Evaluation of Small Molecules and Metal Complexes that Modulate Amyloid-β Aggregation of Relevance to Alzheimer's Disease

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Abstract

Alzheimer’s disease (AD) is the most common form of dementia, representing 50-75% of all cases worldwide. AD is a multifaceted disease that is characterized by increased oxidative stress, metal-ion dysregulation, and the formation of intracellular neurofibrillary tangles and extracellular amyloid-β (Aβ) aggregates. Cu(II), Zn(II) and Fe(II) have been shown to play a role in the aggregation and toxicity of the Aβ peptide, leading to the formation of reactive oxygen species (ROS) for Cu(II) and Fe(II). Different approaches have been used in this thesis to decrease the formation of ROS, modulate peptide aggregation and the interaction between bioavailable metal ions and the Aβ peptide.

In the first approach, metal-protein attenuating compounds (MPACs) were designed to bind dysregulated metal ions thereby limiting metal ion binding to the Aβ peptide. The ability of 8-hydroxyquinoline Schiff-base ligands to inhibit peptide aggregation in the presence of Cu(II), and their antioxidant activity measured by a Trolox equivalent antioxidant capacity (TEAC) assay are described. The ligands were shown to form complexes with Cu(II), 8-H₂QT in a 1:1 metal:ligand ratio, and 8-H₂QH and 8-H₂QS in a 1:2 metal:ligand ratio. The second approach investigated herein describes the use of metal complexes that are able to bind to the peptide, with potential to modulate aggregation and limit ROS formation. We report the high affinity binding of the Fe(III) 2,17-bis-sulfonato-5,10,15-tris(pentafluorophenyl)corrole complex FeL1 to the Aβ peptide (K₆ ~ 10⁻⁷) and the ability of the bound FeL1 to act as a catalytic antioxidant in both the presence and absence of Cu(II) ions. Overall, FeL1 is shown to bind to the Aβ peptide, and modulate peptide aggregation. In addition, FeL1 forms a ternary species with Aβ-Cu(II) and impedes ROS generation. Finally, we report a series of four Ru(III) complexes, inspired by the antimetastatic NAMI-A complex. These complexes bind to Aβ, and were shown to modulate peptide aggregation. Overall, we highlight the promise of discrete metal complexes to limit the toxicity pathways of the Aβ peptide.

Keywords: Alzheimer’s Disease; Amyloid-β; metal-protein attenuating compounds (MPAC); metal complexes; metal binding
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<tr>
<td>CFSE</td>
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<td>Metal-protein attenuating compounds</td>
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<td>nm</td>
<td>Nanometers</td>
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<td>Definition</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<td>Nuclear magnetic resonance</td>
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<td>O-GlcNAc</td>
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<td>O$_2$•⁻</td>
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<td>OEC</td>
<td>Trianion of 2,3,7,8,12,13,17,18-octaethylcorrole</td>
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<td>•OH</td>
<td>Hydroxyl radical</td>
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<td>P-Tau</td>
<td>Phosphorylated tau protein</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PBT2</td>
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<td>ppm</td>
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<td>QTWIMS-TOF-MS</td>
<td>High Definition hybrid quadrupole traveling wave ion mobility time-of-flight mass spectrometer</td>
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<td>RNS</td>
<td>Reactive nitrogen species</td>
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<td>ROS</td>
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<td>rpm</td>
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<td>Superoxide dismutase</td>
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<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
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<td>Scanning TEM</td>
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TBS  
Tris-buffered saline  
TBS-T  
Tris-buffered saline containing 0.1% Tween-20  
TDP-43  
TAR DNA-binding protein 43  
TEAC  
Trolox-equivalent antioxidant capacity  
TEM  
Transmission electron microscopy  
Thr  
Threonine  
ThT  
Thioflavin-T  
Tyr  
Tyrosine  
UV-Vis  
Ultraviolet-visible  
XAS  
X-ray absorption spectroscopy  
2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide  
XTT  
2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide  
λ_{em}  
Wavelength of emission  
λ_{ex}  
Wavelength of excitation  
λ_{max}  
Wavelength of maximum absorption
Chapter 1. Introduction

1.1. Neurodegenerative Diseases

Neurodegenerative diseases (ND) are chronic and progressive disorders that share several common features, such as neuronal death in specific areas of the brain, synaptic damage and the accumulation of misfolded protein aggregates, causing memory and cognitive impairments, and eventually death.\textsuperscript{1-3} Protein misfolding is common in ND, with genetic, neuropathological, cellular and biochemical studies supporting the theory that protein aggregation plays a role in these diseases.\textsuperscript{4} Aggregation-prone proteins in NDs are: amyloid-\(\beta\) (A\(\beta\)) and tau in Alzheimer's disease (AD), \(\alpha\)-synuclein (\(\alpha\)-Syn) in Parkinson disease (PD), TAR DNA-binding protein 43 (TDP-43) in amyotrophic lateral sclerosis (ALS) and prion protein in prion disease (PrD). These proteins differ in function, sequence, expression level and size, however they have all been observed to misfold from their native state, with most forming intermolecular \(\beta\)-sheet-rich structures, leading to a loss of protein function.\textsuperscript{1, 2} These aggregates can vary from oligomeric species (ranging from dimers to larger protofibrils) to larger fibrillar structures, to which oligomers have demonstrated higher toxicity than the fibrillar structures in a number of diseases.\textsuperscript{2}

1.2. Alzheimer’s Disease (AD)

Dementia is a process in which a ND leads to memory and cognitive decline, typically occurring above the age of 65. In 2018, 50 million people worldwide were estimated to be affected by dementia.\textsuperscript{5} This number is expected to drastically increase over the next 30 years, reaching 152 million by 2050, in accordance with a global increase in life expectancy.\textsuperscript{5, 6} AD is the most common form of dementia, representing 50-75\% of the cases.\textsuperscript{7} AD is classified as a proteinopathy and is characterized by the extracellular accumulation of A\(\beta\) aggregates (from oligomers to plaques) and intracellular formation of neurofibrillary tangles (NFTs) of tau.\textsuperscript{8} The Food and Drug Administration (FDA) classifies AD severity in three stages for the design and evaluation of clinical trials.\textsuperscript{9} In Stage 1, patients have pathophysiological abnormalities, but no cognitive decline. Stage 2 represents patients that already show subtle cognitive decline but no functional
impairment. Finally, Stage 3 patients show subtle or more evident cognitive decline and mild functional impairment, and start to have problems performing daily tasks.\textsuperscript{10}

Progressive loss of memory and impairment in cognition evaluation is used in clinical diagnosis of AD,\textsuperscript{11} however, definitive diagnosis of AD requires \textit{post-mortem} examination of the brain. The development of molecular neuroimaging agents could allow for a diagnosis \textit{ante-mortem}.\textsuperscript{12} Positron emission tomography (PET) agents for the detection of amyloid-\(\beta\) deposits are clinically available, such as \(^{18}\text{F}\)-labelled tracers florbetapir, flutemetamol and florbetaben (\textbf{Figure 1.1}), however tau PET ligands are still in clinical development.\textsuperscript{8} The FDA-approved drugs for treatment of AD are the acetyl cholinesterase (AChE) inhibitors donepezil, rivastigmine, and galantamine (\textbf{Figure 1.1}), and the N-methyl-D-aspartate (NMDA) receptor antagonist memantine (\textbf{Figure 1.1}). These drugs ameliorate the symptoms of the disease in its early stage, however disease modifying therapies are needed to provide more effective AD treatments.\textsuperscript{11,13-17} There are currently 64 agents in Phase II and 26 agents in Phase III clinical trials for AD, to which 30\% and 54\% respectively are anti-amyloid (immunotherapy, \(\beta\)-secretase inhibitors and anti-aggregation) and 14\% and 4\% respectively are anti-tau (immunotherapy and anti-aggregation).\textsuperscript{18} Efforts to develop new disease modifying therapies is of great interest for AD treatment.

\textbf{Figure 1.1} Structures of FDA-approved drugs for PET imaging of the brain (florbetapir, flutemetamol, florbetaben), acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine) and the NMDA antagonist memantine.
1.2.1. Amyloid-β Hypothesis

The Amyloid Hypothesis has long been the dominant theory to explain the cause of AD, postulating that Aβ peptide oligomers and aggregates trigger a neurotoxic cascade in the brain. The Aβ peptide is a proteolytic cleavage product of the amyloid precursor protein (APP) by β- and γ-secretases, through the amyloidogenic pathway, producing fragments from 38 to 43 amino acids, forming in majority Aβ1-40 (~90%) followed by Aβ1-42 (~9%) (Figure 1.2). This transmembrane protein can also be cleaved via a nonamyloidogenic pathway, when cleaved by α- and γ-secretases, forming products that have been hypothesized to have a role in brain development or in adult brain processes, such as synaptic plasticity and neurodegeneration protection. Genetic mutations of the APP gene have been associated with higher production of Aβ1-42 and are linked to early-onset familial AD. A decrease in the level of Aβ1-42 in cerebrospinal fluid (CSF) was shown to be detected before the increase in tau and hyperphosphorylated tau (P-tau), supporting the hypothesis that Aβ plays a role in other brain processes leading to the disease.

Figure 1.2 Diagram of the transmembrane protein APP and the amyloidogenic pathway involving its cleavage by β- and γ-secretases producing Aβ1-40 and Aβ1-42 peptides (red). The full amino acid sequence for Aβ1-42 is also shown.

Aβ monomers mostly exist as random coil structures, however, when these monomers fold to form α-helix and β-strand structures they can interact with other folded monomers via hydrophobic interactions. These interactions typically occur first at the
hydrophobic C-terminal and/or at the self-recognition site (from Leu$^{17}$ to Ala$^{21}$, Figure 1.3), leading to aggregate formation.$^{24}$ In addition, metal-ions such as Fe, Cu and Zn, have been shown to interact with the Aβ peptide, modulating its aggregation and increasing toxicity (further details in Figure 1.3 A and Section 1.2.4).$^{25}$ The Aβ peptide can be found in the brain in three general forms: membrane associated, aggregated, and soluble.$^{26}$ Soluble, oligomeric Aβ species have been shown to have higher toxicity,$^{21,26,27}$ and are better correlated with memory impairment and AD progression, leading to loss of dendritic spines and their synaptic connections.$^{28-30}$

Figure 1.3 A) Aggregation process of the Aβ peptide including metal-ion interactions. B) Structure of Aβ$_{1-42}$ fibrils (PDB 5KK3) showing the 15-42 core structure and unstructured N-terminus (residues 1-14).

Figure 1.4 Example compounds currently in clinical trial as treatments for AD.

Clinical trials of Aβ therapeutics targeting either plaque formation or production of Aβ (β- or γ-secretase) have failed, leaving many researchers to question the relevance of the amyloid hypothesis.$^{23,31}$ The majority of these trials involved patients that had a high
plaque burden, and some researchers suggest that at this late stage Aβ has initiated several disease processes, such as oxidative stress, making treatment with a single agent difficult. The development of multitarget agents may provide a benefit at later stages of the disease. For example, the asthma drug Cromolyn sodium was shown to clear plaques, reduce tangle formation and to target genes related to inflammation. Cromolyn sodium in combination with Ibuprofen (Figure 1.4) is now in a Phase III clinical trial for AD. Another example is the drug Ladostigil (Figure 1.4), that acts as a multitarget agent by inhibiting two enzymes, AChE and monoamine oxidase A and B. This compound failed a Phase III clinical trial, although it has entered a Phase II clinical trial for patients with mild cognitive impairment. Another approach is to test a potential drug on people considered to be at risk of developing dementia before any sign of the disease is detected. For example, the Aβ-targeting antibody Solanezumab failed a Phase III clinical trial on patients with mild AD, however, the treatment group declined less on the Alzheimer Disease Assessment Scale (ADAS) in comparison to the placebo group. Solanezumab is now being tested in a prevention trial on healthy people at risk of AD, either due to inherited mutations or brain plaque load. Another Aβ-targeting antibody, Aducanumab produced by Biogen, had its phase III clinical trial halted earlier this year due to data analyses that indicated that treat had no significant effect on memory loss and disorientation. However, in October this year the company submitted Aducanumab for FDA approval due to new evidence that high concentrations of the drug over an extended period of time showed a decrease in cognitive decline.

1.2.2. Tau Hypothesis

Tau is a phosphoprotein regulated by a number of kinases and phosphatases that participates in the stability of tubulin assemblies as one of the microtubule associated proteins (MAP). Tau can be found as six isoforms, with isoforms 3R and 4R (3-repeat and 4-repeat, respectively, Figure 1.5) mainly in axons of neurons in the human adult brain under physiological conditions. The longest isoform of tau found in humans (2N4R, 441 amino acids) is hydrophilic due to the low proportion of hydrophobic amino acids when compared to other proteins of similar length. There are four major domains in tau: N-terminal acidic projection domains (N, 1-150), proline-rich domain (PRD, 151-243), microtubule binding domain (MTBD, 244-369), and C-terminal tail (C, 370-441). In the microtubule binding domain there are four imperfectly repeated motifs (R1-4) separated
by flanking regions, two of these repeats (R2 and R3) have been shown to form β-sheet structures.\textsuperscript{43} Tau is subject to many different post-translational modifications, such as glycation, acetylation, oxidation, nitration, isomerization, O-Linked N-acetyl-D-glucosamine (O-GlcNAc) acylation, ubiquitination and most commonly, the phosphorylation of Ser, Thr and Tyr.\textsuperscript{44} Tau contains 85 potential phosphorylation sites, mostly found in the PRD, and its phosphorylation and isoform expressions are crucial for cytoskeletal plasticity in early development during embryogenesis. Only one isoform is found during this stage, while adults present all six isoforms and reduced phosphorylation of tau.\textsuperscript{45}

![Diagram of tau isoforms](image)

**Figure 1.5** Representation of the six human tau isoforms (3R and 4R) and their domain representations: N-terminal acidic projection domains (N, 1-150), proline-rich domain (PRD, 151-243), microtubule binding domain (MTBD, 244-369) and C-terminal tail (C, 370-441) (modified from Guo et al., 2017).\textsuperscript{43}

Dysregulation of kinases and phosphatases can result in hyperphosphorylation of tau, which causes tau to lose its function, leading to toxicity.\textsuperscript{45} Interestingly, tau phosphorylation is influenced by O-GlcNAc modification, with increased O-GlcNAcylation shown to limit accumulation of tau aggregates.\textsuperscript{46} P-tau polymerizes into paired helical filaments (PHF) and straight filaments (SF), commonly known as NFTs in neurons, forming neurotoxic aggregates that can be released into the extracellular environment upon neuronal death and trigger the activation of microglial cells.\textsuperscript{47, 48} P-tau can interact with actin leading to its destabilization, synaptic impairment and damage to mitochondrial integrity, causing extensive damage in the cell.\textsuperscript{23} Normal cell functions such as morphology, axonal transport and synaptic function are impaired by the formation of NFTs, triggering neurodegeneration. Mutations in the tau gene (chromosome 17) results in the expression of tau that is more prone to hyperphosphorylation, and has been linked to frontotemporal dementia and parkinsonism (FTDP).\textsuperscript{48} Metal ions, such as Zn(II), Cu(II),
and Fe(II), have been shown to also play a role in tau aggregation and hyperphosphorylation. The binding of these metal ions was observed in the MTBD region and it was shown to lead to the generation of reactive oxygen species (ROS).  

### 1.2.3. Oxidative Stress Hypothesis

Approximately 20% of the oxygen supplied by the respiratory system is consumed by the brain, thus the brain is the most susceptible organ in the human body to oxidative stress. With aging there is a general increase in oxidative stress, and increased ROS and reactive nitrogen species (RNS) are observed. Superoxide anion (O$_2^{-}$) and hydrogen peroxide (H$_2$O$_2$), are by-products of normal metabolism, and enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase have a regulatory role in scavenging these ROS species. However, when present in excess O$_2^{-}$ and H$_2$O$_2$ can lead to the formation of the highly reactive hydroxyl radical (•OH) catalyzed by Fe(II) and Cu(II) ions via Fenton chemistry (See Section 1.2.4). The balance between ROS production and antioxidant defense is critical for cellular function, and shifts towards production of ROS in AD. In fact, mitochondrial dysfunction is observed in transgenic mouse models of AD, leading to an increase in the production of ROS in the brain. There is significant evidence that oxidative stress plays a role in AD, and a correlation between higher levels of Aβ and elevated oxidation products has been reported in the hippocampus and cortex in the AD brain.

### 1.2.4. Metal-ion Dysregulation

Metal-ions such as Fe(II/III), Zn(II), Cu(I/II), Mn(II), Mg(II), and Ca(II) are essential for the functioning of critical processes in a healthy brain. These biologically relevant metal-ions (also known as biometals) are tightly regulated in a healthy individual, however, an imbalance in their homeostasis can affect brain function. Metal dyshomeostasis can occur by disruption in the transport/utilization and/or the absence of specific metal binding proteins, and is observed in a number of ND. Amyloid plaques have been described as ‘metallic sinks’ on account of the remarkably high concentrations of Cu (0.4 mM), Fe (0.9 mM), and Zn (1.0 mM) found within these deposits in AD brains, compared to aged-matched controls with concentration of Cu (0.08 mM), Fe (0.3 mM), and Zn (0.4 mM).
A nuclear magnetic resonance (NMR) solution structure of the Aβ1-40 peptide (Figure 1.6) shows the metal-binding amino acid (aa) side-chains in the N-terminus, the central hydrophobic region (aa 17-21) and easily oxidized Met35 that are important to metal-ion binding, aggregation and biomolecule oxidation. The three Aβ His residues (His6, His13, and His14) are involved in the coordination of metal-ions, with dissociation constants ($K_d$) of \( \sim10^{-10} \) M for Cu(II) and \( \sim10^{-5} \) M for Zn(II).\(^{58, 67-71}\) Furthermore, residues Asp1, Tyr10, and Glu11 also play a role in the binding of Aβ to Cu(II) and Zn(II).\(^{72-75}\) Fe is typically found in the brain as naturally occurring Fe porphyrins (heme, 95% of all Fe) or bound to biomolecules, such as ferritin.\(^{76}\) Interestingly, depletion of complex IV (cell enzyme containing heme-a) occurs due to the binding of heme-a to Aβ in the brain of AD patients.\(^{77}\) The binding of Cu(II) to Aβ depends on the pH of the solution, and at a pH lower than 7.8 the majority Cu(II) is bound to two His and Asp1 (component I), while higher pH leads to the loss of one His and binding of a deprotonated amide (Ala2) (component II) (Figure 1.6).\(^{78}\) This metal-ion coordination to Aβ modulates its aggregation pattern, and potentiates the neurotoxicity of Aβ via redox-cycling and the production of ROS in the presence of dioxygen, playing an important role in oxidative stress.\(^{68, 69, 79-88}\) The Aβ-Cu(II) complex was shown recently to generate ROS, such as \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), and \( \cdot\text{OH} \) by reducing \( \text{O}_2 \) in the presence of cholesterol and vitamin C.\(^{79, 80, 89-92}\) Due to the metal-ion dyshomeostasis observed in AD and the involvement of these metal-ions in protein aggregation, it is hypothesized that metal-ion dysregulation plays a significant role in AD development.\(^{93}\) Two approaches for the prevention of metal-ion binding to Aβ will be further discussed below. The first is the use of ligands that can bind to Cu(II) and Zn(II), limiting their interaction with the Aβ peptide, and the second discrete metal complexes that target Aβ peptide metal-binding residues and/or peptide aggregation.
1.3. Metal Binding Agents

The role of metals in AD has lead to the investigation of chelating agents, compounds able to bind to metal ions, as therapeutic agents for AD. These ligands typically have moderate binding affinity for metal-ions, in order to minimize alteration of metalloenzyme function, and have shown promise in restoring normal homeostasis and prevention of metal-induced Aβ aggregation. One of the first compounds investigated as a chelating agent was desferrioxamine (DFO, Figure 1.7), which was reported to slow the progression of AD. However, a 24 month clinical trial on patients with probable AD showed
that there was no significant difference in intelligence and memory between groups, although the rate of decline of daily living skills was smaller for the treatment group when compared to no treatment or placebo. A lipophilic chelator DP-109 (Figure 1.7), used for chelating Ca(II), was shown to reduce amyloid plaque formation, after 3 months of treatment of human APP transgenic mice. This chelating agent was shown to cross the blood-brain barrier (BBB) and bind to free Zn(II) in synaptic vesicles. The bicyclam JKL-169 was shown to reduce Cu(II) concentration in the cortex of rats, while the Cu(II) concentration in blood remained the same, similar to Clioquinol (HCQ) (Figure 1.7). HCQ and the second generation 8-hydroxyquinoline (8-HQ) derivative PBT2 (Figure 1.7) have been extensively studied as potential AD therapeutics, and properties such as lipophilicity and high selectivity for Zn(II) and Cu(II) has resulted in promising results in both animal models and preliminary clinical trials. Even though PBT2 reduces Aβ aggregation, limits Aβ oligomer toxicity, and redistributes metal ions (Cu(II) and Zn(II)) into neurons, the most recent clinical trial failed when treatment with PBT2 did not show significant difference compared to placebo in a 12-month double-blind phase II study.

![Figure 1.7 Example structures of chelating agents DFO, JKL-169, 8-HQ, HCQ, DP109 and PBT2.](image)

### 1.4. Multifunctional Ligands as Therapeutics

Due to the multi-factorial characteristics of AD, and the lack of therapeutic success of the FDA-approved drugs, a new drug design strategy was developed – the multi-target-directed-ligand (MTDL) approach. In this approach, molecules are designed to target two...
or more of the pathogenic steps that lead to the symptoms of the disease, such as Aβ plaque deposition, NFT formation, oxidative stress, metal-ion dysregulation and cholesterol metabolism. Often MTDLs are prepared by combining two different pharmacophores that have been identified as having therapeutic potential in the context of AD. Several structural scaffolds, such as 8-HQ (Figure 1.7), curcumin (Figure 1.8), along with the FDA-approved drugs donepezil, tacrine, and rivastigmine (Figure 1.1), have been used in the development of new multifunctional compounds.

Figure 1.8 Structures of ligands derived from 8-HQ, ρ-l-stilbene, and phenol triazole as potential MPACs for AD.

Considerable research effort has been invested in the development of MTDL targeting metal-Aβ interactions, modulating peptide aggregation and formation of ROS. These multifunctional compounds are termed metal-protein attenuating compounds (MPAC). Glycosylated tetrahydrosalens, such as GL₃ (Figure 1.8), were shown to act as potential pro-drugs for AD treatment. The presence of the β-glucosidase enzyme leads to cleavage of the glucose moieties, affording the tetradentate ligand (HL₃). HL₃ was shown to bind to Cu and Zn, decrease metal-induced Aβ aggregation, and display potent antioxidant activity. The bidentate ligand L2-NO (Figure 1.8), based on the structures of ρ-l-stilbene (an amyloid binding scaffold) and clioquinol, was developed to optimize the interaction with the Aβ peptide while incorporating a metal binding unit. The ligand was shown to modulate the aggregation of Aβ in the presence of Cu(II), and to limit •OH formation by Aβ-Cu(II). L2-NO was demonstrated to interact with Aβ by NMR and isothermal calorimetric titration (ITC), and was predicted to cross the BBB. The same group has more recently reported a second-generation ligand L2-b (Figure 1.8) and a radiolabelled analogue was shown to cross the BBB. Interestingly, this ligand does not
prevent Cu binding to the peptide, instead it forms a ternary complex with Aβ and Cu, likely inhibiting ROS generation. Recently, a series of ligands based on phenol triazole were developed as MPACs for AD. The most promising ligand, POH (Figure 1.8), was shown to interact with Aβ by NMR, to bind to Cu(II) thereby modulating the peptide aggregation pattern, and limit Aβ peptide toxicity in neurons.

1.5. Inorganic Medicinal Chemistry

Metal ions can have a structural or functional role in biological processes, and their presence is vital for many functions in biological systems. Medicinal inorganic chemistry is a field that focuses on the application of metal complexes as therapeutic and diagnostic agents for metal sensing, photooxidation, release of signalling molecules, cellular imaging, protein labelling as well as DNA probes. Inorganic chemistry has been present in medicine for over 5000 years, with Cu being used to sterilize water in Egypt around 3000 BC, gold was used in a number of treatments in Asia over 3500 years ago and mercurous chloride (Hg₂Cl₂) was used during the Renaissance period in Europe. Interestingly, when it comes to applying inorganic compounds to biology, chemists are not limited to the naturally bioavailable set of endogenous metals and can take advantage of the properties of biologically "exotic" elements, including 2nd and 3rd row transition metals, as well as lanthanide elements. For example, in 1969 the cytotoxicity of the Pt(II) complex cisplatin (cis-[Pt(NH₃)₂Cl₂], Figure 1.9) was discovered, bringing attention to this important research field and leading to the development of many metal complexes with therapeutic activity. Cisplatin toxicity and resistance led to the development of a new generation of Pt compounds, such as carboplatin and oxaliplatin (Figure 1.9).

There are currently many metal-containing drugs with therapeutic potential, such as Pt (anticancer), Ag (antimicrobial), Au (antiarthritic), Bi (antiulcer), Sb (antiprotozoal), V (antidiabetic), and Fe (anticancer and antimalarial) (Figure 1.9). Metal complexes can interact with proteins and nucleic acids in unique ways in comparison to organic compounds, restoring function that was lost due to misfolding or introducing a new function not found naturally. The photophysical, magnetic, or radioactive properties of metal compounds make them suitable for imaging applications such as luminescence, magnetic resonance, PET, and single-photon emission computed tomography (SPECT).
In order for the approval of metal-based drugs for use in humans it is critical to understand the mechanism(s) of biological action of metal compounds for both therapy and diagnosis. However, in many cases the mechanism(s) of action of metal-based drugs are not fully elucidated. For example, only recently has the crucial role of the two major serum proteins albumin and transferrin to the pharmacological action of metal complexes, as well as their overall toxicity profile, been fully appreciated. In addition, metallothioneins, which are small cysteine-rich intracellular proteins primarily involved in...
storage and detoxification of soft metal-ions, can be important to the pharmacological action of metal complexes.

1.5.1. Metal Complexes as Potential Therapeutics for AD

The design of metal complexes that interact with specific proteins related to ND has primarily focused on the Aβ peptide in AD. Complexes of several metal-ions, such as Pt(II/IV), Co(III), Rh(III), Ir(III), Ru(II/III), and Fe(II) have been studied as potential therapeutic agents for AD, and are described below. These complexes have been shown to interact with either monomeric or aggregated forms of the Aβ peptide, and modify peptide aggregation and toxicity in cells and animal models.

