Small Molecule Agents that Target Amyloid-β Aggregation in Alzheimer’s Disease

by

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Abstract

Alzheimer’s disease (AD) is the most common form of dementia and currently there is no cure. AD is characterized by the formation of two pathological hallmarks; aggregated forms of the amyloid-β (Aβ) peptide called Aβ plaques and hyperphosphorylated tau proteins, called neurofibrillary tangles (NFT). Aβ is enzymatically cleaved from the amyloid precursor protein (APP) to afford a 38-43 amino acid residue peptide with Aβ_{1-40} and Aβ_{1-42} being the most common. Plaque deposits have been shown to contain abnormally high concentrations of dysregulated metal ions, specifically Cu, Zn, and Fe. Metal-Aβ interactions have been shown to increase the rate of Aβ aggregation leading to increased neurotoxicity and oxidative stress. Specifically, Cu-Aβ species in stoichiometric amounts produce soluble, oligomeric species, which are hypothesized to play a role in AD.

This thesis presents several strategies to influence metal-Aβ interactions in order to mitigate peptide aggregation and overall toxicity. Three triazole-based ligand scaffolds are presented that were designed to exhibit a range of properties including metal binding, peptide interactions, and antioxidant capabilities. Chapter 2 describes a series of pyridine-triazole ligands that have an affinity for the N-terminus region of the Aβ peptide where metal binding occurs. Chapter 3 builds from the previous chapter by extending the aromatic ring system to present a series of quinoline-triazole ligands. This framework demonstrated interactions with the peptide in the hydrophobic region (residues 17-21) of the peptide and was able to influence Cu-Aβ aggregation. Finally, chapter 4 describes a series of phenol-triazoles that compete with Aβ for binding of free Cu, interact in the hydrophobic region of the Aβ peptide, and exhibit antioxidant properties. Chapter 5 presents a new strategy to prevent Aβ aggregation through the use of KP1019, a Ru(III) anticancer agent. KP1019 readily binds to the Aβ peptide within 2 hours, modulating Aβ aggregation by producing non-toxic aggregates as demonstrated in a human neuroblastoma cell line (SH-SY5Y).

Keywords: Alzheimer’s disease; multifunctional ligands; Amyloid-β; metal binding
To my mother Donna, who raised me to be the man I am today.
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# Table of Contents

Approval .................................................................................................................. ii  
Abstract .................................................................................................................. iii  
Dedication ................................................................................................................ iv  
Acknowledgements .................................................................................................. v  
Table of Contents .................................................................................................. vii  
List of Tables ......................................................................................................... xi  
List of Figures ....................................................................................................... xi  
List of Acronyms .................................................................................................... xx  

## Chapter 1. Introduction ......................................................................................... 1  
1.1. Medicinal Inorganic Chemistry ........................................................................ 1  
1.2. Inorganic Chemistry in Neurodegenerative Disease ........................................ 3  
1.3. Hallmarks and Hypotheses of AD .................................................................... 4  
  1.3.1. Characteristics of AD .................................................................................. 4  
  1.3.2. Amyloid Cascade Hypothesis ...................................................................... 7  
  1.3.3. Tau Hypothesis ............................................................................................ 8  
  1.3.4. Cholinergic Hypothesis .............................................................................. 10  
  1.3.5. Metal Ion Hypothesis ................................................................................. 11  
1.4. Current Therapeutic Agents and Strategies .................................................... 16  
  1.4.1. Approved Cognitive Drugs .......................................................................... 16  
  1.4.2. Immunotherapy ............................................................................................ 17  
  1.4.3. Targeting Secretase Inhibition ..................................................................... 18  
  1.4.4. Aβ Plaque Imaging Agents ......................................................................... 19  
  1.4.5. Metal Binding Agents .................................................................................. 20  
1.5. Multifunctional Metal-binding Therapeutics ................................................... 21  
  1.5.1. Linkage, Incorporation, and Responsive Strategies ...................................... 22  
  1.5.2. Aβ Aggregation Regulated by Metal Complexes ....................................... 25  
  1.5.3. Blood Brain Barrier (BBB) Permeability .................................................... 26  
1.6. Thesis Goals .................................................................................................... 27  

## Chapter 2. Dual Function Triazole-Pyridine Derivatives as Inhibitors of Metal-induced Aβ Aggregation 

2.1. Introduction ..................................................................................................... 29  
2.2. Experimental .................................................................................................. 31  
  2.2.1. Syntheses .................................................................................................... 31  
  2.2.2. Metal Binding Studies ................................................................................... 34  
  2.2.3. X-ray Crystallography ................................................................................ 34  
  2.2.4. Determination of Acidity Constants by UV-vis ......................................... 36  
  2.2.5. Determination of Acidity Constants by NMR ............................................ 36  
  2.2.6. Two-dimensional (2D) 1H-15N transverse relaxation optimized spectroscopy (TROSY)-heteronuclear single quantum correlation (HSQC) NMR measurements .................................................. 37  
  2.2.7. Docking Studies using AutoDock Vina ....................................................... 37  
  2.2.8. Turbidity Measurements ............................................................................. 38
4.2.10. Transmission Electron Microscopy .................................................. 85
4.2.11. CCA Anti-Oxidant Assay .................................................................. 85
4.2.12. Trolox-Equivalent Anti-Oxidant Capacity (TEAC) Assay .................. 86
4.3. Results and Discussion ........................................................................ 87
  4.3.1. Design and Synthesis ......................................................................... 87
  4.3.2. Drug-Like Properties and BBB Permeability ...................................... 87
  4.3.3. Job’s Plot ............................................................................................ 88
  4.3.4. Ligand Speciation Measurements ...................................................... 89
  4.3.5. Metal Stability Constants ................................................................... 92
  4.3.6. Ligand-Peptide Interactions via 2-D \(^{1}H-{ }^{15}N\) SOFAST-HMQC NMR ...... 95
  4.3.7. Molecular Docking Simulations .......................................................... 100
  4.3.8. Native Gel Electrophoresis, Western Blotting, and TEM .................... 103
  4.3.9. Antioxidant Assays ........................................................................... 106
4.4. Conclusions ......................................................................................... 110

Chapter 5. Modulation of the A\(\beta\) peptide aggregation pathway by KP1019
limits A\(\beta\)-associated neurotoxicity.......................................................... 112
5.1. Introduction ......................................................................................... 112
5.2. Experimental ...................................................................................... 114
  5.2.1. Thioflavin-T Assay .......................................................................... 114
  5.2.2. Transmission Electron Microscopy .................................................... 115
  5.2.3. EPR Measurements and Simulations ................................................. 115
  5.2.4. Dot Blot Assay .................................................................................. 115
  5.2.5. Native Gel Electrophoresis and Western Blotting .............................. 116
  5.2.6. Alamar Blue Cell Viability Assay ...................................................... 117
5.3. Results and Discussion ....................................................................... 118
  5.3.1. Effect of KP1019 on A\(\beta\) Peptide Aggregation ..................................... 118
  5.3.2. A\(\beta\) Peptide Coordination to KP1019 .............................................. 120
  5.3.3. Native Gel Electrophoresis/Western Blotting ................................... 122
  5.3.4. KP1019 Rescues Differentiated SH-SY5Y Cells from A\(\beta\)\(_{1-42}\) –
induced Toxicity ......................................................................................... 124
5.4. Conclusions ....................................................................................... 126

Chapter 6. Future Directions ...................................................................... 128
6.1. Cell Viability and BBB-Permeability Studies ......................................... 128
6.2. Ligand Design Strategies .................................................................... 129
6.3. Cu-A\(\beta\) Catalysis ............................................................................... 131
6.4. Concluding Remarks ......................................................................... 134

References ............................................................................................... 135
Appendix A. Supplementary Information for Chapter 2 .............................. 160
Appendix B. Supplementary Information for Chapter 5 ............................. 172
List of Tables

Table 1.1. FDA approved drugs for treating the symptoms of AD............................... 17
Table 1.2. Summary of various immunotherapeutic agents and their clinical trial results. .................................................................................................................. 18
Table 1.3. Physicochemical properties of FDA-approved amyloid imaging agents for AD. .................................................................................................................. 20
Table 2.1 Crystallographic data for [Cu(L1)Cl₂] and [Zn(L3)(NO₃)₂(H₂O)].............. 35
Table 2.2. pKₐ values and speciation at physiological pH as determined by UV-vis and NMR spectroscopy. .............................................................. 41
Table 3.1. Summary of the Lipinski’s rules for drug-likeness and determination of the LogBB to predict BBB permeability. ...................... 59
Table 3.2. pKₐ values and speciation at physiological pH as determined by variable pH UV-vis and NMR spectroscopy titrations. ................... 61
Table 4.1 Summary of the Lipinski’s rules for drug-likeness and determination of the LogBB to predict BBB permeability. ...................... 88
Table 4.2 Phenol-triazole pKₐ values and speciation at physiological pH as determined by variable pH UV-Vis and ¹H NMR spectroscopy data fit using HypSpec, HypNMR, and HySS............................................................... 91
Table 4.3 Summary of the stability constants determined via variable pH UV-vis titrations and fit using the programs HypSpec and HySS.......................... 94
List of Figures

Figure 1.1. Select examples of approved drugs that contain metal ions. .................... 2

Figure 1.2. Enzymatic processing of the APP leads to the formation of the Aβ peptide. APP is first cleaved by either α-secretase or β-secretase, leading to the sAPPα or sAPPβ cleavage product, respectively. If β-secretase cleaves a portion of the APP, Aβ will be produced upon final cleavage by γ-secretase. The Aβ monomer can aggregate under various physiological stimuli to create oligomers, fibrils, and finally, plaque deposits in the brain. 53 ........................................... 6

Figure 1.3. Cu²⁺-Aβ coordination is dependent on the pH environment. At pH < 8, Component I is the dominant coordination mode. Under basic conditions (pH ≥ 8), Component II predominates. ........................................... 12

Figure 1.4. General schematic of metal ion release and re-capture at the synaptic cleft. In the presynaptic neuron, a Zn-vesicle is loaded with Zn²⁺ and migrates to the cell surface to release Zn²⁺ into the synaptic cleft. Zn²⁺ binds to the NMDA-R receptor, which triggers a response to ATP7A to release Cu into the synaptic cleft. At the same time, APP processing is occurring, liberating the Aβ peptide into the synaptic cleft as well. It is possible for metallothionein-3 (MT-3) to sequester most of these metal ions, but if this process is impaired, then metal-Aβ interactions can occur, leading to Aβ aggregation. .............................................................................. 13

Figure 1.5. Chemical structures of four FDA-approved therapeutics for AD that aid in decreasing the rate of cognitive decline. ........................................... 17

Figure 1.6. Chemical structures of secretase inhibitors used to regulate the production of Aβ. AZD3293/LY3314814 and MK8931 target BACE while semagacestat was used as a γ-secretase inhibitor. All three compounds have been or are currently involved in clinical trials. .......... 19

Figure 1.7. Chemical structures of three FDA-approved Aβ plaque imaging agents........................................................................................................... 20

Figure 1.8. Metal-binding AD therapeutics that have undergone clinical trials. .......... 21

Figure 1.9. Summary of the three multifunctional metal-binding approaches used to combat AD. Linkage: A metal sequestration site anchored to an Aβ peptide-targeting vector. Incorporation: The Aβ peptide targeting vector has been modified to include a site for binding metals. Responsive: A non-active compound will be “turned-on” in the presence of a disease hallmark producing the active therapeutic agent................................................................. 22
Figure 1.10. Chemical structures of compounds that use the linkage approach towards treating AD. A benzothiazole moiety is used to direct the compound towards Aβ aggregates while the other end of the molecules has a metal binding site to regulate metal-Aβ interactions. ................................................................. 23

Figure 1.11. Chemical structures that utilize frameworks that are known to interact with Aβ species and incorporate a metal binding site to regulate metal-Aβ interactions. ........................................................................................................ 24

Figure 1.12. Chemical structures that mask a metal binding site. (Top) A boronate ester is released when exposed to H₂O₂ to afford a strong metal binding agent. (Bottom) A glycosylated deferiprone derivative shows improved brain uptake via GLUT1 transporters. When in the brain, hydrolysis by β-glucosidase will afford a metal binding site. .................................................................................................................. 25

Figure 1.13. Metal-based therapeutics that inhibit Aβ aggregation ........................................... 26

Figure 1.14. Chemical scaffolds that are presented in Chapters 2-5........................................... 28

Figure 2.1. Examples of bifunctional agents that incorporate or link a metal binding site into known Aβ-targeting vector such as Thioflavin-T (ThT) or (E)-Stilbene. ........................................................................................................ 30

Figure 2.2. Chemical structures of compounds L1 – L4 ................................................................ 31

Figure 2.3. Solution speciation studies of L2. (Left) Variable-pH UV-vis spectra of L2 (pH range 2 (Blue) – 12 (Red); [L2] = 50 µM). (Right) Solution speciation diagram for L2. ........................................................................................................ 41

Figure 2.4. UV-vis spectra of L1 (50 µM, black) incubated with metal ions (50 µM) in CH₃CN. L1 + CuCl₂ (Red), L1 + ZnCl₂ (blue). .................................................................................................................. 42

Figure 2.5. ORTEP diagram of [Cu(L1)Cl₂] showing 50% probability thermal ellipsoids. Selected bond lengths (Å) and angles (deg): Cu(1)–N(1) = 2.062(2); Cu(1)–N(2) = 2.024(3); Cu(1)–Cl(1) = 2.2647(8); Cu(1)–Cl(2) = 2.2417(9); Cu(1)–O(1) = 2.808(3); N(1)–Cu(1)–N(2) = 79.31(10); Cl(1)–Cu(1)–O(1) = 86.80(8); N(2)–Cu(1)–Cl(2) = 92.32(7); Cu(1)–Cl(1)–Cu(2) = 94.86(3); Cl(1)–Cu(1)–O(1) = 163.22(8). ................................................................. 43

Figure 2.6. ORTEP diagram of [Zn(L3)(NO₃)_2(H₂O)] showing 50% probability thermal ellipsoids. Selected bond lengths (Å) and angles (deg): Zn(1)–O(3) = 2.0348(19); Zn(1)–O(6) = 2.0864(18); Zn(1)–O(7) = 2.503(2); Zn(1)–O(9) = 2.1036(19); Zn(1)–N(1) = 2.174(2); Zn(1)–N(2) = 2.0749(19); O(3)–Zn(1)–O(6) = 88.49(8); O(3)–Zn(1)–O(7) = 142.21(8); O(6)–Zn(1)–O(7) = 54.70(7); O(3)–Zn(1)–O(9) = 95.61(8); O(6)–Zn(1)–O(9) = 92.02(7); N(1)–Zn(1)–N(2) = 76.36(7); O(3)–Zn(1)–N(2) = 129.71(8); O(7)–Zn(1)–N(2) = 86.56(7); O(9)–Zn(1)–N(1) = 166.67(7) ................................................................. 43
Figure 2.7.  NMR studies of L1 against $^{15}$N-labeled Aβ$_{1-40}$ in the SDS condition. (a) 2D $^1$H-$^1$N TROSY-HSQC NMR spectra of L1 (0, 1, 5, and 10 eq., indicated by black, blue, red, and green, respectively) with the peptide (200 mM SDS-d$_{25}$, 20 mM NaPi, pH 7.3, 7% v/v D$_2$O). (b) Calculated $^1$H and $^{15}$N chemical shifts of Aβ$_{1-40}$ in the presence of 10 eq. of L1. Peaks which could not be resolved, due to overlap or absence, are indicated by an asterisk (*). ................................................................. 45

Figure 2.8. Docking of small molecules with Aβ$_{1-40}$ monomer in the presence of SDS (PDB 1BA4)$^{272}$ predicted by AutoDock Vina. Cartoon depictions of (a) L1 (light blue), (b) L2 (yellow), (c) L3 (carboxylate form, magenta), and (d) L4 (protonated form, light pink) with Aβ$_{1-40}$ in the presence of SDS (PDB 1BA4) in the same confirmation. (e) Surface depiction of L1 – L4 interacting with Aβ$_{1-40}$. ................................................................. 46

Figure 2.9. Degree of Aβ$_{1-40}$ aggregation as measured by UV-vis measurements. Data represents the mean absorbance of quadruplicate trials at 405 nm of peptide in the presence of metal ions and ligands incubated for 45 mins. at 37 °C at pH 6.6 and 7.4 for Cu(II) and Zn(II), respectively. Error bars represent the standard deviation of the average absorbance value. See experimental section for details......................................................... 48

Figure 2.10. TEM images of samples incubated with 25 µM Aβ$_{1-40}$, 25 µM metal ions, and 50 µM L1 were incubated for 24 h at 37°C. Top: TEM images of Aβ$_{1-40}$ + CuCl$_2$, (left); Aβ$_{1-40}$ + CuCl$_2$ + L1 (right). Bottom: Aβ$_{1-40}$ + ZnCl$_2$ (left); Aβ$_{1-40}$ + ZnCl$_2$ + L1 (right)......................................................... 48

Figure 2.11. TEM images of the samples containing metal-free Aβ$_{1-40}$ species (25 µM) and L1 (50 µM). TEM images of metal-free Aβ$_{1-40}$ species (left) and of metal-free Aβ$_{1-40}$ species treated with L1 (right) (Conditions: 24 h at 37 °C). ......................................................................................... 49

Figure 3.1. A comparison between chemical scaffolds synthesized in Chapter 2 (pyridine) vs. Chapter 3 (quinoline). ................................................................. 51

Figure 3.2. Chemical structures of QOH (top), QMorph (middle), and QTMorph (bottom)............................................................................................................ 57

Figure 3.3. Degree of Aβ$_{1-40}$ aggregation in the presence of Cu determined by UV-vis measurements at 405 nm. Data represents the mean absorbance of quadruplicate trials. Error bars represent the standard deviation of the average absorbance values. Conditions: 150 µM ligand, 25 µM CuCl$_2$, 25 µM Aβ$_{1-40}$, 0.1 M PBS pH 6.6 buffer, incubation at 37°C under constant agitation for 45 mins. followed by measurement at 405 nm using a 96-well plate reader. ....... 58

Figure 3.4. (Top) Variable pH UV-vis spectra of QMorph (left), QOH (middle), and QTMorph (right) from pH 2 (red) – 12 (blue). (Bottom) Solution speciation diagrams of QMorph (left), QOH (middle), and QTMorph (right) indicating that each ligand is predominantly neutral at physiological pH (pH 7.4). ................................................................. 61
Figure 3.5. (Left) 2D SOFAST-HMQC NMR spectra of 80 µM Aβ_{1-40} and 0-10 eq. QMorph (Red = 0 eq. QMorph, Blue = 10 eq. QMorph). (Top Right) Chemical shift changes in relation to specific Aβ_{1-40} residues. The dotted line represents the average CSP for the entire experiment and the long dashed line represents one standard deviation above the average CSP. Residues denoted with * indicates the inability to resolve that specific residue. (Bottom Right) Solution NMR structure of Aβ_{1-40} (PDB 2LFM) with residues possessing a CSP > 0.02ppm highlighted in red and residues with a CSP between 0.01 – 0.02 are highlighted in yellow. ................................................................. 64

Figure 3.6. (Left) 2D SOFAST-HMQC NMR spectra of 80 µM Aβ_{1-40} and 0-10 eq. QT Morph (Red = 0 eq. QT Morph, Blue = 10 eq. QT Morph). (Top Right) Chemical shift changes in relation to specific Aβ_{1-40} residues. The dotted line represents the average CSP for the entire experiment and the long dashed line represents one standard deviation above the average CSP. Residues denoted with * indicates the inability to resolve that specific residue. (Bottom Right) Solution NMR structure of Aβ_{1-40} (PDB 2LFM) with residues possessing a CSP > 0.02ppm highlighted in red and residues with a CSP between 0.01 – 0.02 are highlighted in yellow. ................................................................. 66

Figure 3.7. (Left) 2D SOFAST-HMQC NMR spectra of 80 µM Aβ_{1-40} and 0-10 eq. QOH (Red = 0 eq. QOH, Blue = 10 eq. QOH). (Top Right) Chemical shift changes in relation to specific Aβ_{1-40} residues. The dotted line represents the average CSP for the entire experiment and the long dashed line represents one standard deviation above the average CSP. Residues denoted with * indicates the inability to resolve that specific residue. (Bottom Right) Solution NMR structure of Aβ_{1-40} (PDB 2LFM) with residues possessing a CSP > 0.02ppm highlighted in red and residues with a CSP between 0.01 – 0.02 are highlighted in yellow. ................................................................. 67

