Discovery and Characterization of Multi-Target Directed Ligands as Inhibitors of Amyloid-β Aggregation and Regulators of Alzheimer's Disease

Jui-Heng Tseng
University of South Carolina - Columbia

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DISCOVERY AND CHARACTERIZATION OF MULTI-TARGET DIRECTED LIGANDS AS INHIBITORS OF AMYLOID-β AGGREGATION AND REGULATORS OF ALZHEIMER’S DISEASE

by

Jui-Heng Tseng

Bachelor of Science
National Tsing Hua University, 2007

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University of South Carolina

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Accepted by:

Melissa A. Moss, Major Professor
James Chapman, Committee Member
Jay Potts, Committee Member
Ehsan Jabbarzadeh, Committee Member
Lacy Ford, Vice Provost and Dean of Graduate Studies
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ABSTRACT

Alzheimer’s disease (AD) is the most common neurodegenerative disease in the world. As the disease advances, symptoms include confusion, language skill breakdown, and long term memory loss – and culminate in death. Existing therapies for AD provide symptomatic benefits but cannot halt or slow the disease pathogenesis.

Increasing evidence reveals the multi-factorial nature of AD. Numerous factors, including amyloid-β (Aβ) aggregation, calcium homeostasis dysregulation, and oxidative stress, contribute to disease development and interplay with each other. Therefore, multi-target directed ligands (MTDLs) may present an effective AD treatment. Among all factors, Aβ aggregation is suggested to play the most crucial role. Compounds containing aromatic centers have been proposed as effective Aβ aggregation inhibitors; however, the influence of functional groups on the aromatic ring and the number of aromatic structures has not been comprehensively explored. Furthermore, the addition of functional groups presents an opportunity to endow therapeutic compounds with additional properties that can target other pathogenic pathways.

The work presented here examines three potential MTDLs for AD. First, experimentation investigates inhibitory capabilities of a group of FDA-approved calcium channel blockers, dihydropyridines, in Aβ aggregation. Results identify all selected dihydropyridines as inhibitors of Aβ aggregation that exhibit distinct mechanisms. These mechanistic differences alter the morphology of Aβ aggregates, which may be related to cytotoxicity. In addition, naphthalimide analogs were identified as novel inhibitors of Aβ
aggregation. Several naphthalimide analogs with distinct lengths of the carbon linker were tested, and those with a two-carbon linker showed a significant inhibition by delaying the onset of aggregation. By introducing hydroxyl groups on the phenyl ring, the inhibitory capability of this compound was further enhanced and antioxidant activity was endowed. Finally, anthocyanidins, a group of polyphenols, were investigated for their ability to both intervene with Aβ aggregation and perform as antioxidants. Results indicate that anthocyanidins exhibit potent antioxidant activities, and inhibit the earlier stages of Aβ aggregation. Furthermore, the inhibitory capability is related to the number of hydroxyl groups. Together, this study provides insight into the effective properties of dihydropyridines, naphthalimides, and anthocyanidins as novel promising MTDLs for the pathogenesis in AD.
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LIST OF SYMBOLS

M  Molar, abbreviation for SI unit mole/L

p  p-value test statistic

\( R_H \)  Hydrodynamic radius
LIST OF ABBREVIATIONS

AD ................................................................. Alzheimer’s disease
ANS .......................................................... 8-Anilino-1-naphthalenesulphonic acid
ANT .......................................................... Anthocyanidin
APP .......................................................... Amyloid precursor protein
Aβ .......................................................... Amyloid-β protein
AUC ...................................................... Area under curve
BBB ........................................................ Blood brain barrier
BSA ........................................................ Bovine serum albumin
CSF ........................................................ Cerebrospinal fluid
DLS ........................................................ Dynamic light scattering
DHP ........................................................ Dihydropyridine
ECL ........................................................ Enhanced chemiluminescent substrate
HFIP ....................................................... 1,1,1,3,3,3-Hexafluoro-2-isopropanol
HRP ........................................................ Horseradish peroxidase
DMSO ..................................................... Dimethyl sulfoxide
ISF ........................................................ Interstitial fluid
IC₅₀ ........................................................ Half maximum inhibitory concentration
MAP ........................................................ Microtubule-associated protein
NaCl ........................................................ Sodium chloride
NaOH ........................................................ Sodium hydroxide
NF-κB ..................................................... Nuclear factor-κB
NFT ................................................................. Neurofibrillary tangle
NDGA ...................................................... Nordihydroguaiaretic acid
ORAC ........................................................ Oxygen radical absorbance capacity
PBS ........................................................ Phosphate buffer saline
PBS-T ................................................... PBS with tween
Phe .......................................................... Phenylalanine
PHF ........................................................ Paired helical filament
ROS ......................................................... Reactive oxygen species
SDS-PAGE ........................................ Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC ........................................................ Size exclusion chromatography
SEM ....................................................... Standard error of the mean
TEM ....................................................... Transmission electron microscopy
ThT ........................................................ Thioflavin T
CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 Alzheimer’s Disease

Alzheimer’s disease (AD), the most common neurodegenerative disease, was first described by Alois Alzheimer in November, 1906[1]. Currently, there are 5.2 million Americans living with AD, and AD is the sixth leading cause of death in the United States. In 2013, over $200 billion was spent on the health care, long-term care and hospice service for AD patients. Furthermore, throughout the coming decade the baby boom generation is projected to increase the total number of people in the United States with AD by 10 million[2]. It is predicted that in 2050, every 33 seconds another person in the United State will develop AD. As the disease advances, symptoms include the loss of human qualities- memory, reasoning, language, and emotional stability – and culminate in death[3].

With the exception of less than 5% of cases where genetic mutations have been linked to early onset AD, the cause of AD is still debatable[4,5]. Physiologically, AD is characterized by the deposition of intracellular neurofibrillary tangles (NFTs) and extracellular amyloid plaques, consisting of amyloid-β protein (Aβ), in the brain[6]. Tau protein, which comprises NFTs, is an axon specific microtubule-associated protein (MAP) primarily expressed in neurons. Tau proteins are abundant within the central nervous system and interact with tubulin to assist microtubules in forming and stabilizing tight bundles[7]. Six different tau isoforms are produced, and these proteins maintain
equilibrium in phosphorylation[8]. However, due to either mutations within tau protein or the disruption of calcium homeostasis, tau proteins undergo hyperphosphorylation[9]. Hyperphosphorylated tau aggregates to form paired helical filaments (PHFs), the aggregated form of tau and the main component within NFTs, inside neuron cell bodies in the brain. Once sequestered in PHFs, tau is no longer able to bind tubulin. As a result, microtubules become instable and axonal protein transport within neurons is disrupted, causing dysfunction in physiological communication between neurons and finally neuronal death[10]. NFTs not only exist in AD but in several other diseases such as Parkinsonism[11,12]. Furthermore, several findings suggested that NFT formation is an event downstream of Aβ aggregation[13]. Therefore, Aβ is considered the primary influence leading AD pathogenesis.

1.2 Amyloid-β Protein

Aβ, the main component of amyloid plaques, is a protein of 36-43 amino acids that is produced via multiple proteolytic cleavages of the amyloid precursor protein (APP), which are mediated by the enzymes β- and γ- secretase[14]. APP is an integral membrane protein expressed in many tissues and concentrated in the synapse of neurons. Although its function is unclear, APP has been implicated in synapse formation and neuroprotection[15,16]. APP can also be cleaved by another secretase, α-secretase. This cleavage occurs within the Aβ region, leading to release of protein fragments innocuous to the human body. The two most abundant isoforms of Aβ, Aβ_{1-40} and Aβ_{1-42}, are created by the differential location of the γ-secretase cleavage site (Figure 1.1). Aβ_{1-40} is the more common of the two isoforms; however, Aβ_{1-42} is more amyloidogenic. Aβ_{1-42} is
more prone to form Aβ oligomers, hypothesized to be highly toxic, and is thus more closely associated with disease states[15].

Aβ₁₋₄₀ monomers are non-toxic and found in plasma, cerebrospinal fluid (CSF), and brain interstitial fluid (ISF) of normal people[17,18]. Aβ levels are tightly regulated by amyloid degrading enzymes, such as the insulin-degrading enzyme and nephrlysin[19]. In addition to degradation, Aβ monomers can also be cleared via the lysosomal pathway and by certain transporters expressed at the blood brain barrier (BBB)[20,21]. The failure of these Aβ clearance mechanisms lead to the accumulation of excess Aβ monomers as well as mutations within the APP gene that result in increased Aβ production. This accumulation initiates a series of self-assembly steps culminating in insoluble Aβ aggregates. These insoluble Aβ aggregates, characterized by their β-sheet structure, deposit extracellularly between neurons to form amyloid plaques. In 1992, Hardy and Higgins introduced this phenomenon as the amyloid cascade hypothesis, which proposes that aggregated forms of Aβ initiate a series of pathological events that culminate in AD[22] (Figure 1.2). The amyloid cascade hypothesis is the leading hypothesis of the pathology of AD. After more than twenty years of examination, it has been slightly modified but the concept is still commonly accepted and supported by experimental data[23]. There are also several genetic clues indicating Aβ’s role in AD. Thirteen separate point mutations in or near the Aβ gene that all lead to increased production or increased aggregation have been identified and correlated to an increased risk for the disease as well as atypical early onset[24]. In addition, animal models have been designed to overexpress Aβ, and these genetically modified mice not only show cognitive defects that correlate with the build-up of aggregated forms of the protein, but
the succeeding reduction of Aβ levels restores cognitive ability[25]. Although the
evidence from these studies provides support for the amyloid cascade hypothesis, there is
not yet an established mechanistic link between Aβ and AD.

1.3 Multi-Factorial Nature of AD

The amyloid cascade hypothesis also reveals the multi-factorial nature of AD.
Several pathological factors including inflammatory response, ion homeostasis
disruption, oxidative stress, and decreased levels of neurotransmitters have been
identified and considered as subsequential events of Aβ aggregation[26-30]. These
different mechanisms occur concurrently during progression of the disease and are
proposed to be highly interrelated[31]. For example, oxidative stress is believed enhanced
by inflammatory responses[32]; Aβ aggregation and oxidative stress positively feedback
with each other[33]. As a result, there are many attractive targets for the development of
AD therapeutics; however, Aβ aggregation is believed to play the most crucial role in the
pathogenesis and has been widely investigated. The brief introduction of three therapeutic
targets of interest is in the following sections.

1.4 Aβ Aggregation

Aβ monomers, which exhibit a random coil conformation, can fold into β-sheet
conformation and self-assemble to form insoluble aggregates. It is essential to understand
the kinetics mechanisms of Aβ aggregate formation, which define the nature and toxicity
of Aβ aggregates. Aβ aggregation occurs via a nucleation-dependent pathway, which
exhibits three characteristic features: the critical reaction concentration, a lag phase
before polymerization, and the lag phase abrogation in the presence of nucleation seeds[34,35].

Several researchers have postulated that Aβ aggregation involves a multi-step pathway with several interconnected steps: rapid unstable monomer folding, cooperative aggregation of monomers into a “nucleus”, elongation of the nucleus by addition of monomers, and lateral association of filaments into fibrils, as shown in Figure 1.3A. This model is extensively accepted and utilized by Aβ researchers[36,37]. When aggregates are formed from monomers, the protein presents a lag phase of nucleation, which is considered the rate limiting step and probably involves soluble oligomeric Aβ. Larger soluble aggregates were identified along the fibril formation pathway between the nuclei and fibrils[38]. These soluble aggregates are much shorter in length (~200 nm) and more narrow in diameter (6-8 nm) compared to fibrils (1 mm in length and 20 nm in diameter)[39]. To form mature fibrils, soluble aggregates can grow by two parallel mechanisms simultaneously: elongation via monomer addition, which increases the length of soluble aggregates while the diameter remains the same, and direct lateral aggregate - aggregate association, where the aggregate radius and length both increase[36]. These distinct mechanisms for fibril formation render Aβ impervious to multiple points of inhibitor intervention.

Although the connection between Aβ aggregates and AD pathogenesis is still not clear, there are some widely accepted concepts. Not all of Aβ species, including monomers, oligomers, soluble aggregates, and fibrils, are toxic to neurons. Monomeric Aβ, which is found in both normal and AD brains, has been identified to be nontoxic[40]. While insoluble fibrils were considered to be toxic by early research, more recent results
have shown that amyloid plaque burden does not correlate with cognitive loss [41,42]. These findings indicate that other Aβ aggregates, such as oligomers and soluble aggregates, might have higher neurotoxicity than fibrils. In addition, it is possible that AD pathogenesis is initiated by more than one Aβ species.

1.5 Inhibition of Aβ Aggregation

Since Aβ aggregation is hypothesized to be the most critical step of the pathogenic process of AD, the strategy that to inhibit Aβ aggregation has emerged as one promising approach to treat AD. Numerous compounds have been identified as inhibitors of Aβ aggregation; however, the mechanistic interaction between Aβ and these compounds is still not clear.[43] To gain insight into the mechanism of inhibition, it is necessary to understand the structure of Aβ. While this structure of Aβ has not been resolved by crystallography, several structures have been predicted by different techniques, including nuclear magnetic resonance (NMR) spectroscopy and computer simulation[44-46]. Petkova et al. have proposed a widely used structural model for Aβ fibrils using solid state NMR (SS-NMR), as shown in Figure 1.4A[47]. In this model, residues 1-8 are structurally disordered, while residues 12-24 and 30-40 adopt β-strand conformations and form parallel β-sheets by internal hydrogen bonding. Figure 1.4B shows the secondary structure for a single Aβ\(_{1-40}\) monomer within the fibril. Residues 25-29 contain a 180° bend of the protein backbone that brings the two β-sheets in contact through sidechain-sidechain interactions. A single cross-β unit is a double-layered β-sheet structure with a hydrophobic core and a hydrophobic face. The only charged residues in the core are D23 and K28, which form a salt bridge to stabilize the β-sheet structure.
Fibrils with minimum mass-per-length and diameter contain two cross-β units with their hydrophobic faces juxtaposed.

This model contains information relevant to the design of Aβ aggregation inhibitors. For example, compounds that interact with the hydrophobic core would disrupt monomer-monomer interaction, thereby destabilizing the formation of small oligomeric aggregates or nuclei. Compounds that recognize residues 12-24, which are included in the interaction between fibril units, would interfere with lateral association, while compounds forming hydrogen bonds with amino or carboxyl groups of residues 12-24 or 30-40 are expected to inhibit soluble aggregate elongation.