**Pt complexes**

Pt(II) phenanthroline complexes, Pt(phen)Cl₂, and Pt(ϕ-phen)Cl₂ (Figure 1.10) were reported to bind to the Aβ peptide, altering its aggregation pattern and limiting its neurotoxicity. The Pt(II) phenanthroline complexes were compared with cisplatin, with the former binding to His in the N-terminus region and reducing peptide neurotoxicity, while cisplatin targeted Met³⁵ and was shown to be inactive. The free ligands and Pt(II) salts exhibited a low affinity for Aβ, indicating that the planar hydrophobic phenanthroline ligand was necessary for the observed activity. NMR, X-ray absorption spectroscopy (XAS), mass spectrometry (MS), and molecular modelling investigations further confirmed that the planar hydrophobic ligand stabilized histidine-protein adducts. NMR experiments showed that the phenanthroline ligand interacts with the protein via non-covalent interactions, while the Pt(II) center binds directly to two His residues, potentially limiting the coordination of Cu(II) and Zn(II). Other Pt(II) complexes containing planar hydrophobic ligands have been investigated by Hureau et al., such as Pt(ϕ-MePy)(DMSO)Cl (Figure 1.10). This complex bound to the Aβ peptide similarly to the phenanthroline analogues, although the results suggest binding of the Pt complex to just one His instead of two. Cu(II) and Zn(II) typically bind to Aβ His residues, and the presence of Pt(II) complexes modulated the metal binding properties of Aβ, although Aβ-Cu ROS generation was not inhibited completely. Electron paramagnetic resonance (EPR) analysis shows a change in the peptide Cu(II) binding site in the presence of Pt(ϕ-MePy)(DMSO)Cl, with the initial two His Cu(II) coordination (component I) shifting to only one His bound (component II).
aggregation enhancement, however the presence of the Pt(\(\phi\)-MePy)(DMSO)Cl complex limited Zn-induced A\(\beta\) aggregation.\(^{145}\) A bifunctional complex, containing one or two cyclen metal-binding moieties attached to a bipyridine Pt(II) binding unit (Figure 1.10, PC1) was shown to limit Cu/Zn aggregation and associated toxicity through His binding and metal scavenging ability.\(^{147}\)

![Figure 1.10](image_url) The structure of representative Pt complexes that interact with the A\(\beta\) peptide, disrupt metal binding, and show promise in cell and animal models.

It is possible to tune the therapeutic effects of these Pt(II) complexes via alteration of ligand exchange kinetics and overall charge of the complex.\(^{148}\) Pt(IV) complexes, such as Satraplatin (Figure 1.10), exhibit slow ligand exchange kinetics, making them biologically stable and orally bioavailable.\(^{149-151}\) With that in mind, Barnham et al.\(^{152}\) synthesized an orally bioavailable Pt(IV) pro-drug with a hydrophobic diamine, and its Pt(II) analogue (Pt1 and Pt2, respectively, Figure 1.10) as modulators of A\(\beta\) peptide aggregation and toxicity. The Pt(IV) complex showed increased brain uptake in comparison to the Pt(II) complex, and upon reduction to Pt(II), was shown to limit peptide aggregation and toxicity in cortical neurons. The treatment of an APP/PS1 mouse model of AD (Figure 1.10, Pt1) showed a statistically significant reduction in CSF A\(\beta\)\(_{1-42}\) levels and reduction in plaque load. Thus, the Pt(IV) pro-drug strategy was shown to be promising for the development of Pt-complexes that cross the BBB and selectively target A\(\beta\).
**Co complexes**

A Co(III) Schiff base complex (Co(III)-acacen, Figure 1.11) was shown to bind to one or two His of Aβ1-16, with a preference for His\(^6\) and one of His\(^{13/14}\), according to NMR studies and density functional theory (DFT) calculations.\(^{69}\) Aβ1-42 aggregated differently in the presence of Co(III)-acacen, with a concentration-dependent stabilization of high MW oligomeric species (30 to 160 kDa). The presence of the complex also led to a reduction of the binding of the Aβ1-42 peptide to differentiated hippocampal neurons. In a different approach, researchers have shown that Co complexes can induce Aβ peptide cleavage.\(^{153, 154}\) Firstly, a series of Co(III)-cyclen complexes (Figure 1.11) were shown to induce cleavage of monomers and oligomers of Aβ1-40 and Aβ1-42, while the formation of fibrils limited the percentage of cleavage fragments observed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry.\(^{153}\) In a more recent report by Lim et al., a Co(II) complex of a tetra-N-methylated cyclam (Co(II)-TMC, Figure 1.11) was shown to decrease the Aβ1-40 peptide monomer concentration by 60% through amide bond hydrolytic cleavage.\(^{154}\) The control complex Co(II)-EDTA did not induce cleavage, while Co(II)(NO\(_3\))\(_2\) induced cleavage of Aβ1-40 at different sites than those produced by the Co(II) complex (Figure 1.11). Co(II)-TMC can bind covalently to Aβ and alter the aggregation of both Aβ1-40 and Aβ1-42. Interestingly, the complex was shown to protect cells from the toxicity induced by Aβ and was demonstrated to cross the BBB.
Figure 1.11 The structure of Co(III)-acacen, Co(II)-TMC and Co(III)-cyclen.

Ir and Rh complexes

A series of cyclometallated Ir(III) and Rh(III) complexes (Figure 1.12, A and B) were shown to bind covalently to the Aβ peptide, leading to a reduction in fibril length for Aβ_{1-40}. Electrospray ionization-MS (ESI-MS) measurements indicated 1:1 Aβ-Rh(III) adduct formation. Interestingly, the Rh(III) complex, as opposed to the Ir(III) complex, exhibited a more pronounced effect on aggregation, almost completely inhibiting the formation of high molecular weight species for Aβ_{1-40}. As for the Ir(III) complexes investigated, complex B (Figure 1.12) containing the smaller aromatic ligand of the series showed the most pronounced disruption of the fibrillization process when compared to the complex with the ligand phenylquinoline, possibly due to steric effects. These complexes demonstrated enhanced emission in the presence of fibrils, and the Ir(III) complexes containing the more bulky ligands were more suited for cellular labelling applications. More recently, the same group developed derivatives based on complex B in Figure 1.12, in which a third bidentate ligand was incorporated, instead of exchangeable H₂O ligands. All twelve complexes prepared by Lu et al. interacted with Aβ_{1-40} monomers and fibrils differently, however complex C in Figure 1.12 showed the highest affinity for fibrils. Even though no covalent binding was observed between the Ir(III) complex C and peptide, the complex completely inhibited aggregation of Aβ_{1-40}. These results show that non-covalent interactions, when strong enough, can inhibit Aβ_{1-40} aggregation. The presence
of monomers or fibrils of Aβ_{1-40} led to an increase in complex luminescence, possibly due to hydrophobic interactions with the peptide, protecting the complex from non-radioactive decay by solvent quenching. Lastly, the Ir(III) complex C was shown to be neuroprotective against Aβ_{1-40} toxicity in human neuroblastoma SH-SY5Y cells and mouse primary cortical cells. Overall, these studies show that Group 9 complexes in a variety of different geometries bind to the Aβ peptide and can limit associated aggregation and toxicity.

![Figure 1.12](image)

**Figure 1.12** The structure of cyclometallated Rh(III) and Ir(III) derivatives.

**Fe complexes**

Most of the focus on Fe complexes and their influence on the development of AD has centred on naturally occurring Fe porphyrin complexes. Increased production of heme-a and heme-b (Figure 1.13) has been observed in the brain of AD patients, while there is depletion of complex IV (cell enzyme containing heme-a) due to the interaction of free heme with Aβ. Several studies have shown that heme binds to Aβ leading to a red-shift in heme Soret band. In one of these studies, different mutants as well as different fragments of the Aβ peptide were incubated with heme in order to determine which residues are involved in the binding among His, His{^{13/14}}, or Tyr. The single Tyr^{10}Gly mutant of Aβ_{1-16} (Figure 1.14) showed a red-shifted Soret band indicating binding, while the spectrum of fragments or mutants of Aβ without His residues were the same as heme alone, suggesting that His is essential for binding of heme to Aβ. For the double mutants that contained only one His, either 13 or 14, a similar change in the spectra was observed when compared to wild type Aβ. However, the mutant that only contained His
showed no significant change in the spectrum, suggesting that binding in the heme-Aβ complex is likely to occur at either His\textsuperscript{13} or His\textsuperscript{14}. In a different study, residues Phe\textsuperscript{19} and Phe\textsuperscript{20} were shown to be important for the interaction between the peptide and heme.\textsuperscript{161} These studies also showed that the heme-Aβ complex acts as a peroxidase, with higher activity in comparison to heme alone. Interestingly the Aβ\textsubscript{10-20} fragment (Figure 1.14), even though it shows similar binding in comparison to Aβ\textsubscript{1-16} and Aβ\textsubscript{1-40}, exhibited the same peroxidase activity as heme alone, suggesting that a residue in the 1-9 region must be involved in the peroxidase activity of the complex.\textsuperscript{160} Arg residues have been shown to be present in the active site of the peroxidase enzyme and to play a crucial role in the activity.\textsuperscript{162, 163} In fact, rodent Aβ (that differs from human Aβ in the amino acids Arg\textsuperscript{5}Gly, Tyr\textsuperscript{10}Phe and His\textsuperscript{13}Arg, Figure 1.14) has been shown to bind less effectively to heme than human Aβ, with little to no change in the peroxidase activity when compared to heme alone.\textsuperscript{164} However, it is important to note that even though the catalytic activity of heme-Aβ (0.042 \text{ s}^{-1}) is higher than that of heme alone (0.01 \text{ s}^{-1}), it is still very low when compared to that of horseradish peroxidase (HRP, 45.5 \text{ s}^{-1}).\textsuperscript{165} The heme-Aβ complex was also shown to have increased pronitrative activity in NO\textsubscript{2}-H\textsubscript{2}O\textsubscript{2} dependent enolase nitrotyrosination, which could lead to impairment of protein function.\textsuperscript{165, 166}

![Figure 1.13](image.png) The structure of heme a and heme b.
### Figure 1.14

Full sequence of human and rat Aβ (hAβ and rAβ) highlighting the aa that differ, and the single and double mutants of Aβ1-16. Amino acids involved in metal-ion binding and peptide oxidation are shown in red for hAβ1-42 and hAβ1-16, and aromatic amino acids important for Aβ-heme interaction are shown in blue (Modified from Pramanik et al., 2011).

<table>
<thead>
<tr>
<th>Amino Acid Sequence</th>
<th>Single Mutant</th>
<th>Double Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAβ1-42</td>
<td>DAEFRHDGVEVHHQKLFFAEDVGSNKGAIIGLMVGVVIA</td>
<td>G(^6)</td>
</tr>
<tr>
<td>rAβ1-42</td>
<td>DAEFGHDSGEVEHQLKEAEDVGSNKGAIIGLMVGVVIA</td>
<td>A(^{13})</td>
</tr>
<tr>
<td>hAβ1-10</td>
<td>YEVHHQKLFF</td>
<td>A(^{13})</td>
</tr>
<tr>
<td>hAβ1-16</td>
<td>DAEFRHDGVEVHHQQK</td>
<td>G(^6)</td>
</tr>
</tbody>
</table>

Heme was also shown to change the aggregation pattern of Aβ with a decrease in Thioflavin T (ThT) fluorescence, and formation of smaller fibrils as shown by transmission electron microscopy (TEM). Both heme and Cu are known to bind to Aβ, and are found co-localized in Aβ plaques. Thus, the concurrent binding of both Cu and heme to the peptide was investigated. The absorption features of Aβ bound to both heme and Cu(II) were identical to Aβ bound to heme only, while EPR demonstrated that both the Cu and the heme were bound to the peptide with no observable interaction between the two metal centers. Interestingly, the electrochemical properties of the heme-Aβ-Cu species matched the individual Cu and heme Aβ complexes, and the peroxidase activity of the heme-Aβ-Cu species was similar to that of the heme-Aβ. Dey et al. concluded that under physiological conditions both heme and Cu(II) could be bound to Aβ, and both species have biologically-accessible redox responses that could generate ROS and associated oxidative stress.

A series of chiral metallosupramolecular Fe complexes (Figure 1.15), have been investigated for their ability to bind and limit aggregation of Aβ. The compounds were found to bind to the peptide, with the S enantiomer for Fe1 and R enantiomer for Fe2 showing increased interaction with the peptide. An NMR study of the complex Fe2, showed a significant shift in the signal for residues Phe\(^{19}\) and Phe\(^{20}\) (present in the
hydrophobic self-recognition region responsible for peptide-peptide interactions),\textsuperscript{24} suggesting $\pi-\pi$ interactions between the complex and the peptide. Interestingly the NMR shifts differed among the enantiomers, suggesting shape-specific interactions. Atomic force microscopy (AFM), a ThT assay, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) showed that the complexes $\text{Fe1}$ and $\text{Fe2}$ led to a decrease in peptide aggregation. In addition, the complexes acted as SOD mimics and ROS scavengers. The complexes were shown to limit the toxicity of $\text{A}\beta$ in PC12 cells in a concentration-dependent manner, with an enhanced effect observed for derivatives exhibiting a stronger interaction with the peptide. Finally, in an \textit{in vivo} study, the complexes were shown to cross the BBB, exhibiting potential as therapeutics for AD.

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{Fe1_Fe2.png}
\caption{The structure of chiral metallosupramolecular Fe complexes ($\text{Fe1}$ and $\text{Fe2}$).}
\end{figure}

\textbf{Ru complexes}

The interaction of Ru complexes with the $\text{A}\beta$ peptide followed initial reports on Pt, and have highlighted the role of ligand design in peptide binding and associated toxicity. In general, Ru compounds are considered to be less toxic in comparison to Pt compounds, and were thus attractive candidates for further development.\textsuperscript{168} Valensin \textit{et al.} first reported a \textit{fac}-[Ru(CO)$_3$Cl$_2$($N^1$-thz)] complex (\textbf{Figure 1.16}) that selectively targeted His residues on $\text{A}\beta$.\textsuperscript{169} Peptide adducts were confirmed using ESI-MS, with selective His ligation determined from NMR experiments. Further testing of the anticancer candidates
PMru20, NAMI-A, and KP1019 (Figure 1.16) showed that the axial ligands had a significant effect on the biological properties.\textsuperscript{170} PMru20 was determined to limit Aβ aggregation to the greatest extent, and in addition protected rat cortical neurons from both Aβ1-42 and the truncated Aβ25-35 (without His) toxicity. Further investigation of KP1019 showed a concentration-dependent effect of this Ru(III) complex on Aβ1-42 aggregation, and formation of His-adducts \textit{via} EPR spectroscopy.\textsuperscript{171} For KP1019, and likely other Ru(III) complexes, significant interference in the standard ThT aggregation assay was observed, and thus alternate aggregation assays are advised, such as dynamic light scattering and/or gel electrophoresis/Western blotting. Interestingly, KP1019 was found to promote the formation of amorphous high molecular weight aggregates of the Aβ peptide. Finally, KP1019 was determined to have a concentration-dependent rescuing effect on human neuroblastoma (SH-SY5Y) cells in the presence of the Aβ1-42 peptide. Of note, in the case of both PMru20 and KP1019, pre-incubation of the complex with the Aβ peptide was necessary to show a protective effect in cells, highlighting the need for new Ru compounds that selectively target the Aβ peptide.
Figure 1.16 The structure of representative Ru complexes that target His residues of the Aβ peptide, modulating aggregation and limiting toxicity.

A series of Ru(II) polypyridyl complexes (Figure 1.16) were investigated for their ability to interact with Aβ peptide.\textsuperscript{172-174} Due to the hydrophobic nature of the bpy/phen ligands, these complexes can form π-π interactions with the peptide.\textsuperscript{172, 173} The complex [Ru(bpy)\textsubscript{3}]\textsuperscript{2+} has been shown to generate ROS under visible light irradiation (Figure 1.16), which leads to the break down of preformed Aβ aggregates into less-amyloidogenic fragments.\textsuperscript{172} The Ru(II) complex containing an extended polypyridyl ligand ([Ru(bxbg)]\textsuperscript{2+}, Figure 1.16), allowed for interaction with AChE, with inhibitory values similar to that of the FDA approved drug tacrine.\textsuperscript{173} This complex completely inhibits Aβ aggregation as well, demonstrated by ThT fluorescence and TEM. Another series of Ru(II) complexes ([Ru(Apy)]\textsuperscript{2+}, Figure 1.16) were shown to protect against ROS and had an inhibitory effect against AChE.\textsuperscript{174} Interestingly, their luminescence increases in the presence of Aβ aggregates, allowing for the visualization of these species within 3 hours of aggregation, while a ThT signal only appears after 24 hours, making this a promising compound for the visualization of Aβ aggregates in the early stages of fibrilization.
1.6. **Thesis overview**

In this thesis two approaches were evaluated for the modulation of Aβ aggregation, and as possible therapeutics for AD. In Chapter 2, 8-hydroxyquinoline Schiff base derivatives (Figure 1.17) were developed as MTDL, preventing oligomer formation induced by Cu(II) and demonstrating antioxidant activity. Chapters 3 and 4 describe the use of metal complexes capable of binding to the Aβ peptide, potentially through one of the His residues, and their modulation of peptide aggregation. Chapter 3 details studies on an amphiphilic Fe(III) corrole complex (Figure 1.17) that forms an adduct with Aβ simultaneously to Cu(II), preventing the formation of ROS by Aβ-Cu(II). This complex was also shown to prevent the formation of large MW peptide aggregates. Chapter 4 investigates the influence of a series of Ru(III) complexes derived from NAMI-A (Figure 1.17) on peptide aggregation, to which a more significant effect occurs for the complexes with larger more hydrophobic ligands. Finally, Chapter 5 discusses future research directions based on the studies detailed herein.
Figure 1.17 The structure of the compounds studied in Chapters 2-4.
Chapter 2. 8-Hydroxyquinoline Schiff-base Compounds as Antioxidants and Modulators of Copper-Mediated Aβ Peptide Aggregation


L.M.F. Gomes performed the syntheses, stability constant determinations, gel electrophoresis and Western blotting. R.P. Vieira performed the acidity constant calculations, the antioxidant assay, metal binding assay and turbidity assays with L.M.F. Gomes. C. Dyrager completed the DFT calculations and M.C.P. Wang executed the TEM imaging. J.G. Da Silva determined the crystal structure for the compounds.

2.1. Introduction

Dementias are progressive pathophysiological disorders characterized by neuronal cell loss and severe cognitive impairment. The higher prevalence of these neurodegenerative processes in the elderly and the increased life expectancy in many countries represents a significant burden on healthcare systems around the globe. There are over 50 million people worldwide displaying dementia symptoms and this number is expected to more than triple by 2050 (152 million). Alzheimer's Disease (AD) is the most common type of dementia and is characterized by oxidative stress, misfolded proteins, neuronal cell loss, and eventually death. The mechanism underlying the causes and progression of AD is subject to enormous research efforts, and the search for new and effective therapies is justified by the lack of effective treatment options.

Diagnosis of AD, as opposed to other forms of dementia, requires post-mortem examination of the brain to determine the severity of neuropathological hallmarks of the disease; amyloid-beta (Aβ) plaques and neurofibrillary tangles. Neurofibrillary tangles are intracellular fibrillar aggregates of oxidatively-modified and hyperphosphorylated microtubule-associated protein tau. Aβ-plaques are extracellular deposits of fibrils and amorphous aggregates of the Aβ peptide (vide infra). The amyloid hypothesis has long been the dominant theory to explain the cause of AD, postulating that Aβ plaque depositions or partially aggregated soluble Aβ trigger a neurotoxic cascade causing AD.
pathology. Soluble forms of Aβ better correlate with memory impairment and AD progression in comparison to Aβ plaques, however, nearly all aggregated forms exhibit toxicity. Metal ions such as Cu(II), Zn(II), and Fe(III) exhibit a relatively high binding affinity with the Aβ peptide, and this process can modulate aggregation, induce the formation of toxic oligomers and reactive oxygen species (ROS) leading to oxidative stress. Aβ plaques are the most prominent pathological feature of AD, however soluble oligomeric forms of the peptide (approximately 1% of total Aβ in brain) have been found to show a better correlation with disease progression. When Zn(II) binds to Aβ there is an increase in the formation of non-fibrillar aggregates without causing an oxidative cascade, whereas the Cu(II)-Aβ interaction generates non-fibrillar aggregates (oligomer stabilization) and oxidative damage likely via ROS generation. Cellular toxicity studies in neuroblastoma cell lines have shown that Zn(II) reduces Aβ neurotoxicity, while Cu(II) significantly increases Aβ neurotoxicity. For these reasons, despite the fact that both Cu(II) and Zn(II) precipitate Aβ, it has been postulated that the Zn(II)-Aβ interaction exhibits an overall protective effect in the brain. We have thus chosen to focus on modulating the Cu(II)-Aβ interaction in this work.

The development of metal-protein attenuating compounds (MPAC) is a promising therapeutic approach for AD treatment. Targeting the metal-Aβ interaction in the extracellular environment could normalize the distribution of both metal ions and Aβ peptide in brain tissue and cerebrospinal fluid. Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline, HCQ, Figure 2.1) is a small, lipophilic and bioavailable metal chelator with a high affinity for Zn(II) and Cu(II) and has been reported to cross the blood-brain barrier (BBB) efficiently in the Tg2576 mouse model, an AD mouse model with elevated levels of Aβ and plaques formation. HCQ is the archetypical MPAC, and has shown promise as an AD therapeutic in both animal models and preliminary clinical trials. A second generation 8-hydroxyquinoline derivative, PBT2 (Figure 2.1), demonstrated therapeutic potential in AD murine models and Phase II clinical studies. This compound has been shown to reduce Aβ aggregation, limit Aβ oligomer toxicity, and redistribute metal ions (Cu(II) and Zn(II)) into neurons. Unfortunately treatment with PBT2 did not show a significant difference in a 12-month double-blind phase study when compared to placebo. A number of other chemical scaffolds have shown promise as MPAC’s for AD therapy.
bases derived from 8-hydroxy-2-quinolincarboxaldehyde as new MPAC for AD therapy. Hydrazones, semicarbazones, and thiosemicarbazones are Schiff-bases that have shown a broad range of pharmacological application and their mechanisms of action frequently involve metal-chelating properties \textit{in vivo}.\textsuperscript{202} Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, Figure 2.1) is a promising Schiff base that exhibits its pharmacological activity via Fe chelation and is currently being investigated in several Phase II clinical trials for cancer.\textsuperscript{203-207} Furthermore, it has shown effectiveness in preventing or reducing ROS accumulation and concomitant oxidative damage in both AD-derived and age-matched olfactory neuroepithelial cells.\textsuperscript{208}

![Figure 2.1](image.png)

\textbf{Figure 2.1} Chemical structures of clinically relevant AD compounds (Top) and ligands investigated in this work (bottom).

Herein, I have studied the novel Schiff base compound 2-[(8-Hydroxyquinolinily)methylene] acetohydrazide (\textbf{8-H}_2\textbf{QH}), and previously synthesized compounds 2-[(8-Hydroxyquinolinily)methylene] hydrazinecarboxamide (\textbf{8-H}_2\textbf{QS}) and 2-[(8-Hydroxyquinolinily)methylene] hydrazinecarbothioamide\textsuperscript{110} (\textbf{8-H}_2\textbf{QT}), where H\textsubscript{2}L stands for the neutral compound (\textbf{Figure 2.1}). These compounds can bind transition metals in bidentate, tridentate or tetradeutate coordination modes.\textsuperscript{110, 209, 210} Characterization of the Cu(II) binding and A\textsubscript{B} interaction properties of \textbf{8-H}_2\textbf{QH} and \textbf{8-H}_2\textbf{QS} are reported along with the evaluation of their ability to modulate A\textsubscript{B} aggregation.
2.2. Results and Discussion

2.2.1. Synthesis and Characterization

One new acetohydrazone ligand, 8-H$_2$QH, was synthesized by condensation of acethydrazide with 8-hydroxyquinoline-2-carboxaldehyde (Figure 2.2). 8-H$_2$QS and 8-H$_2$QT have been previously prepared.\textsuperscript{209, 210} 8-H$_2$QH was characterized by elemental analysis, which is in agreement with the proposed formula. The infrared spectrum (IR) of the hydrazone displays an absorption at 1678 cm$^{-1}$, which was assigned to $\nu$(C=O), and the absorption at 1594 cm$^{-1}$ was attributed to $\nu$(C=N) of the iminic bond, confirming hydrazone formation.\textsuperscript{211} Hydrazones have been reported to exist as tautomeric enolimines with the speciation dependent on electronic and structural effects, including inter- and intra-molecular hydrogen bonding.\textsuperscript{212, 213} Both the keto and enol forms were observed by proton and carbon-13 nuclear magnetic resonance ($^1$H NMR and $^{13}$C NMR) for 8-H$_2$QH in solution in a 1:0.42 for keto:enol forms (Figure 2.3). However, the X-ray structure indicates that in the solid state this compound exists only in the keto form.

![Figure 2.2 Synthesis of 8-H$_2$QH. The keto form is shown.](image)

![Figure 2.3 Tautomeric forms of 8-H$_2$QH.](image)

2.2.2. Crystal Structure Determination

The ORTEP of 8-H$_2$QH is shown in Figure 2.4. 8-H$_2$QH crystallizes with two independent molecules of the hydrazone (A and B, Figure A1) per asymmetric unit. Since
the geometrical parameters of the two molecules are similar (see Table 2.1 and Table A1), we will further describe molecule A here.

![Chemical structure of molecule A]

**Figure 2.4** ORTEP for 8-H$_2$QH with thermal ellipsoids at the 50% probability level. Hydrogen atoms are drawn as circles of arbitrary radii.

In A the C9–N2 and C10–O2 interatomic distances indicate double bond character (Table 2.1). The C=N–N–C(=O)C skeleton is almost planar (rms deviation from the least-squares plane of 0.0066 Å). The molecule adopts the EE conformation in relation to the C9–N2 and N3–C10 bonds, as indicated by the C2–C9=N2–N3, and N2–N3–C10–O2 torsion angles of 179.4(1)$^\circ$ and 179.1(1)$^\circ$ respectively.

