compared to single-target drugs whose efficacy is questionable for such a complex multifaceted disease. The compounds under investigation mentioned above for the

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treatment of AD explore multifunctional mechanisms to target the different biological pathways for a desired therapeutic agent for AD patients (29).
Chapter 3
Rational design of *sym*-Triazine Compounds for Multitarget Inhibition of Cholinesterases and Aβ in Alzheimer's Disease

3 Introduction

*sym*-Triazines (3.1), a six-membrane ring with a closed system of π electrons comprise of alternating nitrogen and carbon atoms with alternating single and double bonds (Fig. 3.1). They differ from the aromatic system of the benzene ring by the presence of the nitrogen atoms containing six unpaired electrons. The electron symmetry is destroyed in the triazine ring due to the greater electron density on the nitrogen atoms compared to that of the carbon (143-145).

The decrease in the stability of the *sym*-triazine compared to benzene allows for addition reactions such as, with hydrogen chloride to yield $2C_3H_3N_3.3HCl$ and with silver to yield $AgNO_3.2C_3H_3N_3$ and $AgNO.C_3H_3N_3$ (144). It can also undergo substituted reactions for C-halogenated triazines such as, 2, 4, 6-trichloro-1, 3, 5-triazine (cyanuric chloride, CC) (3.2) (Fig. 3.1). However, unlike benzene, triazine is incapable of electrophilic substitutions including nitration and sulphonation because of the decreased electron density on the carbon atoms. Due to this, it is not possible to synthesize C-nitro derivatives by substituting a chlorine atom in cyanuric chloride by a nitro group or by oxidizing amino derivatives of triazine (145, 146).

![Chemical structures of *sym*-triazine (3.1) and cyanuric chloride (CC) (3.2)](adapted from Mur, 1964 (145)).

**Fig. 3.1:** Chemical structures of *sym*-triazine (3.1) and cyanuric chloride (CC) (3.2) (adapted from Mur, 1964 (145)).
Hydrolysis reactions of unsubstituted symmetrical sym-triazine demonstrates its instability, especially involving mineral acids, which generates formic acid and ammonia during instantaneous reactions (143, 144). Ring opening of the triazine ring also occurs when it is treated with primary or secondary amine, which can result in the formation of substituted formamidine. Aluminium chloride can be used to weaken or break the C-N bond in triazine (145, 147).

Most reactions of sym-triazines are nucleophilic substitutions of chloro-triazines, in particular, CC (148, 149). CC is often used as a starting material due to the facile displacement of its chlorine atoms, which has been demonstrated using a number of nucleophiles in the presence of hydrochloride acceptors such as sodium carbonate, sodium bicarbonate and sodium hydroxide (145). CC is especially advantageous as it decreases in reactivity with successive substitutions (150, 151). Consequently, the number of arm substitutions can be controlled by various factors including reaction duration, solvent and base strength, the nucleophilic structure, steric factors and substituents already present in the sym-triazine ring. Temperature is a primary factor governing CC substitutions. Generally mono- substitutions were performed at approximately 4°C, di-substitutions at room temperature and tri-substitutions above 60°C. The commercial availability and low cost of CC render it a practical choice for the synthesis of sym-triazine derivatives (150, 152).

sym-Triazine derivatives have shown to possess various biological activities (150). Previous studies have indicated its properties as anti-cancer (153, 154), anti-microbial (155) as well as potential AD drugs (156-159). An efficient sym-triazine based inhibitor of Aβ aggregation or a beta-sheet breaker was discovered through a high-throughput fluorescent based screen conducted by Kim and her co-workers (156). The compound was selected based on its ability to prevent misfolding of the green fluorescent protein (GFP) when fused with Aβ_{1-42} (156). Previous studies

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done by the group demonstrated the inability of GFP to fluoresce in the presence of Aβ1-42 and Aβ1-42-GFP fusions expressed in *Escherichia coli* (*E.coli*) generated non-fluorescent colonies. Mutations in the Aβ1-42 sequence were able to retard aggregation in the fusion and hence, caused proper folding of the GFP to yield green fluorescent colonies (*160, 161*).

This artificial genetic system in *E.coli* was used to search for small molecule inhibitors that can retard Aβ1-42 aggregation in the Aβ1-42-GFP fusion (*156*). Based on this study, the compound 3.3 was found to be an effective Aβ1-42 aggregation inhibitor (Fig. 3.2). Further *in vitro* studies utilizing the ThT-based fluorescent assay proved 3.3 compound’s property in inhibiting the beta-sheet structure of Aβ1-42. Furthermore, electron microscopy studies showed modulation of the Aβ1-42 structure when conjugated with this sym-triazine compound due to the absence of the fibril structure of Aβ1-42 (*156*). 3.3 also demonstrated in further studies to have protective effects against hydrogen peroxide (*158*) and Aβ (*157*) induced damages in human SK-N-MC neuroblastoma cell lines. Moreover, cytotoxicity analysis in SK-N-MC cells indicated that the compound’s interaction with the hydrophobic region of Aβ1-42 fibrils (KLVFF amino acid sequence) decreases the toxicity of Aβ aggregates (*159*).

![Chemical structure of the sym-triazine molecule 3.3](image)

**Fig. 3.2:** Chemical structure of the sym-triazine molecule 3.3 that demonstrated beta-sheet breaking activity in Aβ1-42 in the fluorescent-based screen and was chosen for further *in vitro* studies (Adapted from Kim *et al*, 2006 (*156*)).

Considering the promising Aβ inhibition properties seen in the studies mentioned above, our laboratory has developed a library of novel sym-triazine-derived compounds capable of paralleled modulation Aβ aggregation and AChE/BuChE hydrolytic activity.
3.1 Reaction Scheme

Using an efficient synthetic route, sym-triazine based methoxy compounds, acids, acyl chlorides, esters, and ester-salt compounds were developed using CC (3.2) as a starting material (Fig. 3.3).

Fig. 3.3: Synthetic routes for new ester and ester-salt sym-triazine derivatives. sym-Triazine based methoxy compounds, acids, acyl chlorides were formed as intermediates. iReactions involving formation of sym-triazine acids 3.6, 3.7, and 3.8. iiReactions involving formation of sym-triazine acyl chloride 3.9, 3.10, and 3.11. iiiReactions involving formation of sym-triazine esters 3.12, 3.15, 3.13, 3.16, 3.14, and 3.17. ivReactions involving formation of sym-triazine ester salts 3.18, 3.21, 3.19, 3.22, 3.20, and 3.23 (6).