Figure 3.8. Chemical scaffolds that have been used in 2D SOFAST-HMQC NMR experiments to determine specific amino acid residue interactions with the Aβ_{1-40} peptide. .................................................................. 68

Figure 3.9. Comparison of all three quinoline derivatives using 2D SOFAST-HMQC NMR to determine which amino acid residues each quinoline-triazole interacts with. E3 and V18 appear to be shifted in all three cases. ................................................................. 68

Figure 3.10. Molecular docking pose of QMorph with the NMR solution structure of Aβ_{1-40} (PDB: 2LFM). (Left) The quinoline ring interacts with the side chain of V18, while the triazole ring is within the van der Waals radius of the E3 side chain. (Right) QMorph has a favourable pose that resides in a hydrophobic region on Aβ_{1-40}. Red = positively charged region; Blue = negatively charged region; Yellow = neutral region. ................................................................. 70
Figure 3.11. Molecular docking pose of QTMorph with the NMR solution structure of Aβ₁₄₀ (PDB: 2LFM). (Left) The quinoline ring interacts with the side chain of V18, while the triazole ring is within the van der Waals radius of the E3 side chain. (Right) QTMorph has a favourable pose that resides in a hydrophobic region of the Aβ₁₄₀ peptide. Red = positively charged region; Blue = negatively charged region; Yellow = neutral region. ................................. 71

Figure 3.12. Molecular docking pose for QOH with the NMR solution structure of the Aβ₄₀ peptide (PDB: 2LFM). The quinoline ring is found to be within the van der Waals radius of the H13 side chain along with the phenyl side chain of F20. The OH group of QOH is also within the van der Waals radius of the D23 side chain (left). Overall, QOH resides in a hydrophobic region of the Aβ₄₀ peptide (right). Red = positively charged region; Blue = negatively charged region; Brown = neutral region. ........................................... 72

Figure 3.13. Native gel electrophoresis of Aβ₁₄₂ in the absence and presence of varying equivalents of quinoline-triazole derivatives after incubation for 24 hours. No changes in the aggregation profile of Aβ₁₄₂ are observed by any of the quinoline-triazole derivatives. ............ 73

Figure 3.14. Native gel electrophoresis of Aβ₁₄₂ in the absence and presence of 1 eq. or 1.4 eq. Cu²⁺ and 5 eq. quinoline-triazole derivatives. Conditions: 25 µM Aβ₁₄₂, 25 (1 eq.) and 35 (1.4 eq.) µM Cu²⁺, 5 eq. ligand, PBS pH 7.4, 24 hour incubation at 37 °C. ............................................. 75

Figure 3.15. TEM analysis correlating the morphological changes of Aβ₁₄₂ in the absence and presence of Cu and ligands. A: Aβ₁₄₂; B: Aβ₁₄₂ + 1 eq. CuCl₂; C: Aβ₁₄₂ + 1.4 eq. CuCl₂; D: Aβ₁₄₂ + 1 eq. CuCl₂ + 5 eq. QOH; E: Aβ₁₄₂ + 1 eq. CuCl₂ + 5 eq. QMorph; F: Aβ₁₄₂ + 1 eq. CuCl₂ + 5 eq. QTMorph; G: Aβ₁₄₂ + 1.4 eq. CuCl₂ + 5 eq. QH; H: Aβ₁₄₂ + 1.4 eq. CuCl₂ + 5 eq. QMorph; I: Aβ₁₄₂ + 1.4 eq. CuCl₂ + 5 eq. QTMorph. .................................................................................. 77

Figure 4.1. Structure of the three phenol-triazole ligands, POH, PMorph, and PTMorph.................................................................................................................. 80

Figure 4.2. Coumarin-3-carboxylic acid (left) in the presence of Cu under aerobic aqueous conditions can react with hydroxyl radicals to produce the fluorescent 7-hydroxycoumarin-3-carboxylic acid (right). ........................................................................................................ 86

Figure 4.3. Job plot analysis of PMorph + CuCl₂. Various mole fractions of CuCl₂ and PMorph were combined in solution (PBS pH 7.4) and their absorbance at 320 nm measured to determine the most favourable stoichiometry between the metal and ligand. The intersection of the slopes is at 32% mole fraction Cu, suggesting a 2:1 ligand:Cu complex is present. ........................................................................... 89
Figure 4.4. (Top Row) Variable pH UV-Vis titration of PMorph (Left), POH (Middle), and PTMorph (Right) demonstrating a clear transition from the protonated phenol form (red) to a deprotonated phenolate (blue). (Middle Row) Using HypSpec, HypNMR and HySS, variable pH data was fit to determine the acidity constants and the overall speciation of each ligand. Each ligand is neutral at physiological pH. (Bottom Row) Chemical structures of PMorph (Left), POH (Middle), PTMorph (Right).

Figure 4.5. (Left) Variable pH UV-Vis titration of PMorph (75 µM) and CuCl$_2$ (37.5 µM) where the red spectra represents pH 2 and the blue spectra pH 12. (Right) Using HypSpec and HySS, the variable pH data was fit to a model including a 1:1 and 2:1 ligand:Cu species along with free Cu and a Cu(PMorph)$_2$OH component. At physiological pH 7.4, very little free Cu exists and a combination of 1:1 and 2:1 ligand:Cu species exist.

Figure 4.6. (Left) Variable pH UV-Vis titration of POH (75 µM) and CuCl$_2$ (37.5 µM) where the red spectra represents pH 2 and the blue spectra at pH 12. (Right) Using HypSpec and HySS, the variable pH data was fit to a model including a 1:1 and 2:1 ligand:Cu species along with free Cu and a Cu(POH)$_2$OH component. At physiological pH 7.4, very little free Cu exists and a combination of 1:1 and 2:1 ligand:Cu species exist.

Figure 4.7. (Left) Variable pH UV-Vis titration of PTMorph (75 µM) and CuCl$_2$ (37.5 µM) where the red spectra represents pH 2 and the blue spectra at pH 12. (Right) Using HypSpec and HySS, the variable pH data was fit to a model incorporating a 1:1 and 2:1 Ligand:Cu ratio along with free Cu and a Cu(PTMorph)$_2$OH component. At physiological pH 7.4, a small amount of free Cu exists and a combination of 1:1 and 2:1 Ligand:Cu species are primarily observed.

Figure 4.8. Comparison of pCu values for several reported phenol-based derivatives.\cite{210,215}

Figure 4.9. 2-D $^1$H-$^{15}$N SOFAST-HMQC NMR experiments using $^{15}$N-labeled Aβ$_{1-40}$ and 0-10 eq. PMorph. (Top) Chemical structure of PMorph. (Left) 2-D $^1$H-$^{15}$N SOFAST NMR spectra showing the assignment of specific amino acid residues in the Aβ$_{1-40}$ peptide. (Top Right) Summary of the specific amino acid residues that have shifted at 10 eq. PMorph. The dotted line represents the average CSP while the dashed line is the average + one standard deviation, which was used to identify which CSP were statistically relevant. (Bottom Right) Aβ$_{1-40}$ solution NMR structure highlighting specific amino acid residues that have significant CSP shifts (Red, > 0.02 ppm shift) and moderate CSP shifts (Orange, between 0.01 and 0.02 ppm).
Figure 4.10. 2-D $^1$H-$^{15}$N SOFAST-HMQC NMR experiments using $^{15}$N-labeled Aβ$_{1-40}$ and 0-10 eq. PTMorph. (Top) Chemical structure of PTMorph. (Left) 2-D $^1$H-$^{15}$N SOFAST NMR spectra showing the assignment of specific amino acid residues in the Aβ$_{1-40}$ peptide. (Top Right) Summary of the specific amino acid residues that have shifted at 10 eq. PTMorph. The dotted line represents the average CSP while the dashed line is the average + one standard deviation. (Bottom Right) Aβ$_{1-40}$ solution NMR structure highlighting specific amino acid residues that have significant CSP shifts (Red, > 0.03 ppm shift) and moderate CSP shifts (Orange, between 0.015 and 0.03 ppm). ............................................................ 97

Figure 4.11. 2-D $^1$H-$^{15}$N SOFAST-HMQC NMR experiments using $^{15}$N-labeled Aβ$_{1-40}$ and 0-10 eq. POH. (Top) Chemical structure of POH. (Left) 2-D $^1$H-$^{15}$N SOFAST NMR spectra showing the assignment of specific amino acid residues in the Aβ$_{1-40}$ peptide. (Top Right) Summary of the specific amino acid residues that have shifted at 10 eq. POH. The dotted line represents the average CSP while the dashed line is the average + one standard deviation, which was used to identify which CSP were statistically relevant. (Bottom Right) Aβ$_{1-40}$ solution NMR structure highlighting specific amino acid residues that have significant CSP shifts (Red, > 0.03 ppm shift) and moderate CSP shifts (Orange, between 0.02 and 0.03 ppm). ................................................................................. 99

Figure 4.12. Summary of the 2D $^1$H-$^{15}$N SOFAST NMR experiment for the phenol-triazole series of ligands, demonstrating which amino acid residues have CSP’s associated with each ligand. D7, F19, D23, G33, M35, and V36 were found to have statistically relevant CSP’s across all three investigated ligands. .................................................. 100

Figure 4.13. Docking pose of PMorph with the NMR solution structure of Aβ$_{1-40}$ (PDB: 2LFM). .................................................................................................. 101

Figure 4.14. Docking pose of PTMorph with the NMR solution structure of Aβ$_{1-40}$ (PDB: 2LFM). .................................................................................................. 102

Figure 4.15. Docking pose of POH with the NMR solution structure of Aβ$_{1-40}$ (PDB: 2LFM). .................................................................................................. 102

Figure 4.16. Native gel electrophoresis of Aβ$_{1-42}$ in the absence and presence of 1 eq. CuCl$_2$ and the absence and presence of 3 eq. ligand. Conditions: 25 µM Aβ$_{1-42}$, 25 µM CuCl$_2$, 75 µM ligand, 0.1 M PBS pH 7.4 buffer, 37 °C incubation for 24 hours. ........................................ 104

Figure 4.17. TEM images of Aβ$_{1-42}$ in the absence and presence of 1 eq. CuCl$_2$ and 3 eq. phenol-triazole ligand. Various morphologies are observed from fibrillar, uniform structures to amorphous aggregates. (1) Aβ$_{1-42}$ alone; (2) Aβ$_{1-42}$ + 1 eq. CuCl$_2$; (3) Aβ$_{1-42}$ + 3 eq. POH; (4) Aβ$_{1-42}$ + 3 eq. PMorph; (5) Aβ$_{1-42}$ + 3 eq. PTMorph; (6) Aβ$_{1-42}$ + CuCl$_2$ + 3 eq. POH; (7) Aβ$_{1-42}$ + CuCl$_2$ + 3 eq. PMorph; (8) Aβ$_{1-42}$ + CuCl$_2$ + 3 eq. PTMorph. .............................................................................. 106
Figure 4.18. Chemical structure of ABTS used in the TEAC assay. .......................... 107

Figure 4.19. Trolox-equivalent anti-oxidant capacity (TEAC) values at 1, 3, and 6 minutes for Trolox, PBT2, glutathione, and each phenol-triazole ligand. Each experiment was completed in triplicate and error bars represent the ± SD for the average TEAC values. .......................... 108

Figure 4.20. Under reducing conditions, O$_2$(g) is reduced by Cu$^+$ through several steps to the highly reactive hydroxyl radical. The hydroxyl radical will specifically react at the 7-position on coumarin-3-carboxylic acid to produce a fluorescent product. .......................... 109

Figure 4.21. A fluorogenic assay monitoring the production 7-hydroxyCCA over time. In the absence of any exogenous ligands, free Cu exhibits Fenton-like chemistry to produce hydroxyl radicals, which react with CCA to form a fluorescent product. When POH, PMorph, or PTMorph are introduced into a solution containing CCA, Cu, and ascorbate, a distinct inhibition in fluorescent product is observed. .......................... 110

Figure 5.1. Examples of metal-based Aβ aggregation inhibitors.......................... 113

Figure 5.2. Chemical structure of KP1019 .......................................................... 114

Figure 5.3 (Left) 24 (red) and 48 (blue) hour aggregation profiles of Aβ$_{1-42}$ (5 µM) analyzed by monitoring the ThT fluorescence (5 µM). Varying equivalents of KP1019/Aβ$_{1-42}$ (0.25 – 2 eq.) were compared against a known inhibitor (Congo red, 2 eq. as a control) of Aβ peptide aggregation. (Right) TEM images of Aβ$_{1-42}$ morphology sampled from the ThT assay after 48 hours of incubation. A: Aβ$_{1-42}$ only; B: Aβ$_{1-42}$ + 2 eq. Congo red; C: Aβ$_{1-42}$ + 0.25 eq. KP1019; D: Aβ$_{1-42}$ + 0.5 eq. KP1019; E: Aβ$_{1-42}$ + 1 eq. KP1019. Conditions: PBS pH 7.4 buffer, 37 °C, constant agitation for 48 hours. Scale bars are 500 nm. .......................................................... 119

Figure 5.4 Frozen-solution EPR spectra of KP1019 (1500 µM) and Aβ$_{1-28}$ (500 µM) incubated for 2 hours (left) and 24 hours (right) in PBS pH 7.4 buffer. The experimental spectra were deconvoluted into KP1019, and Aβ$_{1-28}$ coordinated KP1019 (KP1019-Aβ$_{1-28}$) spectral components via simulation. Experimental conditions: frequency 9.38 GHz microwave power 2.0 mW, time constant 40.96 ms, modulation amplitude 6 G, average of 10 scans of 2 minutes, temperature 20 K. .......................................................... 121

Figure 5.5 24 hour inhibition experiment of Aβ$_{1-42}$ (25 µM) aggregation probed by native gel electrophoresis/western blotting using the 6E10 primary antibody and TEM imaging. (Left) Lane 1 0.25 eq. KP1019/Aβ$_{1-42}$; 2: 0.5 eq. KP1019/Aβ$_{1-42}$; 3: 1 eq. KP1019/Aβ$_{1-42}$; 4: 2 eq. KP1019/Aβ$_{1-42}$; 5: Aβ$_{1-42}$ only (Right) A: Aβ$_{1-42}$ only; B: 0.25 eq. KP1019/Aβ$_{1-42}$; C: 0.5 eq. KP1019/Aβ$_{1-42}$; D: 1 eq. KP1019/Aβ$_{1-42}$. Scale bar = 500 nm. .......................................................... 123
Figure 5.6 Native gel electrophoresis/western blotting of the Aβ1-42 disaggregation experiment. Aβ1-42 (25 µM) was incubated for 24 hours, followed by treatment with 0.25 – 2 eq. KP1019 for a further 24 hours. (Left) Lane 1 0.25 eq. KP1019/Aβ1-42; 2: 0.5 eq. KP1019/Aβ1-42; 3: 1 eq. KP1019/Aβ1-42; 4: 2 eq. KP1019/Aβ1-42; 5: Aβ1-42 only (Right) A: Aβ1-42 only; B: 0.25 eq. KP1019/Aβ1-42; C: 0.5 eq. KP1019/Aβ1-42; D: 1 eq. KP1019/Aβ1-42. Scale bar = 500 nm.

Figure 5.7. Toxicity studies using differentiated SH-SY5Y human neuroblastoma cells. Cells were treated with Aβ1-42 (20 µM) or pre-incubated KP1019-Aβ1-42 for 24 hours and then the cell viability measured using an Alamar Blue assay. Varying equivalents of KP1019-Aβ1-42 were incubated for 5 minutes (yellow), 2 hours (orange), or 24 hours (red) and then exposed to cells for a further 24 hours. Alamar Blue reagent was then incubated with cells for 2 hours at 37 °C, followed by fluorescence measurements to determine cell viability. Data are means ± SEM; n = 4. The t-test analysis of experimental data compared to Aβ1-42 identifies values of p < 0.0001 except for *p < 0.1.

Figure 6.1. Incorporation of a dimethylamino-moiety will aid in targeting and interacting with Aβ species.

Figure 6.2. Several examples of Ru(III) complexes that have demonstrated hydrophobic interactions with human serum albumin.

Figure 6.3. Several Ru(III) complexes that incorporate a ligand that has an affinity for Aβ species.

Figure 6.4. General scheme of using Cu-Aβ species as a catalyst to produce active therapeutic/diagnostic tools.

Figure 6.5. Fluorogenic assay evaluating Cu-Aβ1-40 species as a catalyst for the production of a triazole-based fluorophore. Conditions: 100 µM N3/Alkyne, 20 µM Cu2+, 0 and 20 µM Aβ1-40, 20 µM ascorbate, constant agitation in a 96-well plate reader at 37 °C. Each experiment was performed in quadruplicate.

Figure 6.6. (Black) HPLC trace of 2-ethynylpyridine with a retention time of 8.2 minutes. (Red) HPLC trace of reaction mixture of 1:1 N3/Alkyne (125 mM), 1:1 Cu2+:Aβ1-40 (50 µM), and 1 mM ascorbate after incubation for 60 minutes. (Blue) HPLC trace of synthesized L1 product with a retention time of 2.8 minutes.
**List of Acronyms**

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<th>Acronym</th>
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<tr>
<td>$^{1}$H</td>
<td>Proton</td>
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<tr>
<td>2D</td>
<td>Two-dimensional</td>
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<tr>
<td>Å</td>
<td>Angstrom</td>
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<tr>
<td>ABTS</td>
<td>2',2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)</td>
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<td>ACh</td>
<td>Acetylcholine</td>
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<td>Acetylcholinesterase</td>
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<tr>
<td>AChR</td>
<td>Acetylcholine receptor</td>
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<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<td>BSA</td>
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<td>CCA</td>
<td>Coumarin-3-carboxylic acid</td>
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<td>CDCl$_3$</td>
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<tr>
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<td>Carrier-mediated transporter</td>
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<td>Desferrioxamine</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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dt  Doublet of triplets
DTPA  Diethylenetriaminepentaacetic acid
E  Glutamic acid
EPR  Electron paramagnetic resonance
Et₂O  Diethyl ether
F  Phenylalanine
FDA  Food and Drug administration
G  Glycine
GLUT-1  Glucose transporter-1
H  Histidine
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMQC  Heteronuclear multiple quantum coherence
HR-ESI-MS(+)  High-resolution electrospray ionization mass spectrometry positive mode
hsA  Human serum albumin
HSQC  Heteronuclear single quantum coherence spectroscopy
hsTf  Human serum transferrin
Hz  Hertz
I  Isoleucine
iPrOH  Isopropanol
J  Coupling constant
K  Kelvin
K₂CO₃  Potassium carbonate
L  Leucine
M  Methionine
m/z  Mass/Charge
MAP  Microtubule-associated protein
MeOH  Methanol
MHz  Megahertz
mM  Millimolar
MRI  Magnetic resonance imaging
MT-3  Metallothionein-3
M_w  Molecular weight
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<td>N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>ND</td>
<td>Neurodegenerative diseases</td>
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<tr>
<td>NEt&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Triethylamine</td>
</tr>
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<td>NFT</td>
<td>Neurofibrillary tangles</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<td>Trflate</td>
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<tr>
<td>PAMPA</td>
<td>Parallel artificial membrane permeability assay</td>
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<tr>
<td>PAS</td>
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<td>Phosphate-buffered saline</td>
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<td>Protein database</td>
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<td>Presenilin enhancer-2</td>
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<td>Positron emission tomography</td>
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<td>PHF</td>
<td>Paired helical filaments</td>
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<tr>
<td>ppm</td>
<td>Parts per million</td>
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<tr>
<td>pTau</td>
<td>Phosphorylated tau protein</td>
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<td>Arginine</td>
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<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>Retardation Factor</td>
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<td>RMT</td>
<td>Receptor-mediated transporter</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>Singlet</td>
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<td>Serine</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SDS-&lt;sup&gt;d&lt;/sup&gt;25</td>
<td>Deuterated sodium dodecyl sulphate</td>
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<tr>
<td>SEM</td>
<td>Standard error measurement</td>
</tr>
<tr>
<td>SOFAST</td>
<td>Band-selective optimized-flip-angle short-transient</td>
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<tr>
<td>t</td>
<td>Triplet</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>TBS-T</td>
<td>Tris-buffered saline containing 0.1% Tween-20</td>
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<td>td</td>
<td>Triplet of doublets</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox-equivalent antioxidant capacity</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
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<td>Thioflavin-T</td>
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<tr>
<td>TPSA</td>
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<td>Triazole-pyridine</td>
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<td>Valine</td>
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<td>Y</td>
<td>Tyrosine</td>
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<tr>
<td>µ-PIXE</td>
<td>Micro-particle-induced X-ray emission</td>
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Chapter 1. Introduction


1.1. Medicinal Inorganic Chemistry

The application of inorganic chemistry in medicine has provided an alternative toolbox for scientists to use in diagnosing and treating a wide range of diseases. Medicinal inorganic chemistry can be divided into two main categories: drugs that target metal ions in some form, or metal-based drugs in which the central metal ion is essential for the clinical application.\(^1\)\(^2\) The era of modern medicinal inorganic chemistry began with the discovery of *cis*-platin in 1969 as an antitumour agent against both sarcoma 180 and leukaemia L1210 tumor cells in mice (Figure 1.1).\(^3\) Due to this initial landmark study, *cis*-platin is now used as a front-line treatment for several cancers including testicular, lung, ovarian, and cervical cancers.\(^4\) Two other Pt-containing compounds were later developed with similar efficacies and reduced side effects.\(^5\) Other precious metals have long been used in therapeutic applications, and as an example, Au-based therapies have been well documented in ancient Chinese medicine.\(^6\)\(^7\) More recently, Auranofin was developed in the 1930’s as a treatment for rheumatoid arthritis (Figure 1.1).\(^8\) By inhibiting the enzymatic activity of thioredoxin reductase, a number of Au complexes have shown promise as therapeutics for several cancers, rheumatoid arthritis, and Sjögren’s syndrome.\(^9\) Alternately, many diagnostic agents exploit the physical properties
of metal ions to provide information in a non-invasive manner. For example, nuclear medicine utilizes several metal isotopes ($^{99m}$Tc, $^{64}$Cu, $^{67/68}$Ga) for clinical diagnoses. Another class of metal-based diagnostics are the Gd$^{3+}$ magnetic resonance imaging (MRI) contrast agents such as Magnevist™ (Figure 1.1). These agents take advantage of the magnetic properties associated with the Gd$^{3+}$ ion to image the body in a non-invasive manner.