In the molecular packing of 8-H$_2$QH various NH⋯O hydrogen bonds forming centrosymmetric dimers were observed. The NH⋯O and CH⋯O hydrogen bonds involving A and B lead to the formation of an infinite two-dimensional (2D) network (Figure A2). The low solubility of the compound may be attributed to these H-bonds as well as to π-π interactions in the solid state.

**Table 2.1** Selected bond lengths (Å) and torsion angles ($^\circ$) for the 8-H$_2$QH structure.

<table>
<thead>
<tr>
<th>Selected bonds</th>
<th>Bond length (Å)</th>
<th>Torsion angles</th>
<th>Angles ($^\circ$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1–C2</td>
<td>1.328(2)</td>
<td>O1–C8–C8A</td>
<td>118.0(1)</td>
</tr>
<tr>
<td>C2–C9</td>
<td>1.464(2)</td>
<td>C8–C8A–N1</td>
<td>116.5(1)</td>
</tr>
<tr>
<td>C9–N2</td>
<td>1.280(2)</td>
<td>N1–C2–C9</td>
<td>115.7(1)</td>
</tr>
<tr>
<td>N2–N3</td>
<td>1.363(2)</td>
<td>C2–C9–N2</td>
<td>119.8(1)</td>
</tr>
<tr>
<td>N3–C10</td>
<td>1.358(2)</td>
<td>C9–N2–N3</td>
<td>116.5(1)</td>
</tr>
<tr>
<td>C10–O2</td>
<td>1.230(2)</td>
<td>N2–N3–C10</td>
<td>120.3(1)</td>
</tr>
<tr>
<td>C10–C11</td>
<td>1.503(2)</td>
<td>N3–C10–O2</td>
<td>119.8(1)</td>
</tr>
<tr>
<td>C8–O1</td>
<td>1.358(2)</td>
<td>N3–C10–C11</td>
<td>122.9(1)</td>
</tr>
</tbody>
</table>

**2.2.3. Ligand Speciation Studies**

Speciation studies were performed via UV-Vis in 5% DMSO in 0.1 M NaCl for 8-H$_2$QH, 8-H$_2$QS and 8-H$_2$QT in a range of pH varying from 2 to 12. In the pH range
evaluated, there are four possible species (H₃L⁺, H₂L, HL⁻, L²⁻, where H₂L is neutral) as shown in Figure 2.5. Fitting the variable pH UV-Vis data for the compounds affords three pKₐ values for each compound (Table 2.2), with the speciation diagrams shown in Figure 2.6.

![Figure 2.5 Protonation states of the 8-hydroxyquinoline Schiff-base ligands.](image)

The pKₐ values for HCQ have been reported as pKₐ₁ 3.17 ± 0.11 and pKₐ₂ 8.05 ± 0.08 at 25 °C. The pKₐ₁ refers to the deprotonation of the pyridine nitrogen and pKₐ₂ refers to deprotonation of the hydroxyl oxygen. The pKₐ₁ values for the 8-hydroxyquinoline derivatives studied are in accordance with the value reported in the literature for HCQ. The pKₐ₁ value for 8-H₂QH is lower than those measured for 8-H₂QS and 8-H₂QT. 8-H₂QH displays a tautomeric equilibrium in solution (Figure 2.3), that increases the electron-withdrawing effect of the hydrazone moiety on the quinoline scaffold. Consequently, the mesomeric stabilization of the quinoline is also increased. Electron-withdrawing groups, especially at position 2 of the quinoline, will decrease the electron density on the ring thus reducing the donating ability of the quinoline nitrogen atom. The pKₐ₂ values for 8-H₂QH, 8-H₂QS, and 8-H₂QT are higher than the value reported for HCQ. HCQ has two electron-withdrawing groups in the o- and p- positions (I and Cl), which lowers the basicity of the quinoline hydroxyl. Interestingly, the pKₐ₃ value for 8-H₂QT is significantly less than the values for 8-H₂QH and 8-H₂QS. We attribute this difference to the stabilizing effect of the sulfur atom on the dianionic form of 8-H₂QT. This stabilizing effect likely plays a role in the different metal:ligand ratio in the Cu(II) complex of 8-H₂QT in comparison to 8-H₂QH and 8-H₂QS (vide infra). It is important to note that the major species at physiological pH is neutral H₂L for all three ligands.
Table 2.2 pK\textsubscript{a} and logP\textsubscript{calc} values of compounds 8-H\textsubscript{2}QT, 8-H\textsubscript{2}QH and 8-H\textsubscript{2}QS. pK\textsubscript{a} data were analyzed using the HypSpec program (Protonic Software, UK), and logP\textsubscript{calc} was calculated using ALOGPS 2.1 software.\textsuperscript{217,218}

<table>
<thead>
<tr>
<th>Compound</th>
<th>pK\textsubscript{a1}</th>
<th>pK\textsubscript{a2}</th>
<th>pK\textsubscript{a3}</th>
<th>logP\textsubscript{calc}</th>
<th>logP\textsubscript{exp}</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-H\textsubscript{2}QT</td>
<td>3.36 ± 0.01</td>
<td>9.51 ± 0.01</td>
<td>11.75 ± 0.01</td>
<td>1.67 ± 0.31</td>
<td>-</td>
</tr>
<tr>
<td>8-H\textsubscript{2}QH</td>
<td>2.87 ± 0.03</td>
<td>9.53 ± 0.03</td>
<td>13.50 ± 0.01</td>
<td>1.62 ± 0.58</td>
<td>-</td>
</tr>
<tr>
<td>8-H\textsubscript{2}QS</td>
<td>3.25 ± 0.02</td>
<td>9.63 ± 0.01</td>
<td>13.16 ± 0.01</td>
<td>1.12 ± 0.29</td>
<td>-</td>
</tr>
<tr>
<td>HCQ\textsuperscript{a}</td>
<td>3.17 ± 0.11</td>
<td>8.05 ± 0.08</td>
<td>-</td>
<td>3.43 ± 0.21</td>
<td>3.24\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}\textsuperscript{[214]}

The log\textsubscript{x} n-octanol/water values (logP\textsubscript{calc}, ALOGPS 2.1 software, Table 2.2) were calculated to evaluate the order of Gibbs free energies of solvation. The calculated values were not statistically different, suggesting equivalent solubility behavior in physiological fluids for all derivatives. However, evaluation of the absolute values of logP\textsubscript{calc} suggested a trend of hydrophobicity: 8-H\textsubscript{2}QT > 8-H\textsubscript{2}QH > 8-H\textsubscript{2}QS. 8-H\textsubscript{2}QT exhibited the lowest aqueous solubility in this work. The calculated values for HCQ are in accordance with the experimental data.\textsuperscript{214}

Figure 2.6 Speciation diagrams of 8-H\textsubscript{2}QT (A), 8-H\textsubscript{2}QH (B) and 8-H\textsubscript{2}QS (C). F\textsubscript{L} = fraction of species. H\textsubscript{2}L = neutral species. Diagrams were simulated using the HySS2009 program (Protonic Software, UK).
2.2.4. Metal Binding Properties

UV-Vis spectroscopy was used to probe the solution binding of \(8\text{-H}_2\text{QS}\), \(8\text{-H}_2\text{QH}\) and \(8\text{-H}_2\text{QT}\) with Cu(II) and compare to the solid state characterization data. Changes in the intensity of the ligand-based transitions along with the observation of new absorptions at ca. 449 nm and 438 nm indicated metal binding to ligand \(8\text{-H}_2\text{QH}\). Similar shifts of these peaks for \(8\text{-H}_2\text{QT}\) and \(8\text{-H}_2\text{QS}\) were also observed upon treatment with Cu(II) indicating metal binding to the ligands. Job plot analysis (Figure 2.7) suggests the formation of a 1:1 metal:ligand compound for \(8\text{-H}_2\text{QT}\) in agreement with previous reports of Cu complex formation for this thiosemicarbazone and analogues.\(^{110, 209}\) Interestingly, a 1:2 metal:ligand ratio was determined for \(8\text{-H}_2\text{QS}\) and \(8\text{-H}_2\text{QH}\) with Cu(II), matching the solid-state characterization data for the complexes. The Job plot results, and the resulting metal:ligand stoichiometries in solution, can be correlated to the pKa values for the three ligands. The high pKa values for \(8\text{-H}_2\text{QS}\) and \(8\text{-H}_2\text{QH}\) (>12) suggest lower probability of deprotonation of the N–H Schiff base moiety leading to preferential formation of 1:2 metal:ligand complexes where only the phenolate has been deprotonated. On the other hand, the lower pKa value for the \(8\text{-H}_2\text{QT}\) derivative leads to an increased stabilization of the dianionic form, inducing neutral 1:1 metal:ligand complex formation, with a tetradeinate doubly deprotonated ligand. This proposition is in accordance to the presence of sulfur, a more polarizable atom in comparison to the oxygen atoms that are present in the \(8\text{-H}_2\text{QS}\) and \(8\text{-H}_2\text{QH}\) frameworks.
Figure 2.7 Job plots of 8-H$_2$QS (▼ / ∆ 420 nm), 8-H$_2$QH (● / ○ 449 nm) and 8-H$_2$QT (■ / □ 404 nm) with Cu(II) acetate (20% DMSO in HEPES buffer pH 7.4). The dashed lines for 8-H$_2$QH ($\chi = 0.33$ Cu(II)) and 8-H$_2$QS ($\chi = 0.35$ Cu(II)) indicate a 1:2 Cu(II):L stoichiometry. The dashed line for 8-H$_2$QT ($\chi = 0.52$ Cu(II)) indicates a 1:1 Cu(II):L stoichiometry.

2.2.5. Theoretical Calculations of the Cu(II) Complexes

We further investigated the Cu(II) complexes of the 8-hydroxyquinoline ligands by theoretical calculations. A previously reported X-ray structure of Cu(8-QT) displays a 1:1 metal:ligand stoichiometry, in accordance with our solution analysis (vide supra). The optimized geometry of Cu(8-QT) is in good agreement with the experimental metrical parameters, with coordination sphere bond lengths predicted within ±0.06 Å (Figure 2.8 A). Based on these results we employed the same level of theory to investigate the structures of Cu(8-HQH)$_2$ and Cu(8-HQS)$_2$, and compared the results to the reported 1:2 metal:ligand structure for Cu(CQ)$_2$. The X-ray metrical values for Cu(CQ)$_2$ and the computed values are shown in Table 2.3. The predicted coordination sphere metrical parameters for Cu(8-HQH)$_2$, Cu(8-HQS)$_2$, and Cu(CQ)$_2$ are within ±0.06 Å of the reported data for Cu(CQ)$_2$. The computed structures of all 4 Cu complexes are shown in Figure 2.8. Of interest is the considerable distortion away from a square planar geometry predicted for both Cu(8-HQH)$_2$ and Cu(8-HQS)$_2$ (dihedral angle = 42° for both structures) in comparison to the reported structure for Cu(CQ)$_2$ (dihedral angle = 0°). To further investigate the effects of crystal packing and steric interactions of the o-8-hydroxyquinoline substituents on the dihedral angle, we calculated the optimized geometry of Cu(CQ)$_2$ at the same level of theory. The optimized geometry of Cu(CQ)$_2$ displays a dihedral angle of 17° (Figure 2.8 B), suggesting that while crystal packing plays a role in flattening the
reported Cu(CQ)$_2$ structure, the extended Schiff-base groups of the 8-H$_2$QH and 8-H$_2$QS ligands likely lead to the large predicted tetrahedral distortion observed for the corresponding 1:2 metal:ligand complexes.

![Figure 2.8](image)

**Figure 2.8** Density functional theory (DFT) optimized geometry of: A Cu(8-QT) displaying a distorted square planar geometry; B Cu(CQ)$_2$ displaying a distorted square planar geometry, possessing a planar torsion angle of 17°; C Cu(8-HQH)$_2$ displaying a distorted square planar geometry with a metal coordination sphere torsion angle of 47°; and D Cu(8-HQS)$_2$ displaying a distorted square planar geometry with a planar torsion angle of 42°. See Experimental Section for calculation details.

**Table 2.3** Comparison of experimental (Cu(CQ)$_2$) and calculated (Cu(8-HQH)$_2$, Cu(8-HQS)$_2$, Cu(CQ)$_2$) coordination sphere bond lengths. See Experimental Section for calculation details.

<table>
<thead>
<tr>
<th>Bond Lengths (Å)</th>
<th>Experimental Cu(CQ)$_2$</th>
<th>Calulated Bond Lengths (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu – N1</td>
<td>1.984</td>
<td>2.016</td>
</tr>
<tr>
<td>Cu – N2</td>
<td>1.963</td>
<td>2.016</td>
</tr>
<tr>
<td>Cu – O1</td>
<td>1.915</td>
<td>1.888</td>
</tr>
<tr>
<td>Cu – O2</td>
<td>1.923</td>
<td>1.888</td>
</tr>
</tbody>
</table>

*[^219]*

### 2.2.6. Antioxidant Capacity – TEAC Test

Evidence of oxidative stress is widespread in AD, with early neuronal and pathological changes showing indications of oxidative damage.[^220] The brain is
particularly susceptible to oxidative damage due to the high rate of metabolic activity coupled with relatively low antioxidant levels and low tissue regenerative capacity.\textsuperscript{193} We thus studied the antioxidant activity of \textit{8-H}_2\textit{QH}, \textit{8-H}_2\textit{QS} and \textit{8-H}_2\textit{QT} via the Trolox Equivalent Antioxidant Capacity (TEAC) assay.\textsuperscript{119, 201, 222} The ability of the compounds to quench the ABTS\textsuperscript{••} radical cation (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) was compared to Trolox, a water-soluble analog of (±)-\textit{α}-tocopherol (Figure 2.9). The Schiff-bases exhibited TEAC values that were equivalent to (±)-\textit{α}-tocopherol and enhanced in comparison to both PBT2 and HCQ. The potent antioxidant properties observed for the Schiff-bases in this test in comparison to the other hydroxyquinoline derivatives is likely due to the negative inductive effect of the Cl and I ring substituents in PBT2 and HCQ, which decrease the stability of a hydroxyl radical, reducing the antioxidant properties of these derivatives. In addition, the increased stabilization of the phenoxyl radical through the extended conjugation promoted by the semicarbazone, thiosemicarbazone and acetohydrazone moieties in the Schiff-bases may also contribute to the increased antioxidant properties of \textit{8-H}_2\textit{QH}, \textit{8-H}_2\textit{QS}, and \textit{8-H}_2\textit{QT}.

![Figure 2.9](image-url) Figure 2.9 Trolox Equivalent Antioxidant Capacity (TEAC) values at 1, 3, and 6 min for (±)-\textit{α}-tocopherol, PBT2, Clioquinol (HCQ), \textit{8-H}_2\textit{QH}, \textit{8-H}_2\textit{QS} and \textit{8-H}_2\textit{QT}. Error bars represent ± SD above and below the average TEAC value (determined in triplicate).
2.2.7. Inhibition of Aβ Aggregation via Turbidity Measurements

A turbidity test was carried out as a preliminary evaluation of the ability of 8-hydroxyquinoline derivatives to modulate or even suppress the aggregation of the Aβ₁-₄₀ peptide induced by Cu(II), providing information about the extent of peptide aggregation in solution on a short timescale (45 min). This test is completed by light scattering measurements at 405 nm. ¹⁸⁰ ²⁰¹ 8-H₂QT was not soluble under the test conditions (5% v/v DMSO in HEPES buffer pH 7.4), in agreement with its absolute logP<sub>calc</sub> value, which suggests higher hydrophobicity in comparison to 8-H₂QH and 8-H₂QS. Therefore, the turbidity test was carried out for 8-H₂QH and 8-H₂QS. The pH value was adjusted to pH 6.6 to maximize the Cu(II)-induced aggregation process.¹⁸⁰ Aggregation induced by Cu(II) was significantly inhibited by compounds 8-H₂QH and 8-H₂QS with respect to the negative control (Cu(II) and peptide) (Figure 2.10). In addition, there was a significant difference between 8-H₂QS and diethylenetriamine pentaacetic acid (DTPA, positive control) with respect to aggregation, suggesting that this derivative exhibits a greater inhibitory activity than the positive control under the test conditions; 8-H₂QH, exhibits similar aggregation inhibition in comparison to DTPA. The higher anti-aggregating effect displayed by 8-H₂QS in comparison to 8-H₂QH in this test may be attributed to its higher hydrophilicity in comparison to 8-H₂QH (suggested by logP<sub>calc</sub> values), and / or increased H-bonding interactions of the carboxamide function.

![Figure 2.10](image-url) Degree of Aβ₁–₄₀ aggregation as measured by UV-Vis measurements. Data represent the mean absorbance of quadruplicate trials at 405 nm of peptide in the presence of Cu(II) ions, and Cu(II) and ligands at pH 6.6. Error bars represent ± SD above (and below not shown) the average absorbance value.
2.2.8. Monitoring Aβ Aggregation via Gel Electrophoresis and Western Blotting

It is possible to obtain a more detailed picture of the extent and pathways of Aβ aggregation by using gel electrophoresis, Western blotting, and transmission electron microscopy (TEM) analysis techniques. The lower molecular weight, soluble Aβ species can be visualized by gel electrophoresis and Western blotting, while higher molecular weight and insoluble Aβ aggregates can be revealed by TEM analysis. The aggregation process of the more neurotoxic and oligomer-forming Aβ<sub>1-42</sub> peptide was probed by the aforementioned techniques in the presence of the 8-hydroxyquinoline compounds and Cu(II). Gel electrophoresis and Western blotting and the corresponding TEM images are shown in Figure 2.11. Aβ<sub>1-42</sub> was used at 25 µM for all the samples. Lane 2 contains Aβ<sub>1-42</sub> only, while Lanes 4, 6 and 8 contain Aβ<sub>1-42</sub> in the presence of 8-H<sub>2</sub>QH, 8-H<sub>2</sub>QS, and PBT2 respectively. The pattern of Aβ<sub>1-42</sub> aggregation does not change in the presence of 3 eq. of the ligands showing that the ligands alone do not influence the aggregation process in this assay over 24 hrs. The pattern of Aβ<sub>1-42</sub> aggregation in the presence of Cu(II) (1 eq.) shows reduced aggregate formation (Lane 3), in accordance with prior reports of Cu(II)-induced oligomer formation. Lanes 5, 7, and 9 contain, additionally to Aβ<sub>1-42</sub> and Cu(II), the ligands 8-H<sub>2</sub>QH, 8-H<sub>2</sub>QS, and PBT2 respectively. The addition of 8-H<sub>2</sub>QH, 8-H<sub>2</sub>QS, or PBT2 alters the pattern of aggregation to match that of Aβ<sub>1-42</sub> only (Lane 2). These results suggest that the ligands sequester Cu(II), likely restricting the formation of Cu(II)-containing oligomers in this assay. The TEM images are in accordance with the gel assay, showing large molecular weight aggregates for all Lanes except Lane 3 (Aβ<sub>1-42</sub> and Cu(II)). Interestingly, the gel assay for PBT2 (Lane 9) visually shows less large molecular weight aggregates in comparison to both 8-H<sub>2</sub>QH (Lane 5) and 8-H<sub>2</sub>QS (Lane 7) suggesting that PBT2 does not inhibit Cu(II)-Aβ<sub>1-42</sub> peptide interactions to the same extent as the 8-H<sub>2</sub>QX series.
2.3. Summary

In this Chapter I synthesized and characterized a new acetohydrazone (8-H$_2$QH) derived from 8-hydroxyquinoline, its Cu(II) complex and also the Cu(II) complex of 8-hydroxyquinoline semicarbazone (8-H$_2$QS), as part of the evaluation of new MPACs as AD therapeutics. The solution speciation (pKa values) suggest suitable physicochemical properties (neutral, water soluble) for CNS-targeting compounds, and the metal:ligand binding studies demonstrated the ability of the ligands to bind Cu(II), under physiological conditions: 24 hours with agitation in PBS at 37 °C, [Aβ] = [M] = 25 µM. The scale bar in each TEM image represents 200 nm.

Figure 2.11 Gel electrophoresis/Western blots and TEM images of Aβ$_{1-42}$ aggregation experiments. Top: (1) protein reference; (2) Aβ$_{1-42}$; (3) Aβ$_{1-42}$ + Cu(II); (4) Aβ$_{1-42}$ + 8-H$_2$QH (3 eq.); (5) Aβ$_{1-42}$ + Cu(II) + 8-H$_2$QH (3 eq.); (6) Aβ$_{1-42}$ + 8-H$_2$QS (3 eq.); (7) Aβ$_{1-42}$ + Cu(II) + 8-H$_2$QS (3 eq.); (8) Aβ$_{1-42}$ + PBT2 (3 eq.); (9) Aβ$_{1-42}$ + Cu(II) + PBT2 (3 eq.); bottom: TEM images of the same samples. Conditions: 24 hours with agitation in PBS at 37 °C, [Aβ] = [M] = 25 µM. The scale bar in each TEM image represents 200 nm.
conditions. The antioxidant capacity of these ligands was tested, along with drug candidates PBT2 and HCQ. \(8\text{-H}_2\text{QH}\) and \(8\text{-H}_2\text{QS}\) displayed significantly higher antioxidant capacity when compared to PBT2 and HCQ. The chelating abilities of the Schiff-bases and their subsequent effects on A\(\beta\) peptide aggregation were evaluated. An initial turbidity assay with A\(\beta_{1-40}\) was used to evaluate the influence of the ligands on the short-term Cu(II)-induced aggregation of the peptide. \(8\text{-H}_2\text{QS}\), \(8\text{-H}_2\text{QH}\) and DTPA (positive control) each presented a statistically significant decrease in aggregation in comparison to the negative control, A\(\beta_{1-40}\) in the presence of Cu(II). Gel electrophoresis/Western blotting and TEM images were used to evaluate the influence of the Schiff-base ligands and PBT2 on the aggregation of A\(\beta_{1-42}\), both in the presence and absence of Cu(II). The Western blotting showed that the pattern of A\(\beta_{1-42}\) aggregation in the presence of \(8\text{-H}_2\text{QH}\), \(8\text{-H}_2\text{QS}\), and PBT2 was similar to that of peptide only. The TEM results were in accordance with these observations. A\(\beta_{1-42}\) in the presence of Cu(II) showed a different pattern of aggregation, exhibiting the presence of oligomers (<15 kDa), while high molecular weight aggregates (>130 kDa) were inhibited. In the presence of \(8\text{-H}_2\text{QH}\) or \(8\text{-H}_2\text{QS}\), and Cu(II) the pattern of aggregation was similar to peptide only, suggesting that the Schiff-base ligands limit Cu(II)-induced oligomer formation via metal complexation. A similar result was observed for PBT2, however less high molecular weight aggregates were observed suggesting that this derivative does not restrict Cu(II)-induced oligomer formation to the same extent as \(8\text{-H}_2\text{QH}\) and \(8\text{-H}_2\text{QS}\) in this assay. In summary, \(8\text{-H}_2\text{QH}\) and \(8\text{-H}_2\text{QS}\) were found to influence metal-induced A\(\beta\) aggregation and exhibit antioxidant capacity similar to vitamin E. Overall, the 8-hydroxyquinoline derivatives show promise for modulating metal-A\(\beta\) peptide interactions.

### 2.4. Experimental

All common chemicals were purchased from Sigma-Aldrich and used without further purification. The syntheses of the novel ligand 2-[(8-Hydroxyquinolinyl)methylene]acetohydrazide (\(8\text{-H}_2\text{QH}\)) and previously synthesised ligands 2-[(8-Hydroxyquinolinyl)methylene]hydrazinocarboxamide (\(8\text{-H}_2\text{QS}\)), 2-[(8-Hydroxyquinolinyl)methylene]hydrazinocarbothioamide (\(8\text{-H}_2\text{QT}\)), and their Cu(II) complexes were performed using previously described methodologies.\(^{110,209}\) PBT2 was synthesized according to a reported method.\(^{226}\) The ligand \(8\text{-H}_2\text{QS}\) was previously obtained in its hydrochloride form,\(^{210}\) herein the neutral form was prepared. The Cu(II)
complex of 8-H$_2$QT (Cu(8-QT)) was synthesized as reported.\textsuperscript{209} The A$\beta$$_{1-40}$ and A$\beta$$_{1-42}$ peptides were purchased from 21st Century Biochemicals (Marlborough, MA, USA). The 10-20\% Tris-tricine mini gels were purchased from BioRad and membranes from PALL – Life Sciences. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV-600 instrument. Mass spectra (positive ion) were obtained on an Agilent 6210 time-of-flight electrospray ionization mass spectrometer. Electronic spectra were obtained on a Cary 5000 spectrophotometer. Magnetic susceptibilities were measured on a Johnson Matthey MSB/AUTO balance. Elemental analyses were performed on a Perkin Elmer CHN 2400 analyzer. Infrared spectra were recorded on a Perkin Elmer FT-IR Spectrum GX spectrometer using KBr plates ($4000 – 400$ cm$^{-1}$) and CsI/nujol ($600 – 200$ cm$^{-1}$).

2.4.1. Synthesis of 2-[(8-Hydroxyquinolinyl)methylene]acetohydrazide (8-H$_2$QH)

8-hydroxy-2-quinolincarboxaldehyde (0.173 g, 1 mmol) was suspended in ethanol (10 mL) and treated with an excess of acethyldrazide (0.081 g, 1.1 mmol). The reaction mixture was subsequently refluxed for 4 hours. The light yellow precipitate that formed was washed with water, ethanol and diethyl ether, air-dried and isolated in 89\% yield. Mp: 224.1 – 225.1 °C. Elemental analysis; Found (calcd) for C$_{12}$H$_{11}$N$_3$O$_2$: C, 62.83 (62.87); H 4.95 (4.84); N 18.26 (18.33). $^1$H NMR (DMSO-d$_6$): $\delta$ 11.75 (Enol), 11.68 (Keto) (s,1H); 9.83 (Keto), 9.81 (Enol) (s,1H); 8.35 (Enol), 8.22 (Keto) (s, 1H); 8.32 – 8.29 (Enol and keto) (m, 1H); 8.02 (Enol and keto) (app dd, 1H); 7.45 – 7.37 (Enol and keto) (m, 2H); 7.13 – 7.10 (Enol and keto) (m, 1H); 2.28 (Keto), 2.02 (Enol) (s, 3H), $^{13}$C NMR (DMSO-d$_6$): $\delta$ 172.4 (Keto), 166.0 (Enol) (C); 153.4 (Enol and keto) (C), 151.8 (Enol), 151.5 (Keto) (C); 145.8 (Enol), 143.1 (Keto) (CH); 138.1 (Enol and keto) (C); 136.5 (Enol and Keto) (CH); 128.8 (Enol), 128.7 (Keto) (CH); 128.2 (Enol), 128.1 (Keto) (C); 117.8 (Enol and keto) (CH); 117.6 (Enol), 117.3 (Keto) (CH); 112.2 (Enol), 112.1 (Keto) (CH); 21.8 (Enol), 20.3 (Keto) (CH). IR (KBr): $\nu$(OH) 2928, $\nu$(CO) 1678, $\nu$(CN$_{em}$) 1594, $\nu$(CN$_{qu}$) 1632, $\rho$(qui) 720 cm$^{-1}$.

