Characterization of a Novel Tau Aggregation Inhibitor Isolated from Fungal Secondary Metabolites

By

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Characterization of a Novel Tau Aggregation Inhibitor Isolated from Fungal Secondary Metabolites

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Abstract

Alzheimer's disease is the 6th leading cause of death in the U.S. and the cost of care is billions of dollars per year. Tau aggregation is a pathological hallmark in neurodegenerative diseases known as tauopathies, which includes Alzheimer's disease. Currently there are no approved drugs that can inhibit or reverse tau aggregation. Natural products, such as ones attained from fungi, have been utilized directly as drugs or more commonly as chemical scaffolds to produce biomedically relevant compounds. Previously it was found that secondary metabolites produced from *Aspergillus nidulans* were capable of inhibiting tau aggregation and provided a new chemical scaffold that was used to semi-synthetically produce compounds known as azaphilones. In the present study, more secondary metabolites produced from *Aspergillus nidulans* were provided to find novel chemical scaffolds that had tau aggregation inhibition (TAI) activity. One compound in particular, ANTC 15, stood out because it was structurally similar to the azaphilones but had an isoquinoline core structure. ANTC 15 was tested for TAI activity and it could inhibit the formation of tau aggregates and disassemble previously formed aggregates *in vitro*.

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Introduction

Chapter 1: Introduction

1.1 Alzheimer's disease background

In 1906 Dr. Alois Alzheimer described a disease with symptoms of memory loss, continual cognitive decline, and behavioral issues coupled with pathological hallmarks of plaques and neurofibrillary tangles that were discovered post-mortem[1]. Today this disease is known as Alzheimer's disease (AD), which affects over 5 million Americans and is the 6th leading cause of death. It is also the most common form of dementia with 1 in 10 people 65 years and older having the disease[2]. Hundreds of thousands of people die each year with the disease and the annual projected cost of care for 2017 is $259 billion[2]. AD is also one of two diseases in the top ten leading causes of death in which the number of deaths per year has been increasing. Since 2000, there has been an 89% increase in the number of deaths due to AD[2]. There have been estimates that in 2050 there will be as high as 16 million people living with the disease at a cost of $1.1 trillion[2]. There are three main stages to AD; mild AD, moderate AD, and severe AD. In the mild stage people may be able to live independently or work, but could be experiencing minor memory issues such as forgetting familiar words or troubles planning and organizing. As the disease progresses these issues become more severe to include personality and behavioral changes to a point where it is severe enough to require round-the-clock care[3]. People that suffer AD also have major physiological changes. The cortex of the brain shrinks and the ventricles become enlarged due to progressive neuronal loss[4]. The neuronal loss has been hypothesized to be the result of two main pathological hallmarks. These hallmarks are the presence of extracellular senile plaques consisting of the peptide amyloid beta and intracellular neurofibrillary tangles composed to the microtubule-associated protein tau.

Diagnosing AD is extremely difficult, but tests have been developed to effectively do so. These techniques include looking at medical history, physical exams, neurological exams, brain imaging, biomarkers in the cerebrospinal fluid or blood, and genetic testing[5]. No single test is able to diagnose AD, but when a battery of tests is used an accurate diagnosis can be given.

1.2 Amyloid Introduction

In AD, the occurrence of Aβ plaques are a pathological hallmark of the disease. Aβ plaques consist of cleaved Amyloid Precursor Protein (APP), which is a transmembrane protein expressed in neuronal tissues. The normal function of this protein is not well understood, but recently there has been evidence shown that APP and APP-like proteins can regulate transcription, synaptic functions, and can function as cell surface receptor-like proteins[6]. What has been teased out through years of research is how this protein is involved in Aβ plaque formation. APP can be cut by multiple different secretase enzymes to produce amyloidogenic and non-amyloidogenic species. If it is cut in the membrane by γ-secretase and extracellularly by α-secretase it produces a non-amyloidogenic fragment known as P3. APP becomes amyloidogenic when it is cleaved by a β-secretase and a γ-secretase to create a fragment known as Aβ. The Aβ fragment can either be 40 or 42 amino acids long, with Aβ42 being more prone to aggregation and more toxic due to the extra 2 hydrophobic amino acids[7]. The γ-secretase cleavage can also produce a fragment even less prone to aggregation known as Aβ38, which recently has been an interest for amyloid therapeutics[8]. Aβ has been hypothesized to be the driving force of AD due to the deposits of extracellular Aβ plaques and known genetic risk factors associated with Aβ production can create further downstream effects, which is known as the amyloid cascade hypothesis.

1.3 Alzheimer's disease Genetic Risk Factors

Extracellular senile plaques found in AD are composed of the aggregated Aβ42 oligomers. These oligomers adopt beta sheet structure, aggregates into fibrils, and eventually become large extracellular plaques[9]. The majority of people develop AD sporadically, but about 5% of the cases are inherited through genetic mutations and known as early onset or familial AD[10]. Sporadic AD generally manifests itself after the age of 65, but familial AD patients begins to show symptoms in their 30's, 40's, and 50's and the disease progresses to severe AD around age 60. These mutations are on chromosome 21, chromosome 14, or chromosome 1 which alter APP, presenilin-1 (PSEN1), and presinilin-2 (PSEN2) respectively[11]. PSEN1 and PSEN2 are part of the catalytic domain of γ-secretase and mutations in either of these can affect cleavage of APP[12]. It has been reported that people with Down syndrome develop AD earlier than people without down syndrome with the hypothesis that having an extra chromosome 21 produces an overexpression of APP and increasing the rate of pathology accumulation[13]. Pathogenic missense mutations near the secretase cleavage sites can increase the rate of Aβ production or increase the ratio of Aβ42 to Aβ40 can also occur[14]. In sporadic AD, the apolipoprotein E (ApoE) allele has been associated with protection against AD or as a genetic risk factor. ApoE ε2 may provide protection, ApoE ε4 increases the risk, and having two copies of ApoE ε4 gives an individual an even higher risk of developing AD[15].

1.4 Amyloid Cascade Hypothesis

The amyloid cascade hypothesis has been around since the early 1990's and Aβ aggregation was thought to be the driving force of the disease because of the genetic risk factors associated with the disease. The amyloid cascade hypothesis proposes that the accumulation and oligomerization

of Aβ42 and less commonly Aβ40 is the pathological driving force of the disease. The development of plaques in the limbic and association cortices produces synaptic dysregulation and has further downstream effects, which includes microglial and cytokine activation, oxidative stress, altered kinase and phosphatase activity, formation of neurofibrillary tangles, and eventually widespread neuronal dysregulation and neuronal loss[16].

Although much research and many potential therapies have gone to clinical trials, no Aβ therapy has been able to prevent, stop, or slow the pathology[17]. Aβ therapies would most likely be more useful to the 5% of early onset AD cases due to the strong genetic associations with Aβ and plaque accumulation. The other 95% of AD cases occur sporadically and although there are senile plaques present they may not play as major of a role as originally thought. The accumulation of Aβ plaques have been seen postmortem of people that showed no cognitive decline or dementia[18]. Due to this, there has been an increasing interest in the other pathological hallmark of AD, which is the presence of intracellular neurofibrillary tangles (NFTs).

1.5 Tau Introduction

In the 1980's it was discovered that neurofibrillary tangles found in AD are composed of a microtubule-associated protein called tau[19]. Later it was also shown that tau aggregation plays a major role in multiple neurodegenerative diseases, including AD, which are known today as tauopathies[20]. The tau protein was originally discovered in 1975 by Dr. Weingarten as an essential protein needed for the stabilization and assembly of microtubules[21]. Tau is a part of a family of protein known as microtubule-associated proteins (MAPs) that interact with microtubules and the cytoskeleton[22]. Tau is said to be a natively unfolded protein and since its

discovery, a lot of research has been done to understand its structural, functional, and biochemical properties and how that relates to disease[23].

1.6 Tau Structure

Tau is mainly expressed in neurons and the gene is made up of 16 exons, which is located on chromosome 17[24]. Full length tau in the central nervous system is 441 amino acids long and has 4 microtubule binding repeat regions, but through alternative mRNA splicing tau exists in 6 different isoforms[25]. These isoforms are characterized by the presence or absence of exons E2 and E3 located in the N-terminus and E10 (also known as microtubule binding repeat R2) as shown in figure 1.1. The tau nomenclature identifies the presence or absence of exons E2 or E3 denoted as 0N, 1N, or 2N. The splicing of E3 is dependent on E2 and is never present without it[26]. The other half of the nomenclature describes the presence or absence of exon E10 to explain whether that isoform has 4 or 3 MTBR regions and is denoted as 3R or 4R. In the brain of the fetus only the 0N/3R isoform is present, but tau is developmentally regulated. In the adult brain, all 6 isoforms are present with the 3R and 4R isoforms roughly being equal, but the 0N, 1N, 2N isoforms are present as ~37%, ~54%, and ~9% of total tau[25, 27]. Tau is considered to be an unfolded protein or intrinsically disordered with monomeric tau having very little secondary structure, but utilizing NMR there is evidence that within the MTBRs there are some β-strands[28, 29]. Besides the MTBRs, tau also has other important structural features. When tau binds to the microtubules, the acidic N-terminal region projects outward and is known as the projection domain, which includes E2 and E3, and has interactions with other proteins[30]. There is also a proline-rich region of tau which comes right before the MTBRs. This region has shown to have a binding motif of PXXP with serines or threonines next to the proline residues that bind to fyn

and src tyrosine kinases[31]. Another interesting aspect of tau is that all the isoforms have a slight positive charge overall, but the distribution of the charges is something to make note of. The proline-rich region and the MTBRs are positively charged to bind to and stabilize microtubules, but the N-terminal projection domain is negative with many acidic residues. Although tau is natively unfolded and highly flexible, it has been shown to adopt a global hairpin structure when unbound and in solution[32]. Utilizing NMR and FRET-based assays, it was determined that the N-terminus and C-terminus overlap over the middle of the MTBRs creating this hairpin or paperclip structure. The structure and function of tau is important to its aggregation in disease and it is still not well understood if tau aggregation is a toxic gain of function or a toxic loss of function.