Most compounds showing inhibitory capability toward Aβ aggregation are aromatic compounds, such as resveratrol, coumarin, and nicotine[48-50]. It is hypothesized that the aromaticity plays an important role by breaking the hydrophobic interaction between Aβ monomers. Aromatic compounds can interact with the two phenylalanine residues at positions 19 and 20 via π-π stacking interactions. Another finding that the derivatives of pentapeptide, KLVFF (residues 16-20 of Aβ), can inhibit Aβ aggregation supports this speculation[51,52]. Therefore, to achieve better inhibitory capability toward Aβ aggregation, aromatic compounds have been modified and functionalized on their aromatic center.

1.6 Calcium Homeostasis in AD

Calcium overload can initiate the process of neuronal apoptosis[53]. Calcium entry along L-type calcium channels leads to both calcium overload and mitochondria disruption, which activates apoptotic cell death[54], as shown in Figure 1.5. About two
decades ago, Mattson et al. first showed that Aβ enhances the level of cytoplasmic calcium[55]. Further studies have indicated that this increase in cytoplasmic calcium is mainly attributed to an influx of extracellular calcium across the plasma membrane[56]. Recent studies in transgenic mice strongly support this idea by showing that calcium levels are increased in neurons within brain regions that exhibit extensive amyloid plaque deposition[57].

Although the means by which calcium influx is induced by Aβ is still unclear, several possible mechanisms have been proposed. The most accepted theory suggests that Aβ may bind to plasma membranes to form artificial channels that allow calcium passage[58,59]. Lal et al. have shown that Aβ oligomers can form small annular structures within lipid membranes[60]. Another mechanism by which Aβ can disrupt calcium homeostasis is related to its ability to form reactive oxygen species (ROS). Excess ROS induce membrane lipid peroxidation, which changes membrane properties and influences the functions of membrane transporters and ion channels, leading to an elevated level of intracellular calcium[61]. This membrane-related oxidative stress also occurs within mitochondrial membranes to cause mitochondria dysfunction.

Some groups have also studied the effect of calcium dysregulation on the production and metabolism of Aβ. An increased level of cytoplasmic calcium, either from influx through the plasma membrane or the release from the endoplasmic reticulum (ER), is able to enhance the production of Aβ[62,63]. In contrast, inhibition of the reuptake of cytoplasmic calcium into the ER diminished Aβ production[64]. Together, this evidence confirms a relationship among AD, Aβ and cytoplasmic calcium. While the mechanism is
still debatable, many drugs that block the entrance of calcium into neuronal cells are currently under investigation for AD therapy[65].

1.7 Oxidative Stress in AD

Oxidative stress occurs when excessive generation of ROS cannot be balanced by the antioxidant defense system. ROS are generated from oxygen and nitrogen based molecules with unpaired electrons; thus, ROS are very unstable and reactive and easily cause cellular injury. Typically, oxidative damage to cellular components results in the alteration of membrane properties, such as fluidity, ion channels, and membrane proteins[66].

The brain is particularly vulnerable to oxidative stress due to its comparably low antioxidant level, higher concentration of polyunsaturated fatty acids, and an elevated oxygen requirement of the brain[67]. In AD brain, the oxidative stress, manifested by protein oxidation, DNA/RNA oxidation and lipid peroxidation, is even more furious[61]. Among above, lipid peroxidation is considered especially harmful in mitochondria, the energy source of human body, because decreases in cardiolipin, an important lipid in the inner mitochondrial membrane, lead to mitochondria derangement and eventually apoptosis[66]. Conversely, impaired mitochondrial functions increase the production of ROS, which cause the accumulation of somatic mtDNA mutations[68], that result in mitochondrial respiration deficiencies thought to contribute the cognitive dysfunction in AD[69]. Also, the levels of antioxidant enzymes were found to be decreased in AD brain compared to age-matched controls[70], which can worsen the consequence.
A link between Aβ, oxidative stress, and the ensuring pathological events has also been suggested. Aβ is considered to play an important role in the oxidative process and mitochondrial dysfunction[71]. On the other hand, increased oxidative stress is a major factor in triggering enhanced production and deposition of Aβ in AD[72-74]. Therefore, oxidative stress and Aβ deposition may be interplaying culprits to exacerbate and accelerate AD pathogenesis (Figure 1.6).

1.8 Specific Aims

This study intended to develop compounds that not only inhibit Aβ aggregation, but also regulate other pathological events in AD, such as calcium homeostasis disruption and oxidative stress. A group of dihydropyridines, which are current therapeutic calcium channel blockers, has potential to both inhibit Aβ aggregation and regulate calcium homeostasis in neurons. Their effects to inhibit Aβ aggregation were evaluated and are described in Chapter 3. In addition, a group of novel naphthalimide analogs were investigated for their ability to both inhibit Aβ aggregation and exhibit antioxidant activity (Chapter 4). Finally, a group of polyphenols, anthocyanidins, which commonly exists in our daily diets, were examined for their performances in inhibiting Aβ aggregation and antioxidant activity, as discussed in Chapter 5.
Figure 1.1 APP processing[75]. EC: extracellular; TM: transmembrane; IC: intracellular.
Figure 1.2 Amyloid cascade hypothesis[76].
Figure 1.3 Aβ nucleation dependent aggregation. Monomeric Aβ nucleates in the rate-limiting step of the process. Soluble aggregates are then formed, and insoluble fibril is assembled from soluble aggregates via elongation and association.
Figure 1.4 Structural Model for Aβ₄₀ Fibrils[47]. A) Schematic model for a cross-β unit. B) Central Aβ₁-₄₀ molecule from the energy-minimized system.
Figure 1.5 Calcium dysregulation and neuronal vulnerability[53]. Mitochondria dysfunction is caused by calcium homeostasis dysregulation and leads to neuronal death in AD. MIT: mitochondria; ER: endoplasmic reticulum.
Figure 1.6 Possible involvements of reactive oxygen species in AD pathogenesis[33].
CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Aβ\textsubscript{1-40} and Aβ\textsubscript{1-42} were purchased from Anaspec (Fremont, CA). Thioflavin T (ThT), sodium chloride (NaCl), sodium hydroxide (NaOH), phosphate buffered saline (PBS), 1,1,3,3,3-Hexafluoro-2-propanol (HFIP), and glycine were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were purchased from EMD Biosciences (San Diego, CA). Uranyl acetate and 300 square mesh formvar-carbon supported copper grids were obtained from Electron Microscopy Sciences (Hatfield, PA). 8-Anilino-1-naphthalenesulphonic acid (ANS) was obtained from Research Organics (Cleveland, OH). Surfact-Amps 20 detergent (Tween) and SuperSignal West Pico enhanced chemiluminescent substrate (ECL) were purchased from Thermo Fisher Scientific (Rockland, IL).

Antibodies were purchased from the following vendors: rabbit-anti-Amyloid Fibrils antibody (LOC) from EMD Millipore (Billerica, MA), mouse-anti-Aβ\textsubscript{1-16} antibody (6E10) from Covance (Princeton, NJ), goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate from Bio-Rad (Hercules, CA), and sheep anti-mouse IgG HRP conjugate from GE Healthcare (Piscataway, NJ).
2.2 Preparation of Aβ\textsubscript{1-40} Monomer

Lyophilized Aβ\textsubscript{1-40} protein was stored desiccated at -20 °C until reconstitution in 50 mM NaOH at a concentration of 2 mg/mL to minimize the formation of small aggregates. Remaining aggregates were excluded via size-exclusion chromatography (SEC) on a Superdex 75 HR 10/300 column (GE Healthcare, Piscataway, NJ). SEC, also known as gel filtration, is a chromatographic method to separate molecules based on their size. It is often applied to macromolecule separation, such as proteins. In SEC, molecules with larger size elute earlier since these molecules directly pass through the column and do not enter the pore space of beads.

BSA (2 mg/mL) was employed as a pretreatment to reduce nonspecific Aβ interaction with the dextran matrix of the column. Using a running buffer of 40 mM Tris-HCl (pH 8.0), the elution profile showed a characteristic peak at an elution volume of 14-17 mL corresponding to monomeric Aβ\textsubscript{1-40}, as shown in Figure 2.1. Aβ\textsubscript{1-40} monomer concentrations were determined from absorbance spectra using a calculated extinction coefficient of 1450 M\textsuperscript{-1} cm\textsuperscript{-1} at 276 nm. Purified Aβ\textsubscript{1-40} monomer samples were used fresh or stored at 4 °C for up to 3 days.

2.3 Preparation of Aβ\textsubscript{1-40} Soluble Aggregate

To produce Aβ\textsubscript{1-40} soluble aggregates, purified monomeric Aβ\textsubscript{1-40} (up to 100 μM) was incubated with 40 mM Tris-HCl (pH 8.0) containing 3 mM NaCl at room temperature. Solutions were agitated vigorously by continuous vortexing (800 rpm) to promote aggregation, which was monitored using ThT fluorescence. ThT is a fluorescent dye that interacts with the β-sheet structure of Aβ protein[77]. As a result, ThT
selectively binds to aggregated forms of Aβ, but has no interaction with monomeric Aβ. When associated with Aβ, ThT will exhibit a shifted excitation maximum at 450 nm and a shifted, enhanced emission at 482 nm, in comparison with the excitation at 385 nm and emission at 445 nm of unbound ThT. Measurements were monitored using an LS-45 luminescence spectrometer (PerkinElmer Inc., Waltham, MA) with excitation at 450 nm and emission. Measurements were evaluated by calculating the area under the fluorescence emission spectrum curve (F) from 470 to 500 nm, and were corrected by subtracting the ThT background. Soluble aggregates were separated from fibrils via centrifugation (10 min, 18000 g) and from unreacted monomers via SEC on a Superdex 75 column, where soluble aggregates eluted in the void volume (8-10 mL). Concentrations of Aβ1-40 soluble aggregates, expressed in monomer units, were determined from UV absorbance at 276 nm corrected for light scattering, and equivalent ThT fluorescence measurements were assessed. Measurement of soluble aggregate size via dynamic light scattering (DLS) using a DynaPro-MSX instrument (Wyatt Technology Corporation, Santa Barbara, CA) revealed that this procedure consistently yielded soluble aggregates displaying an average hydrodynamic radius ($R_H$) of ~80 nm. DLS, also known as photon correlation spectroscopy or quasi elastic light scattering, is a technique to determine the size of particles in dilute solution or suspension. In DLS, particles in Brownian motion scatter light from an incident laser beam. Scattered light is measured at a 90° angle, and the temporal fluctuation in scattered light is used to determine particle diffusivity, which can be related to the size of the particle ($R_H$) using the Stokes-Einstein equation.

$$R_H = \frac{k_B T}{6\pi \eta D} \quad (\text{Eq. 1})$$
Here $k_B$, $T$, $\eta$, and $D$ are the Boltzmann constant, absolute temperature, solvent viscosity, and diffusivity respectively. $\text{A}\beta_{1-40}$ soluble aggregates were stored at 4 °C for up to 3 days. Following storage, ThT fluorescence measurements were used to correct for any changes in soluble aggregate concentration, and DLS measurements were used to ensure that soluble aggregates maintained their initial size.

2.4 Preparation of $\text{A}\beta_{1-40}$ Fibrils

Insoluble $\text{A}\beta_{1-40}$ fibrils were prepared from SEC-isolated $\text{A}\beta_{1-40}$ monomers as described previously.[78] 50-100 μM monomeric $\text{A}\beta_{1-40}$ in 40 mM Tris-HCl (pH 8.0) was agitated in the presence of 250 mM NaCl at 25 °C for at least 24 h. Fibrils, also called insoluble aggregates, were separated from monomers and soluble aggregates via centrifugation (14000g, 10 min). Supernatant that contains soluble $\text{A}\beta_{1-40}$ species was removed and the pellet was resuspended in 40 mM Tris-HCl (pH 8.0). Fibril concentration was determined from the fraction of pelleted protein. Fibrils were stored at 4 °C for up to 2 weeks prior to use in the experiments.

2.5 $\text{A}\beta_{1-40}$ Monomer Aggregation Assay

Reaction mixtures containing 20 μM SEC-isolated $\text{A}\beta$ monomer, 150 mM NaCl, 2.5 or 5 % (v/v) DMSO, 40 mM Tris-HCl (pH 8.0), and selected concentrations of inhibitor candidates were prepared in 1.5 mL non-stick microtubes at room temperature. Reactions containing 0 μM inhibitor candidates served as a positive control. Reactions were incubated under continuous agitation at 500 or 800 rpm to promote $\text{A}\beta$ aggregation. During the time course of aggregation, 15 μL aliquots were periodically taken from the
reaction solution and combined with 140 μL of 10 μM ThT for ThT fluorescence measurement. Aggregation of 20 μM Aβ₁-₄₀ monomer displays a time course of ThT fluorescence characterized by a lag phase, followed by a period of rapid growth, and concluding with a plateau as equilibrium is reached. Data is reported as the change in normalized F with time, where normalized F is the ratio of the experimental value of fluorescence to the value of plateau fluorescence in the absence of inhibitor candidates, as shown in Figure 2.2.

To evaluate the inhibitory capabilities of inhibitor candidates at both early and late stages of aggregation, lag time and equilibrium plateau changes were quantified. An extension of lag time demonstrates that an inhibitor candidate is capable of delaying nucleation via interaction with Aβ monomers or oligomers. The lag time extension was determined as the fold increase of the time at which ThT fluorescence is first increased in the presence vs. absence of an inhibitor candidate, Tₑ and Tₖ, respectively. A reduction of the equilibrium plateau indicates that an inhibitor candidate is able to reduce the quantity of aggregates formed. The plateau reduction was determined as the percentage decrease of the plateau ThT fluorescence in the presence of an inhibitor candidate, Pₑ, compared to the plateau ThT fluorescence observed in the absence of inhibitor candidates, Pₖ.