Suitable crystals for X-ray crystallography were grown by evaporation of an ethanol solution of 8-H$_2$QH.
2.4.2. Synthesis of 2-[(8-Hydroxyquinolinyl)methylene]hydrazinecarboxamide (8-H$_2$QS)

This compound has been previously synthesized as the hydrochloride salt.$^{210}$ Semicarbazide hydrochloride (0.123 g, 1.1 mmol) was dissolved in water (10 mL) and treated with an equivalent of sodium acetate (0.090 g, 1.1 mmol). A suspension of 8-hydroxy-2-quinolinicarboxaldehyde (0.173 g, 1 mmol) in ethanol (10 mL) was added and the reaction mixture was refluxed for 4 hours. The light yellow precipitate that formed was washed with water, ethanol and ether, air-dried and isolated in a 95 % yield. Mp: 229.7 – 230.1 °C. Elemental analysis; Found (calcd) for C$_{11}$H$_{10}$N$_4$O$_2$: C, 57.74 (57.39); H 4.32 (4.38); N 23.87 (24.34). $^1$H NMR (DMSO-d$_6$): $\delta$ 10.73 (s, 1H), 9.72 (s, 1H), 8.33 (d, $J$ = 8.7 Hz, 1H), 8.25 (d, $J$ = 8.7 Hz, 1H), 8.05 (s, 1H), 7.42 – 7.35 (m, 2H), 7.08 (dd, $J$ = 1.4, 7.4 Hz, 1H), 6.74 (br s, 2H), $^{13}$C NMR (DMSO-d$_6$): $\delta$ 156.5 (C), 153.2 (C), 152.2 (C), 139.8 (CH), 137.9 (C), 136.0 (CH), 128.5 (C), 127.7 (CH), 118.1 (CH), 117.7 (CH), 111.9 (CH). IR (KBr): $\nu$(OH) 3152, $\nu$(CO) 1720, $\nu$(CN$_{im}$) 1572, $\nu$(CN$_{qui}$) 1578, $\rho$(qui) 722 cm$^{-1}$.

2.4.3. Synthesis of Cu complexes

Bis(2-[(8-Hydroxyquinolinyl)methylene]acetohydrazide) Cu(II) Cu(8-HQH)$_2$

2-[(8-Hydroxyquinolinyl)methylene]acetohydrazide (0.344 g, 1.5 mmol) was dissolved in DMF (5 mL) and treated with 1.2 eq. (0.359 g, 1.8 mmol) of [Cu(OAc)$_2$]·2H$_2$O. The reaction darkened considerably whilst stirring at room temperature for 4 hours. Upon addition of water (5–10 mL) a solid precipitate was collected, washed repeatedly with water (3 x 5 mL) and dried in vacuo to afford a dark red solid (90 % yield). Elemental analysis; Found (calcd) for C$_{24}$H$_{20}$N$_8$O$_4$Cu-0.5H$_2$O: C, 54.89 (54.49); H, 4.01 (4.00); N, 15.86 (15.89). IR (KBr): $\nu$(CO) 1674, $\nu$(CN$_{im}$) 1586, $\nu$(CN$_{qui}$) 1596, $\rho$(qui) 746, $\nu$(MO) 540, $\nu$(MN) 345 cm$^{-1}$. Effective magnetic moment = 1.66 (BM). MS (ES$^+$): m/z (calcd) 521.1011 (521.0140) [M + H$^+$].
Bis(2-[(8-Hydroxyquinolinyl)methylene]hydrazinecarboxamide) 
Cu(II) Cu(8-HQS)$_2$

The reaction was carried out in a similar manner to Cu(8-HQH)$_2$ to afford a light green solid of Cu(8-HQS)$_2$ (99 % yield). Elemental analysis; Found (calcd) for C$_{22}$H$_{18}$N$_8$O$_4$Cu·0.5H$_2$O: C, 50.04 (49.76); H, 3.64 (3.61); N, 20.93 (21.10). IR (KBr): $\nu$(CO) 1666, $\nu$(CN$_{im}$) 1578, $\nu$(CN$_{qui}$) 1556, $\rho$(qui) 756, $\nu$(MO) 530, $\nu$(MN) 476 cm$^{-1}$. Effective magnetic moment = 1.89 (BM). MS (ES$^+$): m/z (calcd) 523.0929 (522.9890) [M + H$^+$].

2.4.4. Crystal structure determination

Single-crystal X-ray diffraction measurements of 8-H$_2$QH were carried out on a GEMINI-Ultra diffractometer (LabCri-UFMG) using a graphite-Enhanced Source Mo K$\alpha$ radiation ($\lambda=0.71073$ Å) at 150 K. Data collection, cell refinement results, and data reduction were performed using the CRYSAIISPRO software.$^{227}$ The semi-empirical from equivalents absorption correction method was applied.$^{227}$ The structure was solved by direct methods using SHELXS-97. Full-matrix least-squares refinement procedure on $F^2$ with anisotropic thermal parameters was carried out using SHELXL-97.$^{228}$ Positional and anisotropic atomic displacement parameters were refined for all non-hydrogen atoms. Hydrogen atoms were placed geometrically and the positional parameters were refined using a riding model. A molecular plot and crystal packing figures were prepared using ORTEP$^{229}$ and MERCURY$^{230}$, respectively. Tables were generated using WINGX suite.$^{231}$ A summary of the crystal data, data collection details and refinement results are listed in Table 2.4.
Table 2.4 Crystal structure and refinement data for 8-H$_2$QH.

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<tr>
<td>c, Å</td>
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<td>Final $R$ indices [$I&gt;2\sigma(I)$]</td>
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2.4.5. Metal binding studies

Metal binding studies were performed by varying the molar fractions of CuCl$_2$ from 0 to 1 (0 to 4.0 x 10$^{-5}$ mol L$^{-1}$) in 20% DMSO/HEPES Buffer pH 7.4 in the presence of ligand and 30 UV-Vis spectra were obtained. An absorbance maximum at 424, 449 and 404 nm wavelength was assigned as interaction of metal and ligand for Cu(8-HQH)$_2$, Cu(8-HQS)$_2$ and Cu(8-QT) respectively for each solution, which gave the determination of the metal:ligand ratio in the complex.

2.4.6. Calculations

Density functional theory (DFT) calculations were used to obtain optimized geometries for the doublet states of the Cu(8-HQH)$_2$, Cu(8-HQS)$_2$, Cu(CQ)$_2$ and Cu(8-QT) complexes. The Gaussian 09 program (revision D.01)$^{232}$ was used with the B3LYP functional$^{233, 234}$ and the 6-31G(d) basis set on all atoms. Frequency calculations at the same level of theory confirmed that the optimized structures were located at a minimum on the potential energy surface.
2.4.7. Determination of acidity constants by UV-Vis

The speciation of $8\text{-H}_2\text{QS}$, $8\text{-H}_2\text{QT}$ and $8\text{-H}_2\text{QH}$ at physiological pH were obtained by the determination of acidity constants through variable pH UV-Vis spectra. Solutions of $8\text{-H}_2\text{QS}$, $8\text{-H}_2\text{QT}$ and $8\text{-H}_2\text{QH}$ (40 µM) were prepared in 5% DMSO in 0.1 M NaCl. A pH electrode was calibrated using a 2-point method (pH 4.01 and 10.01 standard buffers) before obtaining UV-Vis spectra. The pH of the ligand solutions was increased by NaOH to a starting point of ca. pH 12. UV-Vis spectra of the ligand solutions were obtained in the range of 600–190 nm at different pH by addition of aliquots of HCl. At least 30 UV-Vis spectra were obtained in the range of pH 2–12. The HypSpec program (Protonic Software, UK) was used to analyze spectral data.\textsuperscript{235, 236} HySS2009 program (Protonic Software, UK) was used to simulate speciation diagrams for $8\text{-H}_2\text{QS}$, $8\text{-H}_2\text{QT}$ and $8\text{-H}_2\text{QH}$.\textsuperscript{237}

2.4.8. Antioxidant capacity – TEAC test

The ability of the 8-hydroxyquinoline derivatives $8\text{-H}_2\text{QH}$, $8\text{-H}_2\text{QS}$ and $8\text{-H}_2\text{QT}$ to scavenge free radicals was evaluated using the Trolox Equivalent Antioxidant Capacity (TEAC) assay.\textsuperscript{222} These compounds were compared to both PBT2 and HCQ. Natural antioxidants, Vitamin E ((±)-α-tocopherol) and glutathione, were used as positive controls. The relative TEAC values were determined by a decolourization assay with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation. ABTS was dissolved in water (7 mM) and reacted with potassium persulfate (2.45 mM) in the dark for 16 hours to form the colored ABTS radical cation. This stock solution of ABTS was diluted with methanol to an absorbance value of 0.70 (± 0.02) at 744 nm. To start the reaction, solutions of $8\text{-H}_2\text{QH}$, $8\text{-H}_2\text{QS}$, $8\text{-H}_2\text{QT}$, HCQ, and PBT2 in DMSO (20 µL, 3.0–15.0 µM) were added to 2 mL of ABTS solution. The absorbance value was measured in triplicate for each time point (1, 3, and 6 min). The value of absorbance at 744 nm was plotted as a function of compound concentration. The slopes were then compared to the standard, trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), with its TEAC value normalized to 1.\textsuperscript{117, 222}

2.4.9. Turbidity measurements

A 200 µM stock solution of lyophilized synthetic human A$\beta_{1–40}$ (21st Century Biochemicals) was freshly prepared before each trial was performed. Each vial of peptide,
which contained ca. 0.25 mg Aβ<sub>1-40</sub>, was dissolved in 290 µL of deionized water. To achieve complete dissolution of the peptide, sonication for 1 minute followed by a 30 second pause was repeated twice. A 20 mM HEPES buffer solution containing 150 mM NaCl was prepared and treated with Chelex in deionized water at pH 6.6 and used to prepare stock solutions of ligands, Cu(II), and DTPA, as well as the reaction mixtures in the 96-well plates. The turbidity assay was conducted in quadruplicate in flat-bottomed 96-well assay plates (Microtest, BD Falcon). Ligands, Cu(II) and Aβ<sub>1-40</sub> peptide had final concentrations of 150 µM, 25 µM and 25 µM, respectively. Cu(II) solution was prepared from atomic absorption standards (Sigma-Aldrich). Cu(II), Aβ<sub>1-40</sub> and HEPES buffer were first added to the 96-well plate followed by the ligands. The solutions were incubated for 45 minutes at 37 °C under constant agitation, and each well in the 96-well plate was measured at 405 nm using a Synergy 4 Fluorometer plate reader from BioTek. Wells containing ligand, metal and buffer were used as blank and subtracted from corresponding wells. Positive controls containing metal and peptide were used to demonstrate the effect of the absence of ligand on peptide aggregation.

2.4.10. Gel Electrophoresis and Western Blotting

Aβ<sub>1-42</sub> was first monomerized using a reported procedure, which included preparation by dissolving Aβ<sub>1-42</sub> (21st Century Biochemicals) in hexafluoroisopropanol (HFIP) (0.5 mM), sonication for 15 min and incubating overnight in 4°C fridge. The solution was then aliquoted out and evaporated under a stream of N<sub>2</sub>. The monomeric films were stored at -80 °C. The peptide was then dissolved in 1:1 DMSO/ddH<sub>2</sub>O solution and the concentration was assessed with the use of a Thermo Nicolet UV nanodrop at 280 nm and an extinction coefficient of 1450 M<sup>-1</sup>cm<sup>-1</sup>. The amount of DMSO solvent (2.8%) in the eventual incubation solution is small, and has been previously shown to have no effect on aggregation. Aβ solutions were then incubated for 24 hours at 37 °C with continuous agitation at 200 rpm to generate fibrils in the presence of ligands, or ligands and Cu(II) in 0.1M PBS buffer at pH 7.4. A 10 – 20% gradient tris-tricine mini gel was used to separate samples at 100 V for 100 min at room temperature. The gels were transferred to a nitrocellulose membrane on an ice bath for 3 hours at 40 V at 4 °C. The membrane was blocked in a 3% bovine serum albumin (BSA) solution in TBS for 1 hour. The membrane was incubated in a solution (1:2000 dilution) of 6E10 anti-Aβ primary antibody (Covance) overnight at 298 K. After washing 4 X 15 mins with TBS buffer, the membrane
was incubated in a solution containing the secondary antibody (Horseradish peroxidase, Caymen Chemicals) for 3 hours. Thermo Scientific SuperSignal® West Pico Chemiluminescent Substrate kit was used to visualize the Aβ species using a FUJIFILM Luminescent Image Analyzer (LAS-4000).

2.4.11. Transmission Electron Microscopy (TEM)

Samples were prepared from the Western blot assay after the 24 hour incubation time at 37 °C. TEM grids were prepared following previously reported methods. In order to increase hydrophilicity, the Formvar/Carbon 300-mesh grids (Electron Microscopy Sciences) were glow discharged in a vacuum for 15 seconds. Drops of samples (10 µL) were placed onto a sheet of parafilm and the TEM grid was laid on the drop for 5 minutes. The grid was then placed on the first drop of syringe-filtered 5% uranyl acetate and immediately removed, repeated for the second drop, then placed on the third drop to incubate for 1 minute. Excess uranyl acetate was removed using a tissue between drops. The grid was allowed to air-dry for at least 15 minutes. Bright field images were obtained on a Hitachi 8000 STEM with a lanthanum hexaboride thermoionic source operating at 200 kV and at a magnification of 20000x.
Chapter 3. Catalytic Antioxidant for Limiting Amyloid-Beta Peptide Aggregation and Reactive Oxygen Species Generation


L.M.F. Gomes performed the binding constant determinations, gel electrophoresis and Western blotting, TEM imaging, and catalytic antioxidant activity. A. Mahammed completed the syntheses of the Fe corrole. K.E. Prosser performed EPR experiments and simulations. J.R. Smith completed the ESI-MS experiments.

3.1. Introduction

Alzheimer’s disease (AD) is the most common form of dementia, representing between 50-75% of all cases. In 2018, an estimated 50 million people worldwide suffered from dementia, and this number is projected to grow sharply due to increased life expectancy. The lack of effective treatment strategies for AD, coupled with increased incidence, has stimulated extensive research efforts in this important field.

Clinical diagnosis of AD is currently based on progressive loss of memory and impairment in cognition, with final diagnosis requiring post-mortem examination of the brain to determine the severity of two neuropathological hallmarks; Amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs). It is still unclear whether Aβ-plaques, NFTs, or both, are a cause or an effect of the neurodegeneration in AD. NFTs are intracellular aggregates of oxidatively-modified and hyperphosphorylated microtubule-associated protein tau, while Aβ plaques are extracellular and contain the Aβ peptide as the major constituent. The Aβ peptide is a product of the amyloid precursor protein (APP), and a series of cleavage events by α-, β-, γ-secretases, afford the Aβ peptide as predominantly Aβ1-40 or Aβ1-42 (a 40- or 42-residue peptide). In addition, truncation at the N-terminus results in Aβ3(p)-n, Aβ4-ν, and Aβ11(p)-n (where p refers to pyroglutamate) peptides that are also significant components of amyloid deposits. Aβ can be found in three general forms in the brain: membrane associated, aggregated, and soluble. Most of Aβ is
membrane-associated in healthy individuals, but in individuals with AD the aggregated and soluble fractions increase considerably.\textsuperscript{21, 26, 27, 31, 69}

Early neuronal and pathological changes show indications of oxidative damage, indicating oxidative stress is involved in AD.\textsuperscript{221} The cause of oxidative stress in AD has been attributed to a number of factors, including impaired cellular energy metabolism and/or Fenton-type processes involving redox-active metal-ions (Fe, Cu), and metal-containing aggregates.\textsuperscript{175, 250-254} Metal-ions, such as Zn, Cu and Fe, are essential for healthy organisms and brain function, and are tightly regulated under normal circumstances.\textsuperscript{63, 64, 255} However, a change in metal-ion homeostasis in the brain has been associated with protein aggregation, and the generation of reactive oxygen species (ROS) in neurodegenerative diseases such as AD.\textsuperscript{68, 84, 256} Metal-ions are present in increased concentrations in Aβ plaques in comparison to normal brain tissue, with concentrations of ca. 0.4 mM for Cu, 1 mM for Zn, and 0.9 mM for Fe.\textsuperscript{25, 61-66} Metal-ion binding can modify the aggregation pattern of Aβ, disrupt normal metalloenzyme activity, and facilitate the production of ROS.\textsuperscript{68, 69, 84-88} Recent studies have shown that the Aβ-Cu(II) complex exhibits detrimental catalytic ROS generation, particularly so in the presence of cholesterol and vitamin C, and is able to reduce O\textsubscript{2} generating the superoxide anion (O\textsubscript{2}•–), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and hydroxyl radical (•OH).\textsuperscript{79, 80, 89-92}

As a result of the possible role of metal-ion dyshomeostasis in AD, the development of multifunctional metal binding molecules as therapeutics has been actively explored.\textsuperscript{108, 114, 257} Metal-binding agents with additional properties such as radical scavenging, peptide binding and aggregation inhibition, and acetylcholine esterase (AChE) activity have been developed.\textsuperscript{111, 179, 180, 258, 259} In addition, a number of groups have explored the use of discrete metal complexes for the diagnosis and treatment of AD.\textsuperscript{139, 260, 261} In terms of therapeutics, Pt,\textsuperscript{140, 142, 144, 145} Ru,\textsuperscript{170, 171} Ir,\textsuperscript{156, 262} Co,\textsuperscript{69, 153, 154} Re,\textsuperscript{263} Rh,\textsuperscript{155} Mn,\textsuperscript{264, 265} and V\textsuperscript{266} complexes have been investigated for their ability to modify the aggregation of the Aβ peptide, and certain compounds have shown promising results in disease models. For example, an orally-available Pt(IV) complex (Figure 3.1) was shown to cross the blood-brain barrier (BBB), reduce plaque burden, and reduce Aβ peptide levels in a APP/PS1 mouse model.\textsuperscript{152} Therefore, a metal complex that can bind to the Aβ peptide, modulate aggregation, and reduce ROS production is a promising therapeutic for AD.
Corrole ligands are known to bind to metal ions, such as Al, Cu, Fe, Ga, and Au, and the corresponding metal complexes display outstandingly high hydrolytic stability. The Fe(III) complex (FeL1, Figure 3.1) displays excellent catalase activity, superoxide dismutase (SOD) activity, and catalytic activity for the decomposition of peroxynitrite (PN, ONOO⁻). Additionally, FeL1 binds to and protects the cholesterol-carrying lipoproteins from oxidative stress; and oral administration of FeL1 to a mouse model of atherosclerosis leads to a decrease in atherosclerotic lesions. We were thus interested in investigating the interaction of FeL1 with the Aβ peptide, and how this would modulate peptide aggregation and ROS generation. Strong inspiration came from reports by Dey et al., who have shown that heme binds to the Aβ peptide, that one of the three histidine residues (His⁶) of Aβ is ligated to the heme’s Fe, and that the heme-Aβ adduct induces ROS formation. Furthermore, a study that focused on the uptake of Fe complexes by macrophages, which are a major source of ROS, revealed that heme is cytotoxic while FeL1 is cytoprotective. Additionally, FeL1 was reported to have low cytotoxic activity while maintaining cell cycle distribution similar to untreated cancer cells. Herein I report the interaction of FeL1 with the Aβ peptide, how it affects peptide aggregation, and the radical scavenging ability of the FeL1-Aβ adduct, in both the presence and absence of Cu(II) ions.

3.2. Results and discussion

3.2.1. Binding of Aβ His Residues to FeL1

The Fe(III) complex of the amphipolar 2,17-bis-sulfonato-5,10,15-tris(pentafluorophenyl)corrole (FeL1) has very strong affinity to human serum albumin (HSA) and lipoproteins, which is in part due to binding of histidine (His) residues to the
metal ion. The His ligation causes a shift in the Soret band of FeL1 from 390 to 410 nm, as well as the formation of a new band at 620 nm, and the intensity of the latter band is associated with the binding of either one or two axial His residues. There are three Aβ His residues (His6, His13, and His14) and they play an important role in metal-ion binding (Figure 3.2), with dissociation constants (Kd) of ~10^{-10} M for Cu(II) and ~10^{-5} M for Zn(II). In addition, Aβ His residues have been reported to bind to discrete metal complexes such as heme, Ru complexes, and Co complexes. In addition to His binding, residues Asp1, Tyr10, and Glu11 play a role in the coordination of Aβ to Cu(II) and Zn(II).

Prior to investigating the interaction(s) of FeL1 with the Aβ peptide by UV-Vis spectroscopy, its binding to 1-methylimidazole (1-Melm) was examined as to determine the spectral features and binding affinity associated with exogenous imidazole as the axial ligand. Gradual addition of up to 150 eq. of 1-Melm led to a shift in the near UV (Soret) band, a decrease in the band at 533 nm, and the formation of a new band at 620 nm (Figure 3.3). The spectral changes matched those for histidine binding (Figure B1, Appendix B), however in both cases a large excess of ligand is required (150 and 700 eq. respectively) to observe spectral endpoints. A variable pH UV-Vis titration (Figure B2, Appendix B), at a concentration ratio of 1:2 FeL1:1-Melm, together with subsequent data fitting using Hypspec and HySS, provides binding constants of logM(1-Melm) = 5.81 ± 0.01 (where M = FeL1) and a much smaller logM(1-Melm)_2 = 2.57 ± 0.02. Our results are in accord with the higher stability of 5-coordinate mono-axial ligated Fe(III) corroles in comparison to 6-coordinate bis-axial ligated Fe(III) corroles, which is opposite to that

Figure 3.2 Representation of Component I (Ia, Ib, Ic) and Component II, the two major pH-dependent Aβ-Cu(II) binding modes (modified from Borghesani et al, 2018).
reported for Fe(III) porphyrins.\textsuperscript{283} The main reason for this difference is that upon bis-axial ligation Fe(III) porphyrins gain more crystal field stabilization energy (CFSE) as they transform from high spin (HS) to low spin (LS), while Fe(III) corroles only transform from intermediate spin (IS) to LS.\textsuperscript{281, 282}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3a.png}
\caption{UV-Vis spectra of 1-Melm additions to FeL1 (30 µM, black) in PBS buffer (0.01 M, pH 7.4). Grey lines represent additions of 5 eq. of 1-Melm up to 50 eq. (red) with a maximum of absorption at 620 nm for 150 eq. shown in green.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3b.png}
\caption{UV-Vis spectra of FeL1 (30 µM, black) in the presence of 1 eq. of Aβ1-40 (red), Aβ1-16 (green), Aβ1-42 (blue) in PBS buffer (0.01 M, pH 7.4).}
\end{figure}

The studies with 1-Melm provided critical information for examining the interaction of FeL1 with the full length Aβ1-42 and two truncated peptides: Aβ1-16 that contains the metal
binding N-terminus (His\(^6\), His\(^{13}\), His\(^{14}\)), and A\(\beta\)\(_{17-40}\) with the hydrophobic portion of the peptide lacking any His. Addition of A\(\beta\)\(_{17-40}\) to FeL1 did not induce any significant spectral changes, while even a single equivalent of either A\(\beta\)\(_{1-42}\) or A\(\beta\)\(_{1-16}\) led to a red shift and intensity-increase of the Soret band, accompanied by the appearance of a \(\lambda_{\text{max}} = 620\) nm band (Figure 3.4). While this experiment clearly proves the importance of His-Fe binding, the comparison of Figure 3.3 and 3.4 exposes major differences. Importantly, the binding of the protein-provided histidine must be much stronger than that of 1-Melm as full spectra changes are achieved with 1 vs. > 100 eq., respectively. In the former case, the observed spectral changes occur immediately upon mixing, with no further spectral changes apparent after monitoring for 1 hour, and addition of excess A\(\beta\)\(_{1-16}\) (up to 16 eq., Figure B3, Appendix B) did not induce further spectral changes. The last result is also highly relevant to the other spectral difference: while the 533 nm band disappears in the presence of a large excess of 1-Melm (Figure 3.3), the bands at 533 and 620 nm remain of essentially equal intensity starting from a 1:1 (Figure 3.4) to a 1:16 (Figure B3, Appendix B) ratio of FeL1:A\(\beta\)\(_{1-16}\). Taken together, the results show that FeL1 and A\(\beta\) form a 1:1 adduct that relies on only one of the His residues in A\(\beta\). The other two His residues are either too far away to approach the metal center and/or are unable to bind due to steric interference. Previous reports agree with our findings in that axial ligand binding to Fe(III) corroles shows that the 5-coordinate species is stabilized in comparison to the 6-coordinate bis-axial ligated species.\(^{280, 281, 284}\)
Figure 3.5 (Left) Variable pH UV-Vis titration of FeL1 (30 µM) and Aβ1-16 (30 µM) from pH 3.1 (black) to pH 11.5 (blue). The red spectrum represents the maximum absorbance for the FeL1-Aβ complex at pH 8.2. (Right) Using HypSpec and HySS, the variable pH data were fitted to a model including FeL1-Aβ, FeL1-Aβ(H) and a HO-FeL1 component at high pH. At pH 7.4 the majority of FeL1 is bound to Aβ1-16 (>99%).