Figure 1.1

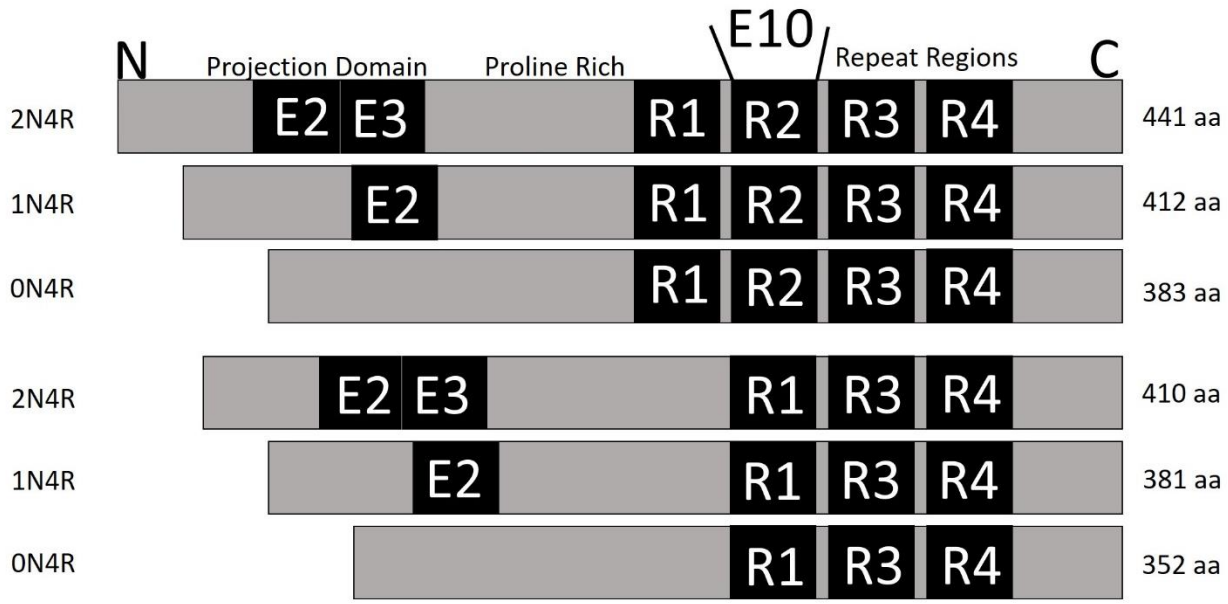

Figure 1.1 Tau isoforms due to alternative mRNA splicing
The human brain has six isoforms of tau generated by alternative mRNA splicing of exons 2, 3, and microtubule binding repeat 2. The tau nomenclature describes the number of N-terminal exons denoted as 2N, 1N, or 0N and the number of microtubule binding repeats present, 4R or 3R. Adapted from Combs et al.[33]

1.7 Tau Function

Tau functions as a microtubule stabilizer as well as promoting α and β tubulin nucleation to form microtubules[34]. Microtubules serve as cytoskeletal support and as a track for molecular cargo to be transported from the cell body to the synapse and vice versa. The microtubule is distinguished by a plus end going toward microtubule growth and a minus end towards the centrosome where the microtubules propagate. Kinesin and dynein are molecular motors that carry cargo from minus to plus and plus to minus respectively[35]. The microtubule binding repeats (MTBRs) regions of tau bind to microtubules for stabilization[36]. Although each individual MTBR has weak binding, there is evidence that they have a cooperative binding effect[37]. Because of this, the 4R isoforms bind more tightly than the 3R isoforms and more efficiently promote the assembly of microtubules. In addition to stabilizing microtubules, tau also influences microtubule bundling[38]. The bundling of microtubules is a critical process involved in cell division and may be important for axonal structure and transport of molecular cargo, which may be affected by the sizes of the tau isoforms. The projection domain of bound tau interacts with the plasma membrane and is involved in anchoring the protein[39]. Tau is also involved in remodeling of the actin cytoskeleton and may assist in mediating microtubule-actin interactions. The proline-rich region has been shown to bind SH3 domains of src family kinases and has been shown to interact with phospholipase isozymes as well[31]. All of these functions of tau are heavily related to the unfolded nature of tau, the acidic N-terminal domain, basic MTBR, and play a role in the aggregation of tau.

1.8 Tau Aggregation

The tau hypothesis suggests that tau aggregation into neurofibrillary tangles is the driving force of AD and other dementias known as tauopathies[40]. Tauopathies are classified as

neurodegenerative diseases with the pathological hallmark of tau aggregation. Tauopathies include AD, Pick's disease, progressive supranuclear palsy, corticobasal degeneration, chronic traumatic encephalopathy, and frontotemporal dementia with Parkinsonism linked to Chromosome 17. Although all of the diseases have a defining characteristic of tau aggregation, they are distinct in the brain regions each affects as well as different forms of pathological tau. When tau becomes pathological it is not known whether aggregation is a toxic loss of function or gain of function. Tau is normally a very soluble protein but in disease it loses its ability to bind to microtubules, which leads to destabilization of microtubules. Pathologically, tau aggregation correlates with the progression of the disease with aggregates in the brain regions that are associated with behavioral and psychological symptoms of the disease. When monomeric tau aggregates, it forms paired helical filaments (PHFs), straight filaments, and twisted ribbons, depending on the disease. In AD PHFs are present and are facilitated through two main hexarepeat amino acid regions in tau, which are ^{275}VQIINK^{280} in the second MTBR and ^{306}VQIVYK^{311} found in the third MTBR[41]. These motifs are essential for tau aggregation and are labeled PHF6* and PHF6 respectively. The 4R isoforms have both regions, but the 3R isoforms are missing VQIVYK motif because of the absence of the MTBR2. When tau aggregates, these motifs form cross-β structures, which make up the core of the filaments[42]. Tau aggregation has been described as a two-step process of nucleation and elongation *in vitro*[43]. The nucleation phase consists of tau monomers coming together to form tau oligomers. These oligomers are able to associate with other tau oligomers and incorporate monomeric tau to form insoluble filaments, which is known as the elongation phase. One of the ways to study tau aggregation and better understand the mechanism of the disease is to aggregate tau in a laboratory setting. Tau is a very

soluble protein and does not aggregate on its own. To aggregate tau *in vitro*, an inducer molecule is used such as heparin, arachidonic acid (ARA), and other polyanionic molecules[44-46].

There has also been debate about what are the actual toxic species of tau aggregation. Initially the hypothesis was the filaments themselves were toxic to neurons. This was explored by taking NFTs from a postmortem AD patient and implanting them in to mice. The mice, however, did not develop AD-like dementia or additional pathology[47]. Additional research was done to suggest that the toxic species is the oligomeric form of tau[48]. There is evidence that neuronal dysfunction is present before NFTs are present, suggesting that a different species of tau aggregation is toxic. It has also been suggested that there is a toxic conformation of tau as well. As previously mentioned, tau sits in a global hairpin conformation with the N and C-terminals overlapping over the middle of the MTBRs. The N-terminal contains a phosphatase-activating domain (PAD). When monomeric tau is in this conformation the PAD is not accessible, but if tau is modified and adopts an "open paperclip" conformation the exposed PAD can activate a signaling cascade, which involves protein phosphatase-1 and glycogen synthase kinase-3[49]. Activation of this pathway can cause phosphorylation of kinesin and inhibit the binding of molecular cargo and disrupt fast axonal transport. This disruption can affect axonal and synaptic processes and can eventually lead to cell death[49].

Tau aggregation seen in tauopathies is still not fully understood. It has been hypothesized and well-studied that monomeric tau undergoes post-translational modifications that could contribute to disease. These modifications are hyperphosphorylation, acetylation, truncation, as well as mutations known as frontotemporal dementia with parkinsonism linked to chromosome 17[50]. Research has shown that these modifications affect aggregation *in vitro* and have implications in

tauopathies. Although tau aggregation and the accumulation of senile plaques are pathological hallmarks of the disease, there are no current treatments targeting this.

Figure 1.2

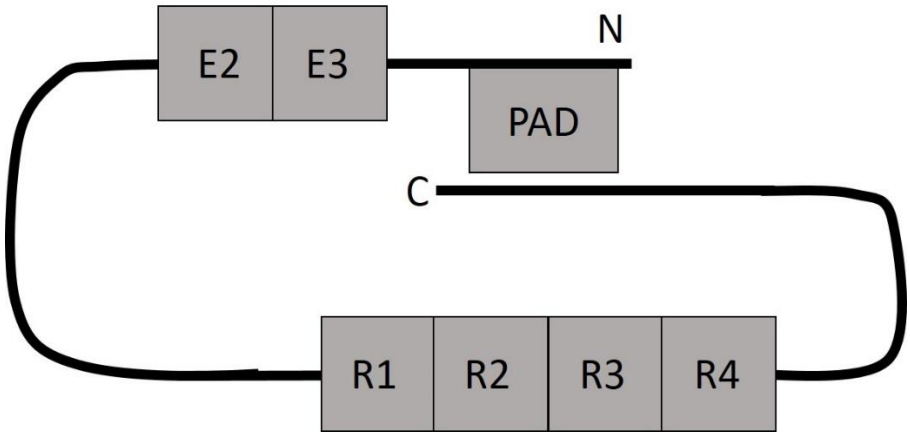

Figure 1.2. Global hairpin conformation of tau
The N-terminal and C-terminal ends of tau interact with each other as well as the interior of the protein in a conformation that has been described as the "global hairpin." This global hairpin is the conformation monomeric tau is in when it is not bound to microtubules. The PAD represents the phosphatase-activating domain, which is accessible when tau is bound to microtubules and during aggregation in disease. Adapted from Combs et al.[51]

1.9 Current Approved Therapies

With the prevalence and cost of AD there are continuous efforts to produce drugs and therapies for it. There are currently 5 drugs approved by the Federal Drug Administration (FDA)[2]. The drugs are known as donepezil, rivastigmine, galantamine, memantine, and the most recent drug approved in 2014 combines donepezil and memantine. The drugs grouped by their mechanism of action and are aimed to improving the quality of life of the individual with the disease by managing its symptoms, such as memory loss. A summary of the drug name, brand name, stage of disease it is used for, drug mechanism, and when it was approved by the FDA can be found in table 1.