2.6 Aβ₁-₄₀ Soluble Aggregate Elongation Assay

One pathway of Aβ soluble aggregate growth is elongation, where soluble aggregates increase their size via monomer addition to both ends of soluble aggregate. This mechanism leads to a longer length than the starting material, indicating that
elongation occurs at both ends of soluble aggregates. The growth of Aβ soluble aggregates via addition of monomer was assayed by incubation of SEC-isolated soluble aggregates with inhibitor candidates followed by incubation with monomer at low ionic strength to isolate and initiate elongation. 1 μM SEC-isolated soluble aggregates in 40 mM Tris-HCl (pH 8.0) were combined with 80 μM of an inhibitor candidate in the presence of 10 μM ThT and incubated at room temperature without agitation for 15 min to allow binding of the inhibitor candidate and soluble aggregates. Elongation was initiated by the addition of 20 μM SEC-isolated monomer and periodically monitored at 20 min intervals via in situ ThT fluorescence measurement. Reactions conducted in the absence of inhibitor candidates served as the positive control. Experiments in which ThT fluorescence of soluble aggregates was monitored in the absence of added monomers or in which ThT fluorescence of monomers was monitored in the absence of added soluble aggregates served as negative controls and reflected the stability of soluble aggregates and monomers, respectively.

For inhibitor candidates that exhibit significant self-fluorescence at the wavelength employed for ThT fluorescence or compete with ThT for Aβ binding sites, DLS was utilized to monitor the increase in $R_H$ during soluble aggregate elongation. These experiments were performed in a manner similar to that described above, with the growth of 2 μM Aβ$_{1-40}$ soluble aggregates in the absence or presence of 80 μM of an inhibitor candidate initiated by addition of 30 μM monomer. Higher concentrations of both Aβ species were used in DLS experiments compared to ThT fluorescence experiments because the change in $R_H$ is less sensitive than the change in fluorescence.
Results are reported as the change in ThT fluorescence or $R_H$ with time, as shown in Figure 2.3A. Elongation rates were determined by regression of the linear portion part of this data. The percentage inhibition was calculated from the percentage decrease of the experimental elongation rate, $E_E$, relative to the elongation rate observed in the absence of inhibitor candidates, $E_C$.

2.7 Aβ$_{1-40}$ Soluble Aggregate Association Assay

Another distinct mechanism of Aβ soluble aggregate growth is association, where soluble aggregates increase their size in the absence of monomers via aggregate-aggregate interaction. This mechanism leads to a larger mass per unit length than the starting material, indicating that association occurs via lateral interaction. The growth of Aβ$_{1-40}$ soluble aggregates by association was isolated by incubating soluble aggregates in the absence of monomeric protein in a high ionic strength buffer. Here, the reaction was monitored by measuring the change in $R_H$ via DLS, as no new β-sheet structures are formed during the association process.

2 μM SEC-isolated Aβ$_{1-40}$ soluble aggregates in 40 mM Tris-HCl (pH 8.0) were combined with 0-10 μM of a filtered inhibitor candidate and incubated without agitation at room temperature for 15 min to allow binding. Association was initiated by addition of concentrated NaCl for a final concentration of 150 mM NaCl and periodically monitored by measurement of the increase in soluble aggregate $R_H$ using DLS. Reactions containing 0 μM inhibitor candidates served as the positive control. Experiments in which $R_H$ of Aβ$_{1-40}$ soluble aggregates was monitored in the absence of added NaCl served as a negative control and reflected the stability of soluble aggregate. Results are reported as the
increase in $R_H$ with time, as shown in Figure 2.3B. Association rates were determined by regression of the linear portion of this data. The percentage inhibition was calculated from the percentage decrease of the experimental association rate, $A_E$, relative to the association rate observed in the absence of inhibitor candidates, $A_C$.

2.8 Transmission Electron Microscopy

Transmission electron microscopy (TEM) was introduced to observe the structure of fibrils that form in the presence of each inhibitor candidate. SEC-purified Aβ$_{1-40}$ monomer was incubated with 150 mM NaCl, 2.5 % (v/v) DMSO, 40 mM Tris-HCl (pH 8.0) in the presence of desired concentrations of inhibitor candidates under agitation at 500-800 rpm. After aggregation reached a plateau, which was recognized by ThT fluorescence of the control reaction, gridded samples were prepared and imaged.

In TEM, images of high resolution, when compared to traditional microscopes, are obtained. In this technique, a beam of electrons is transmitted through a sample and the interaction between the electron beam and electron dense atoms creates an image that is subsequently magnified. In the following studies, negative-staining was used in combination with TEM to visualize the morphology of aggregated Aβ by creating a higher contrast. Negative staining is a method commonly used in TEM that employs a dye that contains electron dense atoms to stain the edges of the protein but not the protein itself.

A 20 μL sample containing aggregated Aβ$_{1-40}$ was placed upon a formvar-carbon supported copper grid and allowed to absorb for 2 min. Excess sample solution was wicked away using a piece of wiper placed at the bottom side of the grid. Sample
application was repeated in this manner until the control grid contained a sufficient quantity of sample for visualization. The gridded sample was then stained with 2% filtered uranyl acetate for 8 min. The staining solution was wicked away from the grid edge, and the grid was allowed to air dry overnight. The dried grid was visualized using a JEOL 200CX transmission electron microscope (JEOL, Peabody, MA) at 120 kV.

2.9 Preparation and Storage of Aβ1-42

Aβ1-42 was employed in oligomerization studies due to its ability to form stable oligomers. Moreover, Aβ1-42 is the earliest species deposited in amyloid plaques. Lyophilized Aβ1-42 was dissolved in HFIP at a concentration of 1 mM and incubated for 1 h. HFIP was then allowed to evaporate overnight. The resulting dried protein, a thin clear film, was stored at -80 °C for up to several months.

2.10 Aβ1-42 Oligomerization Assay

Oligomers were formed using a procedure similar to that described by Klein and colleagues[79]. The dried film of Aβ1-42 was dissolved in DMSO at concentration of 5 mM in the absence or presence of 150 μM of an inhibitor candidate, and subsequently diluted to 10 μM in PBS (pH 7.4) containing 1 μM NaCl to initiate oligomerization. After 30 min, oligomerization was terminated by addition of 0.1% Tween 20. The resulting oligomers were characterized using Western blot, as described below.
2.11 Analysis of Aβ₁-₄₂ Oligomerization via SDS-PAGE and Western Blot

Aβ₁-₄₂ oligomers formed in the absence or presence of inhibitor candidates were separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Equal concentrations of proteins were added to lamelli buffer (1:1) and loaded on a 4-20 % Tris-HCl gel (Bio-Rad, Hercules, CA). The Precision-Plus protein standard (Bio-Rad) was also loaded as an indicator of molecular weight. Proteins were separated by electrophoresis at 120 V until the dye front reached 80 % of the gel length. Following separation, proteins were transferred for 12 min at 14 V onto a 0.2 μm nitrocellulose membrane via semi-dry blot transfer on a Trans-blot SD semi-dry transfer cell (Bio-Rad). The membrane was blocked for overnight at 4 °C using 5 % non-fat dry milk in PBS (pH 7.4) containing 0.1 % Tween 20 (PBS-T). The membrane was then incubated with primary antibody 6E10 (1:1000) in blocking buffer for 1 h at room temperature and washed 3 times at 5 min each with PBS-T. 6E10 is a sequence specific antibody that binds to every species of Aβ from monomer to larger aggregate. The membrane was then incubated with HRP-conjugated goat anti-mouse IgG antibody (1:2000) and Precision Protein StrepTactin-HRP conjugate (1:5000) for 45 min at room temperature and again washed 3 times at 5 min each with PBS-T. Chemiluminescence was enhanced using ECL and images were visualized and acquired using a Bio-Rad ChemiDoc™ XRS+ imaging system. The quantitative analysis of band density was performed using Image Lab™ software (Bio-Rad).
2.12 Determination of Aβ_{1-42} Oligomer Structure Using ANS Fluorescence

1-anilinonaphthalene-8-sulfonate (ANS) is a sulfonated naphthalene with an aniline group. The naphthalene backbone and aniline ring are hydrophobic, which endow ANS an affinity to hydrophobic regions within proteins. The excitation wavelength of free ANS is 350-380 nm, whereas the emission maximum is 500 nm. When ANS binds to protein hydrophobic regions, the emission maximum is blue shifted and the emission intensity is enhanced.[80] As a result, ANS spectroscopy is an effective method to evaluate the exposure of hydrophobic residues on the surface of a folded protein.[81,82]

ANS was solubilized in DMSO at a concentration of 50 mM and stored at 4 °C. ANS stock was diluted in PBS and combined with oligomers formed in the presence or absence of inhibitor candidates without the addition of Tween 20 for final concentrations of 100 μM ANS, 1 μM Aβ_{1-42}, and 100 μM inhibitor candidate. Fluorescence was measured using an LS-45 luminescence spectrometer with excitation at 350 nm, emission from 400 to 600 nm, and excitation and emission slits of 10 nm. Fluorescence values were determined as the integrated area under the emission spectra from 450 to 550 nm with background (buffer or inhibitor candidate with ANS) subtraction.

2.13 Oxygen Radical Absorbance Capacity Assay

An oxygen radical absorbance capacity (ORAC) assay was used to determine the antioxidant capacity of inhibitor candidates. This assay measures the oxidative degradation of the fluorescent molecule fluorescein following mixing with peroxyl radicals, which are one of the most abundant radicals in vivo[83]. Antioxidants present in
the samples delay the degradation of the fluorescent probe until the antioxidant activity in the sample is depleted.

The OxiSelect™ Oxygen Radical Antioxidant Capacity Activity Assay kit (Cell Biolabs, San Diego, CA) was employed. Inhibitor candidates were freshly dissolved in DMSO. Stock solutions were further diluted in DMSO and potassium phosphate buffer (75 mM, pH 7.0) for final concentrations of 1 μM inhibitor candidates and 1 % (v/v) DMSO according to the method described by Ou et al.[84] Trolox (1 μM), a vitamin E analog, served as a standard for the assay. This standard as well as all blanks also contained 1 % (v/v) DMSO. 25 μL of the sample or standard and 150 μL of the 1X fluorescein probe, prepared in assay diluent, were combined in a black sided 96-well plate and incubated at 37 °C for 30 min. After incubation, 25 μL of free radical initiator solution was added. Samples were maintained at 37 °C and fluorescence measured every min for 90 min using a Synergy 2 Multi-Mode microplate reader (BioTek, Winooski, VT) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The area under this curve (AUC) was integrated for all samples and standards using the fluorescence readings. The AUC of the blank was subtracted from all other AUC values to get the net AUC. The net AUC of trolox standards was blank subtracted and plotted versus concentration to obtain a calibration curve from which the equivalent trolox concentration of inhibitor candidates was determined. Equivalent trolox concentrations were normalized for the concentration of inhibitor candidate and reported as an ORAC value, where the standard, trolox, has an ORAC value of 1. Inhibitor candidates with an ORAC value comparable to or above 1 were considered good antioxidants. Inhibitor
candidates with an ORAC value significantly lower than 1 were judged as poor antioxidants.

2.14 Statistical Analysis

All quantitative values are expressed as the mean ± standard error (SEM). Statistical analysis was performed using Prism 5 software (GraphPad Software Inc, San Diego, CA). Differences between independent groups were assessed using a one-way analysis of variance (ANOVA) with a Dunnett’s test. Difference between each experimental group and control was assessed using a paired t-test. Outliers were detected using Grubb’s test. p <0.05 was considered significant.
Figure 2.1 Aβ$_{1-40}$ monomer purification. Monomer (4.3 kDa) was separated from pre-existing aggregates using SEC on Superdex 75, where 40 mM Tris-HCl (pH 8.0) served as running buffer. Aβ$_{1-40}$ monomer elutes at a volume of 14-17 mL, while the void peak (8-10 mL) contains aggregates.
Figure 2.2 Assessment of the capabilities of inhibitor candidates on Aβ₁₋₄₀ monomer aggregation. Monomer aggregation assays were conducted in the absence (control, ○) and presence (experimental, ●) of inhibitor candidates. Aggregation was monitored to evaluate the ability of each inhibitor candidate to modify the lag time to aggregate formation and the equilibrium plateau representing the quantity of aggregate formed. Extension of lag time was determined as the ratio of the lag time in the presence of inhibitor candidates, $T_E$, to the lag time observed for the control, $T_C$, calculated as $T_E/T_C$. Reduction of the equilibrium plateau was determined as the percentage decrease of the fluorescence plateau in the presence of inhibitor candidates, $P_E$, relative to the fluorescence plateau observed for the control, $P_C$, calculated as $[(P_C-P_E)/P_C] \times 100\%$. 
Figure 2.3 Assessment of the capabilities of inhibitor candidates on the growth of Aβ$_{1-40}$ soluble aggregate. (A) 1 or 2 μM SEC-isolated Aβ$_{1-40}$ soluble aggregates in 40 mM Tris-HCl (pH 8.0) were pre-incubated for 15 min alone (positive control, ○) or in the presence of inhibitor candidate (experimental, ●). 20 or 30 μM Aβ$_{1-40}$ monomer was then added to initiate elongation growth. Aβ$_{1-40}$ soluble aggregate (□) or monomer (△) alone served as negative controls, ensuring the stability of these species. Elongation was monitored as an increase with time for either ThT fluorescence (left Y-axis) or $R_H$, monitored via DLS (Right Y-axis). Linear regression (solid lines) was performed to determine the elongation rate. Inhibitory capability was expressed as the percentage decrease of the elongation rate in the presence of inhibitor candidates, $E_E$, relative to the slope of regression observed for the control, $E_C$, calculated as $[(E_C-E_E)/E_C] 	imes 100\%$. (B) 2 μM SEC-isolated Aβ$_{1-40}$ soluble aggregates in 40 mM Tris-HCl (pH 8.0) were incubated
alone (positive control, ○) or in the presence of inhibitor candidate (experimental, ●). 150 mM NaCl was added to induce association. Soluble aggregates in the absence of NaCl (□) served as a negative control, ensuring the stability of this species. Association was monitored using DLS as an increase with time for $R_H$. Linear regression (solid lines) was performed to determine the association rate. Inhibitory capability was expressed as the percentage decrease of the association rate in the presence of inhibitor candidate, $A_E$, relative to the association rate observed for the control, $A_C$, calculated as $[(A_C - A_E)/A_C] \times 100\%$. 


CHAPTER 3

DIHYDROPYRIDINES INHIBIT AMYLOID-β AGGREGATION AND ALTER THE MORPHOLOGY OF AMYLOID-β AGGREGATES

3.1 Introduction

As described in Section 1.5, many small molecules have been reported to modulate the process of Aβ aggregation[85,86]. Particularly, compounds containing aromatic structures are capable of preventing the assembly of Aβ monomer into insoluble fibrils[87]. These compounds are hypothesized to interact with phenylalanine residues within the hydrophobic core of Aβ[88,89] and disrupt π-π stacking of these aromatic side chains, which is believed to be a driving force of Aβ aggregate formation[90].