The stability of the 1:1 FeL1:Aβ1-16 adduct was determined via a variable pH titration (Figure 3.5), which together with subsequent data fitting using Hypspec and HySS, provided binding constants of logM(Aβ1-16) = 11.90 ± 0.01, and logM(Aβ1-16)(H) = 4.90 ± 0.02 (where M = FeL1, and (H) indicates a mono-protonated peptide species). This experiment demonstrates the much higher affinity of FeL1 for the Aβ peptide in comparison to 1-MeIm (see below). At higher pH values (> 9.5) a metal hydrolysis species is likely (modelled as logM(OH), presumably due to the binding of an OH⁻ ligand). As indicated from the speciation diagram (Figure 3.5), the interaction of FeL1 with the Aβ peptide coincides with His deprotonation (reported pKₐ values of 5.72, 6.50, and 6.95). Further analysis of the speciation diagram of FeL1 with Aβ1-16 provides the binding affinity at physiological pH. The concentration of free FeL1 present in solution at a given pH, referred to as pM (p(FeL1) = -log([FeL1]_unchelated)), is a direct estimate of metal-ligand affinity when all species in solution are considered. The calculated value for p(FeL1) is 7.0 ([FeL1] = [Aβ1-16] = 30 µM), which affords a Kₐ value of ~ 10⁻⁷ M. This value shows that the affinity of Aβ for FeL1 is lower than for Cu(II) but larger than for Zn(II).
Figure 3.6 Mass spectrum (ESI-MS) of binding of Aβ1-16 to FeL1 with its chloride and sodium adducts. Across all experiments, species were observed as [FeL1(0-1)-Aβ-Na(0-6)]^{2-3}. Additionally observed was the dimeric species [Aβ2-Cl-Na(0-6)]^{+3} at m/z 1303-1374.

To gain more insight into the binding event, both ¹H NMR and ESI-MS studies were performed. The MS spectrum of a 1:1 FeL1:Aβ1-16 adduct showed multiple m/z peaks consistent with FeL1 binding to Aβ1-16, with the most intense adduct peak corresponding to [FeL1-Aβ1-16]^{2+} (Figure 3.6). FeL1 has been reported to bind to albumin, and in addition the Aβ peptide shows a specific interaction with HSA. Based on these reports we investigated the binding of FeL1 to Aβ in the presence of HSA, and under these conditions observed the [FeL1-Aβ1-16]^{2+} adduct (Figure B4, Appendix B). The ¹H NMR of Aβ1-16 was recorded in the presence of 0.10 and 0.25 eq. of the paramagnetic FeL1. Initially, the signals from the three histidines and the tyrosine were quite sharp and well resolved (Figure 3.7, bottom trace). Addition of FeL1 induced broadening of all signals attributed to the histidines (7.95, 7.05, 7.00 ppm), while those of Tyr^{10} (7.10 and 6.82 ppm) were not affected (Figure 3.7, mid and top traces). Overall, the data are consistent with binding of an Aβ His residue to FeL1, and there is likely no preference for any of the available peptide His residues (His⁶, His¹³, His¹⁴).
Figure 3.7 Changes in the $^1$H spectra of $\text{A}_\beta_{1-16}$ in the presence of $\text{FeL1}$. Shown are spectra obtained at 210 $\mu$M $\text{A}_\beta_{1-16}$, in PBS buffer prepared in D$_2$O pH 7.4 at 25˚C (red) with addition of 0.10 (green) and 0.25 eq. (blue) of $\text{FeL1}$. * His$^6$, His$^{13}$ and His$^{14}$. † Tyr$^{10}$.

3.2.2. FeL1 Binding to Aβ in the Presence of Cu(II)

**UV-Vis Analysis**

The affinity between Cu(II) and $\text{A}_\beta$ is very large ($K_d \approx 10^{-10}$ M) and the inner coordination sphere of Cu(II) in the adduct is usually composed of the N-terminal amine, one carbonyl group and two His (Figure 3.2, component I, major species at pH = 7.4).$^{67-69, 291}$ Although the affinity of $\text{FeL1}$ to $\text{A}_\beta$ was found to be quite large ($K_d \approx 10^{-7}$ M) it is still 3 orders of magnitude lower than that of Cu(II), and thus is not expected to compete for the Cu(II) binding site. Considering, however, that the $\text{A}_\beta$-Cu(II) adduct still has one His residue not involved in Cu binding, concurrent binding of Cu(II) and $\text{FeL1}$ to $\text{A}_\beta$ is possible and of significant interest. This aspect was addressed by combining $\text{FeL1}$ with a preformed $\text{A}_\beta$-Cu(II) complex and also *vice versa*, by adding Cu(II) to a $\text{FeL1}$/protein mixture. Identical results were obtained in both cases (Figure 3.8 and B5, Appendix B) and the corresponding UV-Vis spectra clearly revealed the earlier outlined spectral features associated with axial His binding. This shows that $\text{FeL1}$ binds to the peptide even in the presence of Cu(II) and also provides another independent indication that the inner coordination sphere of $\text{FeL1}$ has only one axial histidine ligand. The formation of the ternary adduct (1:1:1 $\text{FeL1}$:$\text{A}_\beta_{1-16}$:Cu adduct) was further confirmed.
by ESI-MS, with m/z peaks corresponding to \([\text{FeL1-}A\beta\text{-Cu(II)}]^{2+}\) and sodium adducts (Figure 3.9).

**Figure 3.8** UV-Vis spectra of binding of FeL1 (30 µM, black) in PBS buffer (0.01 M, pH 7.4) to 1 eq. of Aβ1-16 with (blue) or without (green) Cu(II) (0.9 eq.).

**Figure 3.9** Mass spectrum (ESI-MS) of binding of Aβ1-16 to FeL1 and Cu(II) with its sodium adducts. Across all experiments, species were observed as \([\text{FeL1}_{(0-1)}\cdot A\beta\cdot Cu_{(0-1)}\cdot Na_{(0-6)}]^{2-3}\). Additionally observed was the dimeric species \([A\beta_{2}\cdot Cl\cdot Na_{(0-8)}]^{3+}\) at m/z 1303-1374.
EPR Characterization

In order to better understand the binding of both Cu(II) and FeL1 to the Aβ peptide, we analyzed the electron paramagnetic resonance (EPR) spectra of Aβ1-16-Cu(II), FeL1-Aβ1-16, and finally FeL1-Aβ1-16-Cu(II). The EPR data for Aβ1-16-Cu(II) are in agreement with previous reports, and simulation of the EPR spectra indicate the existence of both Component I and Component II therein (Figure 3.2 and Figure 3.10). The simulation parameters are detailed in Table 3.1, and an approximate intensity ratio of 0.6:0.4 for Component I:Component II at pH 7.4 is in agreement with the measured pKₐ value of 7.8 ± 0.5 (Figure 3.2) via the Henderson-Hasselbalch equation (Equation 3.1).

\[
\text{pH} = \text{pK}_a + \log_{10} \left( \frac{[A^-]}{[HA]} \right) \quad \text{Equation 3.1}
\]

![EPR spectra](image)

**Figure 3.10** Frozen-solution EPR spectrum (bottom), simulation (red) and spectral deconvolution of Cu(II) in the presence of 1 eq. of Aβ1-16 and Cu(II) at 20 K. **Conditions:** [Aβ1-16] = 550 μM, [Cu(II)] = 500 μM, in PBS buffer (0.05 M, pH 7.4). **EPR parameters:** frequency = 9.38 GHz, microwave power = 2.0 mW, time constant = 40.96 ms, modulation amplitude = 5 G, average of five 1-min scans.
Table 3.1 X-Band EPR simulation parameters.$^a$

<table>
<thead>
<tr>
<th></th>
<th>Aβ-Cu(II) - Component I</th>
<th>Aβ-Cu(II) - Component II</th>
<th>FeL1 (S = 3/2)</th>
<th>Fe bound (S = 1/2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$g_\parallel$</td>
<td>$g_\perp$</td>
<td>$A_{Cu}^{\parallel}$</td>
<td>$A_{Cu}^{\perp}$</td>
</tr>
<tr>
<td>Aβ-Cu(II) FeL1</td>
<td>2.26</td>
<td>2.05</td>
<td>186</td>
<td>0.6</td>
</tr>
<tr>
<td>FeL1-Melm</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FeL1-Aβ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FeL1-Aβ-Cu(II)</td>
<td>2.26</td>
<td>2.05</td>
<td>186</td>
<td>0.7</td>
</tr>
</tbody>
</table>

$^a$) See experimental section for details. $^b$) Component relative abundance

FeL1 in buffer displays a weak intermediate-spin Fe(III) signal at $g_1 = 3.9$ and $g_2 = 2.0$, whereas FeL1-Aβ$_{1:16}$ shows, in addition to this signal, also a rhombic spin system consistent with low-spin Fe(III) ($S = \frac{1}{2}$) species (Table 3.1, and Figure 3.11). The latter is similar to the spectrum for FeL1 in the presence of 20 eq. of 1-Melm (Figure 3.11) and to reported EPR data for other 6-coordinate low spin Fe(III) corroles (with CN$^-$ and pyridine as axial ligands). These data are consistent with contributions from both mono- and bis-axial ligated FeL1 in the EPR experiment, likely due to the increased ligand affinity upon freezing the solutions for EPR analysis. Increased bis-axial ligation to FeL1 is observed for both 1-Melm and Aβ$_{1:16}$ in solution at lower temperatures (10 °C) by UV-Vis (Figure B7, Appendix B), and freezing a 5-coordinate (OEC)Fe(III)(py) corrole (OEC = trianion of 2,3,7,8,12,13,17,18-octaethylcorrole) in pyridine results in a similar spectral pattern with both intermediate and low spin signals. Due to the distinct temperature-dependence of signal intensity for the EPR spectra of the Fe species it is not possible to accurately determine their ratios from these experiments.
Figure 3.11 Frozen-solution EPR spectra (black) and simulations (red) collected at 20 K of FeL1, FeL1-Aβ₁₋₁₆ and FeL1-Melm. The linewidth for the FeL1-Melm g₂ feature (S = 3/2) is decreased by a factor of 2 relative to FeL1. Conditions: [Aβ₁₋₁₆] = 0.55 mM, [FeL1] = 0.50 mM, [1-Melm] = 10 mM in PBS buffer (0.01 M, pH 7.4). Experimental parameters: frequency = 9.38 GHz, microwave power = 2.0 mW, time constant = 40.96 ms, modulation amplitude = 5 G, average of five 1-min scans. *The intermediate spin g₂ signal overlaps with an instrument background signal.

Incubation of both Cu(II) and FeL1 with Aβ₁₋₁₆ affords the ternary species FeL1-Aβ₁₋₁₆-Cu(II) with an EPR spectrum that is essentially the sum of the components Aβ₁₋₁₆-Cu(II) and FeL1-Aβ₁₋₁₆ (Figure 3.12). The simulation parameters are detailed in Table 3.1, with the two Cu(II) species Component I and Component II in a 0.7:0.3 ratio. While this differs slightly from the ratio for Aβ₁₋₁₆-Cu(II) alone, the limited resolution of the spectra suggests minimal change to the Cu-site upon binding of FeL1 to the peptide (vide infra). Similarly, the simulation parameters for the Fe species present in FeL1-Aβ₁₋₁₆-Cu(II) (Table 3.1) are identical to FeL1-Aβ₁₋₁₆ suggesting that while both Cu(II) and FeL1 bind to Aβ₁₋₁₆, the binding sites are independent of one another as far as can be determined through the EPR experiments. This is reminiscent of reported EPR analysis of Cu(II) and
heme with Aβ1-16, which also suggested that while both did bind to the peptide there was no observable interaction between the two metal centres.89

![Graph](image)

**Magnetic field (Gauss)**

**Figure 3.12** Frozen-solution EPR spectrum (bottom), corresponding simulation (red) and spectral deconvolution of FeL1 in the presence of 1 eq. of both Aβ1-16 and Cu(II) at 20 K. **a)** FeL1-Aβ component simulation (multiplied by a factor of 3), **b)** Aβ-Cu(II) Component II simulation **c)** Aβ-Cu(II) Component I simulation **d)** sum of all simulated species and **e)** experimental spectrum. **Conditions:** [Aβ1-16] = 550 μM, [FeL1] = [Cu(II)] = 500 μM, in PBS buffer (0.05 M, pH 7.4). **EPR parameters:** frequency = 9.38 GHz, microwave power = 2.0 mW, time constant = 40.96 ms, modulation amplitude = 5 G, average of five 1-min scans.

### 3.2.3. Influence of FeL1 on Aβ aggregation

Gel electrophoresis and Western blotting, in combination with Transmission Electron Microscopy (TEM), were used to investigate if binding of FeL1 to the Aβ peptide would alter the size distribution of Aβ species and the morphology of the resulting aggregates. The longer Aβ1-42 peptide was employed for this study, as it is most
aggregation prone and neurotoxic.\textsuperscript{64, 223-225, 299} Incubation with low concentrations of \textit{FeL1} (0.1 to 1 eq., for 24 hours) significantly affected the aggregation pattern (\textbf{Figure 3.13 A}). While the A\textsubscript{β1-42} peptide forms mostly high molecular weight aggregates (Lane 1) in the absence of \textit{FeL1}, consistent with previous reports,\textsuperscript{33, 111, 154} \textit{FeL1} exhibits a concentration-dependent effect on the aggregation pattern (Lanes 2-5). Only low molecular weight species were observed after 24 hours with one eq. of \textit{FeL1} (Lane 5). The influence of \textit{FeL1} on A\textsubscript{β1-42} aggregation was further confirmed by TEM (\textbf{Figure 3.13 B-D}). The TEM image for peptide alone shows long fibrils and large aggregate size, matching previous reports.\textsuperscript{33, 154} However, as the concentration of \textit{FeL1} is increased, a reduction in aggregate size is observed, with only small aggregates present with 1 eq. of \textit{FeL1} (\textbf{Figure 3.13 D}). We also investigated the effect of the free ligand \textit{L1} on A\textsubscript{β1-42} peptide aggregation. Under the same experimental conditions, \textit{L1} also displays a concentration-dependent effect on aggregation (\textbf{Figure 3.14}), however aggregate species are observed over a broad molecular weight range. I hypothesize that \textit{L1} alters the aggregation pattern via hydrophobic interactions with the A\textsubscript{β} peptide,\textsuperscript{111, 119, 279, 300} while the covalent interaction of \textit{FeL1} with A\textsubscript{β} His residues results in the preferential formation of low molecular weight species (\textbf{Figure 3.13}).
Figure 3.13 Influence of FeL1 on the aggregation profile of Aβ1-42. A) Gel electrophoresis/Western blot of 25 µM Aβ1-42 and different concentrations of FeL1 in PBS buffer (0.01 M, pH 7.4) after 24 hours incubation with agitation at 37 °C, using anti-Aβ antibody 6E10. Lane 1: Aβ1-42; lane 2: Aβ1-42 + FeL1 (0.1 eq); lane 3: Aβ1-42 + FeL1 (0.25 eq); lane 4: Aβ1-42 + FeL1 (0.5 eq); lane 5: Aβ1-42 + FeL1 (1 eq). TEM images of B) Aβ1-42; C) Aβ1-42 + 0.1 eq FeL1; and D) Aβ1-42 + 1 eq FeL1.
Figure 3.14 Influence of L1 on the aggregation profile of Aβ1-42. Gel electrophoresis/Western blot of 25 µM Aβ1-42 and different concentrations of L1 in PBS buffer (0.01 M, pH 7.4) after 24 hours incubation with agitation at 37 °C, using anti-Aβ antibody 6E10. Lane 1: Aβ1-42; lane 2: Aβ1-42 + L1 (0.1 eq); lane 3: Aβ1-42 + L1 (0.5 eq); lane 4: Aβ1-42 + L1 (1 eq).

3.2.4. Catalytic Antioxidant Activity

FeL1 was reported to exhibit exceptional antioxidant activity for the disproportionation of H₂O₂,²⁷⁰ dismutation of O₂⁻,²⁶⁷ and catalytic activity for the decomposition of peroxynitrite (PN, ONOO⁻).²⁷³ In addition, the antioxidant activity of FeL1 is maintained, and even enhanced, when bound to albumin, lipoproteins, or imidazole since this minimizes formation of the less catalytically-active µ-oxo Fe(IV) dimer.²⁶⁹, ²⁷⁰ This work highlighted that the FeL1-Aβ species can act as a potent antioxidant, and possibly minimize ROS generation from Aβ-Cu(II) when both FeL1 and Cu(II) are bound to the peptide simultaneously.

Catalase Activity

The catalase activity of FeL1 has been demonstrated to exceed that of any other synthetic mimic of the enzyme,²⁷⁰, ²⁷², ³⁰¹ and its activity increases in the presence of excess imidazole. In order to determine the influence of FeL1 binding to Aβ1-16 on its catalase activity, an Amplex Red/H₂O₂ catalase assay was performed. This assay relies on competing with the very fast color producing reaction by adding a complex that catalytically decomposes H₂O₂. A catalase standard curve (Figure 3.15 A) was prepared.
and different concentrations of FeL1 in the presence and absence of Aβ1-16 (Figure 3.15 B) were tested to determine their activity. Both FeL1 and the 1:1 FeL1-Aβ1-16 adduct displayed good catalase activity, with the latter being superior. This shows that His binding of the Aβ peptide to FeL1 results in an enhancement of catalase activity at all concentrations studied (1-5 µM).

Figure 3.15 Catalase activity measured by absorbance of the amplex red reagent ($\lambda = 570$ nm) in the presence of H$_2$O$_2$ (10 µM) after 30 min incubation of catalase enzyme, FeL1 or FeL1-Aβ. A) Catalase standard curve, and B) FeL1 and FeL1-Aβ at different concentrations. [Aβ1-16]=FeL1]=1, 2.5, 5 µM ● Control; ▲ FeL1; ● FeL1-Aβ.

Aβ-Cu(II) ROS Production

Under biologically relevant reducing conditions, which are commonly mimicked by reducing agents such as ascorbate (Asc), Aβ-Cu(II) species are known to produce an array of ROS composed of O$_2$•$^-$, H$_2$O$_2$, •OH. There are many protocols for investigating the multiple steps that lead to these damaging species (Figure 3.16), of which the first one is the oxidation of Asc by Cu(II). This process was followed by monitoring the time course for disappearance of the Asc absorption band at 265 nm (Figure 3.17) in the presence of CuCl$_2$ and FeL1 only, their binary adducts with Aβ1-16, and the ternary adduct formed by combining Aβ1-16 with Cu(II) and FeL1. Consistent with previous reports, the high rate of Asc consumption in the presence of Cu(II) is diminished when bound to Aβ1-16; and consistent with expectations, both FeL1 and FeL1-
Aβ did not promote Asc oxidation. The most revealing result is that the ternary FeL1-Aβ16-Cu(II) complex displayed only slightly enhanced Asc oxidation in comparison to Aβ16-Cu(II). Overall, and in accordance with the EPR results that show that FeL1 does not alter the Cu(II) binding site, this assay suggests that the presence of FeL1 does not significantly affect the reduction of Aβ-Cu(II) by Asc.

Figure 3.16 Reactive oxygen species generated by Aβ-Cu(II) in the presence of Ascorbate and the possible assays to detect them (Modified from C. Cheignon et al., 2018).38b

Figure 3.17 Ascorbate consumption by Cu(II) (black), Aβ-Cu(II) (blue); FeL1-Aβ-Cu(II) (red); FeL1-Aβ (purple); FeL1 (green), measured using UV-Vis spectroscopy (λ = 265 nm) as a function of time in PBS buffer (0.01 M, pH 7.4). [Asc] = 100 µM, [Aβ16] = [FeL1] = 10 µM and [CuCl2] = 9 µM.
Figure 3.18 ROS production by Aβ-Cu(II) in the presence of Ascorbate. A) Amplex red assay for H$_2$O$_2$ formation by UV-Vis absorbance at 570 nm. [Asc] = 400 µM, [Aβ$_{1-16}$] = [FeL1] = [Cu(II)] = 20 µM, [Amplex red] = 100 µM; [HRP] = 0.4 U/mL (where 1 unit is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C). B) CCA assay for •OH formation measured by fluorescence, $\lambda_{ex}$ 395 nm and $\lambda_{em}$ 450 nm. [Asc] = 300 µM, [Aβ$_{1-16}$] = [FeL1] = [Cu(II)] = 40 µM, [CCA] = 100 µM. ● Cu(II); ○ Aβ-Cu(II); ▼ FeL1-Aβ-Cu(II); ▲ FeL1-Aβ; ■ FeL1.

Cu-Aβ species can transform O$_2$ to H$_2$O$_2$ through a series of steps, which can be detected via its reaction with Amplex Red, which forms the intensively colored resorufin (Figure 3.16). Following this process by monitoring the formation of resorufin (Figure 3.18 A) revealed that: a) Cu(II) alone induces the fastest rate of formation of H$_2$O$_2$; b) the binding of Cu(II) to Aβ slows down the process, as reported previously, and c) the
ternary FeL1-Aβ1-16-Cu(II) species shows a reduced rate of H₂O₂ formation and lower amount overall. The latter phenomenon is consistent with FeL1 quenching the H₂O₂ that is produced by the bound Cu(II), due to the good catalase-like activity of FeL1 in both its free form and when bound to the Aβ peptide (Figure 3.15 B). In addition to catalase activity, FeL1 displays exceptional antioxidant activity for the dismutation of O₂•−,267 and thus the complex may also quench superoxide formed as shown in Figure 3.16. Detection of this reactivity using a cytochrome c assay80,249 was challenging due to interference of FeL1 absorption bands.

The last and most damaging step in Scheme 3.2 occurs via the formation of •OH from the reaction of Cu(I) with H₂O₂, which may be detected by the reaction of 3-Coumarin carboxylic acid (3-CCA) with •OH to form the fluorescent 7-hydroxy-3-coumarin-carboxylic acid (7-OH-3-CCA).304 Consistent with previous reports,253 •OH production is quite fast and significant for Aβ-Cu(II) albeit much less than for non-His-coordinated Cu(II) (Figure 3.18 B). The addition of FeL1, to form the ternary FeL1-Aβ1-16-Cu(II) species, resulted in a further 6-fold reduction in the amount of 7-OH-3-CCA. In principle, this may reflect either the lower availability of H₂O₂ due to the catalase-like activity or the direct quenching of •OH by FeL1, or a combination of both. Another possible interpretation is that Cu in the ternary complex is less reactive, which is unlikely considering the minimal interaction of FeL1 with Aβ1-16-Cu(II) binding motif. In any case, the almost complete elimination of hydroxyl radical formation demonstrates that the potent antioxidant activity of FeL1 is maintained when bound to the Aβ peptide.

3.3. Summary

This chapter underlines the ability of FeL1 to target both Aβ peptide aggregation and ROS formation, two factors influencing AD progression. FeL1 and the free corrole ligand L1 influence Aβ aggregation differently; FeL1 stabilizes low molecular weight species while L1 stabilizes aggregates over a broad MW range. FeL1 forms a 1:1 adduct with Aβ via axial binding of one His residue with a moderate binding affinity (K_d of ~10⁻⁷ M), which is weaker in comparison to Cu binding to Aβ (K_d of ~10⁻¹⁰ M) but still stronger than Zn(II) binding (K_d of ~ 10⁻⁵ M).67-69,291 It is interesting to note that FeL1 has a much higher affinity for Aβ peptide His residues than 1-Melm or free His, suggesting significant non-covalent interactions between FeL1 and the Aβ peptide. These results are in
agreement with the specific binding of FeL1 to HDL2 proteins in comparison to other serum constituents, due to the amphipolar character of FeL1. Indeed, L1 was also shown to influence Aβ peptide aggregation likely due to hydrophobic interactions, albeit to a significantly lower extent. In a similar manner, non-covalent π-π stacking interactions, in addition to covalent binding, have been shown to dictate the association of Pt(II)(phenanthroline) complexes with the Aβ peptide.

I have also shown herein that FeL1 binds to the Aβ peptide concurrently with Cu(II). My EPR data suggests no significant change in the Cu-binding site with FeL1 His coordination. This is further corroborated by the ascorbate oxidation assay, which displays only minor changes to the rate of ascorbate oxidation for Aβ-Cu(II) and the ternary species FeL1-Aβ-Cu(II). However, the bound FeL1 acts as an efficient catalase, and decomposes a significant fraction of the H$_2$O$_2$ generated by Aβ-Cu(II) (Figure 3.18 A). In the presence of FeL1 I also observe a decrease in the formation of •OH (Figure 3.18 B), consistent with the result from the Amplex Red assay. My results show that amphipolar FeL1 binds specifically to the Aβ peptide via a His residue in a 1:1 stoichiometry, and this interaction modulates the peptide aggregation pathway. In addition, the peptide-bound FeL1 maintains its exceptional antioxidant activity, limiting ROS formation from Aβ-Cu(II). Overall, our results highlight the promising multifunctional character of FeL1 to limit Aβ peptide aggregation and the formation of damaging ROS, two hallmarks of AD.

### 3.4. Experimental

All common chemicals were purchased from Sigma-Aldrich and used without further purification. The compound Fe(III) 2,17-bis-sulfonato-5,10,15-tris(pentafluorophenyl)corrole (FeL1) was synthesized as reported. The Aβ$_{1–16}$, Aβ$_{17–40}$ and Aβ$_{1–42}$ peptides were purchased from 21st Century Biochemicals (Marlborough, MA, USA), Pepmic Co., Ltd (Suzhou, China) and Cellmano Biotech Limited (Hefei, China), and monomerized before use according to a reported procedure. Aβ$_{1–16}$ was dissolved in double distilled H$_2$O (ddH$_2$O), while Aβ$_{1–42}$ and Aβ$_{17–40}$ were dissolved in DMSO and ddH$_2$O in a 1:1 mixture, unless stated otherwise. The stock peptide solution concentration was determined by absorbance with the use a Thermo Nicolet UV nanodrop and an extinction coefficient of 1410 and 1450 M$^{-1}$cm$^{-1}$ at 280 nm, for Aβ$_{1–16}$, and Aβ$_{1–42}$ respectively. A Bradford assay (BioRad) was used to determine the concentration of
Aβ17–40. Human serum albumin (HSA) was used as a lyophilized powder from Sigma Aldrich. An Amplex red catalase kit was purchased from Thermo Fisher Scientific. 1H NMR spectra were recorded on a Bruker AV-600 instrument. Electronic spectra were obtained on a Cary 5000 spectrophotometer. All ROS assays were measured using a Synergy 4 Multi-Detection microplate reader from BioTek. TEM images were obtained using an OSIRIS FEI scanning TEM (STEM) operating at 200 kV. X-band (9.4 GHz) EPR spectra were collected using a Bruker EMX plus spectrometer equipped with a Premium X microwave bridge and HS resonator. Samples were run in 4 mm outer-diameter quartz tubes.