The first group of drugs is donepezil, rivastigmine, and galantamine, which are all cholinesterase inhibitors. These drugs inhibit the enzyme acetylcholinesterase that hydrolyzes acetylcholine into choline and acetic acid[52]. Acetylcholine is a neurotransmitter involved in memory and learning and in AD there is a decrease in the amount of acetylcholine present in the brain due to neuronal loss[53]. The loss of neurons is in brain areas highly associated with memory such as the cortex, hippocampus, and the entorhinal cortex. The major hypothesis of inhibiting acetylcholinesterase is preventing the breakdown of acetylcholine so more neurotransmitter will be present in the synapse[54]. This will allow more acetylcholine to bind to receptors on the post-synaptic neuron to improve memory.

Memantine, however, works by a different mechanism. This drug works as an N-methyl-D-aspartate (NMDA) receptor antagonist to help regulate the activity of glutamate and calcium[55]. NMDA receptors require the binding of glutamate and are unique from other ionotropic

receptors because of the strong voltage dependence needed to remove the magnesium (Mg^{2+}) blockade to allow ions such as K^+, Na^+, and Ca^{2+} to permeate the post-synaptic neuron. There is a hypothesis that in AD there is overactivation of NMDA receptors, which can cause neuronal damage or even death due to Ca^{2+} excitotoxicity[56]. Memantine is an uncompetitive antagonist meaning it does not cause inhibition by competing with glutamate, but instead acts like Mg^{2+} to block the channel. Unlike Mg^{2+}, however, memantine is not released from the channel due to the binding of glutamate and ions are not able to flow through the channel. Regulating the influx of Ca^{2+} can reduce the damage or death of neurons and preserve synaptic pathways associated with memory[56].

As mentioned previously 5 FDA approved drugs are focused on improving the quality of life by medicating symptoms, but there are issues with these drugs. The main issues are seen with dosing and tolerance. Dosing has been shown to be very individualistic with many patients not responding to the drug until higher doses are given. With higher doses, however, side effects become more likely. The acetylcholinesterase inhibitors have been shown to cause nausea, vomiting, dizziness, and increased bowel movements. Memantine has been known to cause headaches, confusion, loss of appetite, and vomiting. Although most of the drugs are approved for mild to severe stages of the disease, the formation of pathology occurs well before symptoms begin. Currently there are no approved drugs that are targeting the pathology to prevent or slow the progression of the disease.

Table 1.1 FDA Approved Drugs for Alzheimer's Disease

Drug(s)	Brand Name	Approved For	Mechanism	FDA Approved
Donepezil	Aricept	All stages	Acetyl cholinesterase inhibitor	1996
Rivastigmine	Excelon	Mild to moderate	Acetyl cholinesterase inhibitor	2000
Galantamine	Razadyne	Mild to moderate	Acetyl cholinesterase inhibitor	2001
Memantine	Namenda	Moderate to severe	NMDA receptor inhibitor	2003
Donepezil and Memantine	Namzaric	Moderate to severe	Acetyl cholinesterase inhibitor and NMDA receptor inhibitor	2014

1.10 Tau Aggregation inhibitors

Although the current FDA approved drugs do not target pathology, there is research being done to do so. A lot of research has been done targeting the components of the amyloid cascade hypothesis including β-secretase inhibitors, γ-secretase inhibitors, preventing oligomerization of Aβ, increasing the clearance of Aβ, and anti-inflammatory drugs.[57-59] Although many of these Aβ drugs have gone to clinical trials, no drug has been approved[60]. Due to this, efforts have put towards targeting the other major pathology seen in AD, which is tau aggregation and NFTs. Inhibiting tau aggregation or breaking down the existing pathology can be useful in preventing or delaying the progression of AD.

Targeting tau aggregation directly through small molecules has been of interest as an avenue and notably a tau aggregation inhibitor for the treatment of AD has reached phase-3 clinical trials and is a stable reduced form of the methylthionium moiety (figure 1.3)[61].

Figure 1.3

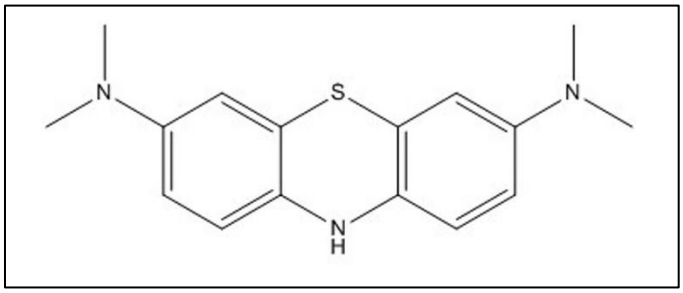

Figure 1.3
The structure of rember (TRX-0237), a tau aggregation inhibitor with a methylthionium moiety and is currently in phase 3 clinical trials[62].

Small molecules are believed to interact with the β-sheet structures or the hexarepeat aggregation motifs in tau to inhibit aggregation. Chemical scaffolds of tau aggregation inhibitors have been identified from high-throughput screen of small molecule libraries that consisted of synthetic, semi-synthetic, and natural products. The scaffolds that had TAI activity were rhodanines, anthraquinones, N-phenylamines, and phenylthiazolyl hydrazides (figure 1.4)[63]. Many of these compound classes were able to inhibit tau aggregation and disassemble preformed aggregates as well.

Figure 1.4

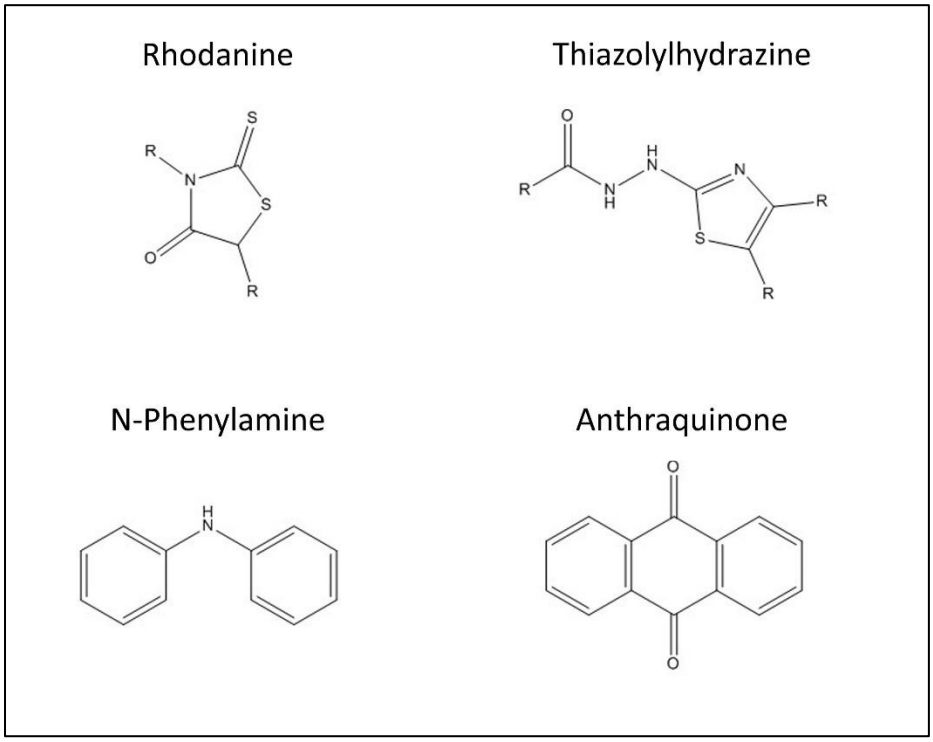

Figure 1.4
Compound classes found in a high-throughput screen of 200,000 compounds from random libraries[63].

Natural products have been extremely useful in medicinal chemistry and drug development. Between 1981 and 2014 of the 1,562 drugs approved by the FDA, unaltered natural products that became drugs was 4%[64]. Although this doesn't seem significant, natural products have been used as scaffolds or insights into synthetic drugs. Synthetic or semi-synthetic drugs that had influence from natural products make up 47% of approved drugs during that time period[64]. One source of biomedically useful natural products have been from fungi, which have given us drugs such as cyclosporin, lovastatin, and penicillin[65]. Many natural products used in medicinal chemistry are secondary metabolites, which are molecules used by the fungus in situations such as defense mechanisms but are not necessarily needed for life cycle purposes[66]. One of these purposes is when fungi encounter invading bacteria they will produce chemicals for antibiosis[67] Bacteria utilize biofilms for adhesion and quorum sensing, but the biofilms are made up of matrix proteins that polymerize similar to β-structures seen in protein aggregation related to AD[68]. Because of this, we hypothesized that fungal secondary metabolites could possess TAI activity.

Dr. Berl Oakley and colleagues have developed genetic tools to access silent gene clusters for expression of secondary metabolites in *Aspergillus nidulans*[69]. The secondary metabolites isolated from their lab contained novel compounds as well as compounds that were from the class of anthraquinones, including the compound emodin which was known to inhibit tau aggregation *in vitro*[63]. The 17 compounds Dr. Oakley isolated from *Aspergillus nidulans* were utilized in tau aggregation inhibition reactions and one of the compounds was a novel inhibitor scaffold known as asperbenzaldehyde[70]. Asperbenzaldehyde was an intermediate to azaphilone biosynthesis, which have known lipoxygenase activity. Asperbenzaldehyde was semi-

synthetically modified to 11 azaphilone derivatives, which all were able to inhibit tau aggregation and 4 were able to also disassemble preformed filaments[71].