Calcium homeostasis is considered one of the pathological events in AD, as discussed in Section 1.6. For the past two decades, it has been considered that hypertension and dementia, two common diseases of the elderly, may be causally linked[91]. Clinical trials have shown several antihypertensive treatments also reduce the incidence of AD[92-95]. Among these treatments, calcium channel blockers are the most effective, reducing AD prevalence by 50 %[95]. Therefore, calcium channel blockers have been emerging for AD therapy.

Dihydropyridines are verified L-type calcium channel blockers currently prescribed for the treatment of hypertension. Dihydropyridines regulate calcium influx across cell membrane by competing with calcium for binding to calcium channels. Dihydropyridines have at least one aromatic structure, further endowing them the
potential to be multi-functional drugs in AD treatment. In this study, we introduced five dihydropyridine calcium channel blockers (Figure 3.1), amlodipine (AML), nimodipine (NMD), nitrendipine (NTD), nicardipine (NCD), and the metabolic product of nicardipine, dehydro nicardipine (DNC), to investigate the interaction between these compounds and distinct steps of Aβ aggregation, including monomer aggregation, soluble aggregate elongation, and soluble aggregate association. The morphological difference of the aggregates formed in the presence of different dihydropyridines was also observed to correlate fibril morphology and inhibitory mechanism.

3.2 Materials and Methods

3.2.1 Preparation of Dihydropyridines

Dihydropyridine derivatives amlodipine, nimodipine, nitrendipine, nicardipine, and dehydro nicardipine were purchased and purified by Dr. James Chapman in Department of Drug Discovery and Biomedical Sciences, College of Pharmacy, University of South Carolina. All dihydropyridines were dissolved DMSO at a concentration of 10 mM. The stock solutions were then stored in -20 °C for up to 3 months.

3.2.2 Inhibition of Aβ_{1-40} Monomer Aggregation by Dihydropyridines

To evaluate the overall inhibitory capabilities of dihydropyridines, monomer aggregation assays were conducted as described in Section 2.5. Dihydropyridines were individually assessed for their inhibitory capabilities at 20 and 100 μM. To determine the IC_{50} of nicardipine, concentrations of nicardipine ranging from 0.001-20 μM were tested
in 4 independent experiments. To evaluate additive effect of inhibitors, varying concentrations of nicardipine ranging from 10-20 μM and amlodipine ranging from 10-100 μM were evaluated.

### 3.2.3 Aβ<sub>1-40</sub> Soluble Aggregate Elongation Assay

Inhibition of Aβ<sub>1-40</sub> soluble aggregate elongation was assessed as described in Section 2.6 with the inclusion of 80 μM dihydropyridines. The effect of nicardipine was assessed using DLS due to its strong self-fluorescence. Other dihydropyridines were evaluated using ThT fluorescence.

### 3.2.4 Aβ<sub>1-40</sub> Soluble Aggregate Association Assay

Inhibition of Aβ<sub>1-40</sub> soluble aggregate association was assessed as described in Section 2.7 with the inclusion of 10 μM dihydropyridines.

### 3.2.5 Transmission Electron Microscopy

Transmission electron microscopy was utilized to evaluate the morphological differences of aggregates formed in the presence of different dihydropyridines. Sample preparation and imaging were performed as described in Section 2.8.

### 3.3 Results

#### 3.3.1 Dihydropyridines Inhibit Aggregation of Aβ<sub>1-40</sub> in a dose-dependent manner

To evaluate the effect of dihydropyridines on Aβ aggregation, an Aβ<sub>1-40</sub> monomer aggregation assay was introduced. Aggregation of 20 μM SEC-purified Aβ<sub>1-40</sub> monomer
was initiated by addition of physiological NaCl and continuous agitation and was monitored using ThT fluorescence to estimate the extent of β-sheet structure in the samples. As shown in Figure 3.2, reactions containing 0 μM dihydropyridines (positive control) showed a typical curve of Aβ aggregate formation: a lag time followed by a rapid growth and a subsequent plateau. When amlodipine was present at a concentration 5-fold in excess of Aβ1-40 monomer, the lag time to aggregate formation was extended slightly and the plateau fluorescence observed at equilibrium was reduced, indicating the inhibition of Aβ1-40 aggregation by amlodipine. Due to its strong self-fluorescence, nicardipine was tested only at a concentration equimolar with Aβ1-40 monomer. Even at this lower concentration of 20 μM, nicardipine showed a stronger inhibition than 100 μM amlodipine. Reactions containing 20 μM nicardipine exhibited a significant lag extension of 2.2-fold and plateau reduction of 70 %.

Table 3.1 summarizes the lag extension and plateau reduction of Aβ monomer aggregation by dihydropyridines at different concentrations. When present at concentrations in excess of Aβ, all selected dihydropyridines were able to inhibit Aβ monomer aggregation, as characterized by increased lag time and reduced plateau; however, all compounds except nicardipine failed to inhibit Aβ aggregation when present at a concentration equimolar with monomer. These results demonstrated that the presence of each these compounds resulted in a dose-dependent decrease in the formation of Aβ1-40 aggregates from monomeric Aβ1-40 protein and that nicardipine exhibited the strongest inhibitory effects.

Nicardipine was furthered evaluated for its potency in inhibiting monomer aggregation. Monomer aggregation assay was conducted with ten distinct concentrations
of nicardipine (Figure 3.3). The half maximum inhibitory concentration (IC$_{50}$) of nicardipine was determined to be 6.28 μM in the presence of 20 μM Aβ monomer. This substoichiometric ratio is lower than previous reported inhibitors.

3.3.2 Select Dihydropyridines Inhibit Mechanistic-Specific Growth of Aβ$_{1-40}$ Soluble Aggregates

Since all dihydropyridines were able to inhibit monomer aggregation, further investigation of later stages within the aggregation pathway was performed. The effect of dihydropyridines on Aβ$_{1-40}$ soluble aggregate elongation was evaluated by ThT fluorescence or DLS. Elongation of SEC-isolated Aβ soluble aggregates was initiated by the addition of monomer. As shown in Table 3.2, among all selected dihydropyridines, dehydro nicardipine was the only compound that showed a significant inhibition in elongation growth (p<0.01). Nimodipine, nitrendipine, and nicardipine all failed to alter the elongation of soluble aggregates, while amlodipine inhibited elongation by reducing the growth rate by 24 % compared with the positive control. Among all selected dihydropyridines, dehydro nicardipine was the only one showed a significant inhibition in elongation growth (p < 0.01).

The effect of dihydropyridines on Aβ$_{1-40}$ soluble aggregate association was evaluated using DLS. Association of 2 μM SEC-isolated Aβ soluble aggregates was triggered via addition of 150 mM NaCl. Results are shown in Table 3.2. The effect of nicardipine and amlodipine were significant compared to control. Nicardipine inhibited association by 18 % compared to the positive control (p<0.01), while amlodipine reduced association by 35 % (p<0.001), presenting a more potent inhibition. In contrast,
nimodipine, nitrendipine, and dehydro nicardipine all promoted this mechanism of soluble aggregate growth. These results demonstrate that different dihydropyridine structures distinctly affect different soluble aggregate growth mechanisms.

3.3.3 Inhibitory Capabilities of Nicardipine and Amlodipine Are Not Additive

The above results demonstrate that all dihydropyridines exhibited different degrees of inhibitory capabilities and acted at distinct points of the aggregation pathway. For example, nicardipine interacted with Aβ monomers or small unordered oligomers since it significantly extended the lag time, which correlates to the nucleation step. In contrast, amlodipine was able to interplay with larger Aβ aggregates as it inhibited both elongation and association growth. Therefore, these two dihydropyridines that act with different Aβ species in distinct stages of aggregation were utilized in combination to examine the potential of compounds to work additively to enhance the inhibitory capability on Aβ aggregation.

Nicardipine and amlodipine were selected for evaluation of their combined effect in monomer aggregation. Three combinations of nicardipine and amlodipine were introduced into the Aβ monomer aggregation assay. However, all three combinations exhibited an almost identical effect: ~1.5 fold lag extension and ~45 % plateau reduction (Table 3.3). Furthermore, this inhibitory capability is very similar to that observed in the presence of 100 μM amlodipine alone. Because the structures of nicardipine and amlodipine are similar, it is speculated these compounds bind Aβ at the same site, with amlodipine exhibiting a higher affinity for Aβ.
3.3.4 Dihydropyridine Inhibition of Elongation and Association Leads to Parallel Changes in Aggregate Morphology

Fibril morphology has been related to Aβ cytotoxicity[96]. As a result, it is important to consider the effect of inhibitors on aggregate structure. To observe the morphology of fibrils formed in the presence of different dihydropyridines, TEM was used to visualize aggregates formed following monomer aggregation assay in the presence of dihydropyridines. TEM is a technique commonly used in the biochemistry field to distinguish morphological differences in protein aggregates[97,98]. The TEM images (Figure 3.4) show that the morphology of Aβ aggregates formed in the presence of dihydropyridines are quite different from that of native Aβ fibrils. These morphological differences can be correlated with the inhibitory capabilities of different dihydropyridines in the later stages of aggregation.

The Aβ aggregates formed in the presence of amlodipine (Figure 3.4, panel B) are thinner and shorter compared to control, corresponding to the ability of amlodipine to inhibit both elongation and association. The aggregates formed in the presence of nitrendipine showed an opposite feature; longer and thicker fibrils were seen (Figure 3.4, panel C), paralleling observations that nitrendipine promoted both elongation and association. Much longer fibrils prevailed in the aggregates formed in the presence of nimodipine (Figure 3.4, panel D), which promoted association but had no effect on elongation. The aggregates formed in the presence of dehydro nicardipine appeared shorter but thicker (Figure 3.4, panel E) compared to control, corresponding to dehydro nicardipine inhibition of elongation but promotion of association. Finally, aggregates
formed in the presence of nicardipine, which inhibited association but promoted elongation, exhibited a longer, thinner morphology (Figure 3.4, panel F).

3.4 Discussion

Many small molecules have been identified as inhibitors of Aβ aggregation[99,100]. Many of these molecules exhibit aromatic structures. Thus, dihydropyridines, which are also aromatic in nature, were investigated for their ability to inhibit Aβ aggregation. Dihydropyridines are calcium channel blockers that have been FDA approved for treatment of hypertension and are thus well described pharmacologically. Furthermore, calcium homeostasis disruption is recognized as one of the pathological events in AD, as discussed in Section 1.6. Therefore, dihydropyridines have the potential to ameliorate AD pathogenesis in two ways, inhibiting Aβ aggregation and regulating calcium homeostasis to prevent neuronal death, to serve as dual-target directed ligands. In this chapter, we explored the ability of selected dihydropyridines to specifically inhibit Aβ aggregation in a mechanistic-specific manner as well as the parallel morphological changes in Aβ aggregates that occur as a result of this inhibition.

An Aβ monomer aggregation assay employing ThT is a common first-step to evaluate the inhibitory capability of compounds[101,102]. Aβ aggregation is a nucleation dependent process, where the nucleation step is rate-limiting and energetically unfavorable[48,103]. Inhibition of Aβ nucleation has the potential to delay the onset of AD. All selected dihydropyridines are able to extend the lag time when present at a concentration in excess of Aβ (Table 3.1). When reduced to an equimolar concentration with Aβ monomer, most dihydropyridines lost their ability to extend the lag time;
however, nicardipine significantly extended the lag time (p<0.001) at equimolar concentration with a 2.2±0.4 fold increase over the control (Table 3.1). Aβ aggregation concludes with an equilibrium plateau, which indicates the extent of β-sheet containing aggregates. All selected dihydropyridine reduced the equilibrium plateau when present at a concentration 5-fold in excess of Aβ monomer; however, most compounds failed to show inhibitory capability when reduced to an equimolar ratio (Table 3.1). Nicardipine was the only compound to retain a significant decrease in the equilibrium plateau (p<0.01) at an equimolar ratio (Table 3.1). It is worth noting that the metabolite of nicardipine, dehydro nicardipine, was a less effective inhibitor of monomer aggregation than other dihydropyridines.

Nicardipine was further investigated to determine the IC$_{50}$ in plateau reduction. This quantitative measure indicates how much of an inhibitor is needed to inhibit a given biochemical function by 50 % (Figure 3.3). An IC$_{50}$ value of 6.28 M was derived in the presence of 20 μM monomer. This IC$_{50}$ value reflects a stoichiometric ratio of inhibitor:monomer of 1:3. Compared to previously reported data[100,104,105] which has considered a 1:1 stoichiometric ratio as effective, this substoichiometric IC$_{50}$ for nicardipine is noteworthy, making nicardipine a very effective inhibitor in monomer aggregation.

Clarification of the mechanism of inhibitor action has proven challenging since Aβ aggregation is a complex process. Progression from monomeric protein to the insoluble fibrils that form amyloid plaques in the brain involves several different intermediates and interconnected mechanisms. The monomer aggregation assay provides only a broad view of a compound’s involvement in Aβ aggregation. For example, all
selected dihydropyridines inhibited monomer aggregation, but from this assay it is not possible to determine at which assembly steps these dihydropyridines intervene. To provide mechanistic insight into the inhibition of Aβ aggregation, soluble aggregate elongation and association assays were utilized to investigate the action of dihydropyridines in the later stages of aggregation.

During Aβ soluble aggregate elongation, Aβ monomer adds to aggregates to form new β-sheet structure. This addition involves the formation of hydrogen bonds between existing aggregates and added monomers. Compounds that bind at sites of monomer addition are able to inhibit elongation growth. In contrast to elongation, association is a lateral interaction between Aβ soluble aggregates that is presumed to involve residues 12-24 of the protein.[47] Thus, binding of a compound within this region would be expected to inhibit aggregate growth by association. Soluble aggregate association was significantly inhibited by amlodipine (p<0.001), indicating that amlodipine plays a role to reduce the lateral interactions between soluble aggregates. Soluble aggregate elongation was inhibited by amlodipine as well, showing that amlodipine is also capable of blocking sites of monomer addition to soluble aggregates. Nicardipine showed a similar mechanistic pattern of significantly inhibiting soluble aggregate association but with weaker capability (p<0.01), but soluble aggregate elongation was not inhibited by nicardipine. Nimodipine, however, promoted soluble aggregate association but had no effect in soluble aggregate elongation. Nitrendipine was observed to promote both association and elongation. Dehydro nicardipine promoted association but significantly inhibited elongation by 51 % (p<0.01), indicating that dehydro nicardipine reduced monomer addition but enhanced lateral interaction between soluble aggregates. Thus,
although these dihydropyridines share similar backbone structures, they intervene at very different points along the Aβ aggregation pathway. In addition, not all dihydropyridines inhibited the later stages of aggregation. However, all selected dihydropyridines did inhibit Aβ aggregation. Thus, some of these compounds may intervene at earlier stages of aggregation.