3.4.1. UV-Vis Binding Assay of FeL1 with 1-Melm and Aβ

Aβ1-16 was dissolved in 1:1 DMSO/ddH2O solution. FeL1 was dissolved in PBS buffer (0.01 M, pH 7.4), and one eq. of the Aβ1-42, Aβ1-16 or Aβ17-40 was added to FeL1 and spectra monitored over time. Spectral changes were observed immediately with no further changes for up to 1 hour. The binding of FeL1 to Aβ1-16 in the presence of Cu(II) was also investigated by UV-Vis. CuCl2 was dissolved in PBS (0.01 M, pH 6.3) buffer and added at 0.9 eq. to the Aβ1-16 peptide (30 µM) and incubated for 10 min to ensure binding. 1 eq. of FeL1 was then added to the solution of the Aβ-Cu(II) species. Adding the reagents in reverse order (FeL1 and then Cu(II)) resulted in the same spectral features. The spectral changes associated with 1-methylimidazole (1-Melm) binding to FeL1 were evaluated in PBS buffer (0.01 M, pH 7.4). To a solution of FeL1, 1-Melm was added in aliquots (up to 150 eq.) until no further spectral changes were observed. A variable pH titration was performed in ddH2O with FeL1 and 2 eq. of 1-Melm to determine a binding constant. Data were collected from 200-900 nm, and small aliquots of 0.1 M NaOH were titrated into the solution to adjust the pH. 25 UV-Vis spectra were collected in the pH 2.6-10.3 range. Spectral data were analyzed using HypSpec (protonic Software UK)235, using the pKa value of 7.0 for 1-Melm.307 Metal speciation plots were created using the HySS2009 program (protonic Software, UK).235 The binding constant of Aβ1-16 with FeL1 was obtained in a similar fashion to 1-Melm, however a 1:1 concentration ratio of the peptide and FeL1 was employed. Data were collected from 200-900 nm, and small aliquots of 0.1 M NaOH were titrated into the solution to adjust the pH; 37 UV-Vis spectra were collected in the pH 3.1-11.5 range. Spectral data were analyzed using HypSpec (protonic Software UK)235, using reported pKa values for Aβ1-16.285
3.4.2. $^1$H NMR Binding Assay of Aβ$_{1-16}$ peptide to FeL1

Deuterated PBS (0.01 M, pH 7.4) buffer was prepared by removal of water by vacuum drying of PBS buffer and dissolving the powder in D$_2$O. Aβ$_{1-16}$ and FeL1 were dissolved in deuterated PBS (0.01 M, pH 7.4) buffer, and the $^1$H NMR spectra of Aβ$_{1-16}$ alone followed by additions of FeL1 in 0.1 and 0.25 eq. were acquired.

3.4.3. Mass Spectrometry of Binding of Aβ$_{1-16}$ Peptide to FeL1 and Cu(II)

ESI-TOF-MS experiments were performed on an Agilent 6130 mass spectrometer connected to an Agilent 1260 HPLC system. Samples were analyzed by direct infusion (1-4 μL) of analyte into a mobile phase of 1:1 water:acetonitrile containing 5 mM ammonium acetate (pH unmodified), flowing at 0.3 mL/min and maintained at 30 °C. All components of the mobile phase were mass spectrometry grade. Nitrogen drying gas was 250 °C, 5 L/min with a nebulizing pressure of 15 psig. Voltages were: capillary 3 kV, fragmentor 175 V, skimmer 30 V, octopole 250 V. Samples were prepared as ~4 mg/mL of total protein (Aβ$_{1-16}$ and/or HSA) in ammonium carbonate (0.02 M, pH 9) buffer with 0 or 1 eq. of CuCl$_2$ and/or FeL1.

3.4.4. EPR Analysis

EPR measurements were conducted using a Bruker EMXplus spectrometer at 20 K at X-band with a PremiumX microwave bridge and HS resonator and a Bruker ER 4112HV helium temperature-control system and continuous-flow cryostat, the Bruker cryostat allows for reproducible sample placement with minimal variance in Q-factor. As a result, by using identical spectroscopic parameters and automatic tuning of the spectrometer it was possible to compare the intensities of the different Cu(II) species in solution. The final concentration of Aβ$_{1-16}$ was 550 μM, and in order to ensure a predominance of peptide-bound metal species, Cu(II) and FeL1 concentrations were 500 μM. Samples were prepared in 270 μL of PBS (0.05 M, pH 7.4), incubated at room temperature for 30 min, and then mixed with 30 μL of glycerol (which acted as a glassing agent) immediately before freezing in liquid N$_2$. The intensity on the Fe(III) EPR signal at 77 K was significantly smaller in comparison to the spectra at 20 K. All spectra were simulated with the Matlab-based program, EasySpin.$^{308}$
3.4.5. Gel Electrophoresis and Western Blotting

An Aβ_{1-42} film was dissolved in 1:1 DMSO/ddH_{2}O to obtain a stock solution with a concentration of approximately 300 µM. Aβ solutions with a concentration of 25 µM were prepared in PBS (0.01 M, pH 7.4) then incubated for 24 hours at 37 °C with continuous agitation at 200 rpm to form aggregates in the presence of different concentrations of L1, or FeL1. Electrophoresis separation of peptide aggregates employed 10-20% Mini-PROTEAN® Tris-Tricine Precast Polyacrylamide Gels from Bio-Rad, at 100 V for 100 min. The gels were then transferred to a nitrocellulose membrane for 1 hour at 100 V at 4 °C, followed by blocking of the membrane in a 3% BSA solution in TBS for 1 hour. The membrane was incubated in a solution (1:2000 dilution) of 6E10 anti-Aβ primary antibody (Biolegends) overnight. After washing 4 × 15 min with TBS buffer, the membrane was incubated in a solution containing the secondary antibody (Horseradish peroxidase, Caymen Chemicals) for 3 hours. Thermo Scientific SuperSignal® West Pico Chemiluminescent Substrate kit was used to visualize the Aβ species using a FUJIFILM Luminescent Image Analyzer (LAS-4000).

3.4.6. Transmission Electron Microscopy (TEM)

Samples were prepared from the Western blot assay after the 24 hours incubation time at 37 °C. TEM grids were prepared following previously reported methods. In order to increase hydrophilicity, the Formvar/Carbon 300-mesh grids (Electron Microscopy Sciences) were glow discharged in a vacuum for 15 seconds. Drops of samples (10 µL) were placed onto a sheet of parafilm and the TEM grid was laid on the drop for 5 minutes. The grid was then placed and immediately removed on the first and second drop of syringe-filtered 5% uranyl acetate, then placed on the third drop to incubate for 1 minute. Excess uranyl acetate was removed using a tissue between drops. The grid was allowed to air-dry for at least 15 minutes. Bright field images were obtained on a FEI Tecnai Osiris STEM at 200 kV.

3.4.7. Catalase Activity Assay

The Amplex red reagent was used in the presence of horseradish peroxidase (HRP) to detect H_{2}O_{2} formation, producing a fluorescent compound, resorufin. FeL1 was dissolved in 0.1 M Tris-HCl buffer, pH 7.5. A fresh 10 mM stock solution of L-ascorbic
acid was prepared in 0.1 M Tris-HCl buffer, pH 7.5 and protected from light until use. The Amplex red assay was performed in quadruplicate in a flat-bottomed clear 96-well plate (Microtest, BD Falcon). A working solution was prepared containing 100 µM Amplex red reagent and 0.4 U/mL HRP, where 1 unit is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C. Different concentrations of catalase, from 0 to 1000 mU/mL, where 1 unit is defined as the amount of enzyme that will decompose 1.0 µM of H₂O₂ per minute at pH 7.0 at 25°C, were used to obtain a standard curve. FeL1 catalase activity was determined for different concentrations of the complex alone and in the presence of Aβ1-16. All compounds were incubated at room temperature with 40 µM H₂O₂ for 30 min, followed by addition of working solution containing Amplex red and HRP and incubated for another 15 min before measuring the absorbance at 570 nm.

3.4.8. Aβ-Cu(II) ROS production

Aβ1-16 was dissolved in ddH₂O and a fresh 10 mM stock solution of L-ascorbic acid was prepared in PBS buffer (0.01 M, pH 7.4) and kept protected from light until use. FeL1 was dissolved in PBS buffer (0.01 M, pH 7.4) and CuCl₂ was dissolved in PBS buffer (0.01 M, pH 6.6). The compounds were dissolved as described here in the following assays, unless stated otherwise.

Ascorbate Assay

The absorbance of an initial concentration of 100 µM of Asc at 265 nm was measured over time at room temperature in a quartz cuvette for each sample. Final concentrations of Aβ1-16 and FeL1 were 10 µM, and Cu(II) 9 µM.

Hydrogen Peroxide Assay

For this assay all components, with the exception of the Aβ1-16, were dissolved in a 0.1 M Tris-HCl buffer, pH 7.5. A similar procedure to the catalase assay was performed using the Amplex red kit, with exception of the addition of H₂O₂ and catalase. The compounds were added to the wells followed by addition of 50 µL of the working solution. L-ascorbic acid was the last component added, initializing the reaction between dioxygen
and Cu(II). The formation of resorufin was monitored by absorbance at 570 nm at RT for 45 min.

**Coumarin-3-carboxylic Acid Assay**

Coumarin-3-carboxylic acid (3-CCA) was used to detect hydroxyl radicals (•OH) produced by Aβ-Cu(II) in the presence of L-ascorbic acid. The CCA assay was conducted in quintuplicate in a flat-bottomed black 96-well plate (Microtest, BD Falcon). 3-CCA was dissolved in PBS buffer (0.01 M, pH 7.4). Cu(II), 3-CCA and L-ascorbic acid had final concentrations of 40 µM, 100 µM and 300 µM, respectively. Production of •OH by Cu(II) was determined in the presence of 1 eq. of Aβ1-16 and FeL1. Reaction of 3-CCA with •OH produces 7-OH-CCA, a fluorescent compound with λ<sub>ex</sub>: 395 nm and λ<sub>em</sub>: 450 nm. The reaction was initiated by addition of L-ascorbic acid and was monitored at room temperature for 45 min. Controls of compounds and 3-CCA without L-ascorbic acid showed no significant fluorescence.
Chapter 4. Investigation of the Interaction of Ru NAMI-A Derivatives with the Amyloid-beta Peptide


L.M.F. Gomes performed the gel electrophoresis and Western blotting, TEM imaging, turbidity assay and cell studies. J.C. Bataglioli assisted L.M.F. Gomes with the ¹H NMR, and Bradford assay. A.J. Jussila assisted L.M.F. Gomes with the cytotoxicity studies for the Ru-N series. J.R. Smith completed the ESI-MS experiments.

4.1. Introduction

Dementias are disorders in which severe cognitive impairment occurs, affecting over 50 million people worldwide. An increase in life expectancy is expected to lead to a sharp increase in the number of dementia cases over the next 30 years. Alzheimer’s disease (AD), the most common type of dementia, represents 60-70% of dementia cases, characterizing a significant burden to healthcare systems around the globe. AD is a neurodegenerative disease where protein misfolding and aggregation combined with oxidative stress causes neuronal cell death, leading to loss of cognition and eventually death. Currently, treatment strategies for most neurodegenerative diseases are very limited, to which the approved treatments for AD only ameliorates the symptoms at early to moderate stages of the disease, making this an important research area.

The major neuropathological hallmarks of AD are the aggregation of two proteins, amyloid-β (Aβ) and tau, with the first forming aggregates (oligomers and plaques) in the extracellular environment of the brain, and the latter forming neurofibrillary tangles (NFTs) in neurons due to hyperphosphorylation and oxidative modifications of tau. It is still unclear if these hallmarks are a cause or an effect of AD, however post-mortem examination of the brain in AD patients has shown that Aβ-plaques and NFTs are present. Interestingly, smaller, soluble Aβ oligomers have been more strongly linked to memory loss and progression of the disease in comparison to plaques. These species have been implicated in the initiation of the processes of oxidative stress, decreased
cerebral blood flow, neuronal hyperactivity, synapse deterioration, and nerve cell death.\textsuperscript{21, 26-28, 30, 69}

As cofactors in metalloenzymes, metal ions such as Zn, Cu and Fe are central to many processes in healthy organisms. However, their dyshomeostasis has been observed in neurodegenerative diseases, such as AD.\textsuperscript{63, 64, 68, 84, 256, 310, 311} A high concentration of these metal ions are present in A\textsubscript{\textbeta} plaques,\textsuperscript{63} where they are found coordinated typically to His\textsuperscript{6, 13 or 14} residues, although Asp\textsuperscript{1}, Tyr\textsuperscript{10}, and Glu\textsuperscript{11} have been shown to be involved in A\textsubscript{\textbeta} peptide metal binding.\textsuperscript{68, 69, 72-75} This binding can modify the aggregation pattern of A\textsubscript{\textbeta}, disrupt normal metalloenzyme activity, and produce toxic reactive oxygen species (ROS).\textsuperscript{68, 69, 84-88}

A number of Pt,\textsuperscript{140, 142, 144, 312} Ru,\textsuperscript{169-171} Co,\textsuperscript{69, 153, 154} and V\textsuperscript{266} metal complexes have shown promise in interacting with the A\textsubscript{\textbeta} peptide and modifying its aggregation. For example, a series of Pt(II) phenanthroline complexes (Figure 4.1) were shown to bind to the peptide, modulating the aggregation and the neurotoxicity of A\textsubscript{\textbeta}.\textsuperscript{140} The phenanthroline ligands were determined to facilitate $\pi-\pi$ stacking interactions with Phe\textsuperscript{4}, Tyr\textsuperscript{10} and Phe\textsuperscript{19} residues present in the hydrophobic region of the peptide, thus positioning the Pt(II) center in proximity to His residues (His\textsuperscript{6, 13, and 14}) for covalent bond formation.\textsuperscript{305} In comparison, cisplatin (Figure 4.1) without large hydrophobic ligands, was shown to interact with Met\textsuperscript{35}.\textsuperscript{140} For the Pt(II) phenanthroline complexes the modulation of aggregation was associated with almost complete rescuing of cell viability in primary cortical neurons, while cisplatin was inactive, demonstrating that the presence of phenanthroline ligands was essential for limiting A\textsubscript{\textbeta} toxicity. Barnham \textit{et al.} suggested that by forming covalent interaction with His residue(s) the Pt phenanthroline complexes inhibit the binding of ROS-generating metal ions to A\textsubscript{\textbeta}, such as Cu(II). This was demonstrated by a decrease in the production of H\textsubscript{2}O\textsubscript{2} by A\textsubscript{\textbeta}-Cu in the presence of these complexes.
Figure 4.1 Structures of Pt(II) phenanthroline complexes, fac-[Ru(CO)$_3$Cl$_2$(N$^1$-thz)$^{2+}$, and Ru(III) complexes PMru20, KP1019 and NAMI-A.

Ru(III) complexes have been investigated in anticancer research based on their cytotoxicity, relatively slow ligand exchange rate (similar to Pt(II)), accessible redox chemistry in physiological conditions, and the ability to tune targeting and pharmacokinetic properties via ligand design. Keppler and coworkers were the first to report the use of Ru(III) complexes with axial azole ligands as cancer therapeutics in the 1990's. One of the most promising agents studied by this group was KP1019 (Figure 4.1), which was tested in a phase I clinical trial. Recently, an analogue of this compound, NKP-1339, with a Na$^+$ counterion to improve water solubility, has been the focus of further development, and has also completed a phase I clinical trial. A second type of structurally similar Ru(III) complexes were also developed during the same time period by
Alessio and co-workers. These compounds have an exchangeable DMSO ligand in place of one of the axial azoles of the Keppler-type complexes. Of these the imidazole complex NAMI-A (Figure 4.1) has been the most studied. This compound demonstrates less cytotoxicity than KP1019, but displays a significant antimetastatic effect, thus NAMI-A type complexes have also been a focus for development as anticancer agents. NAMI-A was the first Ru(III) anticancer drug to be studied in humans, and successfully completed a phase-I clinical trial, although a phase II trial demonstrated that it is only moderately tolerated according to common toxicity criteria (CTC).

The concept of Ru complexes as AD treatment agents was introduced by Valensin et al. with the report of the interaction of fac-[Ru(CO)\textsubscript{3}Cl\textsubscript{2}(N\textsubscript{1}-thz)] (Figure 4.1) with A\textsubscript{B}, showing that the complex loses N\textsubscript{1}-thz and both Cl\textsuperscript{-} ligands and the Ru(CO)\textsubscript{3}\textsuperscript{2+} unit binds to a His of the peptide. The anticancer agents PMru20 and KP1019 were also studied as potential AD therapeutics. PMru20 protected rat cortical neurons from toxicity associated with both A\textsubscript{B}\textsubscript{1-42} and the truncated A\textsubscript{B}\textsubscript{25-35} (without His), likely by limiting peptide aggregation. KP1019 was shown to bind covalently to A\textsubscript{B} by modulating the peptide aggregation pattern of monomeric or pre-formed aggregates and forming soluble high-MW aggregates. KP1019 also limited A\textsubscript{B} toxicity in SH-SY5Y neuroblastoma cells.

A series of Ru(III) pyridine NAMI-A analogues (Ru-N, Figure 4.1) was reported by Walsby et al. to bind to human serum albumin (HSA), to which the use of suitable axial ligands enables tuning of the noncovalent interaction between the complexes and HSA. The Ru-N derivatives exhibited enhanced hydrophobic interactions with HSA when larger, more hydrophobic, axial pyridine-based ligands were incorporated into the NAMI-A type structure. As expected for these types of compounds, their axial DMSO ligand underwent rapid aqueous exchange at physiological pH, with loss of Cl\textsuperscript{-} ligands also observed. These ligand exchange processes also promoted the formation of covalent interactions with HSA, likely to His residues. Based on these observations and the previous studies described above, we hypothesized that alteration of the axial ligand in the Ru-N series would influence the interaction of these complexes with the A\textsubscript{B} peptide, with more effective peptide binding for the larger, more hydrophobic derivatives. The interaction of these Ru(III) complexes with the A\textsubscript{B} peptide and the associated effect on peptide aggregation are described herein.
4.2. Results and Discussion

4.2.1. Binding of Aβ His Residues to Ru-N derivatives

Both non-covalent and covalent interactions of the Ru-N complexes with HSA have been characterized by Walsby et al., using electron paramagnetic resonance (EPR).\textsuperscript{322} HSA has 16 His residues, of which 5 His residues are available at the surface of the protein,\textsuperscript{326} providing binding sites for metal ions. The major species formed upon incubation of HSA with the Ru-N series are His adducts at both the axial and equatorial positions following the loss of DMSO or Cl ligands.\textsuperscript{322} Interestingly, the interaction of NAMI-A with a number of proteins including lysozyme,\textsuperscript{327} carbonic anhydrase,\textsuperscript{328} and human H-chain ferritin\textsuperscript{329} has been studied by X-ray crystallography.\textsuperscript{168} In these studies all of the original ligands of NAMI-A are released, and the resulting Ru(III) center is bound to the protein via His, Asp, and Glu side-chains. However, it has been postulated that the process of crystal soaking, in which NAMI-A crystallizes with the protein, can lead to different binding/speciation in comparison to solution studies.\textsuperscript{324}

To evaluate the nature of the interactions between the Ru-N complexes and the Aβ peptide, $^1$H NMR of Aβ$_{1-16}$ in the presence of paramagnetic (Ru(III), $d^5$, $S = \frac{1}{2}$) Ru-N-1 or Ru-N-4 were obtained at 0.25 and 1 eq. (Figure 4.2). These complexes were selected as they exhibit the largest difference in pyridine ligand size. In addition, the soluble Aβ$_{1-16}$ peptide fragment was used, which include the metal binding amino acid residues. Upon addition of either Ru(III) complex, all signals for the residues in Aβ$_{1-16}$ exhibit a shift, suggesting that an interaction between peptide and complex is occurring. Interestingly there is a significant decrease in the intensity as well as a broadening of the signals of Aβ$_{1-16}$ in the presence of 1 eq the paramagnetic complexes. We do not observe a precipitate in the NMR tube in our experiments. The largest shift (ca. 0.1 ppm) observed is for the His resonance at 7.85 ppm, which suggests binding of a peptide His residue. This mode of coordination has also been reported for interaction of these complexes with HSA.\textsuperscript{322}
Figure 4.2 Changes in the $^1$H spectra of Aβ$_{1-16}$ in the presence of Ru-N derivatives. Shown are spectra obtained at 205 µM Aβ$_{1-16}$, in PBS buffer prepared in D$_2$O pH 7.4 at 25°C (red) with addition of 0.25 eq. (green) and 1 eq (blue) of A) Ru-N-1 or B) Ru-N-4. * His$^6$, His$^{13}$ and His$^{14}$. † Tyr$^{10}$.

To investigate further the interaction between the complexes and the peptide ESI mass spectrometry was performed on solutions of Aβ$_{1-16}$ incubated with either Ru-N-1 or Ru-N-4. The mass spectra (Figure 4.3 and Figure 4.4, respectively) indicate the formation of the adducts [Ru-N-1(Aβ$_{1-16}$)(CO$_3$)$_2$]$^{2-}$ (m/z = 1167.5) and [Ru-N-4(Aβ$_{1-16}$)(CO$_3$)$_2$]$^{2-}$ (m/z = 1250.9), where carbonate (CO$_3^{2-}$) in the adducts is likely derived from the running buffer ((NH$_4$)$_2$CO$_3$) used in the MS experiment. The characteristic Ru isotopic pattern was observed for both peaks (Figure 4.3 and Figure 4.4, C and B, respectively), and the mass of the adducts are consistent with loss of the DMSO ligand from each Ru complex, and subsequent coordination to the Aβ$_{1-16}$ peptide.
The results of the ESI-MS studies herein are consistent with the prior work with HSA, showing adduct formation for both Ru-N-1 and Ru-N-4 with Aβ1-16 via loss of an exchangeable DMSO ligand. In addition, incubation of Ru-N-1 or Ru-N-4 with Aβ1-16 led to a shift and broadening of all of the 1H NMR signals of the Aβ peptide, suggesting an interaction between the Ru(III) complexes and Aβ1-16 (Figure 4.2). Similar line broadening of Aβ 1H NMR signals has been observed in the presence of Cu(II) and an Fe(III) corrole complex (FeL1, Figure 4.1), along with the disappearance or shifting of the His resonances. This has been interpreted as binding of either Cu(II) or FeL1 to His residues present in the hydrophilic portion of the peptide. In another report, broadening of the 1H NMR spectrum of Aβ1-28, and the almost complete disappearance of the aromatic signals for His and Tyr, was observed upon incubation of fac-[Ru(CO)3Cl2(N1-thz)] (Figure 4.1) with the peptide. These results supported Aβ1-28 His binding to the Ru(II) complex with ESI-MS verification of adduct formation. Although all the peptide NMR signals shift upon interaction with the Ru-N complexes in this work, the peptide His resonance at 7.85 ppm undergoes the largest change (ca. 0.1 ppm), which is consistent with what has been observed for metal ions or complexes with Aβ. Interestingly, weak signals attributed to the free pyridine ligand at 7.35 ppm and 7.45 ppm are observed upon addition of 1 eq. Ru-N-4 to Aβ1-16 (Figure 4.2), and these signals increase in intensity at 24 hours for 0.25 eq of Ru-N-4 (Appendix C, Figure C1). Pyridine ligand loss is not observed for the Ru-N-1 complex (Figure 1 and Figure C1), suggesting that pyridine ligand exchange is enhanced for the bulkier hydrophobic Ru-N-4 complex. The presence of the free pyridine ligand of Ru-N-4 upon incubation with Aβ1-16 suggests further ligand exchange processes occur for this derivative in addition to DMSO exchange, similar to the reported X-ray studies, and this difference between Ru-N-1 and Ru-N-4 may play a role in the peptide aggregation process (vide infra).
Figure 4.3 Mass spectrum (ESI-MS) of A) Aβ₁₆ with its chloride and sodium adducts; and binding of Aβ₁₆ to Ru-N-1 with its sodium adducts B) Full spectrum; C) zoomed region of the [Ru-N-1Aβ(CO₃)²⁺]²⁻ adduct. Across all experiments, species were observed as [Ru-N₁⁻Aβ(CO₃)Na⁰⁻]²⁻. Additionally was observed the dimeric species [Aβ₂-Cl]³⁻ at m/z 1303-1374.
Figure 4.4 Mass spectrum (ESI-MS) of binding of Aβ1-16 to Ru-N-4 with its sodium adducts. A) Full spectrum; B) zoomed region of the [Ru-N-4Aβ(CO3)2]2- adduct; and C) zoomed region of the Ru-N-4 with isotopic pattern. Across all experiments, species were observed as [Ru-N(0-1)Aβ(CO3)Na(0-6)]2-. Additionally was observed the dimeric species [Aβ2-Cl]3- at m/z 1303-1374.
4.2.2. Influence of Ru-N series on Aβ aggregation

Several metal complexes have been reported to modulate the aggregation pattern of Aβ upon binding covalently to the peptide.⁶⁹, ¹⁴⁵, ¹⁵², ¹⁷¹, ³³⁰ For example, the binding of the Fe(III) corrole complex FeL1 (Figure 4.1 and Chapter 3) to Aβ lead to the stabilization of low MW oligomeric species,³³⁰ however, binding of KP1019 (Figure 4.1) led to decreased oligomer formation and an increase in high MW soluble aggregates.¹⁷¹ The time dependence of the influence of the Ru-N derivatives on Aβ₁₋₄₂ aggregation was analyzed via gel electrophoresis and Western blotting, in combination with Transmission Electron Microscopy (TEM). The Aβ₁₋₄₂ peptide was chosen for these experiments due to its high propensity for aggregation and significant neurotoxicity.²⁸, ³⁰, ⁶⁴, ²²³-²²⁵, ²⁹⁹ At each time point (3, 6, 11 and 24 hours) a 30 µL aliquot was removed from the stock incubation solution for each treatment and kept at -80 °C until further analysis. An increase in high MW aggregates over time was observed for Aβ₁₋₄₂ alone (Lane 1, Figure 4.5), with a significant decrease in soluble Aβ species at 24 hours, as expected based on prior results.¹⁷¹, ³³⁰ The Ru-N derivatives do not significantly affect aggregation at the 3 hour timepoint. However, at longer timepoints the complexes generate increased soluble higher molecular weight species in comparison to peptide alone. This effect is most pronounced for the complexes Ru-N-3 (lane 4) and Ru-N-4 (lane 5), which have the largest pyridine-derived ligands. The Ru(III) complexes containing smaller pyridine-derived ligands, such as Ru-N-1 (lane 2) and Ru-N-2 (lane 3), show a similar modulation of Aβ aggregation to NAMI-A (lane 6), which could reflect the similar properties of the pyridine, 6-methylpyridine, and imidazole ligands in this assay. Interestingly, the Na[Ru(DMSO)$_2$Cl$_4$] complex without an apical aza ligand also induces the formation of soluble high molecular weight Aβ species after 24 hour incubation (Appendix C, Figure C2), however the molecular weight range is larger (~25-250 kDa), even in comparison to Ru-N-1. Overall, these results indicate that in comparison to the formation of insoluble peptide aggregates for peptide alone at 24 hours, 1 eq. of the Ru-N complexes promotes the formation of soluble high molecular weight aggregates.