1.11 Thesis Overview

Alzheimer's disease, along with the other tauopathies, are devastating diseases that collectively affect millions of Americans[2]. Currently there are no approved drugs that can stop, reverse, or slow down the disease. The current treatments are aimed at improving the symptoms of the disease, but do not target the underlying pathology. It has been estimated that a treatment delaying the disease even by 5 years can save billions of dollars in the cost of care[72]. One of the challenges of finding a treatment that can delay the progression of the disease is even though a lot of research has gone in to understanding these diseases, is we do not know the cause of them. Tau aggregation seems to be a major player in the disease, but what initiates tau aggregation is still unknown. Even though we do not know what causes the initial insult of tau aggregation, aggregation inhibitors are still of interest because of the impact it can have on the people that suffer from the disease since pathology accumulates before cognitive deficits occur.

The goal of this study was to identify natural products that could serve as chemical scaffolds for future drug candidates with the potential of preventing or delaying disease. To do this, secondary metabolites from *Aspergillus nidulans* were screened as tau aggregation inhibitors (TAIs). The lead compound, ANTC 15, was chosen out of 30 compounds isolated by Dr. Berl Oakley and colleagues because of the structural similarity to previous TAIs known as azaphilones. The compound was tested using *in vitro* assays previously used by the lab to assess the compounds ability to inhibit the formation of tau filaments or disassemble pre-formed tau aggregates.

Chapter 2: A Novel Tau Aggregation Inhibitor from Fungal Secondary Metabolites

2.1 Introduction:

Alzheimer's disease (AD) is the most common form of dementia and is the 6th leading cause of death in the United States. Current approved treatments are focused on improving the symptoms of the disease rather than reversing or preventing the pathological hallmarks of the disease. These hallmarks being senile plaques composed of amyloid beta and neurofibrillary tangles composed of the microtubule-associated protein tau. The location and amount of tau aggregation correlates with the progression and severity of the disease[73]. It's for this reason it would be beneficial to identify drugs that could inhibit or reverse tau aggregation.

Fungi have produced biomedically relevant compounds that have been useful for antibiotics, immunosuppressants, and drugs to lower cholesterol[74]. There are biosynthetic pathways in *Aspergillus nidulans* that produce secondary metabolites and were discovered as being a part of silent gene clusters[75]. Developments have been made to manipulate the genome of *A. nidulans* to produce secondary metabolites in a laboratory setting[76]. Previously, these secondary metabolites were tested *in vitro* for tau aggregation inhibition (TAI) activity. One compound that was discovered was asperbenzaldehyde, which was structurally distinct from previous tau aggregation inhibitors[70]. Although it only inhibited tau aggregation and not disassembled previously formed aggregates, it was shown to be an intermediate compound for the synthesis of lipoxygenase inhibitors known as azaphilone compounds[77]. These compounds were synthesized from asperbenzaldehyde and tested as tau aggregation inhibitors. All of the azaphilones tested were able to inhibit tau aggregation, but four compounds were able to disassemble previously formed aggregates as well[71].

In the current study, a compound was investigated that was isolated from *Aspergillus nidulans* and provided by Dr. Berl Oakley. This compound was chosen because of its similarity to the azaphilones previously mentioned. This compound, however, has an aromatic core structure known as an isoquinoline and has an IUPAC name of 7-methyl-3-nonylisoquinoline-6,8-diol, but will be referred to as ANTC 15. The ability of the compound to inhibit the formation of tau aggregates or disassemble preformed filaments was assessed *in vitro* using a standard arachidonic acid induction of tau aggregation technique. ANTC 15 was able to inhibit tau aggregation and disassemble tau aggregates in vitro. Dose-dependence tests of inhibition and disassembly were also performed to see how potent the compound was *in vitro* utilizing the filter trap technique as well as electron microscopy. This study was able to provide a novel chemical scaffold as a tau aggregation inhibitor that could be used in further studies to improve the efficacy of the compounds as well as understand how these compounds are interacting with tau.

2.2 Experimental Procedures:

2.2.1 Chemicals and Reagents

Full length 2N4R tau was expressed and purified using a method previously described[78]. 2N4R tau and cysless 2N4R tau were grown overnight in Luria Broth overnight and IPTG was used induce expression. Cells were lysed through a french press and the protein was isolated using a Ni affinity column and Superdex 200 column. Cysless tau was generated by mutating the cysteine at amino acid 291 to alanine and cysteine at 322 to alanine[79].

2.2.2 Polymerization reactions

Inhibition reactions

2 μM recombinant tau protein was incubated in polymerization buffer which contained 10 mM HEPES (pH 7.64), 5 mM DTT, 100 mM NaCl, 0.1 mM EDTA, and 3.75% ethanol. Compounds dissolved in DMSO were added to the tau solution at final concentrations of 400 μM, 300 μM, 200 μM, 150 μM, 100 μM, 50 μM, 25 μM, 12.5 μM, or 6.25 μM. Compounds were incubated with tau for 20 min at room temperature before adding arachidonic acid (ARA) (Millipore) at a final concentration of 75 μM to initiate tau polymerization. The reactions proceeded at 25℃ for 16 hrs before analysis.

Disassembly reactions

2 μM recombinant tau protein was incubated in polymerization buffer which contained 10 mM HEPES (pH 7.64), 5 mM DTT, 100 mM NaCl, 0.1 mM EDTA, and 3.75% ethanol. ARA was added to the reactions at a final concentration of 75μM, causing tau to polymerize into filaments. After 6 hrs of incubation at 25℃, compounds dissolved in DMSO were added to the tau solution at final concentrations of 400 μM, 300 μM, 200 μM, 150 μM, 100 μM, 50 μM, 25 μM, 12.5 μM, or 6.25 μM. The reactions proceeded at room temperature for 16 hrs before analysis.

2.2.3 Filter trap

Tau polymerization reactions, as described in 2.3.4 were done as previously described[71]. The reactions were diluted to 20 ng/300 μL in Tris-Buffered Saline solution (TBS) and passed through a pre-wetted nitrocellulose membrane (Bio-Rad Laboratories) using vacuum force in a dot-blot apparatus (Bio-Rad Laboratories). The membranes were washed three times with TBS-0.05%Tween20 (TBST) and then blocked in 5% nonfat dry milk in TBST for 1 h. The

membranes were then incubated with primary antibody. Primary antibodies included: Tau 5, 7, 12 antibody mixture [Tau 5 at 1:50,000 dilution, Tau 12 at 1:250,000 dilution and Tau 7 at 1:250,000 dilution], TNT1 at a dilution of 1:200,000, and TOC1 at a dilution of 1:7000 overnight at 4 °C. The membranes were washed three times in TBST, at 5 min each, and incubated with secondary antibody: HRP-linked Goat anti-mouse IgG (Thermo Scientific, Rockford, IL) for Tau 5, 7, 12 and TNT1 and HRP-linked Goat anti-mouse IgM (Thermo Scientific, Rockford, IL) for TOC1 for 1 hr at room temperature. The membranes were washed twice at 5 min each in TBST and a final 5 min wash with TBS. The blots were developed using ECL (enhanced chemiluminescence) Western Blotting Analysis System (GE Healthcare, Buckinghamshire, UK). Images were captured with a ChemiDoc-It^2 Imager and were quantified using the histogram function of Adobe Photoshop 7.0.

2.2.4 Antibodies

Detection of the amount of filaments on the nitrocellulose membrane a mixture of antibodies to recognize the N-terminal region (tau-12), the central region (tau-5), and the C-terminal region (tau-7), which is labeled as Tau 5, 7, 12 or total tau. There two other proposed toxic conformations of tau, which are detected by antibodies Tau-N-Terminal 1 (TNT1) and Tau Oligomeric Complex 1 (TOC1). TNT1 recognizes the phosphatase activating domain (PAD) in the N-terminal region of tau and TOC1 recognizes cross-linked oligomers of tau[80]

2.2.5 Electron Microscopy

Polymerization reaction samples were diluted 1:10 in polymerization buffer and fixed with 2% glutaraldehyde for 5 min. 10 μL of each sample was added to a Formvar carbon-coated grid for 1

min. The grid was blotted on filter paper, washed with water, blotted, washed with 2% uranyl acetate, and blotted before staining with 2% uranyl acetate for 1 min followed by a final blotting on filter paper. The grids were examined with a Technai F20 XT Field emission transmission electron microscope (FEI Co., Hillsboro, OR). Images were taken with the Gatan Digital Micrograph imaging system. The images were collected at a magnification of 3600×[51].

2.2.6 Mass Spectrometry

Figure and mass spectrometry (MS) analysis provided by Dr. Weiss and Farai Rusinga.
Intact protein analysis on a Time-of-Flight (ToF) mass spectrometer (Agilent 6200) with electrospray ionization (ESI) was utilized to obtain masses. Before MS, protein (and compound) samples were pumped onto a C4 desalting trap in 0.1% formic acid using by an isocratic pump (Agilent 1200). After 2 minutes of washing, samples were eluted by a gradient of 0.1% formic acid (bottle A) and 90/10/0.1% acetonitrile/water/formic acid (bottle B) into the ESI source for MS analysis. Agilent MassHunter Acquisition software for acquiring spectra and Agilent Qualitative Analysis software for data analysis.

2.2.7 Statistical analysis

An unpaired t-test was used to compare the values for the filter trap assay. P-values less than or equal to 0.05 were indicated with one asterisk (*), less than or equal to 0.01 with two asterisks (**), and less than or equal to 0.001 with three asterisks (***).