As described above, Aβ aggregation comprises several interconnected mechanisms. Highly effective inhibitors may intervene at multiple points. Alternatively, a combination of inhibitors that work via different mechanisms might prove more effective. Therefore, combinations of different dihydropyridines that intervene at different points along the Aβ aggregation pathway may provide an enhanced inhibition. Such a cocktail treatment is an effective multidrug medication therapy for some diseases such as AIDS and cancer. In AD, there also have been potential cocktail therapies[106] as well as a compound with multi effects[107]. In this study, nicardipine and amlodipine were selected for analysis since nicardipine was the most effective inhibitor in extending lag time and amlodipine was the most effective inhibitor at later stages of aggregation. However, three combinations all failed to achieve a raised inhibition (Table 3.3). Moreover, these three combinations all exhibited a similar outcome in both lag extension and plateau reduction that was comparable with that 100 μM amlodipine alone (Table 3.1). The result may be attributed to the structural similarity of nicardipine and amlodipine. It is likely that nicardipine and amlodipine may share the same binding sites on Aβ. In the future, the combination of inhibitors with distinct inhibitory functions may be more effective for inhibitors with distinct structures.
Aβ aggregates can exhibit morphological differences[108]. To modify the surface structure of Aβ aggregate, causing changes of electrostatic and hydrophobic interactions with neurons, is considered one way to alter cytotoxicity[109]. It is also known that different structures of prion, an amyloid-like protein, possess different morphologies and phenotypes in Creutzfeldt-Jakob disease[110]. To study large Aβ aggregate structures, TEM has been largely employed[111-115], allowing discoveries that Aβ aggregate morphology is influenced by conditions such as pH, hydrophobicity, temperature, agitation speed, in the presence of certain compounds, and point mutations within the Aβ sequence[49,112,116-122]. In this study, aggregates formed in the presence of different dihydropyridines were gridded and visualized using TEM to determine the effect of dihydropyridines on aggregate morphology. Aggregates formed in the absence of dihydropyridines exhibited structures similar to those previously reported[123,124]: stacked and twisted rods, rods with different lengths and thickness (Figure 3.4, panel A). The aggregates formed in the presence of amlodipine showed shorter and thinner rods compared to control (Figure 3.4, panel B). This morphological change correlated with amlodipine inhibition of both later stages of aggregation, elongation and association. In contrast, aggregates formed in the presence of nitrendipine, which promoted both elongation and association, were longer and thicker (Figure 3.4, panel C). Nimodipine, which had no effect on elongation but promoted association, caused the formation of aggregates of a similar length compared to control but with a more stacked appearance (Figure 3.4, panel D). The aggregates formed in the presence dehydro nicardipine were thicker but shorter (Figure 3.4, panel E), which corresponds to its inhibition of elongation but promotion of association. Nicardipine, the most effective inhibitor of monomer
aggregation but a selective inhibitor of association, led the morphology of Aβ aggregates to be much longer and thinner (Figure 3.4, panel F). These results demonstrate that the morphology of Aβ aggregates is influenced by the later stages of aggregation and can be altered by the presence of mechanistic-specific inhibitors.

In summary, this study demonstrated that selected dihydropyridines all exhibit inhibitory capability in Aβ aggregation. Nicardipine was the most effective inhibitor with a substoichiometric IC$_{50}$ value. Mechanistic specific inhibition was also investigated. Only amlodipine exhibited inhibition of both growth mechanisms in the later stages of aggregation. When the additive effects of nicardipine and amlodipine were examined; however, no increased inhibition was observed, possibly due to structural similarity. Last, the morphology of Aβ aggregates, which can influence cytotoxicity, was modified by dihydropyridines. This modification paralleled the performance of dihydropyridines in inhibiting elongation and association. However, dehydro nicardipine, a metabolite of nicardipine was unable to inhibit Aβ aggregation. Thus, if nicardipine is pursued in the clinical trials, it should be protected from metabolization.
Table 3.1 Effect of dihydropyridines on Aβ$_{1-40}$ monomer aggregation$^{a,b}$

<table>
<thead>
<tr>
<th>DHPs</th>
<th>Lag Extension (fold increase)</th>
<th>Plateau Reduction (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 μM</td>
<td>20 μM</td>
</tr>
<tr>
<td>AML</td>
<td>1.5 ± 0.0</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>NMD</td>
<td>1.7 ± 0.7</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>NTD</td>
<td>2.1 ± 0.9</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>NCD</td>
<td>—</td>
<td>2.2 ± 0.4***</td>
</tr>
<tr>
<td>DNC</td>
<td>0.8 ± 0.2</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

a. Aβ$_{1-40}$ monomer aggregation experiments were performed as described in Section 2.5. The data were evaluated and reported as lag extension (fold increase) and plateau reduction (% inhibition) as describe in Section 2.5.

b. Parameters are reported as mean±SEM, n=3

**p<0.01 and ***p<0.001 compared to control
Table 3.2 Effect of dihydropyridines on the growth of pre-formed Aβ_{1-40} soluble aggregates\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>DHPs</th>
<th>Elongation (% inhibition)</th>
<th>Association (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>24 ± 8</td>
<td>35 ± 2***</td>
</tr>
<tr>
<td>NMD</td>
<td>No effect</td>
<td>Promotion\textsuperscript{c}</td>
</tr>
<tr>
<td>NTD</td>
<td>Promotion\textsuperscript{c}</td>
<td>Promotion\textsuperscript{c}</td>
</tr>
<tr>
<td>NCD</td>
<td>Promotion\textsuperscript{c}</td>
<td>18 ± 5**</td>
</tr>
<tr>
<td>DNC</td>
<td>51 ± 23**</td>
<td>Promotion\textsuperscript{c}</td>
</tr>
</tbody>
</table>

a. Aβ_{1-40} soluble aggregate elongation and association experiments were performed as described in Sections 3.2.3 and 3.2.4, respectively. The data were evaluated and reported as % inhibition as describe in Figure 2.3.
b. Parameters are reported as mean ± SEM, n=3
c. Promotion indicates % inhibition < 0
   **p<0.01 and ***p<0.001 compared to control.
Table 3.3 Effect of dihydropyridine combinations on Aβ₁₋₄₀ monomer aggregation⁹,₁₀

<table>
<thead>
<tr>
<th>DHPs</th>
<th>Lag Extension (fold increase)</th>
<th>Plateau Reduction (fold increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCD (μM)</td>
<td>AML (μM)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
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</tr>
<tr>
<td></td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

a. Aβ₁₋₄₀ monomer aggregation experiments were performed as described in Section 3.2.2. The data were evaluated and reported as lag extension (fold increase) and plateau reduction (% inhibition) as described in Section 2.5.

b. Parameters are reported as mean ± SEM, n=3
Figure 3.1 Chemical structures of selected dihydropyridines. (A) amlodipine; (B) nimodipine; (C) nitrendipine; (D) nicardipine; (E) dehydro nicardipine
Figure 3.2 Effect of amlodipine and nicardipine on Aβ1-40 monomer aggregation. SEC-isolated Aβ1-40 monomer diluted to 20 μM in 40 mM Tris-HCl, pH 8.0, containing 150 mM NaCl was incubated alone (control, ○) or in the presence of 100 μM amlodipine (panel A, ●) or 20 μM nicardipine (panel B, ●). Fluorescence values are plotted as the ratio of equilibrium control plateau observed in each independent experiment. Error bars represent SEM, n=3.
Figure 3.3 Effect of nicardipine to reduce the equilibrium plateau for Aβ_{1-40} monomer aggregation. Monomer aggregation was performed as described in Section 3.2.2. with concentrations of nicardipine ranging from 0.001 to 20 μM. The effect of nicardipine on plateau reduction (% inhibition) was calculated as described in Section 2.5 and plotted against nicardipine concentration. An IC_{50} value of 6.28 was derived from non-linear regression of the data by allowing the Hill slope to vary. Distinct figure legends represent different independent experiments.
Figure 3.4 Morphology of Aβ_{1-40} aggregates formed in the presence of dihydropyridines. 20 μM SEC-isolated Aβ_{1-40} monomer in 40 mM Tris-HCl (pH 8.0) containing 150 mM NaCl was aggregated alone (control, panel A) or in the presence of 100 μM amlodipine (panel B), nitrendipine (panel C), nimodipine (panel D), or dehydro nicardipine (panel E) or 20 μM nicardipine (panel F). When aggregation was complete, the reactions were stopped and samples were gridded and visualized by TEM as described in Section 3.2.5. Images are representative of three independent experiments. Images are shown relative to a scale bar of 0.5 μm
CHAPTER 4

NOVEL NAPHTHALIMIDE ANALOGS AS DUAL-FUNCTIONAL LIGANDS INHIBIT AMYLOID-β AGGREGATION AND EXHIBIT ANTIOXIDANT ACTIVITY

4.1 Introduction

Oxidative stress has always been considered one of the pathological events followed by Aβ aggregation in AD and one of the direct factors to trigger neuronal death since it leads to lipid peroxidation on mitochondria, the power plants of cells, as described in Section 1.7. Numerous studies have correlated the beneficial effect of antioxidants on neuroprotection[125,126]. Furthermore, hydroxyl, a commonly found substituent in antioxidants, is thought to create hydrogen bonds with carbonyl groups of residues within the hydrophobic core of Aβ protein that enhance the stability of the compound-protein complex and may increase micro environmental hydrophilicity around Aβ. Thus, hydroxyl groups have been an essential element in AD drug design to endow antioxidant activity and inhibitory capability on Aβ aggregation to potential therapeutics.

As discussed in Section 1.5, it is believed that aromatic compounds are potential Aβ aggregation inhibitors since they may interfere with the two phenylalanine (Phe) residues (residue 19 and 20) within the hydrophobic core of Aβ[127]. Based on this theory, a more expatiated theory was proposed, stating that structures containing two aromatic anchor groups separated by a linker probably are more effective inhibitors since they decrease the stoichiometric ratio of compound to Aβ and create a sterichindrance[43,128]. So far, compounds containing two aromatic groups connected
by a linker have been commonly reported to inhibit Aβ aggregation and these structural elements have been incorporated within design compounds to inhibit the formation of Aβ aggregates[129-131]. In this expatiated theory, there are two critical factors, the type of the aromatic groups and the length of the linker, to influence the inhibitory property of compounds. Different aromatic centers have different binding affinity to Aβ and the substituents in the aromatic center affect the binding affinity as well. Length of the linker also plays an important role in the inhibitory capability of the compounds. As shown in Figure 4.1, either the linker is too short or too long does not lead to the best fit of the small molecule to Aβ but an appropriate length of the linker.

It was shown in our lab that when the 1,8-naphthalimide is on one end of ranitidine analogs, it provided fairly potent inhibitory capabilities in Aβ aggregation no matter what types of aromatic rings at the other end of the structures[132]. Therefore, naphthalimide analogs were selected to be the pattern in the design of Aβ inhibitors. In this study, a group of 1,8-naphthalamide analogs with different length of linker were synthesized by Jie Gao working in Dr. James Chapman’s laboratory to investigate the optimal length of the linker for Aβ aggregation inhibition. The substituents on 1,8-naphthalimide ring and phenol ring were replaced by nitro group and hydroxyl group to observe the alternative effects on promoting Aβ aggregation inhibition and exhibiting antioxidant activity, respectively. Lastly, several structural elements were determined to provide the impetus to develop a new class of multi-target directed ligands for the therapy of AD.
4.2 Material and Methods

4.2.1 Chemical Synthesis of Naphthalimide Analogs

All naphthalimides analogs, compound 1 to compound 8, were synthesized, purified, and characterized by Jie Gao working in Dr. James Chapman’s laboratory.

4.2.2 Aβ_{1-40} Monomer Aggregation Assay with Naphthalimide Analogs

To evaluate the inhibitory capability of naphthalimides analogs, monomer aggregation assays were performed as described in Section 2.5. Naphthalimide analogs were evaluated for their inhibitory capabilities at 100 μM.

4.2.3 Aβ_{1-40} Soluble Aggregate Elongation Assay

Aβ_{1-40} soluble aggregate elongation assay was performed as described in Section 2.6 with the inclusion of 80 μM compound 8. The elongation growth was monitored using DLS since compound 8 is potentially to interference with ThT.

4.2.4 Aβ_{1-40} Soluble Aggregate Association Assay

Aβ_{1-40} soluble aggregate association was performed as described in Section 2.7 with the inclusion of 10 μM compound 8.

4.2.5 Transmission Electron Microscopy

Transmission electron microscopy was employed to evaluate the morphological differences of aggregates formed in the absence or presence of compound 8 and performed as described in Section 2.8.
4.2.6 ORAC Assay

In order to characterize the antioxidant capacity of compound 7 and compound 8, ORAC assay was conducted as described in Section 2.13.