TEM images (Figure 4.6 and Figure C3) of Aβ₁₋₄₂ alone and in the presence of either Ru-N-1 or Ru-N-4 after incubation for 24 hours show different morphologies for the three samples analyzed. Aβ₁₋₄₂ incubated alone led to the formation of large amorphous aggregates, with no fibrils observed on the TEM grid. The presence of the Ru-N
complexes led to an increase in fibril formation, with both Ru-N-1 and Ru-N-4 showing a mixture of fibrils and amorphous aggregates. The size of the amorphous aggregates are however much larger for peptide alone in comparison to treatment with the Ru-N derivatives (Figure C3). These results are consistent with the electrophoresis gels (Figure 4.6) that show that most of the peptide has formed large insoluble aggregates for peptide alone, while the Ru-N series show stabilization of smaller soluble aggregates.

![Gel Electrophoresis Image](image)

**Figure 4.5** Influence of Ru-N derivatives on the aggregation profile of Aβ1-42. Gel electrophoresis/Western blot of 25 µM Aβ1-42 and 1 eq. of Ru-N derivatives in PBS buffer (0.01 M, pH 7.4) at incubation timepoints 3, 6, 11 and 24 hours, with constant agitation at 37 °C, using anti-Aβ antibody 6E10. Lane 1: Aβ1-42; lane 2: Aβ1-42 + Ru-N-1; lane 3: Aβ1-42 + Ru-N-2; lane 4: Aβ1-42 + Ru-N-3; lane 5: Aβ1-42 + Ru-N-4; lane 6: Aβ1-42 + NAMI-A.
The concentration-dependent effect of the Ru complexes (Ru-N-1 and Ru-N-4) on Aβ₁₋₄₂ aggregation was also investigated at the 24-hour timepoint. In this case, the complexes Ru-N-1 and Ru-N-4 were added to Aβ₁₋₄₂ at 0.25, 0.5, 1 and 2 eq. and the aggregation pattern investigated by gel electrophoresis and Western blotting (Figure 4.7). Lane 1 shows high MW aggregates for peptide alone after 24 hours of incubation. The presence of Ru-N-1 has a concentration-dependent effect on aggregation, with aggregates of 25 kDa and higher for 0.25, 0.5 and 1 eq., whereas 2 eq. leads to formation of aggregates of ca. 150 kDa and higher. Interestingly, Ru-N-4 shows a more pronounced concentration-dependent change in Aβ aggregation, with incubation of 1 eq. of Ru-N-4 resulting in aggregates of ca. 150 kDa or higher and 2 eq. affording aggregates higher than ca. 250 kDa in MW. These results indicate a greater shift to high MW aggregates for Ru-N-4 (incorporating the bulky pyridine-derived ligand) than for Ru-N-1.
Figure 4.7 Gel electrophoresis/Western blot of 25 µM Aβ1–42 and different concentrations of Ru-N-1 and Ru-N-4 in PBS buffer (0.01 M, pH 7.4) at 24 hours incubation with agitation at 37 °C, using anti-Aβ antibody 6E10. Lane 1: Aβ1–42; lane 2: Aβ1–42 + 0.25 eq. Ru-N-1; lane 3: Aβ1–42 + 0.5 eq. Ru-N-1; lane 4: Aβ1–42 + 1 eq. Ru-N-1; lane 5: Aβ1–42 + 2 eq. Ru-N-1; lane 6: Aβ1–42 + 0.25 eq. Ru-N-4; lane 7: Aβ1–42 + 0.5 eq. Ru-N-4; lane 8: Aβ1–42 + 1 eq. Ru-N-4; lane 9: Aβ1–42 + 2 eq. Ru-N-4.

Figure 4.8 Chemical structure of pyridine ligands.

In order to determine if the pyridine ligands alone can influence Aβ aggregation, Aβ1–42 aggregation was evaluated at 3, 6, 11 and 24 hours by gel electrophoresis and Western blotting in the presence of 1 eq. of the free pyridine ligands (Figure 4.8). As expected, a decrease in monomeric species and an increase in high MW species is observed for peptide alone over the incubation period (Figure 4.9). Interestingly, the presence of 1 eq. of the pyridine ligands does not significantly change the Aβ1–42 aggregation pattern (Figure 4.9), indicating that the Ru(III) complex, and not the pyridine ligand, is essential for influencing Aβ peptide aggregation.
Figure 4.9 Influence of pyridine ligands on the aggregation profile of Aβ₁₋₄₂. Gel electrophoresis/Western blot of 25 µM Aβ₁₋₄₂ and 1 eq. of pyridine ligands in PBS buffer (0.01 M, pH 7.4) at incubation time points 3, 6, 11 and 24 hours, with constant agitation at 37 °C, using anti-Aβ antibody 6E10. Lane 1: Aβ₁₋₄₂; lane 2: Aβ₁₋₄₂ + L₁; lane 3: Aβ₁₋₄₂ + L₂; lane 4: Aβ₁₋₄₂ + L₃; lane 5: Aβ₁₋₄₂ + L₄.

The Aβ aggregation process in solution can be studied by a number of different methods, including turbidity, dynamic light scattering, and Thioflavin T (ThT) fluorescence. We have previously shown that the Ru(III) complex KP1019 interferes with ThT fluorescence analysis (either by quenching or inhibition of ThT binding), however, turbidity has been shown to be a reliable alternative for the investigation of peptide aggregation in the presence of compounds that disrupt ThT fluorescence. We thus employed turbidity measurements here to investigate the effect of Ru-N-1 and Ru-N-4 on Aβ₁₋₄₂ aggregation in solution. The formation of peptide aggregates in solution over time leads to an increase in turbidity, and the degree of light scattering can be measured by UV-vis measurements. The results of the time-dependent aggregation of Aβ₁₋₄₂ in the presence of Ru-N by electrophoresis and Western blot (Figure 4.5) show that the complexes appear to induce the formation of soluble higher MW aggregates after...
24 hours of incubation. At this time point the aggregation profiles for Ru-N-1 and Ru-N-4 differ indicating an effect of the axial ligands.

In order to further evaluate the influence of the Ru-N series on peptide aggregation in solution, the turbidity of an Aβ_{1-42} solution was measured in quintuplicate in a 96-well plate over the course of 3 hours in the presence and absence of Ru-N-1 and Ru-N-4 (Figure 4.10A). Aggregation was monitored at 500 nm as there is no absorption by either the Ru complexes or the peptide at this energy. As expected, an increase in turbidity was observed for the peptide alone over the 3-hour incubation period. In the presence of the Ru(III) complexes an increase in turbidity was also observed, about at the 2 hour timepoint the presence of Ru-N-1 and Ru-N-4 led to a significant increase in turbidity in comparison to peptide alone, but with no statistical difference between the two complexes. Due to water evaporation from the 96-well plate at longer measurement times, a lid was placed on the plate at 3hrs, and a further single reading taken at the 20 hour timepoint (Figure 4.10B). At the longer timepoint an approximate doubling of the turbidity is observed for solutions containing the Aβ_{1-42} peptide and either Ru-N-1 and Ru-N-4 complexes in comparison to peptide alone. Again, no statistical difference between the two complexes was observed. Overall, the higher turbidity reading for the Ru-N complexes in comparison to peptide alone is likely due to the formation of a large number of soluble aggregates for the former, while fewer insoluble peptide aggregates form for the latter. This conclusion is in accord with the gel studies, and TEM data.
I next investigated the total amount of soluble Aβ₁₋₄₂ peptide in solution after incubation with and without the Ru-N complexes via the Bradford assay. The Bradford assay measures the shift in the absorbance peak for the reagent Coomassie brilliant blue G-250 from 495 nm to 595 nm upon binding to the C-terminus of proteins.³³⁵ Before measurement the samples were centrifuged to remove insoluble fibrils using an established protocol.³³⁶, ³³⁷ I analyzed the change in the concentration of Aβ₁₋₄₂ between 0 and 24 hours of incubation in the presence of the four Ru-N complexes. The peptide alone does not show a significant decrease in the amount of peptide at 24 hours of incubation (Figure 4.11), suggesting that the aggregates at this stage are non-fibrillar. This result is in accord with TEM images showing only amorphous aggregates for peptide alone (Figure 4.6). In contrast, after 24 hours of incubation in the presence of the Ru-N complexes, a decrease in the amount of peptide is observed, suggesting that fibrillar species had formed and were removed via centrifugation. This result is also in accord with fibril formation observed for Ru-N treatments by TEM. Overall, the complexes reduce the amount of measurable Aβ in the sample by 50% after 24 hours, with no significant difference observed across the Ru-N series.
Figure 4.11 Bradford assay of 60 µM Aβ1-42 in the presence of 1 eq. of all four Ru-N complexes in PBS buffer (0.01 M, pH 7.4) at 0 hour (black) and after 24 hours of incubation with agitation at 37 °C (red). Samples were centrifuged at 14,000 g for 5 min prior to absorbance measurement. Statistically significant difference between *Aβ1-42 only and in the presence of the complexes (Ru-N-2, p = 0.0025, Ru-N-1 and Ru-N-3, p = 0.0005, and Ru-N-4, p < 0.0001) **0 hour and 24 hour time point (Ru-N-1, p = 0.0003, and Ru-N-2, Ru-N-3 and Ru-N-4, p < 0.0001). Calculated using 2 way ANOVA.

The Ru(III) complexes investigated in this study have a similar effect to that observed for KP1019, leading to the formation of soluble high MW aggregates in a concentration-dependent manner. Our results also show that the binding of the Ru(III) center is essential for the change in aggregation, since the ligands alone do not exhibit an effect on the aggregation process. The electrophoresis gel/Western blot data suggests a greater influence on aggregation by the Ru-N complexes with larger, more hydrophobic ligands (Ru-N-3 and Ru-N-4). In addition, the complex without the apical Py ligand, leads to a range of soluble species after 24 hour aggregation, with the gel results similar to NAMI-A. The fibrillar structures shown by TEM in the presence of Ru-N-1 and Ru-N-4 when compared to the amorphous aggregates for peptide alone, suggest that binding of the complexes to Aβ promotes fibrillation of the peptide.

Additionally, incubation of the Ru-N series with the Aβ peptide for 24 hours, followed by centrifugation, leads to a 50% decrease in peptide concentration in comparison to peptide alone as determined by a Bradford assay. We employed a centrifugation protocol
to remove insoluble fibrils,\textsuperscript{336, 337} and thus the reduction in peptide measured for the \textbf{Ru-N} treatments is likely due to the removal of fibrillar structures, as observed by TEM. Alternatively, the Bradford assay depends on Coomassie blue binding to basic amino acids (such as His), thus it is possible that \textbf{Ru-N} binding to the peptide leads to the observed reduction in signal. However, we would expect to see a reduction in signal in the initial measurements due to interaction of the \textbf{Ru-N} complexes with the peptide if this was the case.

Overall, the \textbf{Ru-N} series promote the formation of soluble high molecular weight aggregates at 24 hours, while peptide alone leads to almost complete precipitation of the peptide. Only minor differences are observed across the \textbf{Ru-N} series, with the larger more hydrophobic derivatives (\textbf{Ru-N-3} and \textbf{Ru-N-4}) narrowing the size distribution of the soluble aggregates to higher molecular weights (Figure 4.5). TEM analysis (Figure 4.6) of the insoluble aggregates shows that while incubation of peptide alone produces very large amorphous aggregates, \textbf{Ru-N} treatment results in both fibrils and amorphous aggregates, with the amorphous aggregates smaller in size in comparison to peptide alone. It is possible that by stabilizing soluble high molecular weight species, the \textbf{Ru-N} complexes slow down the rate of peptide precipitation, thereby promoting the formation of the more ordered fibrillar structures observed by TEM. Our results suggest that increasing the pyridine ligand size/hydrophobicity even further may afford fibrillar structures exclusively, which could ultimately have a protective effect in AD by promoting the formation of a stable insoluble peptide aggregate with limited potential to furnish toxic oligomeric species.\textsuperscript{338-340}

\subsection*{4.2.3. Cytotoxicity Studies in the SH5Y Neuroblastoma cell line}

The A\textsubscript{β1-42} peptide has been shown to reduce cell viability in the neuroblastoma cell line SH-SY5Y at different concentrations (typically 20-30 \(\mu\)M), and conditions, with reports showing the toxicity of monomers,\textsuperscript{171, 341} oligomers\textsuperscript{342} or aggregates\textsuperscript{154, 343}. The binding of metal complexes, such as KP1019 and Pt(II) phenanthroline complexes, to A\textsubscript{β} have been shown to lead to decrease in A\textsubscript{β} cell toxicity.\textsuperscript{140, 171} The goal of this section was to determine if the Ru(III) complexes exhibited a rescuing effect on A\textsubscript{β} toxicity in the SH-SY5Y cell line.

\textbf{A\textsubscript{β1-42}}

The cytotoxicity of A\textsubscript{β1-42} was determined in the SH-SY5Y neuroblastoma cell line, subcloned from SK-N-SH cells,\textsuperscript{344} containing characteristics of dopaminergic neurons. This cell line has been used extensively to study neuron-like behaviour in response to neurotoxins.\textsuperscript{345} The XTT assay uses a second generation tetrazolium die (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2h-tetrazolium-5-carboxanilide) that is reduced to a soluble, brightly colored orange formazan derivative as marker of viable cells, effectively
used in cell proliferation, cytotoxicity, and apoptosis assays.\textsuperscript{346,347} \(A\beta_{1-42}\) was added to cells in the monomeric form and incubated for 24 or 48 hours and cell viability was determined as shown in Figure 4.12 A and B respectively. In order to ensure that any toxicity observed was deriving from the peptide, the same volume of vehicle (10 \(\mu\)L DMSO and 190 \(\mu\)L of PBS buffer) was added to the cells and plotted next to the concentration equivalent of the peptide. As shown, an increase in vehicle or peptide lead to a decrease in cell viability for both incubation times, indicating that the vehicle exhibits a similar cytotoxic effect. Since toxicity was not observed with the incubation of monomeric peptide, the \(A\beta_{1-42}\) peptide was incubated overnight at 4°C to produce oligomers,\textsuperscript{348} and for 24 hours at 37°C to form aggregates (Figure 4.13 C), however the aggregated forms of the peptide did not show significant toxicity when compared to vehicle. Other attempts to observe the cytotoxicity of \(A\beta_{1-42}\) as reported in the literature demonstrated that under the conditions described in this chapter the peptide does not have cytotoxic effect on the SH-SY5Y cell line.

![Figure 4.12](image-url)  

**Figure 4.12** Cell viability for different concentrations of monomeric \(A\beta_{1-42}\) (red) and respective vehicle (black) in differentiated SH-SY5Y cells at A) 24 hours and B) 48 hours incubation. Cells were differentiated for 5-7 days prior to treatment and incubated for 24 hours with compounds in 5% CO\(_2\) and 100% relative humidity atmosphere. Cell viability was obtained by XTT assay, through absorbance (475 nm) of the soluble formazan formed in the presence of live cells.
Figure 4.13 Cell viability for different concentrations of Aβ1-42 pre-aggregated (red) and respective vehicle (black) in differentiated SH-SY5Y cells at 24 hour incubation with A) oligomers and B) aggregates. Cells were differentiated for 5-7 days prior to treatment and incubated for 24 hours with compounds in 5% CO₂ and 100% relative humidity atmosphere. Cell viability was obtained by XTT assay, through absorbance (475 nm) of the soluble formazan formed in the presence of live cells. C) Electrophoresis gel of pre-formed aggregates, lane 1: oligomers; lane 2: aggregates.

**Ru-N derivatives**

In order to obtain a safe therapeutic for AD, the compound is expected to have minimal cytotoxicity, limiting the drug’s side effects. NAMI-A and it’s derivatives have demonstrated modest cytotoxicity, however they have had success in the prevention of metastases, thus these complexes are of significant interest in cancer research. All Ru-N derivative complexes were tested in a range of 10 to 100 μM (Figure 4.14 A), and do not show significant toxicity up to 100 μM, with exception of Ru-N-4. A lethal dose LD₅₀ (ability of a compound to kill 50 % of the tested cells) of Ru-N-4 was determined to be
45 ± 2 μM, as determined by the fitting of the curve in Figure 4.14B. These results suggest that the Ru(III) complexes have low (Ru-N-4) to very low (Ru-N-(1-3)) toxicity in neuroblastoma cells, and could be considered for further development of a therapeutic agent in AD.

![Figure 4.14](image)

**Figure 4.14** Cell viability for different concentrations of Ru-N derivatives in the differentiated SH-SY5Y cell line. **A)** Cell viability as percentage of control for different concentrations of Ru-N-1 (green), Ru-N-2 (blue), Ru-N-3 (black) and Ru-N-4 (red); **B)** LD₅₀ curve for Ru-N-4. Cells were differentiated for 5-7 days prior to treatment and incubated for 24 hours with compounds in 5% CO₂ and 100% relative humidity atmosphere. Cell viability was obtained by XTT assay, through absorbance (475 nm) of the soluble formazan formed in the presence of live cells.

### 4.3. Summary

This chapter highlights the ability of a series of Ru(III) complexes derived from NAMI-A to interact with the Aβ peptide and modify aggregation, a known hallmark of AD. It has been shown that the DMSO ligand of the Ru-N complexes can readily be exchanged in buffer (likely for H₂O), which provides a binding site for His residues when incubated with proteins, such as HSA. My NMR and ESI-MS results are in accordance with the previous findings of binding of metal ions or complexes to Aβ and support a covalent interaction of the Ru-N complexes with His residues of the Aβ peptide. The effect of changing the size of the pyridine-derived ligands in the Ru-N series on Aβ aggregation was also investigated, and an increase in the size and hydrophobicity of the pyridine-derived ligand leads to larger-sized aggregates. The influence of Ru-N-3 and Ru-N-4 on
peptide aggregation is demonstrated to be greater than that of the smaller complexes Ru-N-1 and Ru-N-2, with a more prominent induction of soluble high MW aggregates, as demonstrated by electrophoresis gel and Western blotting. A concentration-dependent modulation of aggregation was demonstrated for Ru-N-1 and Ru-N-4, where addition of 2 equivalents of the first complex has a comparable effect on peptide aggregation as 1 equivalent of the latter. Interestingly, the aggregation of Aβ₁₋₄₂ alone after 24 hours shows only large amorphous aggregates by TEM, while the presence of 1 equivalent of either Ru-N-1 or Ru-N-4 shows formation of smaller amorphous aggregates as well as fibrils. However, investigation of the aggregation process in solution, by turbidity analysis, does not distinguish between the Ru-N complexes in terms of peptide aggregation. The Ru-N-1 and Ru-N-4 complexes exhibit increased turbidity in comparison to peptide alone at 3 and 24 hours, consistent with formation of a greater number of aggregates in comparison to peptide alone. Interestingly, all four Ru-N complexes exhibit a ca. 50% decrease in peptide concentration in comparison to peptide alone via a Bradford assay. This result is likely due to the removal of insoluble fibrils in the Ru-N samples (observed by TEM) via centrifugation. In this work I have shown that the Ru-N series undergoes ligand exchange and covalent binding to the Aβ peptide, which leads to modulation of the peptide aggregation pathway, promoting the formation of high molecular weight aggregates in solution, with both amorphous and fibrillar aggregate morphology. Further investigation of the pharmacokinetic properties of the Ru-N complexes, and influence of these complexes on the toxicity of Aβ in cell assays, will provide insight into their therapeutic potential.

The neuroblastoma cell line SHSY-5Y was used to determine Aβ₁₋₄₂ cytotoxicity as a monomer for 24 and 48 hour incubation, and as oligomers or aggregates for 24 hour incubation. Under these conditions, the peptide did not show significant cytotoxicity when compared to vehicle for concentrations up to 40 µM, suggesting that under the conditions tested in this chapter the peptide did not exhibit a cytotoxic effect against SHSY-5Y. The same cell line was used to determine the LD₅₀ for the Ru-N series. The complexes do not show cytotoxicity for concentrations lower than 100 µM, with exception of Ru-N-4, that had a LD₅₀ of 45 ± 2 µM. These results demonstrate the ability of the Ru-N complexes to bind covalently to Aβ₁₋₄₂ and modulate peptide aggregation, while having very little cytotoxic effects in the neuroblastoma cell line, making them promising candidates for further evaluation as modulators of Aβ peptide aggregation.
4.4. Experimental

All common chemicals were purchased from Sigma-Aldrich and used without further purification. All Ru complexes, \textbf{Ru-N-1}, \textbf{Ru-N-2}, \textbf{Ru-N-3} and \textbf{Ru-N-4} were synthesized as reported.\textsuperscript{322} The A\textsubscript{\beta}-16, and A\textsubscript{\beta}-42 peptides were purchased from 21st Century Biochemicals (Marlborough, MA, USA), and Cellmano Biotech Limited (Hefei, China), and monomerized before use according to a reported procedure.\textsuperscript{239, 240} A\textsubscript{\beta}-16 was dissolved in double distilled H\textsubscript{2}O (ddH\textsubscript{2}O), while A\textsubscript{\beta}-42 was dissolved in DMSO and ddH\textsubscript{2}O in a 1:1 mixture, unless stated otherwise. The stock peptide solution concentration was determined by absorbance with the use a Thermo Nicolet UV nanodrop and an extinction coefficient of 1410 and 1450 M\textsuperscript{-1}cm\textsuperscript{-1} at 280 nm for A\textsubscript{\beta}-16, and A\textsubscript{\beta}-42 respectively.\textsuperscript{241, 242} Turbidity assays were measured using a Synergy 4 Multi-Detection microplate reader from BioTek. \textsuperscript{1}H NMR spectra were recorded on a Bruker AV-600 instrument. TEM images were obtained using an OSIRIS FEI scanning TEM (STEM) operating at 200 kV.

4.4.1. \textsuperscript{1}H NMR Binding Assay of A\textsubscript{\beta}-16 peptide to NAMI-A derivatives

Deuterated phosphate buffered saline (PBS) (\(0.01\) M Na\textsubscript{2}HPO\textsubscript{4}, 0.001 M KH\textsubscript{2}PO\textsubscript{4}, 0.14 M NaCl, 0.003 M KCl, \(pD\) 7.4) was prepared by removal of water by vacuum drying of PBS and dissolving the powder in D\textsubscript{2}O. A\textsubscript{\beta}-16 was dissolved in deuterated PBS (\(0.01\) M, \(pH\) 7.4), and \textbf{Ru-N-1} and \textbf{Ru-N-4} complexes were dissolved in DMSO-\textit{d}\textsubscript{6} and added to A\textsubscript{\beta}-16 at 0.25 and 1 eq. at 10\% of DMSO and the \textsuperscript{1}H NMR spectra were collected after approximately 15 min of incubation.

4.4.2. Mass Spectrometry of Binding of A\textsubscript{\beta}-16 Peptide to NAMI-A derivatives

ESI-TOF-MS experiments were performed on an Agilent 6130 mass spectrometer connected to an Agilent 1260 HPLC system. Samples were analyzed by direct infusion (1-4 \(\mu\)L) of analyte into a mobile phase of 1:1 water:acetonitrile containing 5 mM ammonium acetate (pH unmodified), flowing at 0.3 mL/min and maintained at 30 °C. All components of the mobile phase were mass spectrometry grade. Nitrogen drying gas was heated to 250 °C, and run at 5 L/min with a nebulizing pressure of 15 psig. Voltages were: capillary 3 kV, fragmentor 175 V, skimmer 30 V, octopole 250 V. Samples were prepared
as \( \sim 4 \text{ mg/mL} \) of total protein (A\( \beta_{1-16} \)) in ammonium carbonate (0.02 M, pH 9) buffer with 0 or 1 eq. of the Ru-N complexes.

### 4.4.3. Gel Electrophoresis and Western Blotting

\( \text{A\( \beta \)} \) solutions with a concentration of 25 \( \mu \text{M} \) were prepared in PBS (0.01 M, pH 7.4) then incubated at 37 °C with continuous agitation at 200 rpm to form aggregates in the presence of Ru-N complexes or pyridine ligands at 1 eq., Samples were collected at 3, 6, 11 and 24 hour time points. Concentration-dependent modulation of \( \text{A\( \beta \)} \) aggregation was also evaluated after 24 hours incubation for Ru-N-1 and Ru-N-4 (0.25, 0.5, 1, and 2 eq.). Electrophoresis separation of peptide aggregates was completed using 8-16% Mini-PROTEAN® TGX Precast Polyacrylamide Gels from Bio-Rad, at 100 V for 100 min. The gels were then transferred to a nitrocellulose membrane for 1 hour at 100 V at 4 °C, followed by blocking of the membrane in a 3% BSA solution in Tris-buffered saline (TBS) (0.02 M Tris, 0.15 M NaCl, 0.003 M KCl) for 1 hour. The membrane was incubated in a solution (1:2000 dilution) of 6E10 anti-A\( \beta \) primary antibody (Biolegends) overnight. After washing 5 × 5 min with TBS, the membrane was incubated in a solution containing the secondary antibody (Horseradish peroxidase, Caymen Chemicals) for 3 hours. A Thermo Scientific SuperSignal® West Pico Chemiluminescent Substrate kit was used to visualize the A\( \beta \) species using a BioRad ChemiDoc™ MP imaging system.