2.3 Results:

The compound utilized in this study was chosen from secondary metabolites obtained from *Aspergillus nidulans*. ANTC 15 was chosen to be studied as a TAI because of its structural similarities to Aza-9, which is a known tau aggregation inhibitor[71] (figure 2.1). Inhibition of tau aggregation reactions and disassembling preformed tau aggregate reactions were carried out *in vitro* as described above. To determine if ANTC 15 could inhibit or disassemble filaments, a final concentration of 200 µM was incubated with 2 µM tau for 20 minutes before adding ARA at a final concentration of 75 µM. The amount of TAI activity was measured by a filter trap assay, which has been previously used to test *A. nidulans* compounds[70].

.

ANTC 15 significantly inhibited the formation of filaments, the toxic open paperclip conformation of tau, and the formation of toxic oligomers (figure 2.2 A). The compound was also able to disassemble preformed filaments and toxic oligomeric complexes (figure 2.2B). Electron microscopy (EM) was also done to show the absence of filaments after the compound had been added (figure 2.2 A and B). It was also able to significantly decrease the signal of the TNT1 antibody by decreasing the presence of the toxic conformation (figure 2.2).

Figure 2.1

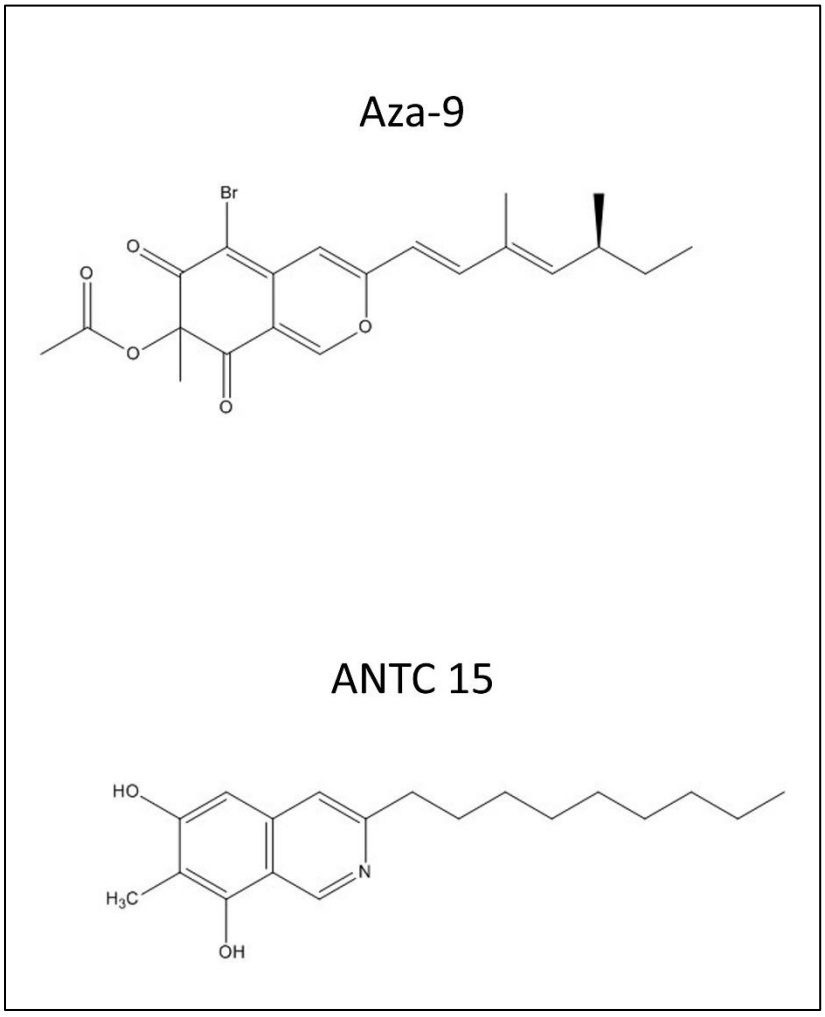

Figure 2.1: Structure comparison of ANTC 15 and known tau aggregation inhibitor Aza-9. Lipoxygenase inhibitors that were previously tested for tau aggregation inhibition activity have an azaphilone score scaffold, and Aza-9 was the most potent. The azaphilone scaffold is characterized by the presence of a pyrone-quinone structure as well as a chiral quaternary center[81]. ANTC 15 was chosen from a set of isolated secondary metabolites due to its similarity to Aza-9, but has an isoquinoline core scaffold to test for tau aggregation inhibition activity. The isoquinoline scaffold is characterized by a benzo-fused pyridine, but with the N in the 2 position[82].

Figure 2.2

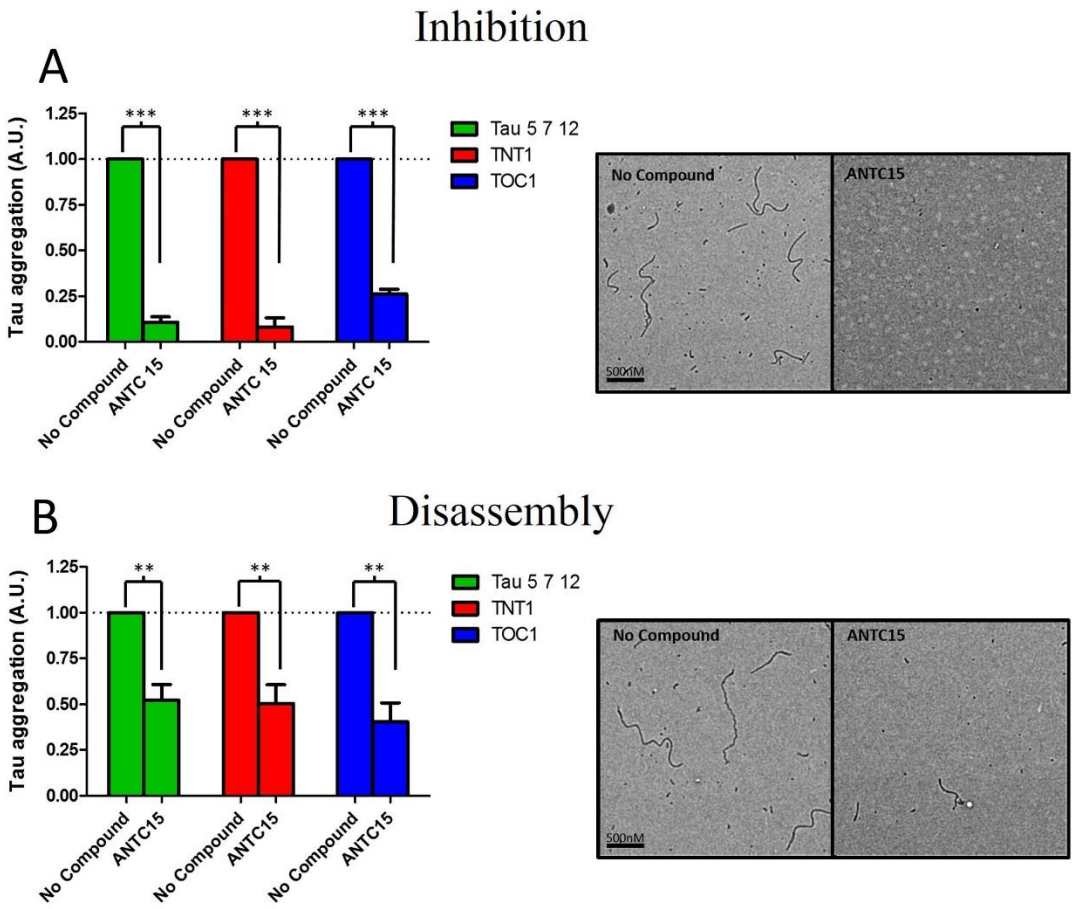

Figure 2.2: Inhibition and Disassembly of ANTC 15 at 200 µM
Inhibition (A) and disassembly (B) reactions were done with a final concentration of ANTC 15 of 200 µM utilizing the filter trap assay. ANTC 15 was able to significantly inhibit and disassemble tau aggregation filaments and the toxic conformations of tau recognized by TNT1 and TOC1. This was also seen with EM images of the reactions. The average of three independent trials ± SD for No Compound and ANTC 15 with Tau 5, 7, 12 (green bars), TNT1 (red bars), and TOC1 (blue bars). *, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.001$

Since the compound was able to significantly inhibit and disassemble filaments and toxic species, I tested to see if there was a dose dependent relationship. IC_{50}'s for inhibition and disassembly *in vitro* were determined using a range of concentrations from 400 µM to 6.25 µM utilizing the filter trap assay. An IC_{50} was used to determine the concentration of ANTC 15 needed to reduce the amount of filaments or toxic species by half. The curves were fit by a dose-response by variable slope with normalized tau aggregation on the y-axis and the log of concentration on the x-axis. For inhibition, the IC_{50}'s were 48.66 µM +/- 29.19 for Tau 5 7 12 (figure 2.3 A), 95.93 µM +/- 69.40 for TNT1 (figure 2.3 B), and 161.25 µM +/- 17.47 for TOC1 (figure 2.3 C). For disassembly, IC_{50}'s were 42.62 µM +/- 17.93 for Tau 5 7 12 (figure 2.3 D), 129.20 µM +/- 85.31 for TNT1 (figure 2.3 E), and 169.20 µM +/- 23.05 for TOC1 (figure 2.3 F). These results were qualitatively validated for inhibition and disassembly by EM images at each of the concentrations showing the decrease of filaments present on the grid as the concentration of ANTC 15 increases (figures 2.4 and 2.5). For inhibition, qualitatively the IC_{50} seems to be between the 100 µM to 50 µM grids (figure 2.4), which is similar to the quantitated IC_{50} for the total tau antibody (figure 2.3 A). Although looking at the disassembly grids the IC_{50} appears to be between 150 µM and 100 µM (figure 2.5), which is a little higher than the quantified IC_{50} (figure 2.3 D). To determine how long it takes for ANTC 15 to disassemble filaments a disassembly time course assay was set up. Tau aggregation was induced with ARA and allowed to proceed for 6 hours. Before 200 µM ANTC 15 was added an EM grid was made of the reaction, then immediately after ANTC 15 was added another EM grid was made. A grid was made at various time points including the next day. The grids showed qualitatively that the amount of filaments before adding ANTC 15 and after addition were comparable and as further EM grids were made, the amount of filaments decreased (figure 2.6). There is a noticeable

difference between the 300 min grid and the Next Day grid and having more time points would be beneficial to understanding how ANTC 15 disassembles filaments. This also showed that ANTC 15 was not interfering with the filaments sticking to the grid because the No compound grid and 0 min grid are comparable.