![cis-Platin, Auranofin, Magnevist™](image)

**Figure 1.1.** Select examples of approved drugs that contain metal ions.

Metal ions have essential roles in the human body providing charge balance, facilitating electron transport, and catalyzing enzymatic transformations. For each application, the metal cation and the atoms immediately surrounding the metal cation (i.e.: the coordination sphere) are tuned specifically. The type, number, and geometry of the ligands (square planar, tetrahedral, octahedral, etc.), commonly in the form of amino acid side-chains, ensure that the active site is maintained. Natural systems provide much of the inspiration for the strategies employed by medicinal inorganic chemists. Thus, the design of active agents uses many of the same features present in biological systems to stabilize metal complexes. In medicinal inorganic chemistry, ligands can be designed to bind the metal ion of interest with appropriate strength, thus regulating ligand dissociation and potential *in vivo* toxicity in the presence of endogenous chelating substances such as phosphate, citrate, and biomolecules such as albumin and transferrin. The ligand plays an integral role in tailoring the agent for the specific application, which allows for the optimization of drug parameters. Kinetics of complex formation, thermodynamic stability, water solubility, overall charge, and the pathway of excretion from the body are all important parameters to consider in the design process. Building on these design elements, recent research has centred on including added
functionality to ligands for specific applications. A multifunctional ligand not only binds a metal ion of choice, but also includes additional features to enhance targeting, reporting, and overall efficacy. The identification of new disease targets and bioactive molecules will continue to drive developments in this area of research.

Continued research into the uptake, transport, and utilization of metal ions in the body has enabled the development of many disease treatment strategies that target and regulate bio-metal activity. Dysregulation of metal ions in the human body can lead to the development of several diseases, such as haemochromatosis, Wilson’s disease, and Huntington’s disease.\textsuperscript{12-14} The dysregulation of metal ions is implicated in several neurodegenerative diseases.\textsuperscript{15} A strategy to combat these diseases is to develop agents that are able to redistribute these metal ions in order to re-establish their proper homeostases.

The development of chemical agents that either utilize the metal centre for medicinal applications or that target metal ions due to a disease state requires a ligand scaffold that will enhance the pharmacokinetic properties of the agent. This thesis focuses on the development of small molecule agents towards the treatment of Alzheimer’s disease (AD). Specifically, chapters 2-4 explore the development of three separate multifunctional ligand scaffolds and their ability to influence metal-peptide interactions associated with AD. Chapter 5 describes how a Ru complex binds to a peptide associated with AD and modulates its neurotoxicity.

1.2. Inorganic Chemistry in Neurodegenerative Disease

Over the past several decades, deaths caused by cancers and stroke have declined while the prevalence of neurodegenerative diseases (ND’s) has increased.\textsuperscript{16} Due to improved healthcare, life expectancy has increased, which has resulted in the increased occurrence of ND’s, such as AD, Parkinson’s disease (PD), and amyotrophic lateral sclerosis (ALS). Currently, there is a serious shortage of effective drugs capable of treating ND’s, and in many cases a definitive diagnosis is only possible post mortem. Without the development of effective therapies, the social and economic burden associated with ND’s may overwhelm our healthcare resources.\textsuperscript{16}
Understanding the roles that metal ions play in the brain (metalloneurochemistry) is a complex area of research that has emerged over the past several decades.\textsuperscript{17-19} When metal ions in the brain become dysregulated, the development of neurodegenerative diseases is possible. The natural folding process of biomolecules such as proteins can be seriously altered in the presence of dysregulated metal ions.\textsuperscript{20} The interaction of dysregulated metal ions with peptides or proteins can lead to a loss of enzymatic function or accelerate aggregation or precipitation, leading to metallic pools that are no longer bioavailable. Metal homeostasis in the human body is critical, especially in the brain, due to the high rate of metabolic activity coupled with relatively low antioxidant levels and low tissue regenerative capacity.\textsuperscript{21}

A number of neurodegenerative diseases have been linked with metal ion dysregulation.\textsuperscript{15, 22} A common factor between neurodegenerative diseases and metal ions is that the interactions of metals with specific biomolecules leads to the aggregation and formation of insoluble structures. For example, PD is characterized by the loss of dopaminergic neurons in the \textit{substantia nigra} and by the formation of inclusion bodies, called Lewy bodies, composed of the protein $\alpha$-synuclein, which have been shown to contain high concentrations of Fe$^{3+}$.\textsuperscript{23-25} AD is characterized by the formation of amyloid plaque deposits, which have been shown to contain elevated concentrations of Cu, Zn, and Fe.\textsuperscript{26-28} As the human body ages, the ability to regulate metal homeostasis naturally decreases, which leads to further prevalence of these diseases.\textsuperscript{15}

### 1.3. Hallmarks and Hypotheses of AD

#### 1.3.1. Characteristics of AD

AD was first described in the literature by the psychiatrist Dr. Alois Alzheimer in 1907 when he detailed the observation of “a peculiar severe disease process of the cerebral cortex”.\textsuperscript{29, 30} Here, Alzheimer documented a female patient who was admitted into a psychiatric hospital in November 1901 and who passed away in April, 1906.\textsuperscript{30} The female patient appeared to have significant cognitive decline, memory impairment, and drastic mood changes. When the female patient had passed away, Dr. Alzheimer performed an autopsy where the two hallmarks of AD were observed, which were...
ultimately determined much later to be extracellular plaques, which are composed of the amyloid-β (Aβ) peptide and intracellular neurofibrillary tangles (NFT), which result from the hyperphosphorylation of the protein, tau (pTau).31, 32

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; Aβ-plaques and neurofibrillary tangles.33 Neurofibrillary tangles are intracellular fibrillar aggregates of oxidatively-modified and hyperphosphorylated microtubule associated protein tau.34 Aβ-plaques are extracellular deposits of fibrils and amorphous aggregates of the Aβ peptide (vide infra).31 It is still unclear as to whether Aβ-plaques, neurofibrillary tangles, or both, are a cause or an effect of the neurodegeneration in AD.35 Recent work using animal models has linked increased oxidative stress to the formation of both of these pathological features of AD,21, 36 and an effective therapeutic agent will likely need to influence both Aβ- and tau-mediated pathologies.37 The Aβ peptide is a proteolytic product of the amyloid precursor protein (APP), an integral membrane glycoprotein38 that may play a role in metal (Cu/Fe) trafficking.39 The enzymes responsible for APP cleavage are termed the α-, β-, and γ-secretases,40 and through a series of cleavage events afford the Aβ peptide as predominantly Aβ1-40 or Aβ1-42 (a 40- or 42-residue peptide) (Figure 1.2). The Aβ peptide has been found in three general forms in the brain; membrane associated, aggregated, and soluble.41 In healthy individuals, most of the Aβ is membrane-associated, but in individuals with AD the aggregated and soluble fractions increase considerably. 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.42-46 Soluble forms of Aβ better correlate with memory impairment and AD progression, however, nearly all aggregated forms exhibit toxicity.47, 48 Recent studies have shown that Aβ aggregates modify the functional properties of nearby neurons,49 and that Aβ clearance following bexarotene administration in murine models leads to behavioural improvement.50

The Aβ peptide is produced via sequential enzymatic cleavage events on the transmembrane amyloid-precursor protein (APP) (Figure 1.2). APP is acted upon by α-secretase or β-secretase to produce either sAPPα or sAPPβ fragments, respectively. In
the case of α-secretase activity, this produces a non-amyloidogenic, non-pathogenic fragment, whereas when β-secretase cleaves the initial APP fragment, this allows γ-secretase to release the final peptide fragment producing Aβ. Various Aβ peptide lengths can be produced ranging from 38-43 amino acid residues, with the 40 and 42 residue lengths being the most common. While Aβ1-40 is the most common form found in the brain, the Aβ1-42 form is more aggregation-prone, due to the two additional hydrophobic amino acid residues (isoleucine and alanine), and is more toxic.20, 27, 31, 51, 52

The Aβ aggregation process is sensitive to many factors, and has been shown to form dimers, trimers, oligomers, fibrils, and, eventually plaques.

![Enzymatic processing of the APP](image)

**Figure 1.2.** Enzymatic processing of the APP leads to the formation of the Aβ peptide. APP is first cleaved by either α-secretase or β-secretase, leading to the sAPPα or sAPPβ cleavage product, respectively. If β-secretase cleaves a portion of the APP, Aβ will be produced upon final cleavage by γ-secretase. The Aβ monomer can aggregate under various physiological stimuli to create oligomers, fibrils, and finally, plaque deposits in the brain.53
Comparatively, the formation of neurofibrillary tangles (NFT) due to tau hyperphosphorylation appears to track closely with clinical symptoms and correlates well with neuronal loss.\textsuperscript{27, 34, 51, 54} The tau protein is responsible for stabilizing microtubule pathways that shuttle nutrients to the neuron.\textsuperscript{55} Upon tau phosphorylation, the protein is liberated from the microtubule scaffold.\textsuperscript{34, 56} If tau is hyperphosphorylated, it can aggregate to form paired helical filaments and eventually produce NFTs, which leads to impaired synaptic plasticity and axonal transport.\textsuperscript{57}

1.3.2. **Amyloid Cascade Hypothesis**

The amyloid cascade hypothesis posits that the production, and resulting aggregation, of the A\(\beta\) peptide is a causal event in the AD pathway.\textsuperscript{33, 58, 59} This hypothesis was first described in the early 1990’s as one of the first rationales for AD etiology.\textsuperscript{59} As research has continued towards developing an AD therapeutic agent using the A\(\beta\) peptide as a target, the amyloid cascade hypothesis has been re-evaluated several times to determine the validity and viability of this strategy.\textsuperscript{60, 61} The production of A\(\beta\), as has been previously described (Figure 1.2), stems from several enzymatic cleavage steps. Once formed, the aggregation of the peptide into various multimers is proposed to be the source of its neurotoxicity. Specifically, the formation of A\(\beta\)-derived diffusible ligands\textsuperscript{62} or soluble, toxic A\(\beta\) oligomers\textsuperscript{63, 64} are thought to be the primary culprits. A\(\beta\) aggregation and accumulation is a result of irregular processing and clearance from the brain parenchyma. Three strategies have been used to target A\(\beta\)-specific pathways:

1. Decrease A\(\beta\) production via inhibition of \(\beta\)- or \(\gamma\)-secretase

2. Expedite A\(\beta\) clearance mechanisms

3. Inhibit the formation of toxic A\(\beta\) aggregates

In order to address AD from the point of view of A\(\beta\) production, several key concerns also need to be addressed. Firstly, determining the extent that A\(\beta\) production should be lowered in order to elicit a therapeutic response is pivotal in developing specific goals for therapeutic agents. Also, it is necessary to determine at what stage of
AD progression that an Aβ-targeting agent will have the most efficacy. One of the drawbacks of the amyloid cascade hypothesis is that Aβ plaque pathology does not correlate well with neuronal impairment and is not able to address the enigmatic relationship between the formation of Aβ aggregates and Tau/pTau. Although Aβ plaque deposition may be present in the brain, this does not always correlate with neuronal impairment. More recently it was reported that the formation of Aβ oligomers correlates with neuronal impairment and toxicity, suggesting that regulating oligomer production could be a therapeutic target.

As the balance between Aβ production vs. processing sways towards the former, hyperaccumulation of Aβ can lead to aggregation and toxicity. Numerous reports have documented the Aβ aggregation process similarly to other protein assembly events where a lag or nucleation phase initiates the process, followed by an elongation phase, and eventually terminating with a plateau or accumulation phase. The lag or nucleation phase is extremely important, as these early events will help to define the aggregation pathway. Several pathways are possible that lead to aggregates with varying molecular weights, morphologies, and neurotoxicity. Although postmortem analysis characterizes AD via the formation of Aβ plaque deposits, as this is thought to be the plateau stage of aggregation, several recent reports suggest that plaques are not the toxic species that causes neuronal impairment throughout the brain. Instead, the formation of soluble Aβ oligomers is thought to play a significant role in the neurotoxicity of AD. The imbalance of Aβ production/clearance is an important factor in AD development; however, the elucidation of the exact mechanism still remains to be discovered. Due to the fact that Aβ aggregation is one of the earliest characterized events associated with AD, many research groups continue to target this hallmark of the disease.

1.3.3. Tau Hypothesis

Tau is an ensemble of six isoforms associated with the microtubule-associated protein (MAP) family. The Tau hypothesis states that the abnormal hyperphosphorylation of the Tau protein leads to the formation of paired helical filaments (PHF) and eventually into NFT, which are found intracellularly in cognitively impaired AD
brains.\textsuperscript{70} Tau plays a role in the structural stabilization of microtubules that are present along the neuronal axon, essentially acting like glue.\textsuperscript{34, 51, 55, 56, 69} When bound to the microtubules, Tau is stabilized allowing for the transportation of nutrients, neurotransmitters, and organelles to be delivered to the neuron and as a dendritic scaffold to facilitate communication between neighbouring neurons. Tau binding to the microtubule is regulated by phosphorylation events.\textsuperscript{71} When Tau is phosphorylated (pTau) it is released from the microtubule and vice-versa. Upon hyperphosphorylation, pTau begins to aggregate into oligomers and amorphous aggregates, eventually maturing into PHF, which are the primary component in NFT's. Tau is essential for the survival of the neuron due to the trafficking of nutrients and signalling agents, and if the neuron is starved due to a decrease in nutrient availability, autophagosomes are produced due to autophagy.\textsuperscript{72}

Several strategies targeting Tau-related pathways associated with AD have been reported.\textsuperscript{73} The development of kinase inhibitors have shown promise, however there are still unknowns associated with which kinases phosphorylate Tau and what are their other substrates. Anti-aggregation agents have also been developed, such as methylene blue, which demonstrated promise in a phase II clinical trial.\textsuperscript{74, 75} Another strategy is to stabilize the microtubules via a similar approach used in cancer. Paclitaxel (Taxol) has been used as a microtubule-stabilizing agent to treat cancer.\textsuperscript{76} One of the difficulties with this approach is the lack of agents that are capable of penetrating the blood-brain barrier (BBB). Another naturally occurring microtubule stabilizing agent is Epothilone D, which was shown to bind to microtubules and stabilize axonal transport.\textsuperscript{77, 78} Epothilone D is able to compete with Tau for microtubule binding and is BBB permeable. With increased research focus on the screening of other natural products that are capable of stabilizing microtubules and/or acting as kinase inhibitors, and that are BBB permeable, a therapeutic agent addressing Tau pathology is hopefully close at hand. Due to the multiple facets associated with AD, a successful disease-modifying drug will have to target both Aβ and Tau hallmarks.
1.3.4. **Cholinergic Hypothesis**

One method of neuronal communication uses the release of acetylcholine (ACh) from the presynaptic neuron and binding to a variety of acetylcholine receptors (AChR) on the postsynaptic neuron. Regulation of this signal is achieved by acetylcholinesterase (AChE) that hydrolyzes the ester bond to produce acetate and choline. Acetylcholine is one of the most important neurotransmitters in the human body and is responsible for maintaining cognition. The cholinergic hypothesis states the correlation between AD-associated dementia and reductions in cortical cholinergic markers (ie: muscarinic and nicotinic AChR, choline acetyltransferase, and acetylcholine) decreases cholinergic activity, which could be associated with memory impairment in AD.\(^\text{79-84}\) A study using positron emission tomography (PET) imaging confirmed that abnormalities in AChR expression occurs in AD patients.\(^\text{85}\)

AChE has also been shown to co-localize in the presence of Aβ plaque deposits.\(^\text{86}\) Several reports studying the *in vitro* relationship between Aβ, AChE, and neurotoxicity have been discussed in the literature.\(^\text{87-91}\) Specifically, the peripheral anionic site (PAS) in AChE interacts with Aβ, increasing its propensity to aggregate, augmenting its neurotoxicity.\(^\text{86, 91, 92}\) Several strategies have attempted to develop AChE inhibitors that target the PAS in order to inhibit AChE activity and to modulate AChE-Aβ interactions.\(^\text{93-96}\)

Nicotinic AChR regulates the production of Aβ by playing a role in APP processing.\(^\text{97, 98}\) Specifically, the sAPPα fragment is favoured when increased expression levels of nicotinic AChR occur. This study was corroborated when Tg2576 transgenic mice, which exhibit increased Aβ plaque deposition, were chronically supplemented with nicotine and shown to have reduced Aβ loads in their brains.\(^\text{99}\) Furthermore, a study of elderly individuals who smoked also exhibited reduced plaque burden in the brain.\(^\text{100}\)

Although a clear interaction between Aβ and AChR has been demonstrated *in vitro and in vivo*, debate still remains whether this is a causal factor in AD pathology or a secondary effect due to earlier trauma. Based on this work, a series of AChE inhibitors have been approved as early-stage treatments for AD (see Section 1.4.1).
1.3.5. **Metal Ion Hypothesis**

Metal ion homeostasis is extremely crucial in the body as metal ions are responsible for electron transfer processes\(^\text{101}\), structural roles\(^\text{102}\), signalling pathways\(^\text{15}\), and O\(_2\) transport.\(^\text{103}\) As such, when the dysregulation of metal ions occurs, severe consequences may result. The metal ion hypothesis states that the dysregulation of metal ions in the brain leads to peptide aggregation and the formation of neurotoxic species.\(^\text{15, 20, 54, 104-106}\) In combination, these factors help to explain several components of AD etiology.