3.2 Experimental Procedure

Thin-layer chromatography (TLC) was performed on Merck Silica Gel 60 F_{254}, 20 x 20 cm plates and visualized using a 254 nm UV lamp. $^1$H-Nuclear Magnetic Resonance (NMR) spectra (400 MHz) (See Appendices) and $^{13}$C-NMR (100 MHz) spectra (See Appendices) were recorded on Bruker Avance III using solvents DMSO-d$_6$, D$_2$O, and CDCl$_3$ and are internally referenced to residual protic solvent signals (δ 2.50, 4.79, and 7.26 δ, respectively). Data for $^1$H-NMR are reported as chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets), J coupling constant (Hz) and assignment. Data for $^{13}$C-
NMR are reported in as chemical shift (δ ppm). Infrared (IR) spectra (See Appendices) were recorded on a Bruker Alpha-P spectrometer with ATR attachment and reported in terms of frequency of absorption (cm⁻¹). Mass spectra (MS) (See Appendices) were obtained using an AB/Sciex QStar mass spectrometer (ESI-TOF) in positive ion mode. Melting points were recorded on a melting point apparatus (Fisher Scientific). Reagents were obtained from commercial vendors and used as received unless otherwise noted (6).

3.2.1 Procedure for sym-Triazine Based Methoxy Compounds

2-chloro-4,6-dimethoxy-1,3,5-triazine (3.4) 2,4-dichloro-6-methoxy-1,3,5-triazine (3.5) (Fig. 3.4): 3.4 and 3.5 can be synthesized following the known procedure by Dudley (6, 162).

![Synthesis of sym-triazine based methoxy compounds 3.4 and 3.5 from CC (3.2). Reactions were carried out using methanol (CH₃OH) as the reagent and sodium bicarbonate (NaHCO₃) as the base.]

3.2.2 Procedure for sym-Triazine Based Acids

![Synthesis of sym-triazine based acids 3.6, 3.7, and 3.8. Reactions were run using oxybenzoic acid, sodium hydroxide (NaOH) and water in acetone. Acidification was performed using concentrated hydrochloric acid (HCl) to yield 3.6, 3.7, and 3.8.]

4-((4,6-dimethoxy-1,3,5-triazin-2-yl)oxy)benzoic acid (3.6) and 4,4′-((6-methoxy-1,3,5-triazin-2,4-diyl)bis(oxy))dibenzoic acid (3.7) (Fig. 3.5): 3.6 and 3.7 can be synthesized following the known procedure by Pogosyan (6, 163).

2,4,6-tris (4′-Chlorocarbonylphenoxy )-1,3,5- triazine (3.8) (Fig. 3.5): 3.8 can be produced in one step by CC (3.2) and 4-oxybenzoic acid; applying the known method by Sklyarskii (6, 164).

3.2.3 Procedure for sym-Triazine Based Acyl Chlorides

![Chemical Structures](image)

**Fig. 3.6:** Synthesis of sym-triazine based acyl chlorides 3.9, 3.10, and 3.11. iiReactions were run at 60°C in thionyl chloride (SOCl₂) using anhydrous chloroform (CHCl₃) as solvent and pyridine as catalyst.

4-((4,6-dimethoxy-1,3,5-triazin-2-yl)oxy)benzoyl chloride (3.9) (Fig. 3.6) (Procedure A): The mixture of 11 g of 3.6 (0.04 mol), 8.82 mL of thionyl chloride (SOCl₂) (0.12 mol) and one drop of pyridine in 100 mL of dry chloroform was refluxed and boiled for 6 hours. The reaction was monitored by thin layer chromatography (TLC) for completion. Chloroform was distilled off at 60 °C until the solution started to turn light yellow. 120 mL of petroleum ether was added to the solution and allowed to precipitate for an hour. The white precipitate was washed with petroleum ether. The white solid had a melting point of 105 °C - 107 °C with a 60.6 % final yield (6).

IR (neat) ν = 3072.00, 1771.45, 1746.00, 1564.14, 1468.49, 1199.02, 806.35 cm⁻¹; ¹H-NMR (400 MHz, CDCl₃) δ 8.21-8.19 (d, J = 8.9 Hz, 2 H), 7.36-7.34 (d, J = 8.9 Hz, 2 H), 4.02 (s, 6 H); ¹³C-
NMR (100.42 MHz, CDCl$_3$) δ 174.1, 173.0, 167.9, 157.1, 133.4, 121.5, 55.9; Calculated C$_{12}$H$_{10}$N$_{3}$O$_{4}$Cl (M +H)$^+$: 295.68, found: 296.0.

4,4'-(6-methoxy-1,3,5-triazine-2,4 diyl)bis(oxy))dibenzoyl chloride (3.10) (Fig. 3.6): 5g (0.01 mol) of 3.7, 11.25mL (moles) of SOCl$_2$, one drop of pyridine in 100 mL of dry chloroform was heated until boiling for 6 hours. Procedure A was followed to provide 3.10 in 79.8 % yield as a yellow powder with a melting range of 133 °C - 135 °C (6).

IR (neat) ν = 3076.93, 1744.13, 1560.86, 1496.04, 1197.93, 815.30 cm$^{-1}$; $^1$H-NMR (400 MHz, CDCl$_3$) δ 8.21-8.19 (d, J = 8.8 Hz, 4 H), 7.35-7.33 (d, J = 8.8 Hz, 4 H), 3.98 (s, 3 H); $^{13}$C-NMR (100.42 MHz, CDCl$_3$) δ 172.8, 169.0, 167.8, 157.0, 133.8, 131.7, 121.4, 56.5, Calculated C$_{18}$H$_{11}$N$_{3}$O$_{5}$Cl$_2$ (M +H)$^+$: 420.2, found: 420.1.

2,4,6-tris(4'-Chlorocarbonylphenoxy)-1,3,5-triazine (3.11) (Fig. 3.6): The mixture of 4.894 g (0.01 mol) of 3.8 and 43.5 mL (0.60 mol) of SOCl$_2$ in 80 mL of dry chloroform and 3 drops of dry pyridine (catalyst) was refluxed under dry conditions. Procedure A was followed to provide 3.11, light yellow powder with 185 - 188 °C melting point and final yield of 88.7 % (6).

IR (neat) ν: 3107.63, 3074.38, 1778.23, 1737.83, 1605.77,1560.62, 1494.60, 1209.28, 1193.44, 1166.72,1085.76,1016.90, 850.96; $^1$H-NMR (400 MHz, CDCl$_3$) δ 8.19-8.17 (d, J = 8.8 Hz, 6H), 7.32-7.29 (d, J = 8.8 Hz, 6H); $^{13}$C-NMR (100.42 MHz, CDCl$_3$) δ 173.4, 167.0, 158.3, 133.5, 131.8, 122.0; Calculated C$_{24}$H$_{12}$N$_{3}$O$_{6}$Cl$_3$ (M +H)$^+$: 544.8, found: 545.0.
3.2.4 Procedure for \textit{sym}-Triazine Based Esters

Fig. 3.7: Synthesis of \textit{sym}-triazine based esters 3.12, 3.15, 3.13, 3.16, 3.14, and 3.17.

Reactions were carried out using a: 3-dimethylamino-1-propanol, b: 1-dimethylamino-2-propanol and c: 2-dimethylaminoethanol in anhydrous tetrahydrofuran (THF) in the presence of triethylamine ((C\textsubscript{2}H\textsubscript{5})\textsubscript{3}N) as a base.