Figure 2.3

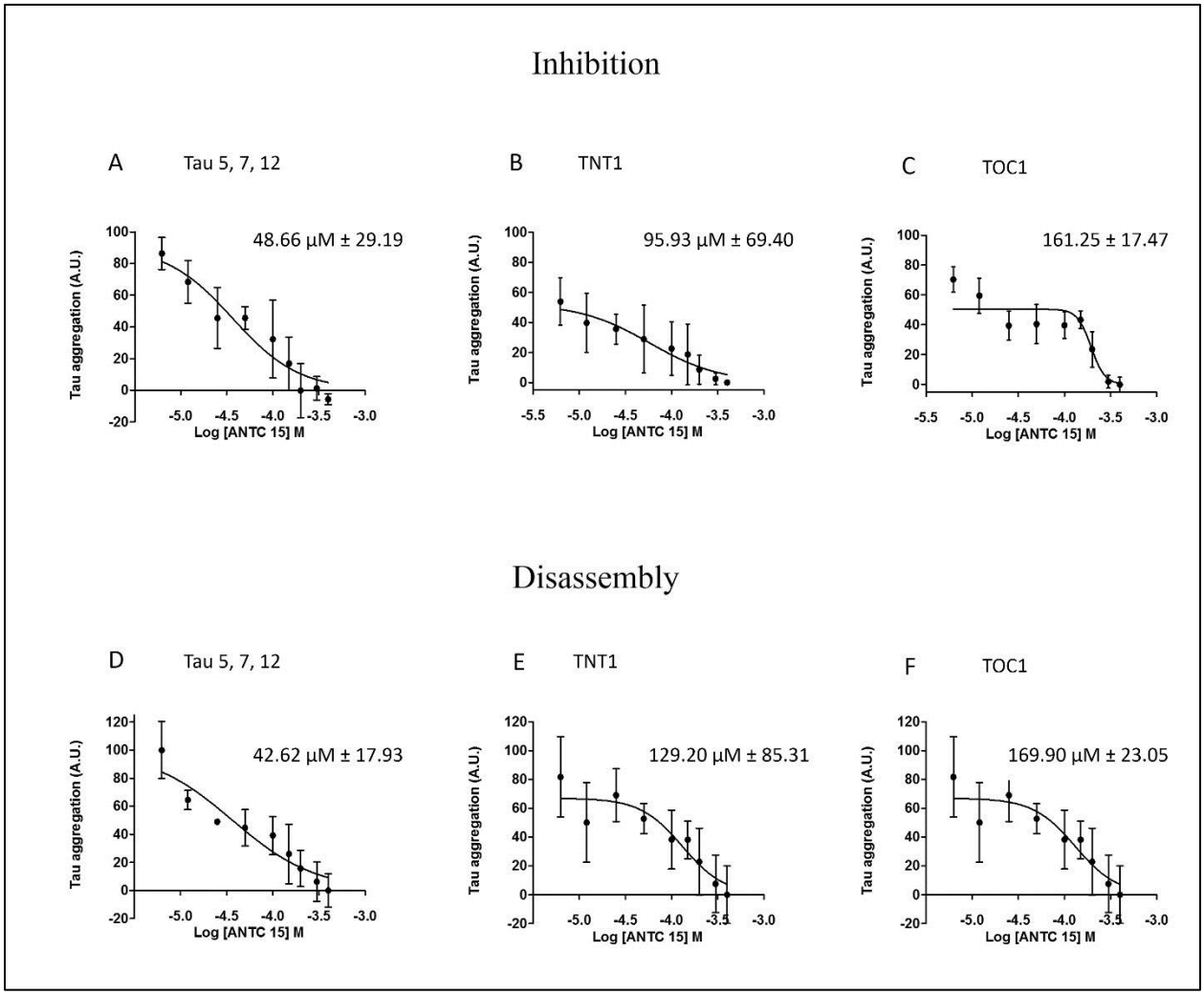

Figure 2.3: IC_{50} of ANTC 15 Inhibition and Disassembly with Filter Trap
IC_{50}'s were determined for inhibition and disassembly reactions by filter trap. 2 µM 2N4R tau and ANTC 15 at several concentrations for the inhibition and disassembly reactions. Antibodies tau 5, 7, 12 (total tau), TNT1, and TOC1 were used to measure the amount of filaments and toxic species. The data was plotted against the log of inhibition concentration and fit to a dose-response curve to determine an IC_{50}. The data points were averaged between trials ± SD.

Figure 2.4

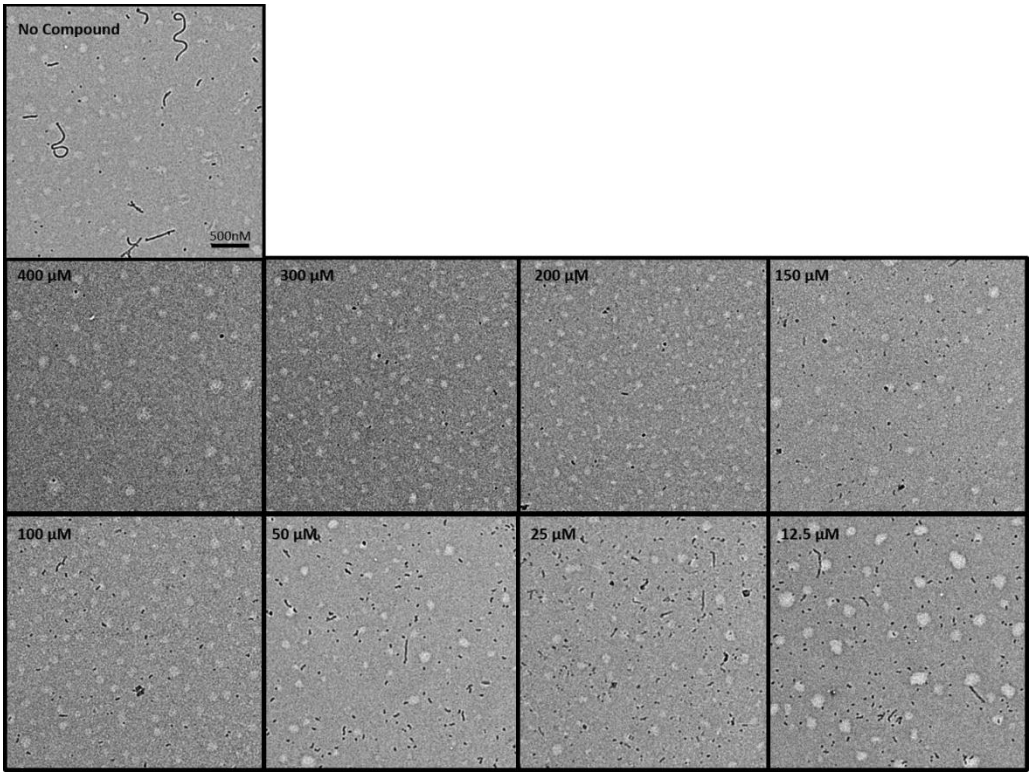

Figure 2.4: Electron Microscopy of ANTC 15 Dose Dependence Inhibition
Inhibition tau aggregation reactions were performed using 2 µM 2N4R tau, ANTC 15 at final concentrations of 400 µM to 12.5 µM incubated with monomeric tau for 20 mins, and 75 µM arachidonic acid to induce polymerization at room temperature overnight. As the concentration of ANTC 15 gets lower, the presence of filaments increases, which qualitatively correlation with the dose dependent filter trap data.

Figure 2.5

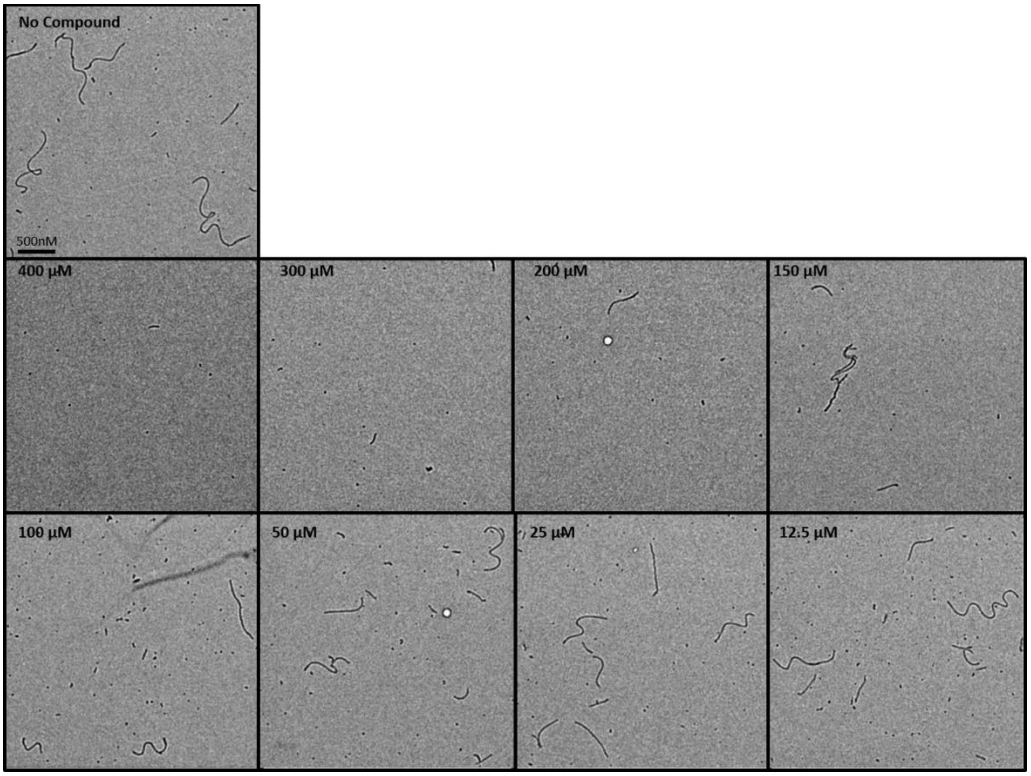

Figure 2.5: Electron Microscopy of ANTC 15 Dose Dependent Disassembly
Disassembly tau aggregation reactions were performed using 2 µM 2N4R tau, 75 µM arachidonic acid to induce polymerization for 6 hours at room temperature, then adding ANTC 15 at final concentrations of 400 µM to 12.5 µM overnight. As the concentration of ANTC 15 gets lower, the presence of filaments increases, which qualitatively correlation with the dose dependent filter trap data.