4.3 Results

4.3.1 Naphthalimide Analogs with a Two-Carbon Linker Exhibit Significant Inhibitory Capability on Both Early and Late Stages of Aβ Aggregation

In order to identify the proper length of the linker connecting the 1,8-naphthalimide and the other aromatic ring, six naphthalimide analogs were provided by Jie Gao in Dr. James Chapman’s laboratory. All structures are listed in Table 4.1. First, Aβ monomer aggregation assay was employed for the purpose of evaluating the overall inhibitory capabilities of these six compounds. The results for all synthesized naphthalimide analogs are summarized in Table 4.1 as well regarding their effect at 100 μM with 20 μM monomer upon both lag extension and plateau reduction. Compound 1 contains a one-carbon linker showed a slight influence upon aggregation on reducing the plateau and no effect on extending the lag time. Compound 2, which was replaced a hydrogen by nitro group on the naphthalimide ring compared to compound 1, exhibited an increased effect on reducing the plateau (p<0.05) but still had no effect to elongate the lag time. A similar comparison of inhibitory capability was observed for compounds containing a three-carbon linker. Compound 4 had no effect on lag extension but reduced the plateau by 22.7±9.7 %. The nitro group substituted compound 5 also had no effect to extend the lag time, but decreased the plateau significantly by 37.3±2.9 % (p<0.01). According to the results above, it was seen that compounds containing a nitro group
exhibited stronger capabilities. This is probably because the electronegative nature of the nitro group makes them easier to interact with Aβ protein. Compounds containing a two-carbon linker or a four-carbon linker both had a relatively potent effect on reducing the plateau (both p<0.01). Compound 6, carrying a four-carbon linker, had no effect on lag extension but it is surprising that compound 3, containing a two-carbon linker, was shown to significantly extend the lag time by 2.4±0.7 over the control (p<0.05). This evidence indicated that the length of the two-carbon linker might be the proper length for 1,8-naphthalimide compounds as Aβ inhibitors with a significant lag time extension and a moderate plateau reduction. This considerable lag time extension gave us a clue that a two-carbon linker between naphthalimide ring and phenol ring is able to make the compound compatible to Phe19-Phe20 in Aβ protein, further interfering with the early stages of Aβ aggregation pathway.

4.3.2 Additional Hydroxyl Groups Enhance Inhibitory on Aβ Aggregation

In order to increase the inhibitory capability on Aβ monomer aggregation and endow the antioxidant activity of compound 3, hydroxyl groups were added to offer compound 7 and compound 8 from Jie Gao to add electronegative centers in the structures and the structures and results are shown in Table 4.2. Compound 7 with one hydroxyl group failed to exhibit lag extension but significantly reduced the plateau by 35.2±9.1 % (p<0.01). Compound 8 with two hydroxyl groups presented significant Aβ aggregation inhibition with a lag extension of 4.9±1.6 fold (p<0.05) and a plateau reduction of 85.2±2.9 % (p<0.001) over the control. The results of compound 7 and 8 indicated that 3-hydroxyl substitution may be more critical for potent inhibitory activities
than 4-hydroxyl substitution on phenol rings. It might also be possible that at least two hydroxyl groups are required to exhibit potent \( \text{A}\beta \) aggregation inhibition.

### 4.3.3 Additional Hydroxyl Groups Also Provide Antioxidant Activity to Naphthalimide Analogs

Oxygen radical absorbance capacity (ORAC) assay has been commonly used to evaluate antioxidant capacities in biological samples in vitro. This method measures the oxidative degradation of fluorescein after being mixed with free radical initiator since free radicals damage the fluorescent molecules, leading to loss of fluorescence. Antioxidants are believed to rescue the fluorescein from being oxidized. In this method, the antioxidant activity is evaluated by comparing the capacity of interest with trolox, a vitamin E analog, exhibiting strong antioxidant activity. By setting the antioxidant capacity of trolox equal to 1, anything larger than 1 means stronger antioxidant activity than trolox and anything less than 1 indicates weaker antioxidant activity than trolox. According to this basis, the antioxidant activity of compound 7 and 8 were evaluated and shown in Table 4.2. By adding one hydroxyl group on the phenol ring, compound 7 showed an ORAC value of 0.51±0.22, indicating a fair capability as an antioxidant. However, if one more hydroxyl group was added on the phenyl ring, compound 8, which contains two hydroxyl groups, showed an improved ORAC value of 1.22±0.33, representing a potent antioxidant activity. It does make sense that more hydroxyls led to a higher antioxidant activity.
4.3.4 The Mechanistic-Specific Inhibition of the two hydroxylated compound

Using monomer aggregation assay, compound 8 has been identified to be the best Aβ aggregation inhibitor among this series of naphthalimides analogs. However, mechanistic-specific assays were necessary to determine the effect of compound 8 on specific stages of aggregate growth that occur along the aggregation pathway between monomer and mature fibril. Here elongation and association were picked since all naphthalimide analogs acted in the later stages of Aβ by reducing the plateau and compound 8 significantly decreased the plateau by 85.2±2.9%.

The Aβ soluble aggregates increase in size via elongation or association was monitored as the change in $R_H$ via DLS as described in Sections 4.2.3 and 4.2.4. The results in compound 8 are shown in Figure 4.2 and 4.3. As shown in Figure 4.2, whereas Aβ monomer or soluble aggregates incubated alone exhibited negligible changes in $R_H$, a steady increase in $R_H$ was observed when soluble Aβ aggregates were incubated in the presence of Aβ monomer. When 2 μM soluble Aβ aggregates were incubated with 80 μM compound 8 before the addition of Aβ monomer to stimulate aggregate growth, the observed elongation rate, was significantly reduced by 75.3±9.5% (p<0.01), illustrating that compound 8 displays significant inhibition on soluble Aβ aggregate elongation.

Figure 4.3 showed whereas soluble Aβ aggregates incubated in low ionic strength buffer alone exhibited negligible changes in $R_H$, a steady increase in $R_H$ was observed when soluble Aβ aggregates were incubated in the presence of NaCl. When 2 μM soluble Aβ aggregates were incubated with 10 μM compound 8 before the addition of NaCl to stimulate aggregate association, the observed association rate was also statistically
proved to reduce by 75.2±11.4 % (p<0.01), indicating a potent inhibition on aggregate association.

In order to monitor morphological changes of Aβ aggregates caused by compound 8, TEM was employed. Figure 4.4 depicts TEM images of the aggregation results when Aβ was incubated alone (control) or in the presence of compound 8 and monitored using ThT fluorescence after the control sample reached plateau. As shown in figure 4.4, after the control sample reached plateau, both samples contained fibrillar aggregates but with some notable morphological differences: aggregates formed in the presence of compound 8 were shorter and thinner; moreover, the cluster was not existed in the aggregates formed in the presence of compound 8, which backed up the fact that compound 8 both inhibited elongation and association.

4.4 Discussion

Since the multi-factorial nature of AD, none of the potential treatments really showed therapeutic effect in human body. The fact has been leading recent drug design to a multi-functional manner[107,133]. Inhibition of Aβ aggregation has emerged as a promising therapeutic strategy for AD, and numerous small molecules have been identified as inhibitors of Aβ aggregation[85,86]. Reduction of oxidative stress has been considered as another hopeful tactic to confront AD and supported by much recent evidence[134]. Compounds containing aromatic structures are to be interested due to their ability to disrupt π-π stacking interactions between aromatic residues especially the two Phe at 19 and 20 and thus, inhibit Aβ aggregation. Furthermore, compounds containing exact two aromatic groups brought together by a linker have been proposed to
be much more effective since only one compound is able to bind two Phe. However, little is known about how functional groups and length of the linker affect the inhibitory capabilities and other therapeutic properties of molecules. Such information can be powerful in the rational design of therapeutics of AD. Thus, in this study, a series of 1,8-naphthalimide analogs, consisted of a naphthalimide ring, and a phenol ring connected by various lengths of linkers, were designed and synthesized as a new type of structural scaffold for multiple potent biological activities at Aβ aggregation and antioxidant effect, which might be favorable for further structural modification and development for the pharmaceutical treatments of AD.

As described in the last chapter, Aβ monomer aggregation assay employing ThT has been commonly employed to evaluate the general inhibitory of inhibitor candidates. In monomer aggregation assay, 20 μM of SEC-purified monomer was aggregated in the absence or presence of 100 μM naphthalimide analogs. As Aβ aggregation inhibitors, the two-carbon linker is identified to possess the optimal length of connection between the 1,8-naphthalimide group and a phenol ring on the other end of the molecules. Simulation was done by Jie Gao to model the length of the linker. It was shown that compound 3 possessed a length of 8.752 Å (Data not shown), which corroborated the hypothesis that the optimal length should be between 8-16 Å proposed by Reinke et al.[128] Their potent activities also support the previously discussed paradigm that the dipeptide Phe19-Phe20 plays an important role in the Aβ aggregation process.

ORAC assay has been introduced to characterize the antioxidant activity in vitro for decades[135]. Although there is no direct evidence that the antioxidant activity in vitro is related to the one in vivo because metabolism and complicated cellular
environment are neglected in the assay, ORAC assay is still largely employed due to its easily-conducting and high-throughput screening feature. Thus, the two hydroxyl containing naphthalimide analogs, compound 7 and compound 8, were evaluated for their potential antioxidant activity using ORAC assay. Trolox served as the standard in the assay since it is vitamin E analog and its water soluble property. The result showed that compound 7, which contains only one hydroxyl, exhibited the ORAC value of 0.51±0.22 while the two hydroxyl containing compound, compound 8, exhibited a high potent value of 1.22±0.33. The addition of two hydroxyl groups offers an effective antioxidant activity on compound 8 in a simplified in vitro environment. However, as discussed earlier in this paragraph, compound 8 will be required to identify its antioxidant activity in vivo using cell model and animal model.

Compound 8 showed a significant inhibition in reducing plateau (p<0.001), which indicated that compound 8 was very likely to interact with larger Aβ aggregates. Moreover, the inhibition of Aβ soluble aggregate growth has been seen for some hydroxyl-containing compounds[78,136]. As a result, compound 8 was evaluated for its capabilities to inhibit later stages of aggregation including elongation and association. It was shown that compound 8 strongly inhibited both mechanisms (Figure 4.2 and 4.3). As discussed in Chapter 3, Aβ monomer adds to form new β-sheet as soluble aggregate elongates. This growth mechanism involves the hydrogen bonds formation between pre-formed aggregates and monomers. The two hydroxyl groups within compound 8 could break the hydrogen bond formation by forming hydrogen bonds with the backbone of Aβ, which may explain the significant inhibition in elongation.
On the other hand, association is the soluble aggregate growth via lateral interaction, which is proposed to relate to the hydrophobic region of Aβ. Aromatic residues Phe19 and Phe 20 are located within this region and are thought to take part in association growth[137]. Therefore, aromatic compounds may be able to bind Aβ soluble aggregates laterally using π-π stacking. The ability of aromatic compounds to bind laterally to Aβ soluble aggregate offers these compounds an opportunity to interrupt the aggregate growth. After binding to Aβ laterally, the extra hydroxyls on compound 8 may be able to create a hydrophilic region within the hydrophobic core of Aβ, further destroying the hydrophobic interaction between soluble aggregates. All the inhibitory property of compound 8 in the later stages of aggregation was confirmed by the TEM image, which showed much thinner and shorter aggregates formed in the presence of compound 8 (Figure 4.4).

In summary, we designed a series of naphthalimide analogs to analyze the structure-activity relationship of how different lengths of linker and substituents to influence the ability to inhibit Aβ aggregation and exhibit antioxidant activity. Compound 8, which has a two-carbon linker with two hydroxyl groups on the phenol ring, showed a strong effect in inhibiting Aβ aggregation especially in the later stages and a potent antioxidant activity. Compound 8 provided the impetus to develop a new class of multi-target directed ligands for the therapy of AD. For example, naphthalimide derivatives have also been shown to attenuate cholinergic dysfunction by slowing neurotransmitter breakdown via inhibition of acetylcholinesterase[138]. These structural elements can be combined to further enhance therapeutic capabilities.
Table 4.1 The structure of 1,8-naphthalimide analogs and their inhibition capability on Aβ_{1-40} monomer aggregation\textsuperscript{a,b}

![Diagram of a 1,8-naphthalimide analog]

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>R</th>
<th>Lag Extension (fold increase)</th>
<th>Plateau Reduction (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>H</td>
<td>NE</td>
<td>17.4±2.9</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>NO\textsubscript{2}</td>
<td>NE</td>
<td>24.4±5.6*</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>H</td>
<td>2.4±0.7*</td>
<td>39.7±8.6**</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>H</td>
<td>NE</td>
<td>22.7±9.7</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>NO\textsubscript{2}</td>
<td>NE</td>
<td>37.3±2.9**</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>H</td>
<td>NE</td>
<td>34.0±5.2**</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Parameters are reported as mean ± SEM, n=3
\textsuperscript{b} NE indicates the results of naphthalimides at 100 μM are not significantly different from the ones of control samples.

\*p<0.05, and \**p<0.01 compared to control
Table 4.2 The structure of 1,8-naphthalimide analogs with a two-carbon linker, and their inhibition capability on Aβ1-40 monomer aggregation, and antioxidant activity\textsuperscript{a,b}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Compound} & \textbf{R1} & \textbf{R2} & \textbf{Lag Extension} & \textbf{Plateau Reduction} & \textbf{ORAC Value} \\
& & & (fold increase) & (% inhibition) & \\
\hline
7 & OH & H & NE & 35.2±9.1** & 0.51±0.22 \\
8 & OH & OH & 4.9±1.6* & 85.2±2.9*** & 1.22±0.33 \\
\hline
\end{tabular}
\end{table}

\begin{flushleft}
a. Parameters are reported as mean ± SEM, n=3  
b. NE indicates the results of naphthalimides at 100 μM are not significantly different from the ones of control samples.  
*p<0.05, **p<0.01, and ***p<0.001 compared to control
\end{flushleft}
Figure 4.1 Structure-activity relationship of typical small molecule Aβ inhibitors containing two aromatic groups connected by a linker[43].
Figure 4.2 Effect of compound 8 on Aβ₁-₄₀ soluble aggregate growth via monomer addition. SEC-isolated soluble Aβ₁-₄₀ aggregates in 40 mM Tris-HCl, pH 8.0, were preincubated for 15 min alone or in the presence of compound 8. Solutions were then diluted for final concentrations of 2 µM soluble Aβ₁-₄₀ aggregates with 0 µM (positive control, □), or 80 µM (▲) compound 8, and 30 µM Aβ₁-₄₀ monomer was added to induce aggregate growth. As negative controls, 2 µM soluble Aβ₁-₄₀ aggregates (◇) or 30 µM Aβ₁-₄₀ monomer (○) were incubated alone. Increases in aggregate size were monitored continuously as changes in R<sub>H</sub> using DLS. Linear regression (solid lines) was performed to determine association growth rates (r²). Results are representative of three independent experiments.
Figure 4.3 Effect of compound 8 on Aβ1-40 soluble aggregate growth via association. SEC-isolated soluble Aβ1-40 aggregates in 40 mM Tris-HCl, pH 8.0, were preincubated for 15 min alone or in the presence of compound 8. Solutions were then diluted for final concentrations of 2 μM soluble Aβ1-40 aggregates with 0 μM (positive control, □), or 10 μM (▲) compound 8, and 150 mM NaCl was added to induce aggregate association. As a negative control, 2 μM soluble Aβ1-40 aggregates (○) were incubated alone and in the absence of NaCl. Increases in aggregate size were monitored continuously as changes in $R_H$ using DLS. Linear regression (solid lines) was performed to determine association growth rates ($r^2$). Results are representative of three independent experiments.
Figure 4.4 Morphology of Aβ_{1-40} aggregates formed in the presence of compound 8. SEC-isolated Aβ_{1-40} monomers in 40 mM Tris-HCl, pH 8.0, were aggregated alone (control, left panel) or with 100 μM compound 8 (right panel). After control reached plateau, the reactions were terminated and samples were gridded and visualized by TEM as described in Section 4.2.5. Results are representative of three independent experiments. Images are shown relative to a scale bar of 0.5 μm.
CHAPTER 5
ANTHOCYANIDINS MODULATE AMYLOID-β AGGREGATION AND SERVE AS ANTIOXIDANTS TO INTERVENE IN ALZHEIMER’S DISEASE PATHOGENESIS

5.1 Introduction

Polyphenols are bioactive compounds characterized by the presence of multiple phenol structural elements. These compounds are frequently found in fruits and vegetables, in which they provide flavor and color. Numerous studies have indicated that polyphenols have a wide range of biological activities, such as antioxidant[139], anti-inflammatory[140], cardiovascular protection[141], and anti-cancer function[142]; among above some of them are considered to be related to AD pathogenesis as described in Chapter 1. A certain amount of epidemiological studies have shown that diets rich in polyphenols result in decreased incidence of AD[143-145]. In particular, a study demonstrated that in developed countries, higher consumption of dietary flavonoid, a subgroup of polyphenols, is associated with lower population rates of dementia[146].