3-(dimethylamino)propyl-4-((4,6-dimethoxy-1,3,5 triazin-2-yl)oxy)benzoate (3.12) (Fig. 3.7) (Procedure B): 2.0 g of 3.9 was dissolved in 40 mL of dry tetrahydrofuran (THF). The solution of 6.76 mmol (0.94 mL) of dry triethylamine and 6.76 mmol (0.80 mL) of 3-dimethylamino-1-propanol in 10 mL of dry THF was added drop wise to the 3.9 solution. The reaction was refluxed and temperature maintained at 40 °C. Reaction was monitored by TLC for completion. The ester slurry was gravity filtered and washed with 3 x 6 mL of dry THF. The combined filtrate was evaporated under vacuum conditions. 50 mL of dichloromethane was used to dissolve the ester residue. The dichloromethane layer was washed with 6 x 50 mL water and the organic layer was collected. The ester solution was dried with anhydrous sodium sulphate (Na\textsubscript{2}SO\textsubscript{4}) overnight. The resulting residue was washed with small portions of petroleum ether (40 - 60 °C) resulting in the formation of a precipitate. Petroleum ether was evaporated 3.12 was collected in 68.6 % yield as a white powder with melting point of 60 °C - 64 °C (6).

IR (neat) \(v = 2953.87, 2822.95, 2764.52, 1709.96, 1567.03, 1468.08, 1264.86, 1220.38, 806.66 \text{ cm}^{-1}\);

\(^1\)H-NMR (400 MHz, CDCl\textsubscript{3}) \(\delta 8.11-8.09 \text{ (d, } J = 8.8 \text{ Hz, } 2\text{H}), 7.27-7.25 \text{ (d, } J = 8.8 \text{ Hz, } 2\text{H}),\)}
1-(dimethylamino)propan-2-yl-4-((4,6-dimethoxy-1,3,5-triazin-2-yl)oxy)benzoate (3.15) (Fig. 3.7): 2.0 g of 3.9 was dissolved in 40 mL of dry THF. The solution of 6.76 mmol (0.94 mL) of dry triethylamine and 6.76 mmol (0.83 mL) of 1-dimethylamino-2-propanol in 10 mL of dry THF was added drop wise to the 3.9 solution. Procedure B was followed to provide 3.15 in 41.6% yield as a white powder with melting point of 48 °C – 52 °C.

IR (neat) ν = 2979.50, 2820.82, 2765.94, 1715.25, 1566.16, 1467.85, 1267.09, 1217.91, 806.42 cm⁻¹; ¹H-NMR (400 MHz, CDCl₃) δ 8.11–8.09 (d, J = 8.8 Hz, 2 H), 7.27–7.25 (d, J = 8.8 Hz, 2 H), 5.38–5.30 (m, 1 H), 4.00 (s, 6H), 2.74–2.69 (dd, 1H), 2.49–2.44 (dd, 1H), 2.28 (s, 6 H), 1.38–1.36 (d, J = 6.4 Hz, 3H); ¹³C-NMR (100.42 MHz, DMSO-d₆) δ 174.5, 172.9, 165.8, 155.8, 131.8, 127.8, 122.9, 63.6, 56.4, 56.1, 45.5, 26.5; Calculated C₁₇H₂₂N₄O₅ ([M +H]⁺): 362.4, found: 363.0.

Bis(3-(dimethylamino)propan-yl)4,4'-(6-methoxy-1,3,5-triazine-2,4-diyl)bis(oxy)dibenzoate (3.13) (Fig. 3.7): 2.0 g (0.0048 mol) of 3.10 was dissolved in 55 mL of THF. 1.32 mL of triethylamine and 0.0095 moles (1.13 mL) of 3-dimethylamino-1-propanol in 18.96 mL of THF was added to the 3.10 solution in THF. Procedure B was followed to provide 3.13 in 53.5% yield as a yellow powder with melting point of 110 °C - 125 °C.

IR (neat) ν = 2948.39, 2856.39, 2768.09, 1710.28, 1578.14, 1466.51, 1268.23, 1207.71, 818.89 cm⁻¹; ¹H-NMR (400 MHz, DMSO-d₆) δ 8.01–7.98 (d, J = 8.8 Hz, 4 H), 7.26–7.24 (d, J = 8.8 Hz, 4 H), 4.35–4.32 (t, 4H), 3.50–3.45 (t, 4H), 3.08 (s, 3H), 2.17 (s, 12H), 1.88–1.81 (m, 1H); ¹³C-NMR (100.42 MHz, DMSO-d₆) δ 172.8, 170.0, 166.0, 157.7, 131.5, 126.5, 123.0, 62.6, 56.0, 53.0, 45.5, 26.8, Calculated C₂₈H₃₅N₅O₇ ([M +H]⁺): 553.7, found: 554.3.

Bis(1-(dimethylamino)propan-2-yl)4,4'-(6-methoxy-1,3,5-triazine-2,4-diyl)bis(oxy)dibenzoate (3.16) (Fig. 3.7): 2.0 g (0.0048 mol) of 3.10 was dissolved in 55 mL of THF. 1.32 mL of triethylamine and 0.009519 moles (1.126mL) of 1-dimethylamino-2-propanol in 18.96 mL of THF was added to the 3.10 solution in THF. Procedure B was followed to provide 3.16 in 34.2% yield as a yellow powder with melting point of 77 °C - 82 °C.
IR (neat) v = 2976.79, 2822.66, 2766.79, 1710.21, 1556.42, 1469.03, 1265.37, 1208.40, 814.06 cm⁻¹; ¹H-NMR (400 MHz, DMSO-d₆) δ 8.05-8.03 (d, J = 8.8 Hz, 4H), 7.29-7.27 (d, J = 8.8 Hz, 4H), 5.55-5.50 (m, 2H), 3.96-3.92 (dd, 2H), 3.69-3.66 (dd, 2H), 3.16 (s, 3H), 2.09 (s, 12H), 1.38-1.36 (d, J = 6.4 Hz, 6H); ¹³C-NMR (100.42 MHz, DMSO-d₆) δ 184.05, 180.47, 159.79, 157.57, 131.21, 125.87, 122.77, 66.24, 53.42, 45.90, 31.01, 19.06; Calculated C₂₈H₃₅N₅O₇ ([M +H]⁺): 553.7, found: 554.3.

2,4,6-tris[(2'-dimethylamino-1'-ethoxy)-4']-1,3,5-triazine (3.14) (Fig. 3.7): 3.0 g (5.51 mmol) of 3.11 was dissolved in 60 mL of dry THF at room temperature conditions. The solution of 2.3 mL of 2-dimethylaminoethanol and 2.5 mL of triethylamine in 10 mL of dry THF was added dropwise to the 3.11 solution. Procedure B was followed however with the temperature for reaction maintained at 15 °C. Compound 3.14 formed had a melting point of 85 °C - 87 °C and final yield of 17 % (6).