A seminal study performed by Lovell and co-workers demonstrated that senile plaques and neuropils from AD brain tissue contain elevated concentrations of Cu, Zn, and Fe.\(^\text{26}\) Micro-Particle-Induced X-ray Emission (µ-PIXE) analysis of the rim and cores of senile plaques showed metal ion concentrations of 400 µM Cu, 1000 µM Zn, and 900 µM Fe, which are 3-5x higher in comparison to age-matched controls. Following this report, several investigations to characterize the metal ion interaction with the A\(_\beta\) peptide were initiated and a large body of work has been produced.\(^\text{28, 107-109}\) The variability of the literature is primarily due to the differences in buffer, pH, ionic strength, metal oxidation state, physical method of analysis, and peptide length and aggregation state used. The current consensus is that the Cu\(^{2+}\)-A\(_\beta\) interaction is dynamic and pH-dependent, resulting in the formation of two types of species called Component I and Component II (Figure 1.3).\(^\text{107, 109-112}\) At physiological pH 7.4 and lower, Component I is the dominant species containing three nitrogen and one oxygen donor atoms from the peptide.\(^\text{113-117}\) The three nitrogen donors are histidine (H) residues residing in the N-terminus (H\(_6\), H\(_{13}\), and H\(_{14}\)), or two histidine residues and the terminal amine nitrogen. The oxygen donor is either a carboxylate from aspartic acid – 1 (D1) or a backbone carbonyl in between residues D1 and alanine - 2 (A2). At pH 8, Component II is the dominant species containing a different three nitrogen, one oxygen coordination sphere.\(^\text{39, 108, 112-114}\) Two possible coordination modes are possible; one contains all three histidine residues and the carbonyl of A2, or one histidine residue, a terminal amine nitrogen, deprotonated backbone amide between D1 and A2, and the backbone carbonyl between A2 and glutamic acid – 3 (E3). A Cu\(^{+}\)-A\(_\beta\) species has been identified using X-ray absorption spectroscopy (XAS), which suggests a linear bis-histidine (two of H\(_6\), H\(_{13}\), or H\(_{14}\)
where two residues are bound at one time.\textsuperscript{118} Zn\textsuperscript{2+}-A\textsubscript{β} species have been studied and are also dynamic systems.\textsuperscript{106, 109, 119-122} Four and six coordinate geometries have been identified where all three histidine residues are bound, along with other possible ligands including either the terminal amine nitrogen or the side chain carboxylate of D1, backbone amide of arginine – 5 (R5), tyrosine - 10 (Y10), E11, or aqua ligands.

\textbf{Figure 1.3.} Cu\textsuperscript{2+}-A\textsubscript{β} coordination is dependent on the pH environment. At pH < 8, Component I is the dominant coordination mode. Under basic conditions (pH ≥ 8), Component II predominates.

Determining the dissociation constant, $K_d$, for each metal with A\textsubscript{β} is very important. Due to the dynamic coordination of each metal ion, and propensity for aggregation, a range of $K_d$ values have been reported.\textsuperscript{123} The Cu\textsuperscript{2+}-A\textsubscript{β} dissociation constant has been reported in the range of $K_d = 10^{-11} - 10^{-7}$ M for 1:1 complexation.\textsuperscript{107, 109, 124-126} Cu\textsuperscript{+}-A\textsubscript{β} complexes have also been reported but are dependent on the length of the peptide variant.\textsuperscript{127} Slightly weaker dissociation constants have been reported for
Zn$^{2+}$-Aβ species in comparison to Cu$^{2+}$, ranging from $K_d = 10^{-9} - 10^{-6}$ M.\textsuperscript{121, 122, 128-130}

Although weak binding of Aβ with Fe$^{2+}$ has been reported, no free Fe has been found bound to the peptide in plaque; instead, co-localization of Fe has been observed associated with other metalloenzymes (eg: ferritin, transferrin, etc.).\textsuperscript{131-134}

Metal ion homeostasis is tightly regulated by metallochaperones throughout the body due to the fact that several metal ions can generate reactive oxygen species (ROS) when not tightly bound, which can lead to oxidative stress. In the brain, the pre-synaptic neuron can release up to 300 μM Zn$^{2+}$ into the synaptic cleft during normal signalling processes (Figure 1.4).\textsuperscript{15, 135-137}

![Figure 1.4.](image)

**Figure 1.4.** General schematic of metal ion release and re-capture at the synaptic cleft. In the presynaptic neuron, a Zn-vesicle is loaded with Zn$^{2+}$ and migrates to the cell surface to release Zn$^{2+}$ into the synaptic cleft. Zn$^{2+}$ binds to the NMDA-R receptor, which triggers a response to ATP7A to release Cu into the synaptic cleft. At the same time, APP processing is occurring, liberating the Aβ peptide into the synaptic cleft as well. It is possible for metallothionein-3 (MT-3) to sequester most of these metal ions, but if this process is impaired, then metal-Aβ interactions can occur, leading to Aβ aggregation.
N-methyl-D-aspartic acid receptors (NMDA-R) located on the post-synaptic cleft bind Zn\(^{2+}\) triggering the activation of the Cu-transporter, ATP7A, to release up to 30 µM Cu\(^{n+}\) (n = 1 or 2) into the synaptic cleft.\(^{138}\) In order to sequester these metal ions, metallothionein-3 (MT-3) is released into the cleft.\(^{139}\) At the same time, APP processing releases Aβ peptides into the synaptic cleft, and if the regulation of metal ions is not maintained, interactions between Cu\(^{2+}\) or Zn\(^{2+}\) with Aβ are possible.\(^{27, 140, 141}\)

Upon coordination of Aβ to metal ions, aggregation of the peptide commences immediately.\(^{28, 107, 142}\) Depending on the metal:Aβ stoichiometry, several aggregation states are possible with different levels of neurotoxicity.\(^{143}\) When Aβ aggregates in the absence of metal ions, definitive fibrillar structures are easily observed using electron microscopic techniques (ie: transmission electron microscope (TEM) or atomic force microscopy (AFM)).\(^{144, 145}\) Upon aggregation of Aβ in the presence of metal ions (ie: Cu or Zn), fibrillar structures are almost completely abolished and exclusive formation of amorphous aggregates are observed.\(^{67}\) Although the overall morphology of these aggregates in the presence of metal ions is similar, the molecular weight of these aggregates can vary significantly depending on the conditions.

Pedersen and co-workers compared the resulting Aβ aggregates depending on the initial Cu\(^{2+}\):Aβ stoichiometry.\(^{146}\) When [Cu\(^{2+}\)] < [Aβ], rapid formation of a nuclear seed occurs followed by a slow elongation phase. In the case of equimolar concentrations, Cu\(^{2+}\)-Aβ oligomeric species dominate, producing amorphous aggregates and eventually producing spherical oligomers at the plateau stage. In the presence of excess Cu\(^{2+}\), ca. 1.4 eq. Cu\(^{2+}\) is able to bind to Aβ resulting in a mixture of oligomeric and high molecular weight species.\(^{146, 147}\) These data suggest that there is a second, weaker affinity Cu\(^{2+}\) binding site on the peptide, which plays a role in Aβ aggregation. In order to provide further evidence into the type of species that are produced, conformationally-specific antibodies that can distinguish between oligomeric and fibrillar structures were employed. Anti-amyloid fibril (OC) and anti-amyloid oligomer (A11) antibodies were used to identify the presence of fibrillar or oligomeric species, respectively.\(^{148, 149}\)
In comparison to Cu levels in the brain, considerably higher Zn concentration occurs due to its role in signalling pathways. As with Cu, Zn-induced Aβ aggregation also leads to the formation of amorphous aggregates.\(^{143}\) Even when Zn-Aβ species are exposed to conditions that favour fibril formation, amorphous aggregates are maintained, suggesting that Zn-Aβ species lie upon a separate, distinct aggregation pathway in comparison to the fibrillar aggregation pathway.\(^{67}\) Interestingly, Zn-induced aggregation is significantly faster in comparison to Cu, suggesting that Zn-Aβ aggregation essentially bypasses the lag phase.\(^{150}\) One hypothesis for the increase in aggregation kinetics has to do with the resulting charge of the Zn-Aβ complex.\(^{28}\) Aβ aggregates more quickly when the pH approaches its isoelectric point (pI), which for Aβ is around 5.5. At physiological pH 7.4, Aβ has a 3- charge therefore, when divalent cations, Cu\(^{2+}\) and Zn\(^{2+}\), bind to the peptide, a -1 charge is observed. Cu\(^{2+}\) is a stronger Lewis acid in comparison to Zn\(^{2+}\) and therefore, is capable of deprotonating amide nitrogens along the peptide backbone, which would increase the negative charge on the complex (ie: Component II of Cu-Aβ complex). In the case of Zn\(^{2+}\), deprotonation of amide nitrogens does not occur and therefore, a complex closer to a neutral species is observed, leading to faster aggregation kinetics.

As described above, the binding of Aβ to metal ions leads to a wide range of neurotoxicity. Soluble, oligomeric Aβ is thought to be the most neurotoxic species due to its ability to travel throughout the brain. In the presence of Cu\(^{2+}\), oligomeric Aβ species are primarily observed.\(^{54, 105, 143}\) These species could exhibit enhanced neurotoxicity in comparison to the apo- form due to the redox nature of Cu in this environment. The formation of ROS (eg: superoxide radical, hydroxide radical, hydrogen peroxide) can be catalyzed by Cu-Aβ species, which can increase the oxidative stress in the brain.\(^{151-154}\) Although our body has the biochemical machinery to process superoxide radicals and \(\text{H}_2\text{O}_2\) employing superoxide dismutase and catalase, respectively, the highly reactive hydroxyl radical is still a major problem. Interestingly, a number of reports suggest that Zn-Aβ species could be neuroprotective or neurotoxic, depending on the stoichiometry. A high Zn concentration leads to a neurotoxic effect, while at low Zn concentration, a neuroprotective effect has been reported.\(^{155-158}\)
Increasing evidence supports the involvement of metals ions in Aβ aggregation and neurotoxicity, suggesting a possible therapeutic target for AD. The changes in brain metal homeostasis appear to be an important step in AD pathology, and understanding how to regulate these metal-Aβ interactions is a subject of current AD research.

1.4. Current Therapeutic Agents and Strategies

1.4.1. Approved Cognitive Drugs

A select few agents have been approved as AD treatments to address the symptoms of AD, specifically affecting the rate of cognitive decline. One approach increases the activity of the neurotransmitter, acetylcholine, in the brain and has been shown to decrease the rate of cognitive decline. This was achieved through the use of AChE inhibitors that increase the residence time of the neurotransmitter on the postsynaptic neuron. Specific therapeutic agents that use this strategy are Donepezil (Aricept), Rivastigmine (Exelon), and Galantamine (Razadyne) (Figure 1.5). An alternative approach is to target N-methyl-D-aspartate receptors (NMDA-R) in neurons responsible for regulating glutamate release. Glutamate is a neurotransmitter that is responsible for trafficking Ca\(^{2+}\) into the cell. In AD, damaged neurons release excess glutamate into the brain, causing chronic overexposure of the cell to Ca\(^{2+}\), expediting cell damage processes. The drug, Memantine (Figure 1.5) has been approved to target NMDA-R and regulate glutamate levels. The appropriate therapeutic intervention depends on the degree of disease progression. Table 1.1 illustrates the various approved therapeutic agents and when they are prescribed based on disease severity.
Donepezil
(Aricept)  
Rivastigmine
(Exelon)  
Memantine
(Namenda)  
Galantamine
(Razadyne)

Figure 1.5. Chemical structures of four FDA-approved therapeutics for AD that aid in decreasing the rate of cognitive decline.

Table 1.1. FDA approved drugs for treating the symptoms of AD.

<table>
<thead>
<tr>
<th>Brand Name</th>
<th>Drug Name</th>
<th>Year Approved</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aricept</td>
<td>Donepezil$^{161-163}$</td>
<td>1996</td>
<td>All</td>
</tr>
<tr>
<td>Exelon</td>
<td>Rivastigmine$^{164, 165}$</td>
<td>2000</td>
<td>All</td>
</tr>
<tr>
<td>Razadyne</td>
<td>Galantamine$^{166}$</td>
<td>2001</td>
<td>Mild-Moderate</td>
</tr>
<tr>
<td>Namenda</td>
<td>Memantine$^{167-169}$</td>
<td>2003</td>
<td>Moderate-Severe</td>
</tr>
<tr>
<td>Namzaric</td>
<td>Donepezil &amp; Memantine$^{170}$</td>
<td>2014</td>
<td>Moderate-Severe</td>
</tr>
</tbody>
</table>

1.4.2. **Immunotherapy**

During the past decade, several unsuccessful immunotherapy clinical trials were completed.$^{171-173}$ For these studies, antibodies were developed that specifically recognize regions and/or conformations of the Aβ peptide with high affinity.$^{174}$ The goal was to bind and remove these biomolecules in order to decrease neuronal impairment.$^{175}$ Several factors are likely associated with the lack of clinical efficacy including limited blood-brain barrier permeability and the patient cohort chosen for the study. The recent development in PET radiotracers for imaging Aβ plaque deposits, has provided a tool to identify disease progression in AD patients enrolled in clinical trials. This will no doubt provide important information on drug efficacy across different disease stages. Table 1.2 summarizes a list of immunotherapeutic agents, how they recognize the Aβ peptide, and what type of clinical trial was performed.
## Table 1.2. Summary of various immunotherapeutic agents and their clinical trial results.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope Recognition</th>
<th>Clinical Trial Phase</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bapineuzumab&lt;sup&gt;176, 177&lt;/sup&gt;</td>
<td>Aβ&lt;sub&gt;1-5&lt;/sub&gt;</td>
<td>Phase III</td>
<td>Binds to monomeric, oligomeric and fibrillar forms of Aβ</td>
</tr>
<tr>
<td>Solaneuzumab&lt;sup&gt;178, 179&lt;/sup&gt;</td>
<td>Aβ&lt;sub&gt;13-28&lt;/sub&gt;</td>
<td>Phase III</td>
<td>Shuttles both Aβ40 and Aβ42 from the brain to the plasma and CSF</td>
</tr>
<tr>
<td>Gantenerumab&lt;sup&gt;180, 181&lt;/sup&gt;</td>
<td>Aβ&lt;sub&gt;1-11&lt;/sub&gt;</td>
<td>Phase III</td>
<td>Binds to Aβ plaques, inducing a phagocytic response by microglia in order to aid in Aβ clearance</td>
</tr>
<tr>
<td>Crenezumab&lt;sup&gt;182&lt;/sup&gt;</td>
<td>Aβ&lt;sub&gt;12-23&lt;/sub&gt;</td>
<td>Phase II</td>
<td>Recognizes oligomeric, fibrillar, and Aβ plaque deposits with a high affinity</td>
</tr>
</tbody>
</table>

### 1.4.3. Targeting Secretase Inhibition

β- and γ-secretase are involved in the proteolytic processing of APP, leading to the production of the Aβ peptide. As such, many research groups have sought to understand how these enzymes work in order to inhibit their activity. Unfortunately, these enzymes have multiple substrates making it difficult to selectively inhibit Aβ production while not resulting in deleterious side effects.

β-site APP cleaving enzyme-1 (BACE-1, β-secretase) is responsible for the first enzymatic cleavage on APP leading to Aβ. It is a transmembrane aspartic acid protease enzyme that is also responsible for the production of myelin sheaths in peripheral nerve cells. Several pharmaceutical companies have invested significant research efforts into developing BACE-1 inhibitors. Recently, Astra-Zeneca and Eli Lilly and Company joined forces to co-develop AZD3293/LY3314814, which has been shown to inhibit BACE-1 and the production of Aβ (Figure 1.6). AZD3293/LY3314814 has just commenced a phase III clinical trial in December 2014 on early stage AD patients with results expected in 2019. Merck Sharp and Dohme Corporation have also initiated research efforts towards developing a BACE-1 inhibitor, which completed phase I clinical trials in 2012. MK8931 had commenced a phase III clinical trial in November 2013 with results expected in early 2018 (Figure 1.6).
γ-secretase is a multi-domain protein complex consisting of 4 subunits: presenilin, nicastrin, anterior pharynx defective-1 (APH-1), and presenilin enhancer 2 (PEN-2).\textsuperscript{184,188} The development of γ-secretase inhibitors has been a challenge due to its enzymatic substrate promiscuity and lack of structural information.\textsuperscript{189-191} γ-secretase is also responsible for NOTCH signalling, which is a key factor in neurogenesis, therefore the development of inhibitors that specifically halt the activity upon APP and not NOTCH are highly desirable.\textsuperscript{192} The presenilin domain is thought to be the catalytic unit responsible for acting upon APP to produce Aβ. This is a target that many researchers are focusing on.\textsuperscript{193,194} Semagacestat was a γ-secretase inhibitor candidate that entered phase 3 clinical trials in 2010 but was halted before the end of the trial due to several complications including increased cognitive impairment, significant weight loss, infections, and development of skin cancers (Figure 1.6).\textsuperscript{195} A primary reason why so many adverse affects were observed is most likely due to the 40+ substrates that γ-secretase reacts with, therefore inhibiting all of their processing most likely lead to the symptoms that were observed.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Chemical_structures.png}
\caption{Chemical structures of secretase inhibitors used to regulate the production of Aβ. AZD3293/LY3314814 and MK8931 target BACE while semagacestat was used as a γ-secretase inhibitor. All three compounds have been or are currently involved in clinical trials.}
\end{figure}

\subsection*{1.4.4. Aβ Plaque Imaging Agents}

Currently, there are three FDA-approved PET imaging agents for AD (Figure 1.7). All three are \textsuperscript{18}F-radiolabeled probes that target Aβ plaque deposits in the brain.\textsuperscript{196} Florbetapir-\textsuperscript{18}F and Florbetaben-\textsuperscript{18}F stem from similar styryl scaffolds that have been shown to bind to Aβ plaques with high affinities (\(K_i = 2.87\) and 2.22 nM, respectively).\textsuperscript{197-199} Flutemetamol-\textsuperscript{18}F is based on a benzothiazole scaffold, demonstrates high binding affinity to Aβ plaques (\(K_i = 0.74\) nM), and excellent pharmacological kinetics.\textsuperscript{200} All of these imaging agents for AD have rapid brain uptake post-injection and rapid washout.
within 1-2 hours\textsuperscript{200-202}. Table 1.3 summarizes their physicochemical properties, demonstrating rapid brain uptake and significant excretion at 2 hours post-injection. Although there are no approved imaging agents for tau hallmarks, the considerable research efforts in this area were recently reviewed\textsuperscript{203}.

**Table 1.3.** Physicochemical properties of FDA-approved amyloid imaging agents for AD.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Brand Name</th>
<th>Company/Year Approved</th>
<th>Binding Affinity (nM)</th>
<th>Uptake/Excretion Kinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Florbetapir</td>
<td>Amyvid\textsuperscript{TM}</td>
<td>Eli Lilly - 2012</td>
<td>2.87</td>
<td>Brain (2 mins.) – 6.8 %ID/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brain (120 mins.) – 1.7 %ID/g</td>
</tr>
<tr>
<td>Florbetaben</td>
<td>Neuroceq\textsuperscript{TM}</td>
<td>Piramal Imaging - 2014</td>
<td>2.22</td>
<td>Brain (2 mins.) – 8.14%ID/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brain (120 mins.) – 2.14 %ID/g</td>
</tr>
<tr>
<td>Flutemetamol</td>
<td>Vizamyl\textsuperscript{TM}</td>
<td>GE Healthcare - 2013</td>
<td>0.74</td>
<td>Brain (5 mins) – 3.67 %ID/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brain (120 mins.) – 0.05 %ID/g</td>
</tr>
</tbody>
</table>

**Figure 1.7.** Chemical structures of three FDA-approved A\textsubscript{β} plaque imaging agents.

### 1.4.5. Metal Binding Agents

Numerous reports identified dyregulated endogenous metal ions as a causal factor in the aggregation of A\textsubscript{β}, leading to increased neurotoxicity\textsuperscript{26, 204}. The development of agents that target dysregulated metal ions in AD commenced in the early 1990’s with desferrioxamine treatment (Figure 1.8)\textsuperscript{205}. Here, a phase 2 clinical trial was performed over a two year period. The goal was to decrease the rate of cognitive decline by using a metal sequestration agent. Promising results were obtained as the non-desferrioxamine
group demonstrated a rate of cognitive decline that was twice as fast as the Alzheimer’s patients that were treated with the therapeutic. This clinical trial created motivation to continue targeting metal-Aβ as an alternative strategy to combat AD.

In the early 2000’s, Bush and co-workers developed Clioquinol, an 8-hydroxyquinoline derivative, that demonstrated remarkable efficacy in animal models (Figure 1.8). Clioquinol binds Cu$^{2+}$ and Zn$^{2+}$ metal ions, interacts with the Aβ peptide near the N-terminus where metal binding occurs and near the hydrophobic self-recognition site (residues 17-21), permeates the BBB, and ultimately reduces brain Aβ deposition. Unfortunately, when Clioquinol was screened in a phase 2 clinical trial, several complications arose including difficulties with purification of Clioquinol from side products. In order to overcome these obstacles, an improved 8-hydroxyquinoline derivative was developed, PBT2 (Figure 1.8). This derivative had equal or better properties in comparison to Clioquinol, while being able to inhibit the formation of oligomeric species in an animal model. A 12-week phase 2 clinical trial was performed where reduced cerebral spinal fluid Aβ$_{1-42}$ concentrations were observed but not plasma Aβ$_{1-42}$ levels. This suggested that central Aβ clearance was occurring and not from the periphery.