Figure 2.6

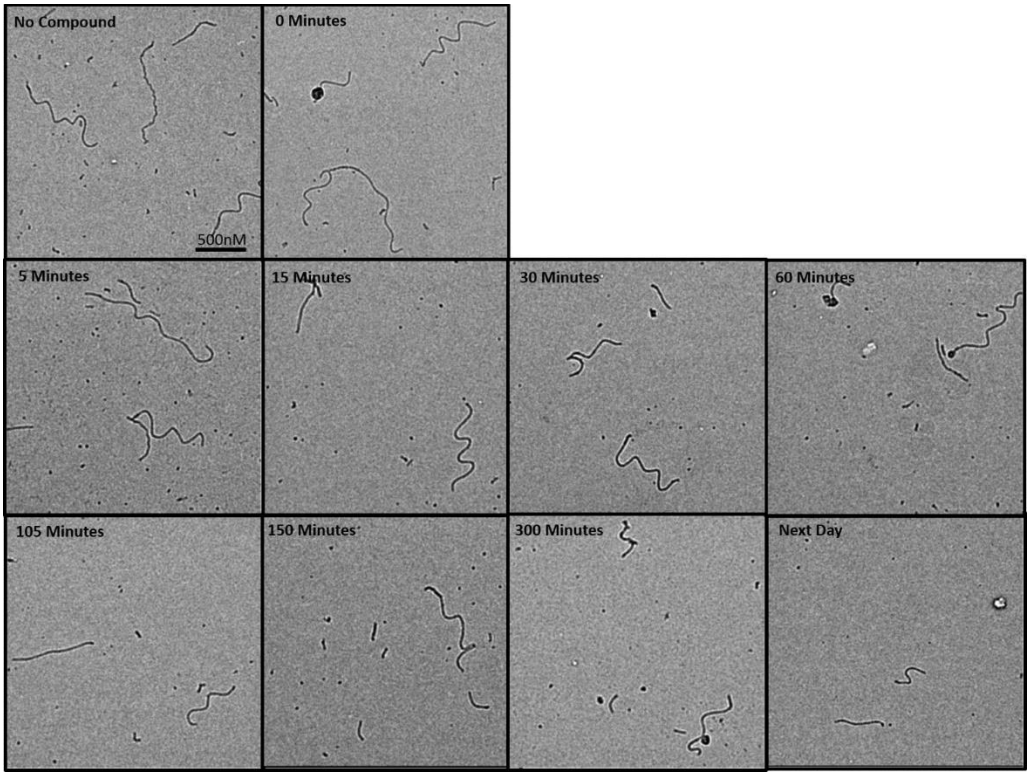

Figure 2.6: Electron Microscopy of Disassembly Time course of 200 µM ANTC 15
Disassembly tau aggregation reactions were performed using 2 µM 2N4R tau, 75 µM arachidonic acid to induce polymerization for 6 hours at room temperature, then a grid was made and labeled No compound. Then adding ANTC 15 a final concentration of 200 µM a grid was made immediately and labeled 0 mins, and grids were continued to be made at the various time points.

Mass spectrometry was utilized to analyze if ANTC 15 was covalently modifying monomeric tau. Covalently modifying monomeric tau could be detrimental because the aggregation motifs are located within the MTBRs and could interfere with tau binding to microtubules. Using electrospray mass spectrometry and intact protein time of flight we expected to see two peaks; a tau mass of 48.013 kDa and an ANTC 15 mass of 301.43 Da. The tau mass was resolved at 47.883 kDa, which is consistent with cleavage of the start methionine and is common in recombinantly expressed proteins[83]. ANTC 15 did not appear to be making a covalent modification because of the appearance of a tau peak and an ANTC 15 peak (figure 2.7). With the possibility that ANTC 15 could be interacting with the cysteines in tau and not the lysines in the aggregation motifs, cysless 2N4R tau was expressed, purified, and used in inhibition reactions. ANTC15 was able to inhibit the formation of filaments as seen by electron microscopy (figure 2.8).

Figure 2.7

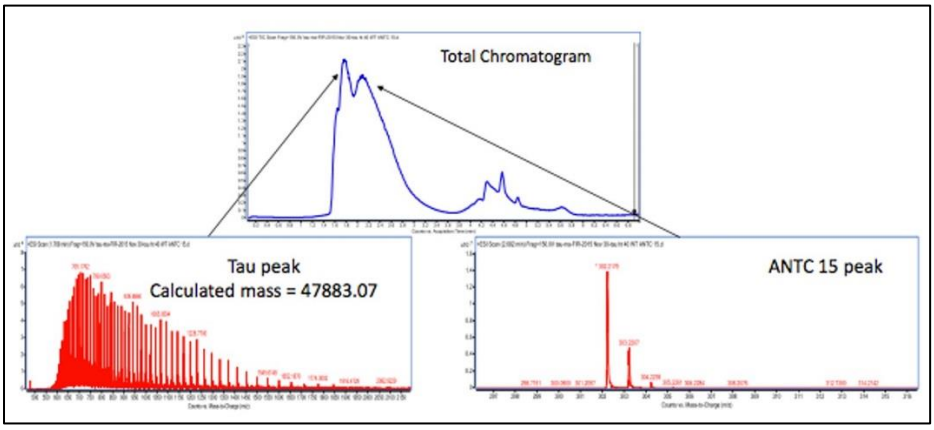

Figure 2.7: Mass spectrometry of ANTC 15 and monomeric tau
2N4R tau mass peak and ANTC 15 mass peak were able to be resolved, suggesting no covalent modification. Resolved 2N4R tau mass is consistent with start methionine cleavage, which is common in recombinantly expressed proteins. Figure and mass spectrometry analysis provided by Dr. Weiss and Farai Rusinga.

Figure 2.8

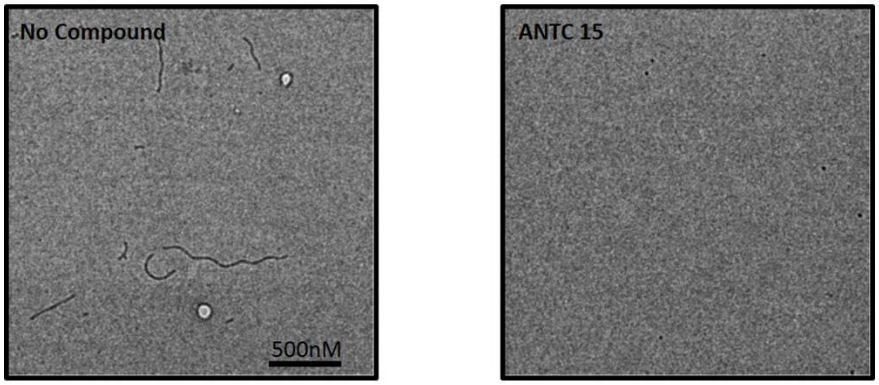

Figure 2.8: Electron Microscopy of Inhibition of Cysless 2N4R tau with 200 µM ANTC 15
Inhibition tau aggregation reactions were performed using 2 µM Cysless 2N4R tau, ANTC 15 at a final concentration of 200 µM incubated with monomeric tau for 20 mins, and 75 µM arachidonic acid to induce polymerization at room temperature overnight. ANTC 15 was able to inhibit the formation of cysless 2N4R filaments.

2.4 Discussion:

Tau aggregation is a major pathological hallmark of AD and a potential therapeutic approach because the accumulation of pathology correlates with the progression and severity of the disease. Previous studies have found that fungal secondary metabolites can produce tau aggregation inhibitors that could then be modified to improve the efficacy *in vitro*. This study was able to characterize a novel tau aggregation inhibitor isolated from *Aspergillus nidulans*, known as ANTC 15. ANTC 15 was able to inhibit the formation of filaments and toxic species of tau aggregation, as well as disassemble preformed filaments and toxic species using filter trap and electron microscopy. ANTC 15 also shows dose dependent responses to inhibition and disassembly of tau filaments and toxic species of tau with IC_{50}'s in the µM range using filter trap. EM grids were also made from the dose dependent inhibition and disassembly reactions to qualitatively compare IC_{50}'s. The inhibition EM grids qualitatively showed an IC_{50} of somewhere between 100 µM and 50 µM, which is consistent with the filter trap IC_{50} of total tau. There was some discrepancy in the in the qualitative EM IC_{50} to the quantified filter trap IC_{50} for disassembly. The disassembly EM grids appear to have an IC_{50} of somewhere between 150 µM and 100 µM, whereas the quantified IC_{50} was closer to 50 µM. This difference could be due to inefficiencies of the filter trap or the EM and could be explored by quantifying the EM grids or doing another assay such as a sandwich ELISA to see which it is more consistent with.