IR (neat) v: 2968.62, 2947.08, 2821.10, 2769.13, 1715.83, 1605.70, 1569.88, 1503.57, 1464.05, 1412.61, 1360.34, 1269.70, 1211.83, 1115.89, 1017.03, 863.99; ¹H-NMR (400 MHz, DMSO-d₆) δ 8.01-7.99 (d, J = 8.8 Hz, 6H); 7.42-7.40 (d, J = 8.8 Hz, 6H), 4.38-4.36 (t, J = 5.6 Hz, 6H), 2.67-2.65 (t, J = 5.6 Hz, 6H), 2.24 (s, 18H); ¹³C-NMR (100.42 MHz, DMSO-d₆) δ 173.3, 166.1, 155.0, 131.8, 128.2, 122.6, 62.9, 57.5, 46.0; Calculated C₃₆H₄₂N₆O₉ ([M +H]⁺): 702.9, found: 704.3.

2,4,6-tris[(1'-dimethylamino-2'-propano)-4'-carbonylphenoxy]-1,3,5-triazine (3.17) (Fig. 3.7): 1.3 g (2.39 mmol) of 3.11 was dissolved in 60 mL of dry THF at room temperature conditions. The solution of 0.725 g (7.16 mmol) of 1-dimethylamino-2-propanol and 0.725 g (7.16 mmol) of triethylamine in 10 mL of dry THF was added dropwise to the 3.11 solution under anhydrous conditions. Procedure B was followed with temperature maintained at 40 °C to provide 3.17 in 64.4 % yield as a white powder with melting point of 76 °C - 82 °C (6).

IR (neat) v: 2978.12, 2947.08, 2821.10, 2769.07, 1711.82, 1593.67, 1566.82, 1502.47, 1461.03, 1412.42, 1360.07, 1265.59, 1210.33, 1160.89, 1014.13, 812.56 cm⁻¹, ¹H-NMR (400 MHz, DMSO-d₆) δ 8.01-7.99 (d, J = 8.8 Hz, 6H), 7.42-7.40 (d, J = 8.8 Hz, 6H), 5.5-5.19 (m, 3H), 2.67-2.63 (dd, 3H), 2.44-2.44 (dd, 6H), 2.24 (s, 18H), 1.30-1.28 (d, J = 6.3, 9H); ¹³C-NMR (100.42 MHz, DMSO-d₆) δ 172.6, 164.7, 153.4, 131.3, 128.4, 122.3, 69.5, 63.7, 46.0, 19.1; Calculated C₃₉H₄₅N₆O₉ ([M +H]⁺): 745.0, found: 746.3.
3.2.5 Procedure for *sym*-Triazine Based Iodine-Ester Salts

![Chemical structures and reactions](image)

**Fig. 3.8:** Synthesis of *sym*-triazine based ester salt compounds 3.18, 3.21, 3.19, 3.22, 3.20, and 3.23. Reactions were carried out at 40°C using iodomethane (CH₃I) in anhydrous THF.

3-((4-((4,6-dimethoxy-1,3,5-triazin-2-yl)oxy)benzoyl)oxy)-N,N,N-trimethylpropan-1-aminium iodide (3.18) (Procedure C): 2.06 mL of iodomethane (0.033 mol) was added drop wise to the solution of 1.20 g (3.3 mmol) of 3.12 in 40 mL of dry THF. The mixture was stirred two hours at 40 °C and left overnight under dry conditions. The resulting precipitate was filtered, washed by 2 × 6 mL of dry THF and 2 × 10 mL of petroleum ether to provide 3.18 in 88.0% yield as a white powder with a melting point of 176 °C - 179 °C (6).

IR (neat) ν = 3002.94, 2954.31, 1717.40, 1554.68, 1459.01, 1266.75, 1215.76, 815.41 cm⁻¹;¹H-NMR (400 MHz, D₂O) δ 8.02-8.01 (d, J = 8.9 Hz, 2H), 7.27-7.25 (d, J = 8.9 Hz, 2H), 4.37-4.35 (t, 2H), 3.88 (s, 6H), 3.46-3.43 (t, 2H), 3.06 (s, 6H), 2.28-2.20 (m, 2H);¹³C-NMR (100.42 MHz, D₂O) δ 173.6, 172.7, 167.6, 155.5, 131.6, 126.7, 122.2, 64.3, 62.5, 56.4, 53.3, 22.7; Calculated C₁₈H₂₅N₄O₅ ([M +H]⁺): 377.3, found: 377.2.

3-((4-((4,6-dimethoxy-1,3,5-triazin-2-yl)oxy)benzoyl)oxy)-N,N,N,2-trimethylpropan-1-aminium iodide (3.21) (Fig. 3.8): 1.60 mL of iodomethane (0.025 mol) was added drop wise to the solution of 0.92 g (0.0025 mol) of 3.15 in 40 mL of dry THF. Procedure C was followed to provide 3.21 in 74.2% yield as a white powder with a melting point of 188 °C - 191 °C (6).
IR (neat) ν = 2997.32, 2952.72, 1721.29, 1558.83, 1467.22, 1256.47, 1220.57, 810.91 cm⁻¹; ¹H-NMR (400 MHz, D₂O) δ 8.05-8.03 (d, J = 8.8 Hz, 2H), 7.27-7.25 (d, J = 8.8 Hz, 2H), 5.64-5.56 (m, 1H), 3.93-3.89 (dd, 1H), 3.47-3.42 (dd, 1H), 3.11 (s, 9H), 1.36-1.34 (d, J = 6.43 Hz, 3H); ¹³C-NMR (100.42 MHz, D₂O) δ 173.6, 172.7, 166.2, 155.8, 132.0, 127.5, 122.2, 67.6, 56.2, 54.2, 54.1, 19.0; Calculated C₁₈H₂₅N₄O₅ ([M +H]⁺): 377.3, found: 377.2.

3,3’-((4,4’-((6-methoxy-1,3,5-triazine-2,4-diyl)bis(oxy))bis(benzoyl))bis(oxy))bis(N,N,N-trimethylpropan-1-aminium) iodide (3.19) (Fig. 3.8): 0.38 mL (6.1mmol) of iodomethane was added drop wise to the solution of 1.3 mmol of 3.13 in 34.6 mL of THF. Procedure C was followed to provide 3.19 in 38.7 % yield as a yellow powder with melting point of 180 °C - 185 °C (6).

IR (neat) ν = 3014.25, 2956.28, 1710.56, 1559.66, 1476.14, 1271.12, 1208.75, 821.05 cm⁻¹; ¹H-NMR (400 MHz, D₂O) δ 7.84-7.82 (d, J = 8.8 Hz, 4H), 7.19-7.17 (d, J = 8.8 Hz, 4H), 4.33-4.29 (t, 4H), 3.8 (s, 3H), 3.65-3.61 (t, 4H), 3.01 (s, 18H), 1.78-1.74 (m, 2H) ; ¹³C-NMR (100.42 MHz, D₂O) δ 181.2, 180.1, 167.3, 155.7, 131.6, 127.1, 122.2, 68.0, 64.0, 53.3, 53.0, 22.5, Calculated C₃₀H₄₁N₅O₇ ([M + 2H]²⁺): 291.9, found: 291.7.