![Clioquinol and PBT2](image)

**Figure 1.8.** Metal-binding AD therapeutics that have undergone clinical trials.

### 1.5. Multifunctional Metal-binding Therapeutics

Due to the many facets associated with AD, the traditional medicinal chemistry approach of “one drug, one target” may not be the most effective strategy in order to create disease-modifying therapeutics. This section will focus on metal binding and
several auxiliary properties that researchers have incorporated into their therapeutic strategy.

### 1.5.1. Linkage, Incorporation, and Responsive Strategies

The development of multifunctional metal-binding AD therapeutic agents falls into three primary categories: linkage of one or multiple functions to a central scaffold, incorporation of additional functionality into an already known scaffold, or a responsive approach where the active conformation of the ligand is initiated upon interaction with a disease hallmark (Figure 1.9).

**Figure 1.9.** Summary of the three multifunctional metal-binding approaches used to combat AD. Linkage: A metal sequestration site anchored to an Aβ peptide-targeting vector. Incorporation: The Aβ peptide targeting vector has been modified to include a site for binding metals. Responsive: A non-active compound will be “turned-on” in the presence of a disease hallmark producing the active therapeutic agent.

The development of compounds that link a targeting vector to a therapeutically-active group is a promising therapeutic strategy to combat AD. Several groups have rationalized that starting with a central scaffold that will target a specific hallmark of AD (eg: Aβ plaques, NFT’s, etc.) and linking a therapeutic moiety could enhance efficacy in treating AD. Several examples include starting with a central scaffold that will target a disease hallmark, followed by appending a separate moiety that could:

1. Sequester endogenous metal ions (ie: Cu, Zn)
2. Scavenge for ROS via antioxidant properties
3. Inhibit AChE activity
4. Inhibit aggregation of Aβ or pTau
I will restrict the discussion to metal-binding multifunctional agents as this is the focus of my thesis research. In one example, Mirica and co-workers utilized the linkage approach to develop two multifunctional ligands (Figure 1.10).\textsuperscript{210} Herein, a benzothiazole scaffold is used as a targeting vector to guide the ligand towards A\(\beta\) aggregates while the appended phenol and pyridine units act as a metal binding unit. The phenol moiety can also act as an antioxidant. While this framework was shown to regulate A\(\beta\) aggregation in the presence of metal ions, the aggregates that were generated under certain conditions were shown to be toxic.

Orvig and co-workers used a similar approach, harnessing a benzothiazole or benzooxazole group as a targeting vector while appending a deferiprone analogue as a metal binding unit (Figure 1.10).\textsuperscript{211} These scaffolds demonstrated antioxidant properties, high affinity for A\(\beta\) aggregates, strong metal binding affinities, and the ligands demonstrated low toxicity in the tested concentration range (EC\textsubscript{50} = 2.5-6.5 \(\mu\)M) in a bEnd.3 mouse neuronal cell line.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{Chemical structures of compounds that use the linkage approach towards treating AD. A benzothiazole moiety is used to direct the compound towards A\(\beta\) aggregates while the other end of the molecules has a metal binding site to regulate metal-A\(\beta\) interactions.}
\end{figure}

A large number of radiolabelled A\(\beta\)-targeting scaffolds have been reported with high affinity towards A\(\beta\) aggregates.\textsuperscript{212-214} As such, several reports incorporated a metal binding site into these scaffolds (Figure 1.11).\textsuperscript{215, 216} Lim and co-workers functionalized the A\(\beta\) imaging agent, \(^{125}\text{I}-\text{IMPY}\), with a metal binding site by installing a pyridine moiety into the framework.\textsuperscript{217} This scaffold was shown to target A\(\beta\) aggregates, and limit A\(\beta\) aggregation. Lim and co-workers also utilized the \(p^{125}\text{I}\)-stilbene framework, an A\(\beta\)
plaque imaging agent, and installed an imine or secondary amine into the framework to create a bifunctional ligand (Figure 1.11).218-220

![Figure 1.11](image_url)

**Figure 1.11.** Chemical structures that utilize frameworks that are known to interact with Aβ species and incorporate a metal binding site to regulate metal-Aβ interactions.

Due to the multifaceted nature of AD, many strategies use a disease hallmark in order to create an active therapeutic, site-specifically, or to aid in trafficking the compound to its intended target. Franz and co-workers have developed a pro-ligand that responds in the presence of oxidative stress, a hallmark of AD (Figure 1.12).221 Phenols that are masked by a boronate-ester are cleaved in the presence of H₂O₂, liberating a metal binding site.

Orvig and co-workers masked a deferiprone or salen metal binding site using a carbohydrate moiety (Figure 1.12).222, 223 Glycosylated pro-ligands were used in order to create compounds that would be BBB-permeable via carbohydrate transporters. Upon gaining access to the brain, glycosylated pro-ligands would be acted upon by enzymatic machinery, cleaving the carbohydrate unit and unmasking the metal binding site.
1.5.2. Aβ Aggregation Regulated by Metal Complexes

With Aβ aggregation thought to be an important factor in AD pathogenesis, many strategies are designed to inhibit this process, one of which is through the use of metal complexes. Barnham and co-workers prepared a simple Pt(II)-phenanthroline complex, which demonstrated Aβ-binding via the N-terminal histidine residues, inhibition of Aβ aggregation and Cu²⁺-Aβ-induced H₂O₂ production, and decreased Aβ₁₋₄₂ neurotoxicity (Figure 1.13, Top Left).²²⁴ Unfortunately, this promising complex showed very little bioavailability and had a lengthy synthetic procedure. A Pt(IV) complex was developed to increase bioavailability (Figure 1.13, Top Right).²²⁵ This improved complex also inhibited Aβ aggregation and an overall decrease in brain Aβ deposition in a transgenic APP/PS1 mouse model. These success stories have motivated other researchers to also investigate the use of metal-based therapeutics towards inhibiting Aβ aggregation.²²⁶-²²⁹
Several Ir$^{3+}$, Rh$^{3+}$, and Ru$^{3+}$-based derivatives have also been evaluated. Man and co-workers synthesized a series of Ir$^{3+}$ and Rh$^{3+}$ cyclometalated complexes that were able to inhibit Aβ fibril formation and upon binding to Aβ$_{1-40}$, produced a luminescent response (Figure 1.13, Bottom Left). Messori and co-workers have developed several Ru$^{3+}$ derivatives (Figure 1.13, Bottom Middle and Right). One such derivative containing a 2-aminothiazole ligand was shown to bind to Aβ, producing an adduct through non-covalent interactions, and protecting primary cortical neurons against Aβ$_{25-35}$ neurotoxicity, a truncated version of Aβ that contains the hydrophobic region. One of the drawbacks of this approach is penetrating the blood-brain barrier; if achieved, this strategy could yield promising results.

![Metal-based therapeutics that inhibit Aβ aggregation.](image)

**Figure 1.13.** Metal-based therapeutics that inhibit Aβ aggregation.

### 1.5.3. Blood Brain Barrier (BBB) Permeability

One of the major impediments to treating neurodegenerative diseases is the difficulty in ensuring that the active therapeutic agent is able to reach the intended target. The BBB regulates the access of chemicals to the brain, and in many cases excludes access of therapeutic agents. Chemical properties, such as molecular weight and number of hydrogen-bonding sites, are key factors in determining whether a compound will be BBB penetrable. Compounds need to be lipophilic in order to penetrate the BBB but require hydrophilic properties in order to be soluble in the body. In order for a compound to permeate the BBB via passive diffusion, the molecular weight should be ≤
400 g/mol and possess ≤ 7 hydrogen-bonding sites.\textsuperscript{234} If passive diffusion does not occur, several strategies have been employed to hijack receptor-mediated transport (RMT) or carrier-mediated transport (CMT) pathways.

CMT takes advantage of active transporters such as the amino acid transporters.\textsuperscript{235} A single transporter is capable of trafficking several different types of amino acid derivatives, making these receptors a target in drug delivery applications. Carbohydrate transporters fall into this category as well, but they are much less accommodating when it comes to ligand scaffold changes. RMT involves the use of a large chaperone molecule that a therapeutic agent can be attached to.\textsuperscript{236} The chaperone molecule will transport the therapeutic agent across the BBB. Attaching agents to transferrin or insulin and hijacking their transport pathways has been exploited to increase drug molecule access to the brain.\textsuperscript{236-238}

\textbf{1.6. Thesis Goals}

The overarching goal of this thesis is to study small molecules capable of targeting dysregulated metal ions in AD pathology. Controlling the interactions of these metal ions with disease hallmarks is a viable therapeutic strategy. Chapters 2-4 highlight three separate ligand scaffolds that demonstrate multifunctional properties towards AD (Figure 1.14). Chapter 2 describes a 1\textsuperscript{st} generation scaffold utilizing a pyridine-triazole framework to bind metal ions, interact with the peptide near the metal binding site, and regulate metal-Aβ interactions. Chapter 3 builds from the previous chapter by extending the aromatic ring to afford a series of quinoline-triazoles. This series was shown to have weak hydrophobic interactions with the peptide and influence Cu-Aβ interactions. Chapter 4 highlights a series of phenol-triazoles, which exhibited the most promising results. This ligand series has an appropriate binding affinity for Cu, is able to regulate Cu-Aβ interactions, and interacts with several residues that reside in the hydrophobic region. Chapter 5 investigates the application of metal complexes (Figure 1.14) to modulate Aβ aggregation and decrease the associated neurotoxicity that is inherent in Aβ aggregates.
Throughout Chapters 2-4, a common theme is apparent through the use of triazolyl-based ligand frameworks. An eventual goal is to use the dysregulated Cu, and Cu-Aβ species, in AD to catalyze the 1,3-dipolar cycloaddition of alkynes and azides to form triazoles, also known as click chemistry. Our goal is to be able to exploit these species as catalysts for the site-specific production of therapeutic agents. An update on the progression of this project and the future applications towards AD is discussed in Chapter 6.

![Chemical scaffolds from Chapters 2-5](image)

**Figure 1.14.** Chemical scaffolds that are presented in Chapters 2-5.
Chapter 2.
Dual Function Triazole-Pyridine Derivatives as Inhibitors of Metal-induced Aβ Aggregation


MRJ performed the syntheses, metal binding measurements, acidity constant determinations, turbidity measurements, and TEM preparation. JRT collected and solved the X-ray crystal structures of the metal complexes. MCPW ran the TEM instrument. IJW, SV, and ASD performed the 2-D TROSY-HSQC NMR experiments and the molecular modeling using AutoDock Vina.

2.1. Introduction

While the role of metal ions in the etiology of AD remains to be completely elucidated, targeting and controlling metal-Aβ peptide species is a viable therapeutic strategy. Metal chelators can solubilize Aβ plaque deposits and, more impressively, show promise as AD therapeutics. An early clinical study with the strong metal-ion chelator desferrioxamine (DFO) showed a significant decrease in the rate of cognitive decline of treated subjects compared with the control group over a 24-month period. However, the long-term use of strong metal chelators that are not tissue-specific likely affects the homeostasis of numerous biometals and the normal physiological functions of essential metal-requiring biomolecules such as metalloenzymes. Recent work with the metal-binding agent clioquinol (Conditional stability constants: $K_c$(Cu): $1.2 \times 10^{10}$ M$^{-2}$; $K_c$(Zn): $7 \times 10^8$ M$^{-2}$) has shown the promise of chelation therapy in both animal models and human trials. Advances have been
made in the targeted delivery of chelating agents for AD therapy via activation by enzymes and hydrogen peroxide. In addition, the design of metal-binding agents that employ Aβ targeting functions also offer a mechanism to improve the activity of this class of compounds. Incorporating or linking a metal coordination site to the Aβ-binding agents, such as thioflavin-T and p-I-stilbene, has afforded a class of bifunctional agents that can influence metal-induced Aβ aggregation.

Figure 2.1. Examples of bifunctional agents that incorporate or link a metal binding site into known Aβ-targeting vector such as Thioflavin-T (ThT) or (E)-Stilbene.

In this work we have studied a series of 1,2,3-triazolyl-pyridine compounds as bifunctional reagents for targeting metal-Aβ species in AD (Figure 2.2). L1 – L4 were synthesized in a modular fashion using Cu-catalyzed alkyne/azide cycloaddition (click) chemistry. In each case the metal binding site is identical, and formed via click chemistry, allowing for the correlation of biological properties with side-chain functional groups. The characterization of the metal chelation and Aβ interaction properties of L1 – L4, along with evaluation of their reactivity toward Aβ aggregation is discussed.
2.2. Experimental

2.2.1. Syntheses

2-Azidoethyl-4-methylbenzenesulfonate\textsuperscript{252}, 1-azido-propanol (1b),\textsuperscript{253} tert-butylazidoacetate (1c),\textsuperscript{254} and 1-azido-5-amino-propane (1d)\textsuperscript{255} were synthesized according to previously reported procedures. Safety Precautions in Handling of Chemicals: Some chemicals are hazardous and toxic, which may result in injury if proper safety precautions are not followed, especially when dealing with azides. To avoid injury, follow safe laboratory practices, wear appropriate protective equipment and use appropriate shielding equipment. Azides tend to decompose violently upon heating, shock and/or friction. For further details, please refer to the MSDS or the Environmental Health and Safety office.

4-(2-azidoethyl)morpholine (1a). Morpholine (1.62g, 18.7 mmol) and triethylamine (3.86 g, 38.1 mmol) were dissolved in 20 mL of CH\textsubscript{3}CN. 2-Azidoethyl-4-methylbenzenesulfonate\textsuperscript{252} (2.30 g, 9.5 mmol) was added and the solution refluxed for 4 days. The solvent was removed in vacuo and the residue was purified by silica gel chromatography (95:5 CH\textsubscript{2}Cl\textsubscript{2}/CH\textsubscript{3}OH eluent) to afford 1a as a yellow oil (1.47 g, 99%). 1H NMR (CDCl\textsubscript{3}, 400 MHz): δ 3.72 (t, J = 4.6 Hz, 4H), 3.35 (t, J = 5.9 Hz, 2H), 2.59 (t, J = 6.1 Hz, 2H), 2.50 (t, J = 4.6 Hz, 4H). FT-IR: 2963 cm\textsuperscript{-1} (w, sh), 2856 cm\textsuperscript{-1} (w, sh), 2810 (w, sh), 2100 (vs, sh), 1650 cm\textsuperscript{-1} (w, sh), 1452 cm\textsuperscript{-1} (w, sh), 1300 cm\textsuperscript{-1} (m, sh), 1117 cm\textsuperscript{-1} (vs, sh). ESI(+)-MS (m/z): [M+H]\textsuperscript{+} Calcd for (C\textsubscript{6}H\textsubscript{13}N\textsubscript{4}O), 157.10; Found, 157.11.

4-(2-(4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl)ethyl)morpholine (L1). In a 20 mL vial, 1a (0.504 g, 3.2 mmol) and 2-ethynylpyridine (0.364 g, 3.5 mmol) were dissolved in 1 mL of isopropanol. In a separate 20 mL vial, CuSO\textsubscript{4} · 5H\textsubscript{2}O (0.041 g, 0.16 mmol) and
L-ascorbic acid (0.290 g, 1.6 mmol) were dissolved in 2 mL of H₂O. Sonication was used to completely dissolve these components. The aqueous solution was added dropwise to the stirring isopropanol solution. The reaction was stirred for 2 hours at 298 K and then Chelex resin was added to remove the Cu catalyst. The solution was filtered after 2 hours to separate the Chelex resin and the solvent was removed in vacuo. The residue was purified by silica gel chromatography (95:5 CH₂Cl₂/CH₃OH eluent) to afford a pale yellow solid (0.584 g, 70% yield). 

1H NMR (D₂O, 600 MHz): δ 8.30 (d, J = 4.3 Hz, 1H), 8.15 (s, 1H), 7.72 (td, J = 7.8, 1.5 Hz, 1H), 7.65 (d, J = 7.9 Hz, 1H), 7.22 (ddd, J = 7.3, 5.2, 0.9 Hz, 1H), 4.51 (t, J = 7.0 Hz, 2H), 3.69 (t, J = 4.1 Hz, 4H), 2.86 (t, J = 7.0 Hz, 2H), 2.53 (t, J = 4.8 Hz, 4H).

13C NMR (D₂O, 151 MHz): δ 148.24, 147.29, 146.18, 137.70, 123.27, 123.01, 120.01, 65.47, 56.24, 51.94, 46.35. HR-ESI(+)MS (m/z): [M+H]⁺ Calcd for (C₁₁H₁₈N₅O), 260.1511; found, 260.1506.

3-(4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl)propan-1-ol (L2). In a 20 mL vial, 1b (0.205 g, 2.0 mmol) and 2-ethynylpyridine (0.207 g, 2.0 mmol) were dissolved in 1 mL of isopropanol. Employing reaction conditions identical to the synthesis of L₁ afforded a pale yellow solid, L2 (0.290 g, 70% yield). 

1H NMR (D₂O, 600 MHz): δ 8.46 (s, 1H), 8.28 (s, 1H), 7.89 (td, J = 7.9, 1.6 Hz, 1H), 7.83 (d, J = 7.8 Hz, 1H), 7.38 (t, J = 6.2 Hz, 1H), 4.54 (t, J = 7.0 Hz, 2H), 3.62 (t, J = 6.2 Hz, 2H), 2.17 (t, J = 6.4 Hz, 2H). 

13C NMR (D₂O, 151 MHz): δ 148.17, 147.39, 146.07, 138.16, 123.44, 123.17, 120.41, 57.63, 46.95, 31.28. HR-ESI(+)MS (m/z): [M+H]⁺ Calcd for (C₁₀H₁₃N₄O), 205.1089; Found, 205.1084.

2-(4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl)acetic acid (L3). In a 20 mL vial, 1c (0.489 g, 3.1 mmol) and 2-ethynylpyridine (0.322 g, 3.1 mmol) were dissolved in 1 mL of isopropanol. Employing reaction conditions identical to the synthesis of L₁ afforded a golden yellow solid. 

1H NMR (CDCl₃, 500 MHz): δ 8.59 (ddd, J = 6.1, 2.2, 1.2 Hz, 1H), 8.25 (s, 1H), 8.18 (dt, J = 9.9, 1.3 Hz, 1H), 7.80 (td, J = 9.6, 2.2 Hz, 1H), 7.23 (ddd, J = 9.4, 6.1, 1.5 Hz, 1H), 5.12 (s, 2H), 1.49 (s, 9H). All material produced in this step was dissolved in 15 mL of dichloromethane and 4.5 mL of trifluoroacetic acid (25 eq.) was added and the reaction stirred overnight. The solvent was removed in vacuo, and multiple additions of diethyl ether induced precipitation of a pale yellow solid. This material was further purified by recrystallization from hot water to produce L3 as white needles (0.611 g, 96% yield). 

1H NMR (D₂O, 600 MHz): δ 9.04 (s, 2H), 8.83 (td, J = 8.1,
1.6 Hz, 1H), 8.62 (dt, \( J = 8.2, 1.0 \) Hz, 1H), 8.21 (ddd, \( J = 7.6, 5.6, 1.2 \) Hz, 1H), 5.57 (s, 2H). \( ^{13} \)C NMR (D\(_2\)O, 151 MHz): \( \delta \) 171.75, 145.55, 143.58, 142.63, 140.53, 126.97, 125.40, 123.74, 53.03. HR-ESI(+)MS (m/z): [M+H]\(^+\) Calcd. for [M+H]\(^+\) (C\(_9\)H\(_9\)N\(_4\)O\(_2\)), 205.0725; Found, 205.0720.