### 4.4.4. Transmission Electron Microscopy (TEM)

Samples were prepared from the Western blot assay after the 24 hour incubation time at 37 °C. TEM grids were prepared following previously reported methods.\(^{180}\) In order to increase hydrophilicity of the Formvar/Carbon 300-mesh grids (Electron Microscopy Sciences), the grids were glow discharged in a vacuum for 10 seconds. Drops of samples (10 \( \mu \text{L} \)) were placed onto a sheet of parafilm and the TEM grid was laid on the drop for 5 minutes. The grid was then placed on a drop of syringe-filtered 5% uranyl acetate and then immediately removed. This process was then repeated for a second drop of 5% uranyl acetate. Finally, the grid was placed on a third drop of 5% uranyl acetate and incubated for one minute. Excess uranyl acetate was removed using a tissue between drops. The grid was allowed to air-dry for at least 15 minutes. Bright-field images were obtained on a FEI Tecnai Osiris STEM at 200 kV.
4.4.5. Turbidity Assay

The turbidity assay was conducted in quadruplicate in flat-bottomed 96-well assay plates (Microtest, BD Falcon). Aβ1-42 peptide and Ru-N complexes had final concentrations of 10 µM. Ru-N complexes were dissolved in DMSO and further diluted to obtain the correct concentration. The absorbance at 500 nm was measured every 10 min for 3 hours at 37 °C under constant agitation using a Synergy 4 Fluorometer plate reader from BioTek. For the 20 h experiment, the samples were incubated at 37 °C with constant agitation with a lid on to prevent evaporation and then the turbidity was measured.

4.4.6. Bradford Assay

The Bradford assay (Thermo Scientific) measures the absorbance at 595 nm of Coomassie brilliant blue G-250 as it binds to protein in duplicate in a flat-bottomed 96-well assay plates (Microtest, BD Falcon). 60 µM solutions of Aβ1-42 in the presence of 1 eq. of Ru-N complexes were incubated at 37 °C for 24 hours under constant agitation. A 30 µL sample was removed at the beginning of the experiment as the 0 hour time point, and kept frozen at -80 °C until time for absorbance reading. Samples were centrifuged prior to reading of the assay to remove insoluble fibrils. (Mok and Howlett, 2006) Measurements of absorbance used a Synergy 4 Fluorometer plate reader from BioTek. Samples were measured in duplicate, and statistics completed using the PRYSM program and ANOVA.

4.4.7. Cytotoxic activity

Cell lines

SH-SY5Y (human neuroblastoma) cells were donated by Prof. Frank Lee (Department of Health Sciences, Simon Fraser University). Cells were grown in DMEM/F-12 nutrient mix (Life Technologies), 1% Penicillin/Streptomycin solution (Life Technologies), 1% MEM non-essential amino acid solution (Gibco), and 10% Fetal Bovine Serum (ATCC) in T-75 tissue culture flasks (VWR) until 90-100% confluency. Cells were detached and separated into single cell with TrypLE express (Life Technologies). Cell pellets were re-suspended in differentiating media containing DMEM/Ham’s F-12, 1% Penicillin/Streptomycin solution, 1% MEM Non-essential amino acid solution, 1% fetal
bovine serum, and 10 μM retinoic acid, and seeded into a 96-well plate at a density of ca. 20,000 cells/well using a haemocytometer.

**Evaluation of cytotoxicity of Aβ₁₋₄₂ or Ru-N derivatives by XTT assay**

Different concentrations of Aβ₁₋₄₂ or Ru-N derivatives were added to the cells after 5-7 days of differentiation in the 96 well plate. The plates were incubated for 24 hours in 5% CO₂ and 100% relative humidity atmosphere. A 500 μM stock solution was prepared in 10% DMSO in media for each complex and further diluted to 100 μL in media in each well, with a maximum of 2 % of DMSO for the highest concentration. Aβ₁₋₄₂ film was dissolved in 10 μL DMSO and 190 μL of PBS buffer and further diluted to 100 μL in media in each well either as a monomer, as an aggregate previously incubated for 24 hours at 37˚C at 200 RPM, or as oligomers previously incubated overnight at 4˚C. Concentrations ranging from 10 to 100 μM of Ru-N derivatives and 10 to 40 μM of Aβ₁₋₄₂ were used to evaluate their toxicity by XTT assay. The XTT assay uses a second generation tetrazolium die (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolum-5-Carboxanilide) that is reduced to a soluble, brightly colored orange formazan derivative as marker of viable cells, and is used effectively in cell proliferation, cytotoxicity, and apoptosis assays. Results are expressed as a percentage of cell proliferation in comparison with control. In the treatment of the Ru-N series, vehicle did not show toxicity.
Chapter 5. Summary and Future Directions

Emily Fan and Michael Silverman contributed to this chapter by the extraction and culturing of rat hippocampal neuron cells.

5.1. Thesis Summary

This thesis summarises two different approaches to modulate Aβ aggregation, one of the hallmarks of AD. Due to the substantial evidence that metal-ions contribute to the aggregation and toxicity of Aβ, the use of multifunctional \( \textbf{8-H}_2\textbf{Q} \) ligands as metal sequestration agents was investigated. Another approach investigated in this thesis was the covalent binding of an Fe(III) corrole, and a series of Ru(III) complexes, to Aβ and the effect of this process on peptide aggregation.

Oxidative stress is related to neurodegenerative diseases such as AD, and the use of anti-inflammatory compounds have been investigated as potential therapeutics for AD. Chapters 2 and 3 describe the use of agents that act as antioxidants. In Chapter 2, the binding of the \( \textbf{8-H}_2\textbf{Q} \) ligands to Cu(II) likely limits the formation of oligomeric Aβ species induced by Cu(II), and suggests that these ligands could hinder Aβ-Cu(II) ROS formation. In addition, the ligands alone act as antioxidants. Chapter 3 demonstrates the ability of the Fe(III) corrole FeL1 to act as a catalase and to bind to Aβ simultaneously with Cu(II), which leads to the disproportionation of \( \textbf{H}_2\textbf{O}_2 \) and •OH produced by Aβ-Cu(II) in the presence of \( \textbf{O}_2 \). These results demonstrate two different approaches that target oxidative stress in AD.

Chapters 3 and 4 described the use of different metal complexes that led to the modulation of Aβ peptide aggregation in two distinct ways. While FeL1 stabilizes the formation of low MW oligomeric species (Chapter 3), in the presence of the Ru-N series high MW aggregates are formed (Chapter 4). FeL1 has a high affinity for the peptide \( (K_d = 10^{-7}) \), and results in the formation of oligomeric species, likely due to the large size of the corrole ligand, and peptide conformation once bound to FeL1. For the Ru-N series, the smaller size of the complex, and the ability for the Ru complexes to bridge individual peptides, could favour higher MW aggregates. The stabilization of aggregates that differ
in comparison to peptide alone may lead to altered interactions of the peptide with cells, ultimately lowering the toxicity of the Aβ aggregates.349-351

During the course of this research new ideas emerged and will be discussed in further detail below. Overall, my results show that the compounds studied herein have potential for being developed into therapeutics for AD, however, issues such as solubility, ability to cross the blood brain barrier (BBB) and selectivity for the Aβ peptide need to be improved. Therefore, there is a need in continuing the investigation of these compounds, as well as structural analogues, in order to obtain a safe and effective therapeutic for AD.

5.2. Chapter 2 – Improving Solubility and Permeability for the H₂QX Ligands

Chapter 2 highlights the ability of Schiff-base ligands derived from 8-H₂Q (see Figure 2.1) to chelate Cu(II), limiting formation of Aβ peptide oligomers induced by Cu(II). These ligands were shown to exhibit antioxidant properties similar to vitamin E in a Trolox equivalent antioxidant assay. The results suggest a potential therapeutic use of these molecules in AD, however, the limited aqueous solubility of these ligands pose a difficulty in their further development. Future work on this series could focus on improving the water solubility of these ligands, allowing for improved bioavailability. Three modifications to the 8-HQX series are proposed below (Figure 5.1). Firstly, the presence of halogens at positions 5 and 7 in the 8-HQ scaffold, such as Cl and I, could be used to improve solubility and membrane permeability, showing a structure similar to HQC and PBT2 (see Figure 2.1).94, 99-106 Another possible modification to the 8-HQX derivatives is the addition of polyethylene glycol (PEG), known as PEGylation. This technique has been shown in the literature to improve pharmacological properties of drugs, due to PEG’s high water solubility, biocompatibility and non-toxicity.352 Finally, an interesting way to enhance brain delivery is by targeting the high consumption level of glucose by the brain,353 therefore a glycoconjugated derivative of 8-HQ could facilitate the delivery of these compounds across the BBB via glucose transporters. Enzymatic cleavage by glucosidase enzymes could then un-mask the metal binding site.354, 355
5.3. Chapter 3 – Influence of FeL1 on Aβ Oligomer Toxicity

Chapter 3 describes the binding of an Fe(III) corrole complex, FeL1, to an Aβ His residue in the presence and absence of Cu(II). The binding of this complex to the peptide modifies the aggregation pattern, stabilizing oligomeric species of low MW. FeL1 also limited Aβ-Cu(II) ROS production. Future work could include determining the influence of FeL1 binding to Aβ on different cell processes, including the cell response to Aβ aggregation. The link between tau hyperphosphorylation and Aβ aggregation will be discussed below.

5.3.1. Aβ Oligomers and Tau Phosphorylation

The species considered to be the most toxic during the process of aggregation of the Aβ peptide are the oligomers (AβO). These soluble species have been shown to interact with glutamate receptors at the membrane on dendrites, increase calcium influx and oxidative stress, promote long-term depression and eventually lead to synapse failure. AβOs have been shown to influence tau phosphorylation as well; therefore we wanted to investigate the effect that FeL1 may have on this process.
Figure 5.2 Immunocytochemistry of rat hippocampal neuron cells incubated for 24 h at 37°C with AβO with or without FeL1. A) Control, B) AβO, C) AβO + FeL1. PHF1 antibody was used to identify P-tau (red), DAP1 antibody was used to identify the cell nucleus (blue), and AβO were prepared with a mix of unlabeled and labeled Aβ1-42 (green).

The presence of FeL1 generates Aβ oligomeric species (Chapter 3), therefore we investigated if these species would induce a different effect on rat hippocampal neuronal cells when compared to AβOs. Neuron cells were incubated with 500 nM of pre-formed AβOs, with and without FeL1 for 24 hours at 37°C. The immunoblotting of the fixed cells show the labeled AβOs (Figure 5.2, B and C green) predominantly associated with the cell membrane. Tau is found mainly associated with microtubules, that maintain the shape of dendrites and axons of neuron cells. Tau phosphorylation (P-tau) was assessed at Ser396 and Ser404 with the antibody PHF1 (Figure 5.2, red), and a small increase in P-tau was observed in the presence of AβOs (1.3 when compared to control). Literature reports have indicated an increase of at least two-fold in tau phosphorylation by AβOs, however we were not able to observe a significant increase under our conditions. It is interesting to note that the presence of FeL1 appears to reduce the binding of AβOs to the cell.
membrane qualitatively, however there is a need to quantify in order to have a proper comparison.

The immunoblot of neuron cell lysates after treatment with AβOs in the absence and presence of FeL1 was obtained as an additional attempt to observe a change in P-tau levels induced by AβOs (Figure 5.3). β-actin was used to normalize the signal and the amount of P-tau compared to total tau was calculated for all of the samples (Table 5.1). The ratio between P-tau and total tau was very similar for all samples, contrary to literature reports in which the P-tau ratio for AβOs is 3 to 4 times higher than that for the control.358

Figure 5.3 Western blot of cell lysate of rat hippocampal neuron cells incubated for 24 h at 37˚C with AβOs with or without FeL1. 1) Control, 2) AβO, 3) FeL1, 4) AβO + FeL1. PHF1 antibody was used to identify P-tau phosphorylated at Ser396 and Ser404, compared to total tau, with signal being corrected by β-actin.

Table 5.1 Amount of P-tau over total tau for cell lysate Western blot of neuron cells treated with AβO.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Treatment</th>
<th>PHF1/Total tau</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>AβO</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>FeL1</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>Aβ-FeL1</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Additional processes could be investigated to further understand the influence of AβOs on neuron cells. FeL1 was shown in Chapter 3 to reduce ROS generated by the reaction of Aβ-Cu(II) with O₂ in the presence of a reducing agent. This process could be monitored in cells to evaluate the influence of the ROS generated by Aβ-Cu(II) and if the presence of the Fe(III) complex would limit ROS formation and toxicity. The binding of FeL1 could alter other processes affected by AβOs, such as calcium influx and disruption
of vesicular transport of brain-derived neurotrophic factor (BDNF).\textsuperscript{349-351} These experiments would provide a better understanding of the influence of FeL1 on the toxicity of AβOs.

5.4. Chapter 4 – Investigation of Ru(III) Complexes on Aβ Aggregation and Toxicity

Ru(III) complexes derived from NAMI-A were investigated in Chapter 4 and shown to influence Aβ peptide aggregation. The Ru-N derivatives were shown to bind covalently to Aβ, possibly through a His residue. Future work for this series could include the determination of which Aβ His residues are involved in binding to the Ru(III) center. As discussed in Chapter 1, Dey et al. have investigated the binding of heme to different mutants of Aβ (see Figure 1.13) and the influence of specific point mutations on the peroxidase activity of the heme-Aβ complex.\textsuperscript{160} The use of Aβ mutants that contain only one, two or no His would give a better understanding of the involvement of His\textsuperscript{5,13,14} in the metal complex-peptide binding. In addition, a mutant at Tyr\textsuperscript{10} can be used to determine if the presence of this residue is necessary to ensure binding to the peptide. Another approach would be subject Ru-N-peptide adducts to trypsin digestion to determine which fragments contain Ru(III), allowing for the determination of the Aβ binding site. With a better understanding of the binding of these Ru-N complexes I can design candidates that show improved solubility and interaction with the peptide, allowing for a more effective potential therapeutic for AD.

5.4.1. Ru(III) complexes

Additional studies could include another series of Ru(III) complexes derived from KP1019 (Ru-K).\textsuperscript{136} Preliminary studies have been completed on the Ru-K series (Figure 5.4), and are detailed here.
Figure 5.4 Structures of Ru(III) complexes derived from KP1019 (Ru-K series) with their sodium counterions.

KP1019 was shown previously to modulate Aβ aggregation, limiting toxicity in neuroblastoma cells.\textsuperscript{171} I investigated a series of Ru(III) complexes derived from KP1019 (Figure 5.4) and their ability to regulate Aβ peptide aggregation. The Ru-K series differs from the Ru-N (Chapter 4) by addition of second pyridyl ligand in place of the DMSO ligand, therefore a similar or enhanced effect on the aggregation of the Aβ peptide was expected. Peptide alone (Lane 1, Figure 5.5) shows a weak signal for soluble aggregates, suggesting that the aggregates are not able to enter the gel, as observed previously (Figure 4.7). The gel data suggests that complexes Ru-K-3 and Ru-K-4 exhibit a larger influence on peptide aggregation, stabilizing species with MW larger than 250 kDa. Further investigation of peptide aggregation could include the Bradford assay, through the shift from 495 nm to 595 nm for the reagent Coomassie brilliant blue G-250 upon binding to the C-terminus of proteins.\textsuperscript{335} These assays could provide an improved understanding of the peptide aggregation process in the presence of the different Ru(III) complexes.\textsuperscript{360} The low aqueous solubility of the KP1019 derivatives poses difficulties for further investigation of the nature of their interaction with Aβ peptide. However, it would be interesting to continue the study of this series of complexes to determine the similarities or differences in Aβ binding of the Ru-K and Ru-N series.
5.5. Experimental

All common chemicals were purchased from Aldrich and used without further purification. All Ru complexes, Ru-K-1, Ru-K-2, Ru-K-3 and Ru-K-4 were synthesized as reported. The Aβ1-42 peptides were purchased from 21st Century Biochemicals (Marlborough, MA, USA), and Bachem Americas (Torrance, CA, USA) and HiLyte™ Fluor 488 labeled Aβ1-42 peptide was purchased from Anaspec (Fremont, CA, USA). All peptides were monomerized before use according to a reported procedure.

5.5.1. AβO Preparation

Aβ oligomeric species (AβO) were prepared following the method of Lambert et al. For the preparation of labeled AβO, labeled Aβ1-42 film was dissolved with 15.1 μL DMSO and this solution was used to dissolve unlabeled Aβ1-42 film. PBS buffer (0.01 M, pH 7.4) was added to solution 57.9 μL. Regular AβO was prepared by dissolving Aβ1-42 film in 1 μL DMSO and 49 μL PBS buffer (0.01 M, pH 7.4). The vials were incubated with or without FeL1 overnight at 4 °C and centrifuged at 14000 g, 4 °C for 10 min. The
concentration of the supernatant was determined by Bradford assay, comparing to a bovine serum albumin (BSA) standard curve.

5.5.2. Treatment of Hippocampal Neuron Cells

Preparation of embryonic day 18 (E18) rat primary hippocampal neuronal cultures followed a procedure established in the literature by Kaech et al.\textsuperscript{361} and were kept in primary neuron growth media from Lonza (Basel, Switzerland). All experiments with animals were approved by and followed the guide-lines set out by the Simon Fraser University Animal Care Committee, Protocol 943-B05. At 10 days \textit{in vitro}, cells were treated with 500 nM of labeled AβOs with or without FeL1 for 18 to 20 hours, fixed in 4% paraformaldehyde and blocked with 10% BSA. Neurons were stained with anti-P-tau PHF1 (1:300, from P. Davies, Albert Einstein School of Medicine, New York, NY) and anti-MAP2 (1:2000; Millipore, Billerica, MA). Finally, the cells were treated with the compatible secondary antibodies conjugated to Cy3 (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) or Cy5 (1:500; Jackson ImmunoResearch Laboratories). The fluorescence images were obtained in a LEICA DMI6000 B microscope.

5.5.3. Immunoblotting

The hippocampal neuron cells were treated with vehicle, AβO or AβO-FeL1 as described above. Cell lysate was obtained with the use of radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitor (Roche). Samples were centrifuged at 14000 g for 5 min, and the concentration of protein in the supernatant was determined by Bradford assay. Protein was separated in a 12% SDS-polyacrylamide gel and transferred onto a Polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked for 1 hour in 5 % BSA at room temperature and incubated with primary antibodies overnight at 4 °C. The membranes were washed with TBS-T (TBS, 0.1% Tween 20, pH 7.4) and incubated with the adequate secondary antibody for 1 hour. Protein bands were visualized with Thermo Scientific SuperSignal® West Pico Chemiluminescent Substrate kit in a FUJIFILM Luminescent Image Analyzer (LAS-4000). Quantification of tau phosphorylation were carried out in ImageJ (National Institutes of Health, Bethesda, MD).
5.5.4. Gel Electrophoresis and Western Blotting

Aβ_{1-42} was dissolved in DMSO and ddH_{2}O in a 1:1 mixture, and its concentration determined by absorbance with the use of a Thermo Nicolet UV nanodrop and an extinction coefficient of 1450 M^{-1}cm^{-1} at 280 nm. Aβ solutions with a concentration of 25 µM were prepared in PBS (0.01 M, pH 7.4) then incubated at 37 °C with continuous agitation at 200 rpm to form aggregates in the presence of Ru-K complexes at 1 eq., samples were collected at 3, 6, 11 and 24 hour time points. Electrophoresis separation of peptide aggregates was completed using 8-16% Mini-PROTEAN® TGX Precast Gels from Bio-Rad, at 100 V for 100 min. The gels were then transferred to a nitrocellulose membrane for 1 hour at 100 V at 4 °C, followed by blocking of the membrane in a 3% BSA solution in TBS for 1 hour. The membrane was incubated in a solution (1:2000 dilution) of 6E10 anti-Aβ primary antibody (Biolegends) overnight. After washing 5 × 5 min with TBS buffer, the membrane was incubated in a solution containing the secondary antibody (Horseradish peroxidase, Caymen Chemicals) for 3 hours. Thermo Scientific SuperSignal® West Pico Chemiluminescent Substrate kit was used to visualize the Aβ species using a BioRad ChemiDoc™ MP imaging system.
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Collin, F.; Sasaki, I.; Eury, H.; Faller, P.; Hureau, C., Pt(II) compounds interplay with Cu(II) and Zn(II) coordination to the amyloid-beta peptide has metal specific consequences on deleterious processes associated to Alzheimer’s disease. *Chem. Commun.* 2013, 49 (21), 2130-2.


Appendix A. Supplementary Information for Chapter 2

Figure A1. ORTEP diagram for the two molecules of \(8\)-\(\text{H}_2\text{QH}\) in the asymmetric unit. Thermal ellipsoids at the 50% probability level. Hydrogen atoms are drawn as circles of arbitrary radii.

Figure A2. Perspective views for the crystal packing of \(8\)-\(\text{H}_2\text{QH}\). In (A) the NH⋯O and CH⋯O hydrogen bonds in 2D-network were presented. In (B) a projection highlighting the interlayer separation was presented.

Table A1. Selected bond lengths (Å) and torsion angles (°) for the \(8\)-\(\text{H}_2\text{QH}\) (molecule B).

<table>
<thead>
<tr>
<th>Bond (Å)</th>
<th>Angles (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N11–C102</td>
<td>1.326(2)</td>
</tr>
<tr>
<td>C102–C109</td>
<td>1.463(2)</td>
</tr>
<tr>
<td>C109–N12</td>
<td>1.280(2)</td>
</tr>
<tr>
<td>N12–N13</td>
<td>1.363(2)</td>
</tr>
<tr>
<td>N13–C110</td>
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<tr>
<td>C110–O12</td>
<td>1.232(2)</td>
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<tr>
<td>C110–C111</td>
<td>1.497(2)</td>
</tr>
<tr>
<td>C108–O11</td>
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<tr>
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</tr>
<tr>
<td>Min</td>
<td>0.12</td>
</tr>
<tr>
<td>Max</td>
<td>68.99</td>
</tr>
</tbody>
</table>

Table A2. Particle size calculated using ImageJ analyser for TEM images of: 2 Aβ_{1-42}; 3 Aβ_{1-42} + Cu^{II}; 4 Aβ_{1-42} + 8-H_{2}QH; 5 Aβ_{1-42} + Cu^{II} + 8-H_{2}QH; 6 Aβ_{1-42} + 8-H_{2}QS; 7 Aβ_{1-42} + Cu^{II} + 8-H_{2}QS; 8 Aβ_{1-42} + PBT2; 9 Aβ_{1-42} + Cu^{II} + PBT2.
Appendix B. Supplementary Information for Chapter 3

Figure B1. UV-Vis spectra of L-Histidine (L-His) additions to FeL1 (30 µM, black) in PBS buffer (0.01 M, pH 7.4). Grey lines represent additions of 10 eq. of L-His up to 700 eq. shown in red.

Figure B2. (Left) Variable pH UV-Vis titration of FeL1 (30 µM) with 1-Melm (60 µM) from pH 3.4 (black) to pH 10.5 (red). (Right) Using HypSpec and HySS, the variable pH data were fit to a model including FeL1(1-Melm) and FeL1(1-Melm)₂. At pH 7.4 the majority of FeL1 is bound to 1-Melm (>99%).
Figure B3. UV-Vis spectra of Aβ_{1-16} titration with FeL1 (30 μM, black) in PBS buffer (0.01 M, pH 7.4). Grey lines represent additions 1 eq. of Aβ_{1-16} up to 16 eq. (blue).
Figure B4. Mass spectrum (ESI-MS) of binding of Aβ₁₋₁₆ to FeL₁ in the presence of human serum albumin (HSA). FeL₁-Aβ₁₋₁₆ and its isotopic pattern inset (above) and free FeL₁ in the presence of HSA (bellow).
Figure B5. UV-Vis spectra of binding of FeL1 (30 µM) and Cu(II) (27 µM) to Aβ1-16 (30 µM) in PBS buffer (0.01 M, pH 7.4). Order of addition varied to observe any potential changes in binding, where the species FeL1-Aβ is shown in black, and the species FeL1-Aβ-Cu(II) are shown in red and blue, with FeL1 or Cu(II) being added first to a solution containing Aβ1-16, respectively.

Figure B6. Frozen-solution EPR spectra collected at 20 K (black) and 77 K (blue) of FeL1 and FeL1-Aβ1-16. Conditions: [Aβ1-16] = 0.55 mM, [FeL1] = 0.50 mM in PBS buffer (0.01 M, pH 7.4). Experimental parameters: frequency = 9.38 GHz, microwave power = 2.0 mW, time constant = 40.96 ms, modulation amplitude = 5 G, average of five 1-min scans.
Figure B7. UV-Vis spectra of the temperature dependence of binding of FeL1 (30 µM) to 1-Melm (600 µM, left) or Aβ1-16 (30 µM, right) in PBS buffer (0.01 M, pH 7.4). Grey lines represent changes of temperature starting at 10 °C (black) to 37 °C (red). The spectral changes are reversible and indicate an increase in bis-axial binding to FeL1 as temperature is lowered.
Appendix C. Supplementary Information for Chapter 4

Figure C1. Changes in the $^1$H NMR spectra of $\text{A}\beta_{1-16}$ in the presence of 0.25 eq. of Ru-N derivatives incubated over time with constant agitation at 37 °C. Shown are spectra obtained from 205 µM $\text{A}\beta_{1-16}$, in pH 7.4 PBS/D$_2$O buffer at 0 h (red) 5 h (green) and 24 h (blue) of A) Ru-N-1 or B) Ru-N-4. *N-4.

Figure C2. Influence of [Ru(DMSO)$_2$Cl$_4$]Na on the aggregation profile of $\text{A}\beta_{1-42}$. Gel electrophoresis/Western blot of 25 µM $\text{A}\beta_{1-42}$ and 1 eq. of Ru(III) complexes in PBS buffer (0.01 M, pH 7.4) at 24 hour incubation, with constant agitation at 37 °C, using anti-$\text{A}\beta$ antibody 6E10. Lane 1: $\text{A}\beta_{1-42}$; lane 2: $\text{A}\beta_{1-42}$ + Ru-N-1; lane 3: $\text{A}\beta_{1-42}$ + Ru-N-4; lane 4: $\text{A}\beta_{1-42}$ + [Ru(DMSO)$_2$Cl$_4$]Na.
Figure C3. Influence of Ru-N-1 and Ru-N-4 on the aggregation profile of Aβ1–42. TEM of Aβ1–42 alone (A), Aβ1–42 with 1 eq. of Ru-N-1 (B) and Aβ1–42 with 1 eq. of Ru-N-4 (C) incubated for 24 hours with agitation at 37 °C.