2,2’-((4,’4-((6-methoxy-1,3,5-triazine-2,4-diyl)bis(oxy))bis(benzoyl))bis(oxy))bis(N,N,N-trimethylpropan-1-aminium) iodide (3.22) (Fig. 3.8): 0.38 mL (6.1mmol) of iodomethane was added drop wise to the solution of 1.3 mmol of 3.16 in 34.6 mL of THF. Procedure C was followed to provide 3.22 in 44.4 % yield as a yellow powder with melting point of 205 °C - 210 °C (6).

IR (neat) ν = 3007.65, 2958.47, 1709.58, 1560.48, 1498.38, 1264.01, 1210.11, 765.47 cm⁻¹; ¹H-NMR (400 MHz, DMSO-d₆) δ 8.13-8.11 (d, J = 8.5 Hz, 4H), 7.48-7.46 (d, J = 8.5 Hz, 4H), 5.56-5.50 (m, 2H), 3.98-3.94 (dd, 2H), 3.72-3.69 (dd, 2H), 3.18 (s, 3H), 2.09 (s, 18H), 1.39-1.37 (d, J = 6.3 Hz, 6H); ¹³C-NMR (100.42 MHz, DMSO-d₆) δ 180.06, 176.57, 174.34, 164.18, 131.60, 127.17, 121.33, 68.47 ,66.71, 53.43, 19.50; Calculated C₃₀H₄₁N₅O₇ ([M + 2H]²⁺): 291.9, found: 291.7.

2,4,6-tris[iodomethylate(2’-trimethylamino-1’-ethoxy)-4’-carbonylphenoxy] 1,3,5-triazine (3.20) (Fig. 3.8): 0.62 g (0.88 mmol) of purified 3.14 was dissolved in 45 mL of dry THF at room temperature conditions. Separately, 0.4 mL (6.2 mmol) of methyl iodide was dissolved in 15 mL
of THF and stirred for 3 h. Procedure C was followed to provide 3.20 with a melting point >185 °C with decomposition and a final yield of 68.0 % (6).

IR (neat) v: 3005.09, 2960.27, 1716.52, 1605.43, 1568.43, 1502.64, 1267.58, 1211.31, 1164.68, 1114.20, 1013.18 cm⁻¹; ¹H-NMR (400 MHz, DMSO-d₆) δ 8.10-8.08 (d, J = 8.8 Hz, 6H); 7.47-7.45 (d, J = 8.8 Hz, 6H), 4.74-4.73 (t, 6H); 3.85-3.83 (t, 6H), 3.23 (s, 27H); ¹³C-NMR (100.42 MHz, DMSO-d₆) δ 173.06, 165.08, 155.43, 131.66, 127.24, 122.32, 64.48, 59.21, 53.47; Calculated C₃₉H₅₁N₆O₉ ([M +H]³⁺): 249.3, found: 249.5.

2,4,6-tris[iodomethylate(1′-trimethylamino-2′-propoxy)-4′-carbonylphenoxy] 1,3,5-triazine (3.23) (Fig. 3.8): 0.65 mL (10.6 mmol) of methyl iodine was added drop wise into 1.16 g (1.56 mmol) solution of 3.17 in 40 mL of dry THF at room temperature conditions. Procedure C was followed to provide 3.23 as light yellow powder with 189 °C - 191 °C melting point and final yield of 89.1 % (6).

IR (neat) v: 3003.32, 2956.57, 1711.91, 1604.65, 1567.36, 1501.60, 1264.14, 1210.76, 1162.85, 1092.82, 1013.12; ¹H-NMR (400 MHz, DMSO-d₆) δ 8.12-8.10 (d, J = 8.8 Hz, 6H), 7.47-7.45 (d, J = 8.8 Hz, 6H); 5.56-5.50 (m, 3H), 3.98-3.93 (dd, 3H), 3.72-3.69 (dd, 3H), 3.30 (s, 27H); ¹³C-NMR (100.42 MHz, DMSO-d₆) δ 173.06, 165.08, 155.43, 131.66, 127.24, 122.32, 64.48, 59.21, 53.47; Calculated C₄₂H₅₇N₆O₉ ([M +H]³⁺): 263.4, found: 263.5.

### 3.3 Discussion

Initial derivatization of CC (3.2) involved the incorporation of methoxy groups (3.4 and 3.5) via nucleophilic aromatic substitution of chlorine with methanol, following procedures established by Dudley (6, 162). The predicted reaction mechanism is shown in Fig. 3.9 (165). The nucleophile, methanol can attack the carbon attached to the leaving group, chloride ion. This nucleophilic attack results in a carbanian intermediate that is resonance-stabilized. The chloride ion then leaves, which regains the aromaticity of the ring (165). Methanol substitution was integral for controlling the number of Aβ and AChE-targeted substitutions within the triazine core. Accordingly, the synthesis of 3.5 was performed at 0°C in order to displace only a single chlorine unit in the triazine ring of CC, leaving two chlorine units available for further derivatization forming the targeted di-substituted species. Similarly, the synthesis of 3.4
required reaction temperatures of 60ºC for the displacement of two chlorine units, leaving a single unit available for derivitization of a mono-substituted triazine. Temperature was a key factor in this reaction as synthesis of 3.5 resulted in a mixture of mono- and di-substituted sym-triazine methoxy compounds when temperature conditions were over 0ºC. The resulting products (3.4 and 3.5) were washed with water to eliminate inorganic salts formed during the reaction (6).

![Reaction mechanism](image)

**Fig. 3.9:** Reaction mechanism for the formation of sym-triazine based methoxy compound (adapted from Bruice, 2001 (165)).

The sym-triazine-based carboxylic acids, 3.6 and 3.7, were subsequently synthesized from the methoxy derivatives, 3.4 and 3.5 respectively, using 4-oxybenzoic acid reported by Pogosyan et al (6, 163). The predicted reaction mechanism is shown in Fig. 3.10 (165). The base, sodium hydroxide (NaOH) can deprotonate the two oxygen molecules on 4-oxybenzoic acid, which can then cause this nucleophile to attack the carbon bearing the chlorine (leaving group) to eventually form the sym-triazine based acids. The tri-substituted acid, 3.8, was directly synthesized from a reaction of 4-oxybenzoic acid with CC (3.2) following conditions and procedures developed by Sklyarskii et al.(164). Sodium hydroxide was employed as a base to synthesize oxybenzoate salts of 3.6, 3.7 and 3.8 followed by the addition of dilute hydrochloric acid (HCl) to the salt solution. This resulted in the precipitation of compounds 3.6, 3.7 and 3.8 that were washed thoroughly with water to yield the final sym-triazine acids. Notably, we have observed that Aβ aggregation could be severely impaired *in vitro* by the presence of hydrophobic poly aromatic compounds, which are capable of disrupting or preventing π-π stacking. Thus, the addition of multiple aromatic functional units by substitution with oxybenzoate salts was performed for rational targeting of Aβ aggregation (6).
Fig. 3.10: Reaction mechanism for the formation of mono-substituted sym-triazine based acid (adapted from Bruice, 2001 (165)).