5-(4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl)pentan-1-amine (L\(_4\)). In a 20 mL vial, 1-azido-5-amino-propane\(^{255}\), 1d (0.327 g, 2.5 mmol) and 2-ethynylpyridine (0.263 g, 2.5 mmol) were dissolved in 1 mL of isopropanol. Employing reaction conditions identical to the synthesis of L1 afforded a residue that was purified by silica gel chromatography (1:1 CH\(_2\)Cl\(_2\)/CH\(_3\)OH with 1% NH\(_4\)OH eluent). Dissolving the residue in acidified CH\(_3\)OH and addition of diethyl ether precipitated L4 as the hydrochloride salt (0.397 g, 67% yield). \(^1\)H NMR (D\(_2\)O, 500 MHz): \( \delta \) 8.24 (ddd, \( J = 6.2, 2.0, 1.1 \) Hz, 1H), 7.99 (s, 1H), 7.66 (td, \( J = 9.8, 2.2 \) Hz, 1H), 7.58 (dt, \( J = 9.9, 1.2 \) Hz, 1H), 7.15 (ddd, \( J = 9.3, 6.2, 1.6 \) Hz, 1H), 4.22 (t, \( J = 8.9 \) Hz, 2H), 2.48 (t, \( J = 9.0 \) Hz, 2H), 1.72 (quintet, \( J = 9.3 \) Hz, 2H), 1.33 (m, 2H), 1.15 (m, 2H). \( ^{13} \)C NMR (D\(_2\)O, 125 MHz): \( \delta \) 148.65, 147.81, 146.65, 138.08, 123.62, 122.97, 120.32, 50.29, 40.21, 30.73, 29.13, 22.96. HR-ESI(+)MS (m/z): [M+H]\(^+\) Calcd for (C\(_{12}\)H\(_{18}\)N\(_5\)), 232.1562; Found, 232.1557.

Scheme 2.1. Synthetic routes for the preparation of L1–L4.
2.2.2. Metal Binding Studies

Metal binding studies were performed by using a 50 µM ligand solution in a 1:1 ligand : metal ratio with either CuCl₂ or ZnCl₂ in CH₃CN. Ligand UV-vis spectra were first obtained, followed by addition of 20 µL of a 5 mM CuCl₂ or ZnCl₂ solution (1 eq.) to the ligand solution to obtain UV-vis spectra of the metal complexes.

2.2.3. X-ray Crystallography

Single crystals suitable for data collection were prepared by dissolving L₁ (0.027 g, 0.10 mmol) and CuCl₂ (0.018 g, 0.11 mmol), separately, in 0.5 mL of CH₃CN and then combining the solutions. Additional CH₃CN was added (ca. 5 mL) to increase solubility of the complex. The solution was sonicated and filtered. The solution was allowed to slowly evaporate to obtain greenish-blue crystals suitable for single crystal X-ray diffraction. L₃ (0.011 g, 0.05 mmol) and Zn(NO₃)₂ (0.016 g, 0.05 mmol) were first dissolved in 1 mL of CH₃CN and then 0.5 mL of H₂O was added to completely solubilize the metal-ligand complex. The solutions were filtered and allowed to slowly evaporate. The crystalline samples were mounted on a 150 µm MiTeGen dual-thickness MicroMount using paraffin oil and data collected at room temperature. Crystallographic information can be found in Table 2. Disorder in C9/C10 for the Cu complex was modelled with floating occupancies to 0.752/0.248. All diffraction data were processed with the Bruker Apex II software suite. The structures were solved with SIR92 and subsequent refinements were performed using CRYSTALS. Diagrams were prepared using ORTEP-3 and POV-RAY.
Table 2.1  Crystallographic data for [Cu(L1)Cl2] and [Zn(L3)(NO3)2(H2O)].

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<th>[Zn(L3)(NO3)2(H2O)]</th>
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Note:  

a [weight] = 1.0/[A_0 x T_0(x) + A_1 x T_1(x) + ... + A_n-1 x T_(n-1)(x)] where A_i are the Chebychev coefficients listed below and x = F_{calc}/F_{max} method of Robust Weighting W = [weight] x [1 – (ΔF/6 x σF)]²

b $R = \Sigma |F_o| - |F_c|/ \Sigma |F_o|$  

c $wR^2 = (\Sigma w(\sigma F_c)^2)^{1/2}$
2.2.4. Determination of Acidity Constants by UV-vis

In order to determine the speciation of L1 – L4 at physiological pH, acidity constants were measured by obtaining variable pH UV-vis spectra. Solutions of L1 – L4 (50 µM) were prepared in 0.1 M PBS buffer at pH 6.6. Before obtaining UV-vis spectra, a pH electrode was calibrated using a 2-point method (pH 4.01 and pH 10.01 standard buffers). NaOH was used to increase the pH of the ligand solutions to a starting point of ca. pH 12. Aliquots of HCl were added to the L1 – L4 ligand solution and UV-vis spectra obtained in the range of 600 – 190 nm. At least 30 UV-vis spectra were obtained in the range of pH 2-12. Spectral data was tabulated and analyzed using the HypSpec program (Protonic Software, UK)\(^{259}\). A model was created for each ligand and simulated to fit the experimental data using selected wavelengths where significant spectral changes were observed. Speciation diagrams for L1 – L4 were simulated using the HySS2009 program (Protonic Software, UK)\(^ {260}\).

2.2.5. Determination of Acidity Constants by NMR

For \( pK_a \) values that could not be measured using UV-vis spectroscopy, variable pH NMR experiments were performed in order to follow changes in chemical shifts based on individual protonation sites of L1 – L4. Solutions of 80 – 100 mg L1 – L4 in 8 mL of D\(_2\)O were prepared. NaOD and DCl were used to vary the pD of the solution. Before NMR samples were prepared, a pH electrode was calibrated using a 2-point method (pH 4.01 and pH 10.01 standard buffers). Once the pH of the solution had stabilized, 500 µL of solution was obtained and placed in an NMR tube. Ten aliquots were prepared and \(^1\)H and/or \(^{13}\)C experiments were run on a Bruker AVANCE III 500 MHz NMR or a Bruker AVANCE II 600 MHz NMR. Chemical shifts for the o-H to the pyridine N and the triazole H were used to determine the \( pK_a \) for the chelation site. Depending on the R-group, the \( pK_a \) was determined by measuring the chemical shifts of the protons in the vicinity of the protonation site over a specific pH range. NMR data was analyzed by HypNMR (Protonic Software, UK). Speciation diagrams for L1 – L4 were simulated using the HySS2009 program (Protonic Software, UK). The equation \( (pK_a (H_2O) = (pK_a (D_2O) – 0.45)/1.015) \) was used to convert pD values to pH values\(^ {261}\).
2.2.6. Two-dimensional (2D) $^1$H-$^{15}$N transverse relaxation optimized spectroscopy (TROSY)-heteronuclear single quantum correlation (HSQC) NMR measurements

A ca. 308 µM solution of $^{15}$N-labeled Aβ$_{1-40}$ (rPeptide, Bogart, GA, USA) was prepared from ca. 0.25 mg of peptide dissolved in 186 µL of an aqueous buffer containing SDS-$d_{25}$ (200 mM), NaPi (20 mM, pH 7.3), and D$_2$O (7%, v/v). Spectra of the peptide solutions were acquired in a Shigemi NMR tube at 298 K. The ligands L1 – L4 were added to the peptide solution from a stock solution in the above buffer (5 mM) to give 1, 5, and 10 equivalents of ligand to peptide. Two-dimensional $^1$H-$^{15}$N HSQC spectra were collected at 298 K, with spectral widths of 14.3 kHz ($^1$H) and 1.7 KHz ($^{15}$N), using 4 transients per free-induction decay, 128 complex points in the $^{15}$N dimension, and 2048 complex points in the $^1$H dimension. Total experiment time for each spectrum was 10 min.$^{217,218,262-264}$ The water peak was referenced to 4.77 ppm at 298 K. $^1$H-$^{15}$N HSQC peaks were assigned by comparison of the observed chemical shift values to those reported in the literature.$^{265}$ Combined backbone $^1$H and $^{15}$N 2D chemical shifts were calculated using Eq. 2.1.$^{266-268}$ 2D spectra were processed using Topspin software (version 2.1) from Bruker and analyzed with Sparky (version 3.112).

$$\Delta \delta_{N,H} = \sqrt{\left(\frac{\Delta \delta_H}{2}\right)^2 + \left(0.2(\Delta \delta_N)\right)^2}$$

(Eq. 2.1)

2.2.7. Docking Studies using AutoDock Vina

Potential molecular conformations of the ligands with Aβ$_{1-40}$ were elucidated in silico using AutoDock Vina,$^{269}$ AutoDockTools4,$^{270}$ and PyRx.$^{271}$ The 2D structures of L1 – L4 were generated in MarvinSketch (ChemAxon, Budapest, Hungary), and the corresponding lowest energy conformations were obtained using ChemAxon’s chemical calculators. The predicted major microspecies for L3 at pH 7.3 were generated using the same calculators. The SDS condition receptor was obtained from an NMR-derived Aβ$_{1-40}$ structure in SDS-$d_{25}$ micelles (PDB 1BA4$^{272}$); all 10 conformations were used for docking. Docking was performed using AutoDock Vina through the PyRx suite. Search space for each receptor conformation was set to the bounds of the peptide and an exhaustiveness of 1024 was used. Docked poses were viewed using PyMol, after which
the conformations that best represented the experimental conditions were chosen based on the relative proximity (< 5 Å) of ligand heavy atoms to the majority of perturbed amino acid residues.\textsuperscript{273}

\subsection*{2.2.8. Turbidity Measurements}

Lyophilized synthetic human Aβ\textsubscript{1-40} (21\textsuperscript{st} Century Biochemicals) was freshly prepared as a 200 µM stock solution before each trial was performed. Each vial of peptide contained 0.25 mg Aβ\textsubscript{1-40}, which 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. Chelex-treated 20 mM HEPES buffer containing 150 mM NaCl was prepared in deionized water at pH 6.6 and 7.4 for both Cu and Zn turbidity assays, respectively, and used to prepare stock solutions of ligands, metals, and ascorbate, as well as the reaction mixtures in the 96-well plates. Each ligand measurement was evaluated in quadruplicate. Ligands, metals, and Aβ\textsubscript{1-40} peptide had final concentrations of 150 µM, 25 µM, and 25 µM respectively in the turbidity assay. Cu and Zn solutions were prepared from atomic absorption standards (Sigma-Aldrich). Turbidity assays were completed in flat-bottomed 96-well assay plates (Microtest, BD Falcon). Metal, Aβ\textsubscript{1-40}, and HEPES buffer were first added to the 96-well plate followed by the ligands. After a 45 minute incubation period in a 37 °C water bath under constant agitation, each well in the 96-well plate was measured at 405 nm using a Synergy 4 fluorometer plate reader from BioTek. Blanks containing ligand, metal, and buffer were also measured and subtracted from corresponding wells. Wells containing metal and peptide were used as a positive control to demonstrate amount of aggregation in the absence of ligand.

\subsection*{2.2.9. Transmission Electron Microscopy (TEM)}

Samples were prepared by incubating the Aβ peptide (25 µM) with and without CuCl\textsubscript{2} or ZnCl\textsubscript{2} (25 µM) and L1 (50 µM) for 24 hours in a 37 °C water bath. TEM grids were prepared following previously reported methods.\textsuperscript{274} Formvar/Carbon 300-mesh grids (Electron Microscopy Sciences) were glow-discharged in air for 15 seconds to increase hydrophilicity. Samples (10 µL) were dropped onto a sheet of parafilm and the
TEM grid was placed on the drop for 5 minutes. Afterward, the grid was stained with syringe-filtered 5% uranyl acetate from $3 \times 30 \, \mu\text{L}$ drops placed onto parafilm. The grid was placed on the first drop of 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 at 200 kV and 9000X magnification.

2.3. Results and Discussion

2.3.1. Design and Synthesis of Triazole-Pyridine (TriPy) ligands

The development of compounds capable of metal binding as well as inhibiting $\text{A}\beta$ aggregation is of considerable interest for AD research. The synthesis of a small library of triazole-pyridine ligands $\text{L1} – \text{L4}$ (Figure 2.2) was completed using a facile Cu-catalyzed alkyne-azide cycloaddition reaction of 2-ethynylpyridine with a number of different azide fragments (Scheme 2.1). Click chemistry allows for the modular synthesis of a number of TriPy ligands with an identical bidentate metal binding function, while varying the peripheral $R$-group. This synthetic pathway permitted the rapid development of a series of metal-binding compounds for study in a number of assays. Four azides containing different functional groups ($R$) were synthesized, and each azide was coupled with 2-ethynylpyridine via click chemistry (Scheme 2.1). To complete the synthesis of $\text{L3}$, trifluoroacetic acid was used to remove the $t\text{Bu}$ group and afford the free carboxylic acid. The synthetic route described allows for the synthesis of $\text{L1} – \text{L4}$ in two to three steps in good to excellent yields (Scheme 2.1.).

2.3.2. Ligand Speciation Measurements

To determine the solution speciation of $\text{L1} – \text{L4}$, variable pH UV-vis (Figures 2.4 and Appendix, Figures S1 – S3), and $^1\text{H}$ NMR (Appendix, Figures S4 – S8) experiments were performed in the pH 2 – 12 range. As shown in Figure 2.4, $\text{L2}$ contains only one protonated species in the pH range studied, which corresponds to the triazole-pyridine unit as protonation clearly affects the $\pi – \pi^*$ transition of the conjugated ring system.
Fitting the variable pH UV-vis data for L2 affords a single pK\textsubscript{a} value of 3.40(6) (Table 1). This pK\textsubscript{a} value is lower than the pK\textsubscript{a} of pyridine (pK\textsubscript{a} = 5.25),\textsuperscript{276} and that of 2,2'-bipyridine (pK\textsubscript{a} = 4.30),\textsuperscript{277,278} and it is possible that hydrogen bonding stabilizes a \textit{trans} geometry of the pyridine-N and triazole-N thus lowering the pKa value for the TriPy ligands relative to pyridine. The low pK\textsubscript{a} value for the pyridine-triazole unit (uncharged at physiological pH) is potentially important for biological uptake of this class of compounds. The variable pH UV-vis spectra of L1, L3, and L4 exhibit similar pK\textsubscript{a} values to L2 for the pyridyl nitrogen (Table 2.1).

To obtain a more complete understanding of the speciation of L1 – L4 at physiological pH, variable pH \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were obtained. Chemicals shifts of protons in the vicinity of a potential protonation site were monitored, and based on the fitted NMR data, L2 was determined to exhibit a single pK\textsubscript{a} (3.31(7)), in agreement with the UV-vis data (Appendix, Figure S5). Thus, at physiological pH (pH 7.4), L2 was determined to be a neutral species in aqueous solution. Fitting the variable pH NMR chemical shifts of L1 allows for the determination of the morpholine pK\textsubscript{a} value (Table 2.1, Appendix, Figure S6). Using NMR, the pK\textsubscript{a} of the morpholine amine was determined to be 4.89(3), while the pK\textsubscript{a} of the pyridine unit was 2.84(8). The pK\textsubscript{a} values for morpholine (8.36) and N-methylmorpholine (7.38)\textsuperscript{279,280} are higher than the value determined for L1, which could be due to steric and/or electronic effects of the triazole-pyridine ring system.\textsuperscript{281,282} Based on the NMR data, L1 is also expected to be a neutral compound at physiological pH. The pyridine pK\textsubscript{a} (2.84(8)) is slightly lower in comparison to that of L2, likely due to the protonated morpholine function, and this value coincides well with UV-vis analysis (Table 1). The carboxylic acid moiety of L3 is expected to be deprotonated at physiological pH, and the variable pH NMR spectra confirm two pK\textsubscript{a} values at 2.3(1) and 3.08(7). By analysis of the UV-vis and NMR (Appendix, Figures S2 and S7) data the former pK\textsubscript{a} is assigned to the carboxylic acid and the latter pK\textsubscript{a} to the pyridine unit, and thus at physiological pH L3 is a monoanionic species. The variable pH NMR data of L4 (Appendix, Figure S8) can be fit to a pK\textsubscript{a} value of 10.3(3), indicating that L4 exists as a monocation at pH 7.4 (via protonation of the primary amine). The peripheral R-groups, thus afford us the opportunity to investigate how charge influences the interactions of L1 – L4 with the A\textbeta-peptide under physiological conditions. In addition, the solution
speciation results show that the bidentate triazole-pyridine binding unit is neutral at physiological pH, which may be beneficial for in vivo applications.

![Figure 2.3](image)

**Figure 2.3.** Solution speciation studies of L2. (Left) Variable-pH UV-vis spectra of L2 (pH range 2 (Blue) – 12 (Red); [L2] = 50 µM). (Right) Solution speciation diagram for L2.

<table>
<thead>
<tr>
<th>UV-vis</th>
<th>NMR</th>
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<tr>
<td>pKₐ(TriPy)</td>
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</tr>
<tr>
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</tr>
<tr>
<td>L2</td>
<td>3.40(6)</td>
</tr>
<tr>
<td>L3</td>
<td>3.45(7)</td>
</tr>
<tr>
<td>L4</td>
<td>3.21(9)</td>
</tr>
</tbody>
</table>

### 2.3.3. Metal Binding Properties

The interactions of L1 – L4 with Cu(II) and Zn(II) ions were probed using UV-vis spectroscopy and X-ray crystallography. Upon addition of metal ions to solutions of ligand in CH₃CN, increased intensity and shifts of the optical bands at ca. 235 nm and 275 nm were observed relative to L1 (Figure 2.4). Similar shifts of these peaks for L2 – L4 (Appendix, Figures S9 – S11) were also observed upon treatment with metal ions.
indicating interaction of the bidentate metal-binding unit with Cu(II) and Zn(II) in each case. Addition of a CuCl₂ solution afforded additional weak bands at ca. 390 nm and 460 nm. The change in intensity of the ligand-based transitions, along with the observation of new optical bands at ca. 390 nm and 460 nm (for Cu) indicated metal binding to ligands L₁ – L₄.

![UV-vis spectra](image)

**Figure 2.4.** UV-vis spectra of L₁ (50 μM, black) incubated with metal ions (50 μM) in CH₃CN. L₁ + CuCl₂ (Red), L₁ + ZnCl₂ (blue).

The metal-binding properties of L₁ and L₃ were further explored by X-ray crystallography. Crystals were obtained by slow evaporation of a solution of 1:1 ligand to metal in CH₃CN and/or H₂O. Single crystals of [Cu(L₁)Cl₂] were produced by slow evaporation from CH₃CN. Figure 2.5 shows the metal complex, with L₁ bound to the Cu centre in a bidentate fashion using two nitrogen atom donors, one from pyridine and the other from the triazole ring with two additional chloride ligands present. Additional weak axial interactions to the Cu(II) centre exist from a neighbouring chloride ligand and a morpholine oxygen atom. (Appendix, Figure S12). L₁ exists in a flat, planar geometry in the crystal structure. In addition to the Cu(L₁) complex, single crystals of [Zn(L₃)(NO₃)₂(H₂O)] were obtained by slow evaporation of a 1:1 mixture of L₃ and Zn(NO₃)₂ in CH₃CN / H₂O. A similar bidentate coordination to Zn(II) was observed for L₃ where two nitrogen donor atoms from pyridine and the triazole, respectively, are coordinated to the Zn(II) metal centre (Figure 2.6). Taken together, the X-ray structures along with UV-vis data supports metal binding of L₁ – L₄ to Cu(II) and Zn(II).
Figure 2.5. ORTEP diagram of [Cu(L1)Cl2] showing 50% probability thermal ellipsoids. Selected bond lengths (Å) and angles (deg): Cu(1)–N(1) = 2.062(2); Cu(1)–N(2) = 2.024(3); Cu(1)–Cl(1) = 2.2647(8); Cu(1)–Cl(2) = 2.2417(9); Cu(1)–O(1) = 2.808(3); N(1)–Cu(1)–N(2) = 79.31(10); Cl(1)–Cu(1)–N(1) = 86.80(8); N(2)–Cu(1)–Cl(2) = 92.32(7); Cu(1)–Cl(1)–Cu(2) = 94.86(3); Cl(1)–Cu(1)–O(1) = 163.22(8).

Figure 2.6. ORTEP diagram of [Zn(L3)(NO3)2(H2O)] showing 50% probability thermal ellipsoids. Selected bond lengths (Å) and angles (deg): Zn(1)-O(3) = 2.0348(19); Zn(1)-O(6) = 2.0864(18); Zn(1)-O(7) = 2.503(2); Zn(1)-O(9) = 2.1036(19); Zn(1)-N(1) = 2.174(2); Zn(1)-N(2) = 2.0749(19); O(3)-Zn(1)-O(6) = 88.49(8); O(3)-Zn(1)-O(7) = 142.21(8); O(6)-Zn(1)-O(7) = 54.70(7); O(3)-Zn(1)-O(9) = 95.61(8); O(6)-Zn(1)-O(9) = 92.02(7); N(1)-Zn(1)-N(2) = 76.36(7); O(3)-Zn(1)-N(2) = 129.71(8); O(7)-Zn(1)-N(2) = 86.56(7); O(9)-Zn(1)-N(1) = 166.67(7).