Furthermore, as shown in Figure 5.1, the fact that polyphenols all have two or more aromatic carbon rings makes them potential inhibitors in Aβ aggregation. Polyphenols such as resveratrol, quercetin, catechin, and nordihydroguaiaretic acid (NDGA) have been reported for their ability to intervene in Aβ aggregation in different stages[48,136,147,148]. However, it is still unclear that the relationship between the capabilities and hydroxyls, including the amount and location. To build the relationship is
able to provide rational design strategies for AD therapeutics and suggestions for AD preventive diets.

Anthocyanidins are a group of flavonoids commonly found in daily diets, such as berries, grapes and purple cabbages. In this study, three anthocyanidins, pelargonidin, cyanidin, and delphinidin were selected to examine their inhibitory capabilities in Aβ aggregation and antioxidant activities. They are all structural similar and the only difference is the numbers of hydroxyls on their phenol rings; thus, a structure-activity relationship was able to build by studying these three anthocyanidins.

5.2 Materials and Methods

5.2.1 Anthocyanidins

Structures are shown in Figure 5.2, pelargonidin (PEL), cyanidin (CYA), and delphinidin (DEL) were purchased from Indofine Chemical Company (Hillsborough, NJ). 5 mM Stock was dissolved in DMSO and prepared freshly each time before the experiments.

5.2.2 Preparation of Aβ1-40 Fibrils

Aβ1-40 fibrils were prepared for ThT detection of Aβ1-40 aggregate in the presence of anthocyanidins as described in Section 2.4.

5.2.3 ThT Detection of Aβ1-40 Fibrils in the Presence of Anthocyanidins

To ensure that ThT detection of aggregated Aβ1-40 was not affected by the presence of anthocyanidins, detection of pre-formed Aβ1-40 fibrils was carried out in the
presence of each anthocyanidins. Aβ₁₋₄₀ fibrils were diluted to a concentration at 2.5 μM in 40 mM Tris-HCl (pH 8.0), containing 10 μM ThT and in the absence (control) or presence of 12.5 μM anthocyanidins and 0.25 % (v/v) DMSO. These concentrations reflect the final concentrations of diluted samples used to monitor Aβ₁₋₄₀ monomer aggregation. Solutions were incubated at room temperature for 15 min to ensure binding, and ThT fluorescence was evaluated as described in Section 2.5. The result is reported as the percentage of the ThT fluorescence of fibrils incubated alone.

5.2.4 Aβ₁₋₄₀ Monomer Aggregation Assay with Anthocyanidins Using Dot Blot

Since ThT fluorescence was affected by the presence of anthocyanidins, dot blot assay was employed to evaluate the inhibitory capability of anthocyanidins. Samples were prepared as discussed in Section 2.5. To determine the extent of fibrillar species of Aβ, a 4 μL sample was removed from each reaction, dotted on a 0.1 μm nitrocellulose membrane, allowed dry for 9 min, and blocked into 5 % nonfat milk dissolved in PBS-T at 4 °C overnight. The second day, the membranes were incubated with primary antibody, LOC (1:5000), in blocking buffer for 1 h at room temperature and washed 3 times for 5 min each with PBS-T. The membranes were then incubated with HRP-conjugated goat anti-rabbit IgG antibody (1:3000) for 45 min at room temperature and again washed 3 times at 5 min each with PBS-T. Chemiluminescence was enhanced using ECL and images were visualized and acquired using a Bio-Rad ChemiDoc™ XRS+ image system. The quantitative analysis of density with identical area elements was performed using Image Lab™ software (Bio-Rad).
5.2.5 Transmission Electron Microscopy

Transmission electron microscopy was employed to evaluate the morphological differences of aggregates formed in the absence or presence of anthocyanidins and performed as described in Section 2.8.

5.2.6 Aβ\textsubscript{1-42} Oligomerization

Aβ\textsubscript{1-42} oligomerization was performed as described in Chapter 2.10 in the absence or presence of 150 μM anthocyanidins. After 30 min incubation, oligomers were analyzed via SDS-PAGE and Western blot as illustrated in Section 2.11.

5.2.7 Determination Aβ\textsubscript{1-42} Oligomer Structure Using ANS Fluorescence

Aβ\textsubscript{1-42} oligomerization was conducted as described in Section 2.10 with the inclusion of 150 μM anthocyanidins. Changes of oligomer structure in solvent exposed hydrophobic regions were determined employing ANS fluorescence following procedures in Chapter 2.12.

5.2.8 ORAC Assay

In order to characterize the antioxidant capacity of anthocyanidins, ORAC assay was conducted as described in Section 2.13.
5.3 Results

5.3.1 ThT Fluorescence is Affected by Anthocyanidins in Monomer Aggregation Assay

Anthocyanidins were first investigated for their ability to interfere with ThT fluorescence measurements of Aβ aggregates. In the assay, 2.5 μM Aβ1-40 fibrils and 10 μM ThT were incubated together in the absence or presence of anthocyanidins for 15 min, and then a ThT fluorescence reading was taken for each sample. Results are shown in Figure 5.3. Incubation of Aβ1-40 fibrils with delphinidin resulted in a less than 10% change in ThT fluorescence compared with the ThT fluorescence observed for fibrils incubated alone, and the difference did not reach significance (p>0.05). Thus, the ThT fluorescence assay can be employed to dependably detect the extent of β-sheet structure for Aβ aggregation in the presence of delphinidin. In contrast, incubation of Aβ1-40 fibrils with pelargonidin and cyanidin resulted in relative fluorescence of 48±2% and 67±6%, respectively. These two both were significantly different from that observed for fibrils incubated alone (p<0.01). The results indicated that pelargonidin and cyanidin did interfere with ThT fluorescence measurements of Aβ aggregates. Therefore, the ThT assay cannot be employed to monitor the effect of anthocyanidins on Aβ1-40 aggregation.

As a result, an alternative way to monitor monomer aggregation assay was introduced: dot blot with immunocytochemistry. This approach has been employed as an effective method to monitor Aβ aggregation.[102,149] Antibody detection of Aβ aggregates can be categorized into two classes: sequence and conformational specific. Sequence specific antibodies, such as 6E10 and 4G8, bind all Aβ species regardless of its secondary structure, and therefore recognize both monomeric and aggregated forms of
Aβ. In contrast, conformational specific antibodies only bind Aβ with certain structural elements and provide the information of the secondary structure of Aβ. For example, A11 antibody recognizes pre-fibrillar oligomer and OC antibody only binds fibrillar but not pre-fibrillar species of Aβ. However, OC antibody was shown to non-specifically interact with monomeric Aβ. Thus, LOC (like OC) antibody was developed to reduce the affinity to Aβ monomer, which makes LOC a perfect indicator in monomer aggregation assay to monitor the extent of fibrillar Aβ[150] when ThT detection is not adequate due to binding competition or interference with inhibitor candidates.

5.3.2 Anthocyanidins Inhibit Aβ1-40 Monomer Aggregation and Modify the Morphology of Aβ1-40 Aggregate

To evaluate the effect of anthocyanidins in Aβ1-40 monomer aggregation, first, 20 μM of Aβ monomer was aggregated alone and monitored using both ThT fluorescence assay and dot blot assay to compare the growth curve obtained from these two techniques. The result is shown in Figure 5.4. It is clear that the two growth curves are overlapped, which indicates these two methods are comparable.

Next the aggregation of 20 μM Aβ1-40 monomer was performed in the presence of 100 μM anthocyanidins. Samples were periodically dotted on nitrocellulose membrane and probed using LOC antibody (Figure 5.5A). The intensity of each dot was plotted as shown in Figure 5.5B. None of the anthocyanidins showed inhibitory capability in reducing the plateau, but they all extended the lag time by different degrees. Consequently, the lag time extension for anthocyanidins was calculated and shown in Figure 5.3C. Pelargonidin, which only contains one hydroxyl within the phenol ring,
modestly elongated the lag time by 1.50±0.29 fold. The two hydroxyls containing anthocyanidins, cyanidin, extended the lag time by 2.33±0.33 fold, which is still insignificant (p>0.05). Delphinidin, containing three hydroxyls in the phenol ring, significantly prolonged the lag time by 3.33±0.67 fold (p<0.01). From the analysis performed above, the tendency that more hydroxyls led to longer lag time was found.

To confirm inhibition of the Aβ₁₋₄₀ monomer aggregation by anthocyanidins and further investigate alterations in aggregate morphology, TEM images were acquired after control sample reached plateau. In the absence of anthocyanidins, Aβ aggregates were formed in various lengths and distinct thicknesses (Figure 5.6A). This morphology is similar to Aβ aggregates observed in previous chapters (Figure 3.4A and Figure 4.4A). While Aβ aggregates were formed in the presence of pelargonidin, the morphology was similar but not that tangled as the aggregates formed alone (Figure 5.6B). Interestingly, aggregates formed in the presence of cyanidin exhibited fewer long fibrils but many short rods (Figure 5.6C), which backed up the fact that cyanidin inhibited monomer aggregation by extending lag time. Furthermore, aggregates formed in the presence of delphinidin, which significantly elongated the lag time, were mostly short fragments of Aβ aggregate (Figure 5.6D). The TEM images shown conformed the performance of anthocyanidins in monomer aggregation assay.

5.3.3 Anthocyanidins Alter the Size and Quantity of Aβ₁₋₄₂ Oligomers

Aβ₁₋₄₂ oligomerization was employed to examine the effect of anthocyanidins in the earlier stages of aggregation as a result of they all were able to extend lag time in monomer aggregation assay. Numerous studies state that Aβ₁₋₄₂ deposits first in amyloid
plaques and has been related to the formation of Aβ oligomers.[151] Also Aβ₁₋₄₂ is more fibrillogenic and forms more stable oligomers. Freshly prepared Aβ₁₋₄₂ oligomerization was induced by adding 1 μM NaCl and the size distribution of oligomers was analyzed using Western blot as shown in Figure 5.7A. The bands below 10 kD are unreacted monomeric Aβ and the bands slightly above 15 kD are tetramer. Results demonstrated that cyanidin reduced the quantity of both large (100-250 kD) and small (25-100 kD) Aβ₁₋₄₂ oligomers while pelargonidin and delphinidin failed to alter oligomerization. It was confirmed by quantifying the relative band intensity (Figure 5.7B). As the oligomers formed in the presence of pelargonidin and delphinidin showed no significant difference compared to control, cyanidin significantly reduced the quantity of large oligomers by 60 % (p<0.01) and small oligomers by 29 % (p<0.05).

5.3.4 Anthocyanidins Increase Aβ₁₋₄₂ Oligomer Hydrophobicity

ANS was employed to characterize changes in Aβ₁₋₄₂ oligomer conformation caused by anthocyanidins. ANS is a naphthalene based compound that has an emission maximum at 500 nm. When ANS binds to the hydrophobic region of protein, it undergoes a conformational change resulting in both blue shift and increased quantum yield. Therefore, ANS spectroscopy has been commonly employed to measure protein hydrophobicity: higher fluorescence, more exposed hydrophobic regions. Aβ₁₋₄₂ oligomers formed in the presence of pelargonidin (Figure 5.8B) exhibited a similar fluorescence as compared to oligomers formed alone (Figure 5.8A), while cyanidin enhanced the hydrophobicity proven by increased fluorescence (Figure 5.8C). Delphinidin further raised the fluorescence, representing much more hydrophobic
oligomers were formed in the presence of delphinidin (Figure 5.8D). To do the quantitative analysis, spectra from 450 to 550 nm were integrated in order to include the normal and shifted peak (Figure 5.8E). It is clear that compounds containing more hydroxyls led to more hydrophobic oligomer structures; however, the observed increases in fluorescence for cyanidin and delphinidin did not reach significance (p>0.05). From the modest increase of hydrophobicity in oligomers formed in the presence of cyanidin, the hydrophobicity did not correlate to the mechanism of Aβ oligomerization.

5.3.5 Anthocyanidins Exhibit Antioxidant Activity

As described previously, oxidative stress plays a role in AD pathogenesis; also, antioxidants have shown to decrease the incidence of AD from epidemiological studies. Anthocyanidins are known antioxidants but their abilities have not been well studied. Here, ORAC assay was employed to characterize the antioxidant activity of anthocyanidins in vitro and results are summarized in Figure 5.9. Pelargonidin, which has four hydroxyls, exhibited an ORAC value of 0.95±0.17. This value is close to that of trolox, indicating pelargonidin is an effective antioxidant. Cyanidin, containing one more hydroxyl than pelargonidin, demonstrated a slightly increased ORAC to trolox, 1.28±0.21; however, it was not significant. Interestingly, delphinidin, the one that carries six hydroxyls, showed a significantly enhanced ORAC value of 4.55±0.04 (p<0.001), revealing a potent antioxidant activity.
5.4 Discussions

As described in previous chapters, designing and developing small molecule inhibitors has become a promising strategy for AD therapy. However, when to start medication is tricky since AD patients are always diagnosed when they are already in the later stages of AD. For this reason, alternative medicine or so-called healthy diet would be a better way to prevent AD because people take food every day, and thus, many researchers have been investigating active pharmaceutical ingredients from daily diet. Anthocyanidin, a sub class of flavonoids, widely presents in berries and grapes. Their aromatic and antioxidant nature make them rising as potential AD therapeutics. Hence, pelargonidin, cyanidin, and delphinidin were picked for further investigation. Picking these three compounds provides not only information about them as alternative medicine but also identification of functional groups that most capable of serving regulators for AD pathogenesis.