The synthesis of the acyl chloride compounds 3.9, 3.10 and 3.11 from 3.6, 3.7 and 3.8, respectively, by nucleophilic acyl substitution reaction involved replacement of the hydroxy groups of the carboxylic acids with chlorine functional units (6). To accomplish this, the carboxylic acid derivatives were treated with thionyl chloride using anhydrous chloroform as the solvent with a pyridine catalyst. The predicted reaction mechanism is shown in Fig. 3.11 (166). The nucleophile, carbonyl oxygen can attack the sulphur of thionyl chloride to form a tetravalent sulphur intermediate. Subsequently, the chloride ion is displaced from the intermediate, which results in an acyl chlorosulphite intermediate and at the same time, also reestablishing the sulphur-oxygen π bond. The chloride ion that was lost from the intermediate can then attack the activated carbonyl group, which eventually causes the displacement of the thionyl chloride group and formation of the sym-triazine based acyl chloride (166). IR spectroscopic data indicated formation of the acyl chloride by a shift of carbonyl and carboxyl absorption peaks as well as the disappearance of the hydroxyl peak. The resulting acyl chlorides were unstable hygroscopic species that could be easily converted back to their corresponding carboxylic acid derivatives simply by exposure to air. Due to this reason, anhydrous chloroform was employed and a drying tube was used to ensure no exposure to air and water (6).
Due to the instability of acyl chlorides, esterification was performed immediately following the formation of the acyl chlorides by nucleophilic addition-elimination or nucleophilic acyl substitution with various combinations of the following compounds: 1-dimethylamino-2-propanol, 3-dimethylamino-1-propanol and 2-dimethylaminoethanol (6). The predicted reaction mechanism is shown in Fig. 3.12 (165). In order to convert the acyl chloride to an ester, the amino alcohol (1-dimethylamino-2-propanol, 3-dimethylamino-1-propanol or 2-dimethylaminoethanol) can attack the carbonyl carbon of the sym-triazine based acyl chloride. A tetrahedral intermediate is formed followed by the expulsion of the chloride ion as it is a weaker base compared to the alkoxide ion (165). This can then result in the formation of the sym-triazine based ester compounds. These compounds were designed as analogues of the native enzyme substrate, acetylcholine, and were thus incorporated into the triazine structure for functional targeting of the AChE active site (6).

All ester compounds demonstrated moderate to high conversion to the esters except for one due to the different temperature conditions employed (6). 3.11 reacted with 2-dimethylaminoethanol at a temperature maintained at 15 °C, which generated the ester, 3.14, in a poor isolated yield of 17%. Hence, reaction conditions were optimized for the formation of esters 3.12, 3.15, 3.13, 3.16 and 3.17 by carrying out their synthesis at 40°C. The triethylamine hydrochloride salt formed during these esterification reactions was eliminated from the solvent THF comprising the ester.
For each esterification reaction, brown, viscous oil resulted after evaporation of THF, which was washed several times with water to allow the esters to precipitate out. Water was eliminated to obtain the solid esters, 3.12, 3.15, 3.13, 3.16, 3.14 and 3.17 in 68.6, 41.6, 53.5, 34.2, 17.0 and 64.4% yields, respectively (6).

![Reaction mechanism for the formation of mono-substituted sym-triazine based ester](image)

**Fig. 3.12:** Reaction mechanism for the formation of mono-substituted *sym*-triazine based ester (adapted from Bruice, 2001 (165)).

Further electrophilic substitution of the ester products with iodomethane (CH$_3$I) was used to generate a series of novel iodine-ester salts, 3.18 in 88.0% yield, 3.21 in 74.2% yield, 3.19 in 38.7% yield, 3.22 in 44.4% yield, 3.20 in 68.0% yield and 3.23 in 89.1% yields (6). The predicted reaction mechanism is shown in Fig. 3.13 (165). The tertiary amide of the *sym*-triazine based esters can attack the methyl group of iodomethane and result in a protonated quartenary ammonium iodide (165). The compounds 3.21, 3.22 and 3.23 were isolated as stereoisomers. These compounds initially precipitated out in THF during the reaction upon addition of CH$_3$I and had to be washed off with THF and petroleum ether to get rid of any impurities and yield the final solid ester-salt compounds. They were initially tested for their solubility in water to confirm that they were salts and not their corresponding esters that are insoluble in water (6).
**Fig. 3.13:** Reaction mechanism for the formation of mono-substituted *sym*-triazine based ester-salt (adapted from Bruice, 2001 (165)).

Reactions for all compounds synthesized were monitored using thin layer chromatography (TLC) in 1:1 acetone and hexane solution and structures were confirmed using IR, MS, $^1$H NMR and $^{13}$C NMR (See Appendices) (6).

### 3.4 Inhibition of AChE, BuChE, and Aβ Aggregation by *sym*-Triazine Derivatives and their Cell Viability Studies

The *sym*-triazines esters and ester-salt compounds containing the hydrophobic aromatic phenyl were analyzed for their activity as Aβ fibrillogenesis inhibitors (6) (Fig. 3.14). The esters and ester-salt compounds were also tested for their inhibition activities against AChE and BuChE. The ester-salt *sym*-triazines differed from their corresponding esters by the positive charge on the nitrogen atom in the ester chain and by an additional methyl group on the positively charged nitrogen. It would be interesting to observe how this affects the *in vitro* AChE inhibition studies as the ester-salt component of the ester-salt *sym*-triazines resemble the structure of ACh (6).
Fig. 3.14: sym-Triazine esters, ester-salt compounds and a negative control (3.24) tested for their activity as AChE, BuChE, and Aβ aggregation inhibitors (6).
3.4.1 Cholinesterase inhibition studies

The inhibitory effects of 12 novel sym-triazine derivatives (Fig. 3.14) on AChE activity was characterized using Ellman's colorimetric assay \((6, 91, 116, 167, 168)\), in which AChE-catalyzed hydrolysis was initiated in the presence of chromogenic agent, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). Enzyme activity was determined by comparing the rate of substrate hydrolysis \((\Delta \text{OD}_{410}/\text{min})\) in the presence or absence of inhibitor. The AChE IC\(_{50}\) values for all sym-triazines are reported in Fig. 3.15, and correspond to the concentration of inhibitor that resulted in a 50% reduction of enzyme activity. Clinically established cholinesterase inhibitor (ChEI), Donepezil, was implemented as a positive control for AChE inhibition. The AChE IC\(_{50}\) for Donepezil was determined to be approximately 0.02 µM, in agreement with previously reported values \((116, 169)\).

All quaternary amines exhibited drastic improvements to inhibition with increasing arm substitutions, with tri-substituted compounds, 3.20 and 3.23, resulting in the most profound inhibition of AChE activity (IC\(_{50}\) 0.3 µM and 2.8 µM, respectively) \((6)\). A negative control lacking the acetylcholine-like substitutions, 3.24 (Fig. 3.14), showed lower activity (IC\(_{50}\) of 153.3 µM) than the least effective quaternary-amine-based sym-triazine, 3.18 (IC\(_{50}\) 38.8 µM), thus emphasizing the relevance of positive charge in addition to acetylcholine-like structures for enhancing targeted inhibition of AChE. Analysis of BuChE IC\(_{50}\) (Table 1) indicated that all compounds possessed substantially higher AChE selectivity (i.e. lower IC\(_{50}\)), suggesting that interaction with the PAS was critical to sym-triazine-mediated cholinesterase inhibition. The most effective BuChE inhibitors, 3.20 and 3.23, exhibited similar activity (BuChE IC\(_{50}\) 3.9 µM and 15.3 µM, respectively) to Donepezil (BuChE IC\(_{50}\) 4.0 µM) \((6)\).