2.3.4. NMR Studies for Ligand-Peptide Interactions

Experiments using 2D ¹H-¹⁵N TROSY-HSQC NMR spectroscopy were employed in order to investigate the interactions of L1 – L4 with metal-free Aβ1-40 monomer in the presence of sodium dodecyl sulfate-d25 (SDS-d25). Previous structural studies have used detergent micelles as a model membrane and also to trap the helical intermediates.
present in the misfolding pathway.\textsuperscript{265, 283-285} It was shown that A\textsubscript{\(\beta\)}\textsubscript{1-40} embedded in SDS micelles is monomeric with residues 15 to 36 forming an \(\alpha\)-helix with a kink from residues 25 to 27, and the \(N\)-terminal residues 1 to 14 are unstructured.\textsuperscript{265, 272, 286} In the presence of SDS, the introduction of L\textsubscript{1} – L\textsubscript{4} (1, 5, and 10 eq.) into the buffered solution (pH 7.3) of monomeric A\textsubscript{\(\beta\)}\textsubscript{1-40} induced changes in chemical shifts in the \(^1\text{H}^{-15}\text{N}\) TROSY-HSQC spectrum of the peptide, particularly in the region between E3 and E22 (Figure 2.8, Appendix, Figure S13). In particular, upon addition of 10 eq. L\textsubscript{1}, significant chemical shift perturbations were observed for the amino acid residues E11, H13, and Q15 (Figure 2.7). These residues are in close proximity to the proposed metal chelating site in A\textsubscript{\(\beta\)}\textsubscript{1-40}, which involve three histidine residues (\textit{i.e.}, H6, H13, and H14).\textsuperscript{219, 263, 287} This suggests that L\textsubscript{1} has the potential to interact with A\textsubscript{\(\beta\)} monomer near the proposed metal binding site and perturb the metal-mediated peptide aggregation.\textsuperscript{217-219, 263} L\textsubscript{2} – L\textsubscript{4} also displayed changes in chemical shifts of amino acid residues in the vicinity of the metal binding site of the peptide, but the shifts were smaller in comparison to L\textsubscript{1} (Appendix, Figure S13). In the case of L\textsubscript{3} (Appendix, Figure S13b), several additional peaks in the HSQC spectrum shift as a result of treatment with 10 eq. L\textsubscript{3}; however, the direction of the shift for residues E11, H13, Q15, F20, and E22 upon titrating with L\textsubscript{3} is in opposition to that observed for the corresponding residues while titrating with the ligands L\textsubscript{1}, L\textsubscript{2}, and L\textsubscript{4}.

Due to the low \(pK_a\) of the carboxylic acid moiety of L\textsubscript{3}, the ligand at 10 eq. may sufficiently lower the pH of the peptide solution leading to the observed spectral changes; thus, it would be a challenge to fully attribute the spectral changes to L\textsubscript{3}. The A\textsubscript{\(\beta\)}\textsubscript{1-40} monomer has been shown to exhibit a more structured conformation upon decreasing pH in the presence of SDS (with the helical structure near residues 15 – 24 being especially sensitive to changes in pH).\textsuperscript{272, 285, 288} NMR titration experiments under the SDS conditions show that all ligands affected residues near the purported metal binding site in A\textsubscript{\(\beta\)}\textsubscript{1-40}, with L\textsubscript{1} inducing the largest chemical shift changes in amino acid residues.
Figure 2.7. NMR studies of L1 against $^{15}$N-labeled Aβ$_{1-40}$ in the SDS condition. (a) 2D $^1$H-$^{15}$N TROSY-HSQC NMR spectra of L1 (0, 1, 5, and 10 eq., indicated by black, blue, red, and green, respectively) with the peptide (200 mM SDS-$d_{25}$, 20 mM NaPi, pH 7.3, 7% v/v D$_2$O). (b) Calculated $^1$H and $^{15}$N chemical shifts of Aβ$_{1-40}$ in the presence of 10 eq. of L1. Peaks which could not be resolved, due to overlap or absence, are indicated by an asterisk (*).

2.3.5. Docking Studies

To better understand and visualize the interactions of L1 – L4 with the monomeric Aβ$_{1-40}$ peptide, the small molecules were docked against the previously reported NMR solution structure of monomeric Aβ$_{1-40}$ (PDB 1BA4$^{272}$) in the presence of SDS micelles using AutoDock Vina (Figure 2.8 and Appendix, Figure S14).$^{269}$ Due to imperfections in scoring functions and limited ability to cope with the dynamic nature of protein-ligand interactions,$^{289, 290}$ multiple receptors (NMR ensembles) were used to account for some level of dynamics in the Aβ$_{1-40}$ monomer. Ligand poses generated by AutoDock Vina were ranked according to predicted binding affinities (Appendix, Table S1).$^{291}$ Of these results, four poses using SDS conditions that supported the observations from NMR experiments (vide supra) were selected as representative theoretical bound conformations (Figure 2.8 and Appendix, Figure S14). Poses that best represented the experimental conditions were chosen based on the relative proximity (< 5 Å) of the ligand’s heavy atoms to the majority of perturbed amino acid residues.$^{273}$ Similar poses were predicted for each ligand among the various peptide conformations.
Docking results suggest that the ligands favour the N-terminal region, clustering near residues E11, Q15, and the putative metal binding site (H6, H13, and H14). Potential non-covalent interactions were identified between the small molecules and the nearby residues that NMR results indicated were likely sensitive to the introduction of ligands. For both L1 and L4, the triazole ring is predicted to interact with the K16 residue, while L2 and L3 are predicted to interact near the metal binding site in the opposite orientation. Overall, these theoretical interactions suggest the interaction of L1 – L4 with monomeric Aβ species.

**Figure 2.8.** Docking of small molecules with Aβ_{1-40} monomer in the presence of SDS (PDB 1BA4) predicted by AutoDock Vina. Cartoon depictions of (a) L1 (light blue), (b) L2 (yellow), (c) L3 (carboxylate form, magenta), and (d) L4 (protonated form, light pink) with Aβ_{1-40} in the presence of SDS (PDB 1BA4) in the same confirmation. (e) Surface depiction of L1 – L4 interacting with Aβ_{1-40}.

### 2.3.6. Inhibition of Aβ Aggregation

Previous reports have demonstrated that the interaction of Cu(II) and Zn(II) with the Aβ peptide facilitates aggregation. Interestingly, metal binding agents could disrupt metal-induced Aβ aggregation via chelation, thus limiting Aβ fibril formation. In this work, a turbidity assay via light scattering measurements (405 nm) was first used to study the ability of L1 – L4 to regulate metal-induced aggregation of the Aβ peptide. This assay provides information about the degree of peptide
aggregation in bulk solution. The lower pH value for Cu(II) was chosen as Cu(II)-induced aggregation is optimal at pH 6.6. The high affinity metal ion chelator, diethylenetriaminepentaacetic acid (DTPA), is used as a positive control, and demonstrates essentially complete inhibition of both Cu(II)- and Zn(II)-induced aggregation in this study. L1 – L4 were shown to inhibit Cu(II)- and Zn(II)-induced Aβ aggregation by 50 – 90% at pH 6.6 and 7.4, respectively (Figure 2.9). Secondly, to further demonstrate that L1 is able to influence metal-induced Aβ1-40 aggregation, analysis of the samples containing metal ions, Aβ1-40, and L1 was conducted by transmission electron microscopy (TEM). As shown in Figure 2.10, upon incubating the Aβ1-40 peptide with either CuCl₂ or ZnCl₂, a significant amount of fibrillogenesis is observed (Figure 2.10). When the ligand L1 was introduced to a metal-peptide solution before incubation, smaller aggregates were observed after 24 hours. This further supports the hypothesis that L1 could alter metal-induced Aβ peptide aggregation.

We also investigated the ability of L1 to influence Aβ1-40 peptide aggregation in the absence of metal ions. Incubation of Aβ peptide for 24 hours leads to fibril formation (Figure 2.11), albeit fibril morphology is smaller and thinner in comparison to Aβ1-40 exposed to CuCl₂ or ZnCl₂. Incubation of the Aβ1-40 peptide with L1 leads to a mixture of fibrillar and unstructured aggregates in comparison to the peptide alone (Figure 2.11). The overall results from studies of both metal-free and metal-induced Aβ aggregation employing L1 suggest that L1 could interact with Aβ1-40 peptides (along with 2D NMR studies, vide supra) and may restrict the formation of Aβ1-40 fibrils in both the absence and presence of metal ions.
Figure 2.9. Degree of $\text{A}\beta_{1-40}$ aggregation as measured by UV-vis measurements. Data represents the mean absorbance of quadruplicate trials at 405 nm of peptide in the presence of metal ions and ligands incubated for 45 mins. at 37 °C at pH 6.6 and 7.4 for Cu(II) and Zn(II), respectively. Error bars represent the standard deviation of the average absorbance value. See experimental section for details.

Figure 2.10. TEM images of samples incubated with 25 µM $\text{A}\beta_{1-40}$, 25 µM metal ions, and 50 µM L1 were incubated for 24 h at 37°C. Top: TEM images of $\text{A}\beta_{1-40}$ + CuCl$_2$, (left); $\text{A}\beta_{1-40}$ + CuCl$_2$ + L1 (right). Bottom: $\text{A}\beta_{1-40}$ + ZnCl$_2$ (left); $\text{A}\beta_{1-40}$ + ZnCl$_2$ + L1 (right).
Figure 2.11. TEM images of the samples containing metal-free Aβ₁-₄₀ species (25 µM) and L1 (50 µM). TEM images of metal-free Aβ₁-₄₀ species (left) and of metal-free Aβ₁-₄₀ species treated with L1 (right) (Conditions: 24 h at 37 °C).

2.4. Conclusion

Four bifunctional ligands L₁ – L₄ containing a triazole-pyridine metal binding scaffold were prepared and studied. The compounds were assembled in a modular fashion using click chemistry to afford a uniform metal-binding unit, while allowing for the installation of different peripheral groups. The protonation states and metal binding properties of L₁ – L₄ were investigated by UV-vis, NMR, and/or X-ray crystallography. Subsequent 2D NMR investigations and docking studies provided important information on the ability of L₁ – L₄ to interact directly with the metal-free Aβ peptide in solution. Turbidity and TEM studies demonstrated their ability to inhibit Aβ aggregation processes in the presence of metal ions. All four ligands were found to influence metal-induced Aβ aggregation, with L₁ considered to be the most promising bifunctional molecule. Overall, the TriPy ligand framework shows promise for disrupting metal-Aβ interactions and peptide aggregation.
Chapter 3.
Quinoline-Triazole Ligands that Influence Cu-based Aβ Aggregation Associated with Alzheimer’s Disease

M. R. Jones performed the syntheses, acidity constant determinations, native gel electrophoresis and Western blotting, TEM imaging. M. Hoarau aided in the syntheses and acidity constant determination. K. Korshavn and A. Ramamoorthy performed the 2-D SOFAST-HMQC NMR experiments. C. Dyrager completed the molecular modelling simulations.

3.1. Introduction

To date, many research efforts have focused on the development of compounds that regulate the aggregation pathway of Aβ. Dysregulated metal ions such as Cu, Zn, and Fe associate with the Aβ peptide increasing its propensity for aggregation. For the redox active metals Cu and Fe, oxidative stress can result. A number of Aβ aggregation pathways display different aggregation intermediates and eventually lead to the end-stage formation of amyloid plaques, which is a distinct hallmark in the brain. Initiation of the aggregation process commences via hydrophobic self-recognition interactions, specifically through peptide residues 17-21. This region has several hydrophobic amino acid residues including alanine, leucine, valine, and phenylalanine. In order to inhibit the Aβ aggregation process, the development of small molecules that limit interactions between these hydrophobic self-recognition sites may be a valid therapeutic strategy. Furthermore, incorporating a metal binding site into the chemical scaffold provides additional functionality to the compounds, which may allow for improved Aβ anti-aggregation activity. The development of multifunctional/target compounds has been extensively evaluated as a therapeutic strategy for many neurodegenerative diseases, including AD. This chapter extends from Chapter 2 by developing small molecules with increased potential for hydrophobic interactions with the
Aβ peptide by exchanging the pyridine ring for a quinoline group (Figure 3.1). Further in-depth analysis of ligand-peptide interactions were achieved via 2-D SOFAST NMR studies and molecular docking simulations using Schrödinger Suite, as well as native gel electrophoresis and TEM imaging. This provides a more complete analysis of the mechanism in which these ligands interact with the peptide in the absence and presence of metal ions. In addition, this chapter focuses on the longer Aβ₁₋₄₂ variant, in comparison to the shorter Aβ₁₋₄₀ variant studied in Chapter 2, which possesses two extra hydrophobic amino acids and is more prone to aggregation and neurotoxicity.²⁷, ⁵¹

Figure 3.1. A comparison between chemical scaffolds synthesized in Chapter 2 (pyridine) vs. Chapter 3 (quinoline).

3.2. Experimental

3.2.1. Syntheses

2-Azidoethyl-4-methylbenzenesulfonate,²⁵² 1-azido-propanol,²⁵³ and 2-ethynylquinoline³⁰² were synthesized according to reported procedures. See Section 2.2.1. for the synthesis of 4-(2-azidoethyl)morpholine.

4-(2-azidoethyl)thiomorpholine. 2-Azidoethyl-4-methylbenzenesulfonate²⁵² (2.004 g, 8.3 mmol), thiomorpholine (1.714 g, 16.6 mmol), and NEt₃ (3.362 g, 33.2 mmol) were dissolved in 10 ml MeCN in a 2-neck round bottom flask under N₂ atmosphere following a modified procedure.²⁵² The solution was allowed to reflux for 4 days. The solution was concentrated in vacuo, dissolved in CH₂Cl₂ and washed with saturated NaHCO₃. The organic layers were combined, dried over MgSO₄, filtered, and concentrated in vacuo. The residue was purified by silica gel chromatography (90:10 CH₂Cl₂ / CH₃OH eluent) to afford 4-(2-azidoethyl)thiomorpholine as a dark yellow oil
(0.891 g, 63%). $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.31 (t, $J = 5.9$ Hz, 2H), 2.79 (t, $J = 4.4$ Hz, 4H), 2.70 (t, $J = 5.6$ Hz, 4H), 2.63 (t, $J = 6.1$ Hz, 2H). FT-IR: 2911 cm$^{-1}$ (w, sh), 2811 cm$^{-1}$ (w, sh).

3-(4-(quinolin-2-yl)-1H-1,2,3-triazol-1-yl)propan-1-ol (QOH). 2-ethynylquinoline (2a) (0.037 g, 0.24 mmol) and 3-azido-1-propanol (0.041 g, 0.40 mmol) were dissolved in 2 ml iPrOH. In 2 ml H$_2$O, CuSO$_4$ (0.003 g, 0.012 mmol) and L-ascorbic acid (0.021 g, 0.12 mmol) were dissolved and added to the organic mixture. The reaction was allowed to stir at 298 K for 3 hours. Chelex® resin was added to the solution and allowed to stir at 298 K for 2 hours in order to remove the Cu catalyst. The Chelex resin was filtered and the supernatant was concentrated in vacuo. The residue was purified by silica gel chromatography (90:10 CH$_2$Cl$_2$ / MeOH) to afford QOH as a brown solid (0.021 g, 34% yield). $^1$H NMR (CD$_3$OD, 400 MHz): $\delta$ 8.67 (s, 1H), 8.41 (d, $J = 8.6$ Hz, 1H), 8.21 (d, $J = 8.6$ Hz, 1H), 8.06 (d, $J = 8.5$ Hz, 1H), 7.94 (d, $J = 8.1$ Hz, 2H), 7.78 (t, $J = 8.4$ Hz, 1H), 7.60 (t, $J = 8.1$ Hz, 1H), 4.66 (t, $J = 7.0$ Hz, 2H), 3.65 (t, $J = 6.1$ Hz, 2H), 2.21 (quin, $J = 6.1$ Hz, 2H). $^{13}$C NMR (CD$_3$OD, 100 MHz): $\delta$ 151.63, 149.26, 149.12, 138.95, 131.50, 129.56, 129.39, 129.25, 128.02, 125.35, 59.46, 48.72, 34.18. HR-ESI(+)-MS (m/z): [M+H]$^+$ Calcd for [M+H]$^+$ (C$_{14}$H$_{15}$N$_4$O), 255.1240; Found, 255.1274.

4-(2-(4-(quinolin-2-yl)-1H-1,2,3-triazol-1-yl)ethyl)morpholine (QMorph). 2-ethynylquinoline (2a) (0.049 g, 0.32 mmol) and 4-(2-azidoethyl)morpholine (0.059 g, 0.38 mmol) were dissolved in 2 ml iPrOH and followed the same procedure as for the formation of QOH. The residue was purified by silica gel chromatography (90:10 CH$_2$Cl$_2$ / MeOH) to afford QMorph as a brown solid (0.059 g, 72% yield). $^1$H NMR (CD$_3$OD, 400 MHz): $\delta$ 8.73 (s, 1H), 8.42 (d, $J = 8.6$ Hz, 1H), 8.23 (d, $J = 8.6$ Hz, 1H), 8.06 (d, $J = 8.5$ Hz, 1H), 7.94 (d, $J = 8.1$ Hz, 1H), 7.77 (t, $J = 7.1$ Hz, 1H), 7.60 (t, $J = 7.1$ Hz, 1H), 4.68 (t, $J = 6.2$ Hz, 2H), 3.70 (t, $J = 4.6$ Hz, 4H), 2.94 (t, $J = 6.3$ Hz, 2H), 2.57 (t, $J = 4.7$ Hz, 4H). $^{13}$C NMR (CDCl$_3$, 400 MHz): HR-ESI(+)-MS (m/z): [M+H]$^+$ Calcd for [M+H]$^+$ (C$_{17}$H$_{20}$N$_5$O), 310.1662; Found, 310.1682.

4-(2-(4-(quinolin-2-yl)-1H-1,2,3-triazol-1-yl)ethyl)thiomorpholine (QT Morph). 2-ethynylquinoline (2a) (0.111 g, 0.72 mmol) and 4-(2-azidoethyl)thiomorpholine (0.118 g, 0.65 mmol) were dissolved in 2 ml iPrOH and followed the same procedure as for the
formation of QOH. The residue was purified by silica gel chromatography (95:5 CH₂Cl₂ / CH₃OH eluent) to afford a brown solid (0.187 g, 89% yield). ¹H NMR (CDCl₃, 400 MHz): δ 8.42 (s, 1H), 8.33 (d, J = 8.5 Hz, 1H), 8.24 (d, J = 8.6 Hz, 1H), 8.07 (d, J = 8.6 Hz, 1H), 7.84 (d, J = 8.5 Hz, 1H), 7.73 (t, J = 8.5 Hz, 1H), 7.54 (t, J = 8.5 Hz, 1H), 4.53 (t, J = 6.0 Hz, 2H), 2.93 (t, J = 4.5 Hz, 4H), 2.81 (t, J = 6.1 Hz, 2H), 2.67 (t, J = 5.4 Hz, 4H). ¹³C NMR (CDCl₃, 151 MHz): δ 150.63, 148.70, 148.16, 136.94, 129.81, 129.15, 127.88, 127.86, 126.42, 123.36, 118.78, 58.33, 55.09, 47.97, 28.05. HR-ESI(+)-MS (m/z): [M+H]⁺ Calcd for [M+H]⁺ (C₁₇H₂₀N₅S), 326.1434; Found, 326.1460.

Scheme 3.1. Synthetic routes for the preparation of QOH, QMorph, and QTMorph.

3.2.2. Turbidity Measurements

See Section 2.2.8. for details.

3.2.3. Predictability of Drug-Like/BBB Permeability Properties

Using the website molinspiration.com, several physicochemical properties were determined in order to predict the drug-like properties and blood-brain barrier (BBB) permeability of each ligand. cLogP, Mᵤ, total potential surface area (TPSA), H-bond acceptors, and H-bond donors were calculated to determine the drug-like properties of
each ligand.\textsuperscript{303} Clark’s equation\textsuperscript{304} (Eq. 3.1) was used to determine the logBB, which is a reasonable indication of permeability through the blood-brain barrier:

\textbf{Eq. 3.1.} \quad \log BB = -0.0148(\text{TPSA}) + 0.152(c\text{Log}P) + 0.139

\subsection*{3.2.4. Determination of Acidity Constants by UV-Vis Spectroscopy}

See Section 2.2.4. for details.