ThT fluorescence has been a powerful and common technique to monitor the growth of Aβ aggregation for decades. However, due to the aromatic structural similarity of ThT and potential inhibitors, they may compete for the binding sites on β-sheet containing Aβ aggregates or even interact with each other. Previously studies have shown the ThT fluorescence assay can be biased by polyphenols such as curcumin, resveratrol, and NDGA[136,152], which disturb the binding between ThT and Aβ aggregates. With these compounds, evaluation of Aβ aggregation using ThT fluorescence leads to false positive conclusions. Since anthocyanidins are phenolic compounds, ThT detection of Aβ fibrils in the presence of distinct anthocyanidins was conducted to investigate the interaction between them. The structure of Aβ fibrils is stable compared to other Aβ
aggregates; therefore, the ThT fluorescence for each sample was supposed to be identical. However, pelargonidin and cyanidin both reduced the ThT fluorescence significantly (p<0.01), indicating that the inhibitory capabilities of these two anthocyanidins could have been overrated if the ThT fluorescence assay was employed (Figure 5.3). As a result, in this study, a conformational-specific antibody, LOC, was employed as an alternative method since ThT assay was not applicable. LOC antibody binds to Aβ fibrillar species but not monomers or oligomers, and also provides the clue of the extent of Aβ aggregation. First, Aβ monomers were aggregated alone and monitored using both ThT and LOC. Both growth curves were alike (Figure 5.4), which indicates fibrillar species and β-sheet containing aggregates are mostly overlapping in the Aβ aggregation process.

In Aβ monomer aggregation assay, no plateau reduction was seen with any anthocyanidins (Figure 5.5B), which suggests that none of them is capable of intervening with the later stages of aggregation. It is unexpected since many polyphenols have been reported to inhibit association[132,136]. However, all anthocyanidins were able to prolong the lag time and showed a trend that more hydroxyls led to longer lag time (Figure 5.5C), representing they all had ability to interact with smaller Aβ species such as monomers and oligomers and this ability is related to the number of hydroxyls. TEM images backed up the aggregation result. The effective inhibitors such as cyanidin and delphinidin held Aβ aggregates into short fragments, while aggregates formed in the presence of pelargonidin were mature (Figure 5.6).

Increasing evidence from AD brain autopsies has suggested that Aβ1-40 and Aβ1-42 both are present in amyloid plaques[153]. Aβ1-40 is the more abundant isoform while Aβ1.
aggregates form earlier and are more toxic[154,155]. Aβ₁₋₄₂ soluble oligomers have been regarded as the most toxic Aβ species[156]; therefore, inhibition of Aβ oligomer formation is probably a strategy to reduce toxicological effects. Consequently, Aβ₁₋₄₂ oligomerization was employed to evaluate the capabilities of anthocyanidins to inhibit the formation of Aβ₁₋₄₂ oligomers (Figure 5.7). Western blot image showed that pelargonidin slightly reduced the size and amount of both large (100-250 kD) and small (25-100 kD) oligomers but the effect is not significant. Delphinidin had a similar performance as pelargonidin. It is noted that cyanidin significantly cut down the amount and size of Aβ oligomers especially in larger oligomers (p<0.01). This result did not conform the data in monomer aggregation that delphinidin was the most effective inhibitor in the earlier stages. There are two possible interpretations. First, the monomer aggregation assay was conducted using Aβ₁₋₄₀ where the oligomerization assay was done with Aβ₁₋₄₂: the two more hydrophobic amino acids at the C-terminus of Aβ₁₋₄₂ are very likely to alter the protein folding and its affinity with anthocyanidins. Another explanation is that delphinidin perhaps did not change the size of oligomers but the structure, and thereby held Aβ in the oligomer state for a longer time. Based on the second possibility, characterization of the structures of Aβ oligomer formed in the presence of anthocyanidins was conducted.

Local condensed and exposed hydrophobic environment is believed the driving force of Aβ aggregation[157]. Harmful proteins present highly hydrophobic surfaces that endow them ability to interact with biological membranes and cause toxic effects[158,159]. Therefore, ANS spectroscopy was employed to compare the hydrophobicity of Aβ₁₋₄₂ oligomers formed in the absence or presence of anthocyanidins.
Oligomers formed in the presence of pelargonidin had the similar hydrophobicity as that formed alone. The one more hydroxyl compound, cyanidin, showed an increased ANS fluorescence while delphinidin enhanced the hydrophobicity of oligomers the most, although no one reached significance (p>0.05). These results suggest Aβ oligomers can be categorized by size and hydrophobicity. Among these two characteristics, hydrophobicity is more likely related to Aβ aggregation.

In AD pathogenesis, oxidative stress has always been considered as a consequence of Aβ aggregation and implicated to cause neuronal death. Compounds with antioxidant activity have been extensively investigated for AD therapy. Some of them showed therapeutic effect in transgenic animals and epidemiological studies[160]. ORAC assay was employed to determine antioxidant activity of anthocyanidins. Pelargonidin exhibited an ORAC value similar to trolox, indicating moderate antioxidant ability. Cyanidin showed a slightly higher ORAC value than trolox but was not significant. Delphinidin, the one with most hydroxyls, demonstrated a potent antioxidant activity due to a significant increase of ORAC compared to trolox.

In summary, the selected anthocyanidins showed potent antioxidant activity and inhibitory capabilities in Aβ aggregation, specifically by extending the lag time. In Aβ oligomerization, cyanidin significantly decreased the amount and size of oligomers while the other two did not showed any effect. The hydrophobicity of Aβ oligomers was also altered but neither anthocyanidins reached significance. Together these results indicate that two or more hydroxyls on the phenol ring are crucial for anthocyanidins to function as regulators in AD pathogenesis in inhibiting Aβ aggregation and serving as antioxidant.
Figure 5.1 Structures of some common polyphenols. Promising inhibitors of Aβ aggregation, including curcumin, resveratrol, and EGCG, are members of polyphenol.
Figure 5.2 Structures of anthocyanidins. All experiments in Chapter 5 were carried out in the absence or presence of pelargonidin (PEL, panel A), cyanidin (CYA, panel B), and delphinidin (DEL, panel C).
Figure 5.3 Effect of anthocyanidins on ThT fluorescence with Aβ₁-₄₀ fibrils. 2.5 µM Aβ₁-₄₀ fibrils in 40 mM Tris-HCl (pH 8.0), containing 0.25 % (v/v) DMSO were incubated with 12.5 µM anthocyanidins and 10 µM ThT at room temperature for 15 min. Relative fluorescence is expressed as a percentage of the fluorescence observed for Aβ₁-₄₀ fibrils incubated in the absence of anthocyanidins. Error bars represent SEM, n=3. **p<0.01, and ***p<0.001 compared to control.
Figure 5.4 Aβ1-40 monomer aggregation monitored using ThT and dot blot. SEC-purified Aβ1-40 monomer was diluted to 20 μM in 40 mM Tris-HCl containing 150 mM NaCl. Aggregation of monomer was induced by continuous agitation and monitored using ThT fluorescence (□) or dot blot employing LOC antibody (○). Error bars represent SEM, n=3
Figure 5.5 Effect of anthocyanidins on Aβ1-40 monomer aggregation. (A) Aβ1-40 monomer diluted to 20 μM in 40 mM Tris-HCl containing 150 mM NaCl was incubated alone (control, ○) or in the presence of PEL (■), CYA (▲), or DEL (●). Extent of aggregation was evaluated by dot blot using LOC antibody detection. (B) Each dot was quantified and reported as the adjusted volume versus aggregation time. Results are representative of three independent experiments. (C) Inhibitory capability was evaluated as the fold increase of lag time observed for the control. Reduction of equilibrium plateau was not observed in any compound. Error bars represent SEM, n=3. **p<0.01 compared to control.
Figure 5.6 Morphology of Aβ$_{1-40}$ aggregates formed in the presence of anthocyanidins. 20 μM SEC-isolated Aβ$_{1-40}$ monomers in 40 mM Tris-HCl containing 150 mM NaCl, were aggregated alone (panel A), or in the presence of 100 μM PEL (panel B), CYA (panel C), or DEL (panel D). After control reached plateau, the reactions were stopped and samples were gridded and visualized by TEM as described under Section 5.2.5. Results are representative of three independent experiments. Images are shown relative to a scale bar of 0.5 μm.
Figure 5.7 Anthocyanidins reduce the size of Aβ_{1-42} oligomers. Aβ_{1-42} oligomers were prepared in the absence (CONT) or presence of PEL, CYA, or DEL. (A) Oligomerization products resolved by SDS-PAGE using 4-20% Tris-glycine gels were transferred to nitrocellulose membrane and probed using 6E10 antibody. Image is representative of four independent experiments. (B) Adjusted volume of aggregate species of 25-100 kD (strip bars) and 100-250 kD (open bars) reported relative to control. Error bars represent SEM, n=4. *p <0.05, **P<0.01 compared to control.
Figure 5.8 Anthocyanidins alter Aβ1-42 oligomer hydrophobicity. Aβ1-42 oligomers were prepared in the absence or presence of PEL, CYA, or DEL, and oligomer hydrophobicity was quantified by ANS fluorescence. Representative spectra of respective background (black) or oligomers (color) formed alone (control, panel A) or in the presence of PEL (panel B), CYA (panel C), or DEL (panel D). (E) Integrated fluorescence from 450 to 550 nm. Relative fluorescence values of oligomers prepared in the presence of anthocyanidins are reported relative to control. Integrated fluorescence from 450 to 550 nm. Error bars represent SEM, n=4.
Figure 5.9 Anthocyanidins exhibit antioxidant activity. ORAC assay was performed to determine the antioxidant activity of anthocyanidins. Error bars represent SEM, n=3-5. ***p<0.001 compared to control
CHAPTER 6

CONCLUSIONS

As the world population ages, the situation of AD is expected to worsen in the next decades. Based on the multi-factorial nature, development of MTDLs has emerged as one of the promising strategies for AD therapeutic. In this study, Aβ aggregation, calcium homeostasis dysregulation, and oxidative stress were chosen as drug targets; three groups of aromatic containing compounds were selected or synthesized to evaluate their properties to serve as MTDLs.

In Chapter 3, dihydropyridines were examined for their capabilities to inhibit Aβ aggregation. All dihydropyridines were able to inhibit Aβ monomer aggregation in a dose-dependent manner; nicardipine was the most effective inhibitor and exhibited a substoichiometric IC$_{50}$ value in plateau reduction. However, the effect in monomer aggregation cannot be replicated in the later stages of aggregation; only amlodipine intervened with both elongation and association growth. Aggregates formed in the presence of dihydropyridines exhibiting distinct morphology were observed employing TEM, and these morphological differences correlate with the performance of dihydropyridines in elongation and association. The results illustrate that dihydropyridines inhibit Aβ aggregation with diverse mechanisms, which lead to Aβ aggregates with different morphology.

The work presented in Chapter 4 provides further knowledge to the “two aromatic groups connected with a linker” model. The naphthalimide containing a two-carbon...
linker showed a superior inhibitory capability in Aβ aggregation compared to the compounds that have other lengths of linkers. The addition of two hydroxyl groups not only endow a moderate antioxidant activity to this compound but also drastically enhance the inhibitory capability of this compound in Aβ aggregation within both early and late stages. This result indicates that once the compound interferes with Aβ aggregation, the addition of hydroxyl groups is able to enhance its ability since the local hydrophilicity produces.

In Chapter 5, a group of polyphenol in daily diet, anthocyanidin, was assessed for their ability to serve as antioxidants and inhibitors of Aβ aggregation. All selected anthocyanidins showed potent antioxidant activity and inhibited monomer aggregation by delaying the onset and this effect is proportional to the numbers of hydroxyl groups within the compounds: more hydroxyls lead to longer lag time. The further investigation of anthocyanidins in the earlier stages was performed. In Aβ oligomerization assay, the compound that reduced the amount of oligomers is not the one with the most hydroxyls. ANS spectroscopy revealed that more hydroxyls lead to more hydrophobic oligomers, indicating that the effect of anthocyanidins in the earlier stages could be related to the hydrophobicity alteration.

To sum up, this study introduces three groups of MTDLs that offer potential strategies for future AD therapeutics development.
CHAPTER 7

FUTURE WORK

In this research, all assays were conducted in solution; neither cell line nor animal model was employed. To connect the study from test tubes to \textit{in vitro} or \textit{in vivo} will be the priority in the future.

\(\text{A}β\) aggregates were altered with all three groups of MTDLs. As discussed previously, \(\text{A}β\) toxicity is related to the structure and morphology of \(\text{A}β\) aggregates. Hence, cell viability assay will be employed to assess the cytotoxicity of aggregates formed in the presence of different inhibitor candidates and the toxic \(\text{A}β\) species will be determined. Antioxidant activity of naphthalimides and anthocyanidins was evaluated employing ORAC assay. However, cellular environment is much more complicated so ORAC assay does not reveal the real antioxidant activity of compounds in a physiological environment. ROS assay, thus, will be introduced to determine the antioxidant activity of potential antioxidants within cells.

Inflammatory response is considered as another pathogenic events followed by \(\text{A}β\) aggregation\cite{161,162}. Furthermore, it has shown that \(\text{A}β\) soluble aggregates are involved in several inflammatory responses, including Nuclear factor-\(κ\)B (NF-\(κ\)B) activation\cite{163}, where \(\text{A}β\) monomers are not able to activate the pathway. Additionally, the levels of NF-\(κ\)B activity are regulated by the levels of ROS\cite{164}. As a result, using confocal microscopy, NF-\(κ\)B activation assay will be utilized to evaluate if the inhibitor
candidates, particularly those potential antioxidants, are capable of deactivating the pathway and give insight for the connections between these pathogenic events.
REFERENCES