3.4.2 Inhibition of Aβ\(_{1-40}\) Aggregation

Targeting of Aβ aggregation was achieved primarily through the addition of multiple aromatic phenyl groups proximal to the triazine core \((6)\). Thus, a significant reduction of Aβ\(_{1-40}\) aggregation was observed by ThT fluorescence, as the number of phenyl-containing substitutions was increased between mono- and tri-substituted derivatives. This measured activity was substantially greater than established penta-peptide-based β-sheet breaker, iAβ5p (58.5 %). ThT studies also confirmed that Donepezil exhibited a near negligible effect on amyloid aggregation.
(6.9%), further highlighting the significance of rational sym-triazine functionalization for β-sheet targeting (6).

Transmission electron microscopy (TEM) studies confirmed the inhibition of fibril formation using quaternary amine-salt converted sym-triazines 3.19, 3.22, 3.20, 3.23 as well as iAβ5p, measured after 4 days of incubation (6). Aβ1-40 controls showed distinct fibrillar networks of defined elongated structure in the absence of sym-triazines. As in fluorescence studies, Donepezil appeared to demonstrate no observable effect on the fibril elongation process. While in the presence of known inhibitor, iAβ5p, a mixture of both globular aggregates and fibrils was observed, suggesting incomplete inhibition concurrent with fluorescence data. Conversely, Aβ1-40 samples containing sym-triazines exhibited only globular features lacking a defined fibrillar component (6).

**Table 1:** Analysis of AChE, BuChE, and Aβ inhibition in vitro by sym-triazine derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield (%)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; AChE (µM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; BuChE (µM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ratio BuChE/AChE</th>
<th>% Inhibition Aβ fibrils 2x[i]&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Inhibition Aβ fibrils 1x[i]&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.12</td>
<td>68.6</td>
<td>89.8 ± 2.9</td>
<td>&gt; 200</td>
<td>-</td>
<td>48.3</td>
<td>5.5</td>
</tr>
<tr>
<td>3.15</td>
<td>41.6</td>
<td>56.4 ± 1.7</td>
<td>&gt; 200</td>
<td>-</td>
<td>46.6</td>
<td>6.6</td>
</tr>
<tr>
<td>3.18</td>
<td>88.0</td>
<td>38.8 ± 1.4</td>
<td>&gt; 200</td>
<td>-</td>
<td>28.8</td>
<td>34.2</td>
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<tr>
<td>3.21</td>
<td>74.2</td>
<td>18.4 ± 1.3</td>
<td>&gt; 200</td>
<td>-</td>
<td>25.2</td>
<td>31.3</td>
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<tr>
<td>3.13</td>
<td>53.5</td>
<td>171.5 ± 8.6</td>
<td>&gt; 200</td>
<td>-</td>
<td>71.3</td>
<td>42.3</td>
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<tr>
<td>3.16</td>
<td>34.2</td>
<td>110.5 ± 4.3</td>
<td>&gt; 200</td>
<td>-</td>
<td>88.2</td>
<td>58.4</td>
</tr>
<tr>
<td>3.19</td>
<td>38.7</td>
<td>20.3 ± 1.5</td>
<td>&gt; 200</td>
<td>-</td>
<td>75.2</td>
<td>41.8</td>
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<tr>
<td>3.22</td>
<td>44.4</td>
<td>9.7 ± 0.4</td>
<td>&gt; 200</td>
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<td>95.2</td>
<td>65.6</td>
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<tr>
<td>3.14</td>
<td>17.0</td>
<td>9.8 ± 0.2</td>
<td>13.9 ± 0.3</td>
<td>1.4</td>
<td>67.9</td>
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<td>3.17</td>
<td>64.4</td>
<td>80.8 ± 2.9</td>
<td>163.8±</td>
<td>-</td>
<td>87.4</td>
<td>68.5</td>
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<td>3.20</td>
<td>68.0</td>
<td>0.3 ± 0.02</td>
<td>3.9 ± 0.2</td>
<td>13.0</td>
<td>70.2</td>
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<tr>
<td>3.23</td>
<td>89.1</td>
<td>2.8 ± 0.1</td>
<td>15.3 ± 0.9</td>
<td>5.5</td>
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<td>3.24</td>
<td>82.1</td>
<td>153.2 ± 12.7</td>
<td>&gt; 200</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Donepezil</td>
<td>23.0</td>
<td>0.02 ± 0.004</td>
<td>4.0 ± 0.4</td>
<td>200.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>iAβ5p</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>58.5</td>
<td>28.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Measured with 100 µM Aβ1-40 incubated with 200 µM (2x) or 100 µM (1x) inhibitor ([i]) at 37 oC for 72 h in 50 mM PBS (pH 7.4) (6).
3.4.3 Cell Viability Studies

Collective analysis of all tested sym-triazine compounds revealed that 3.20 and 3.23 distinctly exhibited high activity in regards to multi-targeted inhibition of both AChE and Aβ pathologies (6). 3.20 and 3.23 possess the maximum number of quaternary amine-derivatized acetylcholine substitutions and thus served as effective structures to be used for comprehensive assessment of the sym-triazines impact to live cells. Propidium iodide (PI) exclusion assay demonstrated that the viability of differentiated SH-SY5Y human neuronal cells was not affected with treatment of up to 400 µM of 3.20 and 3.23, demonstrating a tolerable threshold well above the dosages required in this study for effective inhibition of AChE activity. Fluorescence microscopy demonstrated the absence of cell death morphology in differentiated human neuronal cells treated with 400 µM of 3.20 and 3.23 compared to controls in which cell death was induced by toxic levels of celastrol (170). The results further emphasized the applicability of these sym-triazines to biological samples, and demonstrating a promising potential for further preclinical investigations (6).

3.5 Conclusion

Multi-targeted drug therapies are an innovative approach to address the complex and inter-related nature of AD pathologies (6). We presented the structure-activity studies of a novel class of sym-triazine-based therapies for AD capable of multi-targeted inhibition of both AChE activity and Aβ aggregation in vitro. Rational optimization of triazines resulted in progressive enhancement of activity through successive derivitization. Improved targeting of AChE was achieved through direct incorporation of structural analogues of the native substrate, ACh. Additional directed inhibition of Aβ self-assembly was achieved via integration of multiple, hydrophobic phenyl units that disrupted π-π stacking. Several di-substituted and tri-substituted sym-triazine derivatives possessed comparable or greater activity compared to several commercially available inhibitors in regards to the modulation of both AChE and Aβ activity. Compounds 3.22, 3.14, 3.20 and 3.23 demonstrated a mixed-type mechanism of inhibition and high PAS affinity, which has been associated with the capacity to impede AChE-accelerated Aβ fibril formation. Cell viability studies showed that 3.20 and 3.23 were well tolerated by differentiated human neuronal cells. Our design of sym-triazines with ACh-like substitutions has
generated hybrid molecules that possess multiple beneficial activities to be used as potential candidates for AD therapy (6).
Chapter 4  
Future Directions and Conclusion

4 Future Directions and Conclusion

We have achieved promising results in our lab as a starting point for the development of multi-target-directed compounds for AD. Our sym-triazine ester-salt compounds 3.20 and 3.23 were the most potent inhibitors of Aβ aggregation, AChE and BuChE overall. These compounds can be further analyzed for its antioxidant properties and its ability to inhibit AChE-induced Aβ aggregation. Moreover, we hope to conduct in vivo studies on these compounds to test their blood brain barrier permeability in animal models.