Mass spectrometry was utilized to test whether ANTC 15 was making covalent modifications to monomeric tau. Making covalent modifications to tau via cysteines or lysines, especially in the MTBRs, would be detrimental because it may inhibit tau's ability to bind to and stabilize microtubules. ANTC did not appear to be making a covalent bond because a tau mass and an

ANTC 15 mass were able to be resolved. If the compound was making a covalent modification there would be a shift increase in mass of the tau peak, or a mass could not be resolved. ANTC 15 could also be interacting with cysteines in the protein to produce TAI activity. 2N4R tau only has 2 cysteines, C291 and C322, which are near the aggregation motifs. Cysteines are highly reactive by creating disulfide bonds and are often in enzyme active sites because they are good nucleophiles[84]. Although DTT is added to keep the cysteines reduced, DTT has a half-life of about 2 hours at room temperature. The *in vitro* polymerization of the 2N4R isomer of tau has been kinetically characterized and most aggregation takes place before the half-life of DTT[51]. To give further insight into the mechanism of TAI activity for ANTC 15 cysless 2N4R protein was expressed, purified, and used in inhibition reactions. ANTC 15 was able to inhibit the formation of filaments, which suggests the TAI activity is from the compound interacting with the aggregation motifs.

In this study we have identified a novel scaffold as a tau aggregation inhibitor. Previously it was shown that azaphilones were also tau aggregation inhibitors and 4 compounds were also able to inhibit and disassemble filaments. Of these 4 compounds the most potent was labeled Aza-9 with a disassembly IC_{50} of 56 µM ± 14 with tau 5, 7, 12[71]. The azaphilones were optimized compounds derived from asperbenzaldehyde, which was previously isolated from *Aspergillus nidulans.* The azaphilones will also be chemically difficult to further optimize due to the functional groups that were added, such as the Br and acetate group (figure 2.1). ANTC 15 has not been chemically optimized and has a disassembly IC_{50} of 43 µM ± 18, which is more potent than Aza-9. ANTC 15 is also more easily modified than the azaphilone compounds due to the functional groups and conjugated ring structures. Therefore ANTC 15, and the isoquinoline

scaffold, is an ideal lead compound for further studies to increase the efficacy. After optimization, if the compounds are able to inhibit and disassemble aggregates in the nM range, then moving from *in vitro* characterization to mouse and other model organisms used for AD will be important.

Chapter 3: Conclusions and Future Directions

3.1 Introduction

Alzheimer's disease (AD) is a devastating the disease and is the 6th leading cause of death in the U.S. The symptoms on the disease include problems with memory, thinking, and behavior and worsen as the disease progresses. There are also two pathological hallmarks that occur in AD, which are the presence of senile Aβ plaques and neurofibrillary tangles (NFTs) composed of the microtubule-associated protein tau. Pathology begins to accumulate before symptoms occur and tau aggregation correlates with progression of the symptoms of the disease. Tau aggregation is seen in other diseases known as tauopathies, although the process and mechanism of tau aggregation is not well understood. Current treatments focus on improving the quality of life and target symptoms such as memory and thinking. These treatments are not well tolerated and do not prevent or delay the progression of the disease. Developing a therapeutic that targets tau aggregation could be a good avenue for preventing or delaying disease progression.

3.2 ANTC 15 as a Novel Tau Aggregation Inhibitor

Tau aggregation is a pathological hallmark and a potential therapeutic target to prevent or delay the progression of tauopathies such as AD. Natural products have been utilized directly as therapies, but just as importantly, the scaffolds have been utilized in semi-synthetic drugs and

synthetic natural product mimics. Fungi have historically been a rich source of biomedically useful compounds. Dr. Oakley and colleagues were able to genetically modify the fungus *Aspergillus nidulans* to produce secondary metabolites, which were then tested as tau aggregation inhibitors. Asperbenzaldehyde was a novel chemical scaffold to inhibit tau aggregation and was an intermediate to compounds that were known lipoxygenase inhibitors known as azaphilones. These compounds were semi-synthetically produce from asperbenzaldehyde and were able to inhibit the formation of and disassemble pre-formed aggregates *in vitro*. More compounds were isolated from *Aspergillus nidulans* and ANTC 15 was chosen because of the similarities to the azaphilone compound scaffold, but with a major difference (figure 2.1). Antibodies were used to detect the presence of filaments (tau 5, 7, 12), the open paperclip conformation (TNT1), and toxic oligomers (TOC1). ANTC 15 was able to inhibit the formation of and disassemble previously formed filaments and toxic aggregation species at 200 µM. It was also shown that there was a dose-dependence relationship as well and IC_{50} values were determined to be in the µM range. We hypothesize that these compounds are interacting with the lysine residues in the aggregation motifs VQIVYK and VQIINK denoted at PHF6 and PHF 6* respectively.

It was hypothesized that previous compounds were making a covalent modification at lysine or cysteine residues, especially in the aggregation motifs, which gave the compound tau aggregation inhibition (TAI) activity. Although covalent chemistry has been used in medically relevant drugs in the past, because of the high concentrations of glutathione in cells and the potential irreversible bonds it can make with its intended target, they have been utilized with caution[85]. This has been seen especially for compound in early stages of drug development. Covalent modifications to monomeric tau could also inhibit tau from binding to microtubules

because the two cysteines in tau (C291 and C322) and lysines (K280 and K311) in the aggregation motifs are within the MTBRs. Mass spectrometry was used to see if ANTC 15 was making a covalent modification to tau or not, and when tested there were separate convoluted mass peaks of monomeric tau and ANTC 15 suggesting no covalent modification was made. The TAI activity also seems to not be due to ANTC 15 interacting with the cysteines in tau because it was able to inhibit filament formation with cysless tau, suggesting it is interacting with VQIINK and VQIVYK to produce TAI activity. ANTC 15 is a novel scaffold as a tau aggregation inhibitor that could be used as inspiration as an AD therapeutic.

3.3 Future Directions

Although ANTC 15 is a novel tau aggregation inhibitor we do not completely understand how it interacts with tau to have TAI activity. Looking at ANTC 15, there are several motifs of this molecule that could be explored through structure-activity relationship (SAR) studies. ANTC 15 has an isoquinoline core structure, but the functional groups off of the ring structure as well as the polycarbon tail are areas that are more easily modified through semi-synthetic chemistry (figure 3.1). To understand how each functional group add to the potency of the molecule by modifying the hydroxyl groups to electron withdrawing or electron donating species to better interact with the lysines in the aggregation motifs. Another modification that could be explored is the polycarbon tail by shortening, lengthening, or introducing functional groups to assess how it affects the TAI activity. Understanding how these functional groups play a role in TAI activity will provide insight to changes that can be made to improve the efficacy of the isoquinoline scaffold.

In the present study, ANTC 15 was tested against the longest isoform of tau. The aggregation of the longest isoform has been optimized *in vitro* and utilized frequently in tau aggregation studies. In the brain all 6 isoforms of tau are present and in disease there are known mutations associated with tauopathies known as FTDP-17 mutations that occur in all 6 isoforms. To gain further insight into the mechanism of action and probe tau proteins associated with disease models, it would be beneficial to introduce ANTC 15 to FTDP-17 mutants to see if there is TAI activity. Preliminary studies have been done using ANTC 15 and a known FTDP-17 mutant in the 2N4R isoform. This mutant has the proline at position 301 mutated to a serine, denoted P301S. The P301S mutation has shown reduced ability to promote microtubule assembly in disease[86]. At 200 μM ANTC 15 was able to inhibit and disassemble filament formation shown by EM (figure 3.2). All of the current work was done *in vitro*, but introducing ANTC 15 to *in vivo* models will be important to assess whether it is able to cross cell membranes, or is toxic, or can cross the blood brain barrier. It is essential for potential therapeutic scaffolds to be tested in model organisms before it can be used to treat humans to understand if the drug works as it showed *in vitro* and to make sure there are no unwanted side-effects

Figure 3.1

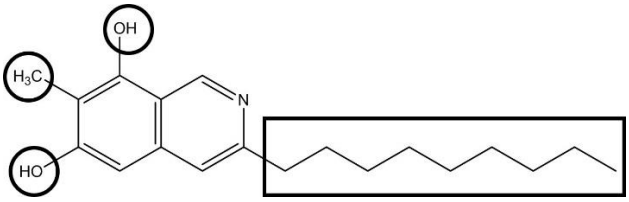

Figure 3.1 ANTC 15 structure-activity relationship (SAR) studies.
ANTC 15 molecule with potential sites to be modified for SAR studies in the black box and circles.

Figure 3.2

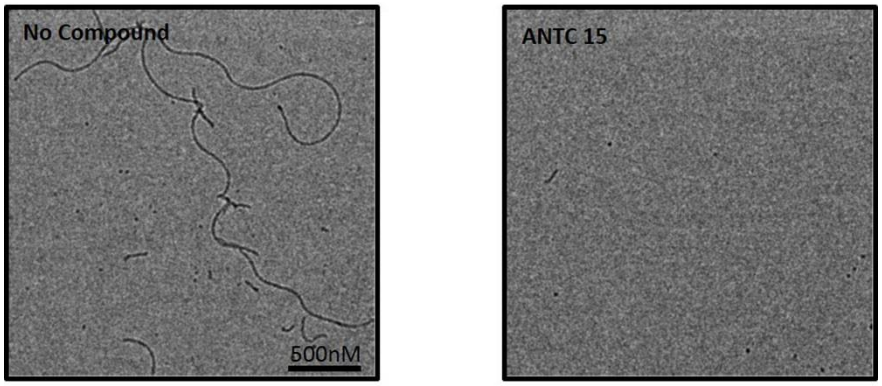

Figure 3.2 Electron Microscopy of Inhibition of P301S 2N4R tau with 200 µM ANTC 15
A proline is changed to a serine in the 2N4R tau isoform at amino acid position 301 (P301S). Inhibition tau aggregation reactions were performed using 2 µM P301S 2N4R tau, ANTC 15 at a final concentration of 200 µM incubated with monomeric tau for 20 mins, and 75 µM arachidonic acid to induce polymerization at room temperature overnight. ANTC 15 was able to inhibit the formation of P301S 2N4R filaments.

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