In order to develop compounds that could hit multiple biological targets involved in AD pathology, other pharmacophores can be incorporated into the sym-triazine scaffold. Chemical moieties that can target the issues with tau hyperphosphorylation, the MAO system, and Ca\(^{2+}\) dyshomeostasis will have a significant potential in AD therapy (29).

N-phenylamine compounds have been shown previously to inhibit toxic tau aggregates in vitro studies (171) and can be a moiety to be incorporated in the sym-triazine core along with the ester-salt chains. This novel compound 4.2 can potentially have multi inhibition properties against AChE, BuChE, Aβ aggregation and tau protein aggregation (Fig. 4.1). 4,6-dichloro-N,N-diphenyl-1,3,5-triazin-2-amine (4.1) was synthesized in our lab (Fig. 4.2) using similar procedure used to synthesize compound 3.5 although a stronger base, sodium carbonate was employed instead of sodium bicarbonate. The compound can be further derivatized to form 4.2 via sym-triazine based acid, acyl chloride, and ester intermediates.

![Fig. 4.1](image-url)  
**Fig. 4.1:** Structure of 4.2 to be developed in order to possess AChE, BuChE, Aβ aggregation and tau protein aggregation inhibitor properties.
Fig. 4.2: Synthesis of 4.1 using diphenylamine. Reaction was carried out at 0°C and sodium carbonate (Na$_2$CO$_3$) was employed as a base and acetone as solvent.

The propargylamine moiety discussed in Chapter 2 was shown in research studies to reside MAO inhibition properties (29, 123). Adding this pharmacophore to the sym-triazine core that also consists of the ester-salt chains, can help target other neurotransmitter systems in addition to ACh, which are involved in AD pathology (29). This novel compound 4.4 can potentially have multi inhibition properties against AChE, BuChE, MAO and Aβ aggregation (Fig. 4.3). The novel compound 4.3 was synthesized using similar procedure used for the synthesis of 3.5 although the base employed was triethylamine instead of sodium bicarbonate. 4.3 will be used to undergo acidification, acylation, and esterification intermediate reactions to synthesize the final 4.4 compound for inhibition studies.

Fig. 4.3: Structure of 4.4 to be developed in order to possess AChE, BuChE, MAO and Aβ aggregation inhibitor properties.
Fig. 4.4: Synthesis of 4.3 using propargylamine. Reaction was carried out at 0°C and triethylamine ((C₂H₅)₃N) was employed as a base and acetone as solvent.

Dihydropyridine compounds have demonstrated neurprotective effects by inhibiting VDCCs (29, 137). Hence, adding the dihydropyridine core in the sym-triazine core that also incorporates the ester-salt chains can be synthesized (compound 4.5) in order to develop a MTDL for AChE, BuChE, Aβ aggregation, and VDCC inhibitor (Fig. 4.5).

Fig. 4.5: Structure of 4.5 to be developed in order to possess AChE, BuChE, Aβ aggregation, and VDCC inhibition properties.

MTDLs are the best approach for the treatment of AD (29). The multifactorial nature of AD, which includes biological factors such as the cholinergic system (55), Aβ aggregation (18), formation of toxic NFTs (21), imbalance in Ca²⁺ homeostasis (20), oxidative stress (19), and activation of the microglia cells (22), make it challenging to treat through single-drug therapy (29). Moreover, the one-molecule-one target paradigm in single-drug therapy involve side effects arising from different bioavailabilites, metabolism, and pharmacokinetics of different drugs that can be obviated with the use of MTDLs (29, 113, 114). Our lab has synthesized novel sym-triazine ester-salt compounds incorporating ACh-like substrate analogues and hydrophobic phenyl moieties through cost-efficient synthetic procedures. These compounds have shown to act as efficient AChE, BuChE and Aβ aggregation inhibitors in vitro making it a MTDL for AD.
They were also well tolerated in human neuronal cell lines indicating that they are non-toxic. We believe these novel compounds have the potential to be lead drug candidates for pharmaceutical analysis in the near future. These compounds can be tested further for its AChE-induced Aβ inhibition activity, antioxidant properties and blood brain barrier permeability. Further modulation of these sym-triazine compounds will be conducted in our lab to incorporate different pharmacophores in order to target different biological factors. We hope to develop and analyze a library of novel sym-triazine compounds that can have a huge impact in AD research and improve the quality of life of AD patients and their caretakers.
References


amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease, *Nat. Med.* 2, 864-870.


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Appendices

Appendix A: $^1$H-Nuclear Magnetic Resonance (NMR) Spectra of sym-Triazine Derivatives

A.1. $^1$H-NMR spectrum of 3.18
A.2. $^1$H-NMR spectrum of 3.13
A.3. $^1$H-NMR spectrum of 3.11
A.4. \( ^1 \)H-NMR spectrum of 3.14
A.5. $^1$H-NMR spectrum of 3.17
A.6. $^1$H-NMR spectrum of 3.20
A.7. $^1$H-NMR spectrum of 3.23
Appendix B: $^{13}$C-Nuclear Magnetic Resonance (NMR) Spectra of *sym*-Triazine Derivatives

B.1. $^{13}$C-NMR spectrum of 3.12
B.2. $^{13}$C-NMR spectrum of 3.21
B.3. $^{13}$C-NMR spectrum of 3.13
B.4. $^{13}$C-NMR spectrum of 3.19
B.5. $^{13}$C-NMR spectrum of 3.11
B.6. $^{13}$C-NMR spectrum of 3.14
B.7. $^{13}\text{C}$-NMR spectrum of 3.17
B.8. $^{13}$C-NMR spectrum of 3.23
Appendix C: Mass Spectra (MS) of *sym*-Triazine Derivatives

C.1. MS of 3.18
C.2. MS of 3.21
C.3. MS of 3.19
C.4. MS of 3.14
C.5. MS of 3.17
C.6. MS of 3.20
C.7. MS of 3.23
Appendix D: Infrared (IR) spectra of sym-Triazine Derivatives

D.1. IR spectrum of 3.18
D.2. IR spectrum of 3.21
D.3. IR spectrum of 3.11
D.4. IR spectrum of **3.14**
D.5. IR spectrum of 3.17
D.6. IR spectrum of 3.20
D.7. IR spectrum of 3.23