\subsection*{3.2.5. Determination of Acidity Constants by NMR Spectroscopy}

See Section 2.2.5. for details.

\subsection*{3.2.6. 2-D SOFAST HMQC NMR Spectroscopy}

2-D band-Selective Optimized Flip Angle Short Transient (SOFAST) Heteronuclear Multiple Quantum Correlation (HMQC) NMR experiments provide rapid residue-specific insight into the protein environment. This is achieved by shaping the preparation pulse and allowing for reduced inter-scan delays required for the spin system to relax. NMR samples were prepared from \textsuperscript{15}N-labeled A\textsubscript{β1-40} (rPeptide) by first dissolving the peptide in 1% NH\textsubscript{4}OH and lyophilizing. The peptide was re-dissolved in 3 \textmu L of DMSO-\textit{d}_6 and diluted into buffer for a final peptide concentration of 80 \textmu M (pH 7.4, 20 mM H\textsubscript{2}PO\textsubscript{4}, 50 mM NaCl, 7% D\textsubscript{2}O v/v, 2.5% DMSO v/v). Spectra were taken at each titration point using a 600 MHz Bruker Avance NMR spectrometer equipped with a triple-resonance z-gradient cryogenic probe at 8 °C with 128 t\textsubscript{1} experiments, 128 scans, and a 100 ms recycle delay. The 2D \textsuperscript{15}N-\textsuperscript{1}H SOFAST-HMQC data were processed using TOPSPIN 2.1 (Bruker) and resonance assignment was performed with Sparky 3.1134. Resonances were assigned based on previous assignments under similar conditions.\textsuperscript{305-307} Chemical shift perturbation (CSP) was calculated using the following equation:

\textbf{Eq. 3.2.} \quad \Delta \delta_{N-H} = \sqrt{(\Delta \delta_{H})^2 + (\Delta \delta_N)^2}

where $\Delta \delta_H$ is defined as the change in $^1$H chemical shift and $\Delta \delta_N$ is defined as the
change in the $^{15}\text{N}$ chemical shift upon addition of ligand to the $^{15}\text{N}$-labeled A$\beta_{1-40}$ peptide. This equation has been previously used in the literature to describe the $\Delta \delta_{N-H}$.  

3.2.7. Molecular Modeling

Molecular docking studies were performed using Maestro from Schrödinger (Suite 2014). The monomeric coordinates for the partially folded A$\beta_{1-40}$ peptide were obtained from the Protein Data Bank (PDB ID: 2LFM), enclosing an NMR-assembly of twenty conformers. The A$\beta_{1-40}$ structures and the ligands (QOH, QMorph, and QTMorph) were prepared and energy minimized according to standard procedures (Protein Preparation Wizard and LigPrep, respectively). Subsequently, the prime energy was calculated for the processed peptide structures in order to confirm that there are no strains or clashes within the systems before the dockings were performed. Flexible dockings were performed with the twenty prepared conformers using the induced fit docking (IFD) protocol with extra precision (XP) settings for glide re-docking using the whole peptide structure as the grid centroid. The docking results were evaluated and compared with the 2D SOFAST-HMQC data in order to find the lowest energy conformations that correlated to the chemical shift perturbation.

3.2.8. Native Gel Electrophoresis and Western Blotting

The ability of QOH, QMorph, and QTMorph to influence the aggregation of A$\beta_{1-42}$ was further evaluated by molecular weight separation on a 10 – 20% gradient tris-tricine gel and visualized using western blotting techniques. Each sample was incubated for 24 hours under constant agitation at 37 °C in a 96-well plate, covered with a lid and sealed with parafilm. Final concentrations, diluted in PBS pH 7.4, were as follows: 25 µM A$\beta_{1-42}$, 25 (1 eq.) and 35 µM (1.4 eq.) CuCl$_2$, 50 µM (2 eq.) and 125 µM (5 eq.) ligand. Samples were loaded onto a 10-20 % gradient tris-tricine gel (Bio-Rad #456-3114) and run at 100 V for 100 minutes in a tricine running buffer, followed by transferring to a nitrocellulose membrane for 3 hours at 40 V in a 4 °C cold room. The membrane was blocked in 3 % BSA solution in tris-buffered saline containing 0.1 % Tween-20 (TBS-T) for 1 hour at room temperature, followed by an incubation with a primary anti-A$\beta$ antibody (6E10) overnight at room temperature under constant agitation. The membrane
was washed 4 x 15 minutes with TBS buffer and then incubated at room temperature under constant agitation for 2 hours with a horseradish peroxidase conjugated goat anti-mouse secondary antibody in 2 % BSA solution. The membrane was washed with TBS buffer for 4 x 15 minutes, incubated with the Thermo Scientific Supersignal West Pico Chemiluminescent Substrate kit (ThermoScientific #34087) for 5 minutes and visualized with a Fujifilm Luminescent imager.

3.2.9. Transmission Electron Microscopy (TEM)

See Section 2.2.9. for details.

3.3. Results and Discussion

3.3.1. Design and Synthesis

The development of compounds that are capable of multiple modes of interaction are of increasing interest in the field of AD therapeutics. A series of quinoline-triazole derivatives were synthesized in a modular fashion using Huisgen’s 1,3-dipolar cycloaddition, also known as click chemistry (Figure 3.2). Herein, a metal binding site was incorporated into the scaffold upon triazole formation with variation of a peripheral R-group. The extended ring system of the quinoline-triazole derivatives in comparison to the pyridine-triazole derivatives (Chapter 2) allowed for the investigation of whether increased hydrophobic interactions between the ligands and the Aβ peptide influences the aggregation process.

Three quinoline-triazole derivatives were synthesized by reacting 2-ethynylquinoline with corresponding azides in the presence of a catalytic amount of CuSO₄ and L-ascorbic acid (Scheme 3.1). The synthesis of 2-ethynylquinoline was initially attempted using trimethylsilylacetylene in a Sonogashira cross-coupling reaction, but the protected alkyne product was found to have a similar Rᵥ value to that of the starting material. An alternative alkyne was chosen, 2-methyl-3-butyn-2-ol, in order to increase the polarity of the Sonogashira product. This allowed for a facile separation using silica gel chromatography as the product possessed a lower Rᵥ value in
comparison to the 2-chloroquinoline starting material. The alkyne was deprotected in toluene using NaOH, which deprotonates the alcohol group and produces acetone as a by-product. The development of compounds that are neutral at physiological pH increase the likelihood of BBB permeability. Based upon the results obtained from the pyridine-triazole derivatives (Chapter 2), azides were specifically chosen that would not be charged at pH 7.4. Based on the positive results of L1 (pyridine-triazole-morpholine) in Chapter 2, a second analog of this type was synthesized by replacing the O-atom in the morpholine ring with a S-atom to produce a thiomorpholine derivative. This modular synthetic route allowed for the construction of three quinoline-triazoles in 34 – 89% yields.

![Chemical structures](image)

**Figure 3.2.** Chemical structures of QOH (top), QMorph (middle), and QTMorph (bottom).

### 3.3.2. Preliminary Studies of Inhibition of Aβ<sub>1-40</sub> Aggregation

Since the initial discovery of the interactions between endogenous metal ions and the Aβ peptide, the development of ligands that are able to favourably regulate the aggregation state and toxicity of the resulting species has become a burgeoning field. Neurotoxic Aβ oligomers are now hypothesized to be important species in AD etiology. More recently, it was shown that Cu-Aβ interactions lead to the formation of Aβ oligomers, which may be more toxic due to the redox-active Cu ion. Building off of the scaffolds that were presented in Chapter 2, a series of quinoline-triazole derivatives were initially studied for their ability to limit Cu-induced Aβ<sub>1-40</sub> aggregation using a turbidity assay (Figure 3.3). When Cu and Aβ<sub>1-40</sub> are incubated at a 1:1 ratio,
significant aggregation is observed via an increase in turbidity, measured at 405 nm. When comparing the degree of Cu-induced Aβ₁₋₄₀ aggregation in the presence of the pyridine analogs or the quinoline derivatives, both systems were able to limit Cu-induced Aβ₁₋₄₀ aggregation to the same degree. A 70-80% inhibition in Cu-induced Aβ₁₋₄₀ aggregation is observed (Figure 3.3). DTPA, a known Cu chelator, is shown to completely inhibit this process. Based on these promising preliminary results, evaluation of this new series of quinoline-triazole derivatives were further analyzed using the more aggregation prone Aβ₁₋₄₂ using more informative physical and biochemical techniques.

![Figure 3.3](image)

**Figure 3.3.** Degree of Aβ₁₋₄₀ aggregation in the presence of Cu determined by UV-vis measurements at 405 nm. Data represents the mean absorbance of quadruplicate trials. Error bars represent the standard deviation of the average absorbance values. Conditions: 150 µM ligand, 25 µM CuCl₂, 25 µM Aβ₁₋₄₀, 0.1 M PBS pH 6.6 buffer, incubation at 37°C under constant agitation for 45 mins. followed by measurement at 405 nm using a 96-well plate reader.

### 3.3.3. Drug-Like Properties and BBB Permeability

In medicinal chemistry, several factors are taken into consideration when evaluating a compound’s drug-like properties. Lipinski’s rule of 5 includes 5 parameters that predict whether a compound will have favourable pharmacokinetic properties, which can then be used to predict BBB-permeability. These parameters include cLogP,
molecular weight, hydrogen bond acceptors and donors, and total potential surface area (TPSA). The cLogP value is the calculated partition coefficient between 1-octanol and water, which provides information about the lipophilicity/hydrophilicity of a compound. Compounds with a molecular weight < 500 g/mol are predicted to be more drug-like.\textsuperscript{303} Ideally, compounds possessing a TPSA under 90 Å\textsuperscript{2} are more likely to permeate the BBB. Finally, drug-like compounds possess < 5 H-bond donors and < 10 H-bond acceptors. All of these characteristics were calculated using the website molinspiration.com. This website has been commonly used by other research groups and has been cited in the literature over 2000 times. Upon determining the drug-like parameters, calculation of the logBB value using Clark’s equation (Eq. 3.1) provided insight into the BBB-permeability of each compound.\textsuperscript{304} LogBB values that are > 0.3 indicate that the compound is highly likely to permeate through the BBB and values < -1.0 indicate a very low probability of BBB access.\textsuperscript{304, 318} The drug-like parameters and logBB values were determined for QOH, QMorph, QTMorph (Table 3.1).

Table 3.1. Summary of the Lipinski’s rules for drug-likeness and determination of the LogBB to predict BBB permeability.

<table>
<thead>
<tr>
<th></th>
<th>QOH</th>
<th>QMorph</th>
<th>QTMorph</th>
</tr>
</thead>
<tbody>
<tr>
<td>M\textsubscript{w}</td>
<td>254.293</td>
<td>309.373</td>
<td>325.441</td>
</tr>
<tr>
<td>MiLogP</td>
<td>1.265</td>
<td>1.502</td>
<td>2.043</td>
</tr>
<tr>
<td>TPSA</td>
<td>63.838</td>
<td>56.082</td>
<td>46.848</td>
</tr>
<tr>
<td>HBA</td>
<td>5</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>HBD</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lipinski’s rules</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
</tr>
<tr>
<td>LogBB</td>
<td>-0.61</td>
<td>-0.46</td>
<td>-0.24</td>
</tr>
</tbody>
</table>

Based on Lipinski’s rules, all three ligands passed the five tests to determine drug-likeness including having a M\textsubscript{w} < 500 g/mol, LogP < 5, and the H-bond donor and acceptors are < 5 and < 10, respectively. With these parameters, LogBB values were calculated using Clark’s equation, which indicate that these ligands have moderate BBB passivation.
3.3.4. Ligand Speciation Measurements

The development of compounds that are neutral at physiological pH is an important consideration for crossing the blood-brain barrier. To determine which species is predominant at pH 7.4, variable pH UV-vis and $^1$H NMR spectra were obtained for each ligand (Figure 3.4) to determine the $pK_a$ values of ligand protonation sites. NMR and UV-vis data were fit using HypNMR and HypSpec, respectively from the HyperQuad suite of programs. The quinoline-$N$ $pK_a$ value ranged from 3.43 – 3.66 (Table 3.2), which is lower in comparison to free quinoline ($pK_a = 4.85$). The $pK_a$ of the quinoline-$N$ is very similar to the $pK_a$ values of the pyridine analogs from Chapter 2 ($pK_a$ Pyridine-$N$ = 3.05 – 3.45) Fitting the variable pH $^1$H NMR data for QMorph and QTMorph using HypNMR provided similar $pK_a$ values for the $N$-atom of the morpholine and thiomorpholine rings (Table 3.1). Interestingly, these values are lower than that of free morpholine and thiomorpholine alone ($pK_a$ free morpholine = 8.35, $pK_a$ free thiomorpholine = 9), which was observed by our group and by others. It has been proposed that the distinct difference in morpholine and thiomorpholine $pK_a$ values can be attributed to the steric and/or electronic effects of the triazole ring.
Figure 3.4.  
(Top) Variable pH UV-vis spectra of QMorph (left), QOH (middle), and QTMorph (right) from pH 2 (red) – 12 (blue). (Bottom) Solution speciation diagrams of QMorph (left), QOH (middle), and QTMorph (right) indicating that each ligand is predominantly neutral at physiological pH (pH 7.4).

QOH has a single $pK_a$ value at 3.66(3), indicating that at physiological pH 7.4, it will predominantly be neutral. QMorph possesses two $pK_a$ values at 3.43(6) and 5.1(1) that are both below pH 7.4. This indicates that the dominant species at physiological pH will be neutral. QTMorph also exhibits two $pK_a$ values at 3.43(3) and 5.4(2) that are both below pH 7.4, indicating a neutral species will predominantly exist at physiological pH. to the fact that all quinoline-triazole derivatives are neutral at pH 7.4 is promising for passive diffusion across the blood-brain barrier.

Table 3.2.  $pK_a$ values and speciation at physiological pH as determined by variable pH UV-vis and NMR spectroscopy titrations.

<table>
<thead>
<tr>
<th></th>
<th>UV-vis</th>
<th>NMR</th>
<th>Speciation (pH 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pK_a$ (Quinoline)</td>
<td>$pK_a$ (R-group)</td>
<td></td>
</tr>
<tr>
<td>QMorph</td>
<td>3.43(6)</td>
<td>5.1(1)</td>
<td>Neutral</td>
</tr>
<tr>
<td>QOH</td>
<td>3.66(3)</td>
<td>n/a</td>
<td>Neutral</td>
</tr>
<tr>
<td>QTMorph</td>
<td>3.43(3)</td>
<td>5.4(2)</td>
<td>Neutral</td>
</tr>
</tbody>
</table>
3.3.5. Ligand-Peptide Interactions via 2-D $^1$H-$^{15}$N SOFAST-HMQC NMR

2-D $^1$H-$^{15}$N SOFAST-HMQC NMR was recently used as a tool to evaluate ligand interactions with macromolecules. Intrinsically, 2-D NMR techniques on peptides and proteins are time consuming due to the stepwise nature of each nuclear dimension. In order to decrease the overall time required to perform each experiment, a decrease in the time period between each scan is achieved by limiting the relaxation time of the system. This decrease in inter-scan delays allows for the collection of data in minutes rather than hours. The faster data acquisition time is especially beneficial for systems related to protein aggregation, as is the case for the $\text{A}_\beta$ peptide. To further aid in avoiding complications due to peptide aggregation, $\text{A}_\beta_{1-40}$, the less aggregation-prone peptide length, was chosen for these experiments. Herein, $^{15}$N-labeled $\text{A}_\beta_{1-40}$ was allowed to interact with 0-10 eq. quinoline-triazole ligands and spectra were obtained at each titration point. Data shown in Figures 3.5 – 3.7 is the average chemical shift perturbation (CSP) accounting for both $^1$H and $^{15}$N dimensions at 10 eq. of each ligand. The main differences between this experiment and that of Chapter 2 is the presence of SDS-$d_{25}$, which was used to maintain a monomeric peptide. A 600 MHz NMR was used to run the SOFAST-HMQC experiments in comparison to the 900 MHz instrument used in Chapter 2 and a significantly lower $\text{A}_\beta_{1-40}$ concentration was used during the SOFAST-HMQC experiments for Chapter 3 in comparison to Chapter 2 (80 µM vs. 308 µM $\text{A}_\beta_{1-40}$). Direct quantitative comparison between the quinoline and pyridine derivatives based on the 2D NMR experiments is not possible due to these differences, but qualitatively comparing which amino acid residues are affected by the presence of the ligands is possible since the goal of the NMR experiment is to identify both the degree of interaction of the ligand, and which residues the ligand interacts with. Finally, evaluating the change in chemical shift is an easier method instead of comparing the Hz values due to the fact that the chemical shift is not dependent on the magnetic field strength of the NMR instrument while the Hz value is dependent upon the magnetic field strength. Residues marked with * were those that could not be resolved. Residues with no chemical shift noted and no * were those amino acids which did not shift at all during the
titration. The aggregation of the Aβ1-40 peptide is hypothesized to initiate via interactions in the hydrophobic region of the peptide, around amino acids leucine 17 (L17) – alanine 21 (A21). Ligands that interact with this region have the potential to limit peptide-peptide interactions and therefore, may inhibit Aβ aggregation processes at an early stage.

**QMorph** exhibited the largest CSP for amino acids valine-18 (V18) and phenylalanine-19 (F19), which are located in the hydrophobic region (Figure 3.5). The reason for **QMorph**’s specific interactions with these amino acid residues is most likely attributed to a combination of hydrophobic and π – π stacking interactions. Several weaker interactions were also observed with residues glutamic acid – 3 (E3), histidine – 13 (H13), phenylalanine – 20 (F20) and valine – 36 (V36). A combination of electrostatic interactions and H-bonding are reasonable explanations to describe the interactions between **QMorph** and the polar amino acids, E3 and H13.

**QTMorph** exhibited interactions, specifically, with valine-18 (V18) and asparagine-27 (N27), but also with E3 in the hydrophilic region (Figure 3.6). Hydrophobic interactions between V18 and **QTMorph** are possible, while with residues E3 and N27, a hydrogen bonding or an electrostatic interaction is plausible via the quinoline-N and carboxylic acid (E3) or amino group (N27).

In comparison to **QMorph** and **QTMorph**, which interacted with only a few specific amino acid residues, **QOH** interacted non-specifically across the Aβ1-40 peptide causing several CSPs with many different amino acids in the hydrophilic and hydrophobic region (Figure 3.7). **QOH** caused marked CSP in residues E3, H13, L17, phenylalanine – 20 (F20), aspartic acid – 23 (D23), and methionine – 35 (M35). Interestingly, the interaction with E3 is common across all three quinoline derivatives, potentially due to the more flexible N-terminus, where several polar, hydrophilic residues exist, allowing for the E3 residue to be exposed at the surface.
Figure 3.5. (Left) 2D SOFAST-HMQC NMR spectra of 80 µM Aβ1-40 and 0-10 eq. QMorph (Red = 0 eq. QMorph, Blue = 10 eq. QMorph). (Top Right) Chemical shift changes in relation to specific Aβ1-40 residues. The dotted line represents the average CSP for the entire experiment and the long dashed line represents one standard deviation above the average CSP. Residues denoted with * indicates the inability to resolve that specific residue. (Bottom Right) Solution NMR structure of Aβ1-40 (PDB 2LFM) with residues possessing a CSP > 0.02ppm highlighted in red and residues with a CSP between 0.01 – 0.02 are highlighted in yellow.

Several other scaffolds have been used to investigate interactions with Aβ using the SOFAST-HMQC NMR technique. The Lim group reported interactions between a quinoline derivative and a series of aminoisoflavones with the Aβ1-40 peptide (Figure 3.8). Interestingly, the 8-hydroxyquinoline derivative demonstrates very specific interactions with only a select number of amino acid residues in the hydrophilic region (phenylalanine-4 (F4), arginine-5 (R5), valine-12 (V12), and glutamine-15 (Q15)) and one residue near the C-terminus (valine–36 (V36)). The absence of interactions with the hydrophobic region of the Aβ1-40 peptide for this derivative is interesting when compared to the quinoline-triazole derivatives presented in this chapter, potentially due to the increased amount of polar groups that are able to interact with the peptide. When
excess amounts of the 8-hydroxyquinoline compound were incubated with Aβ1-40, large CSP changes were observed, suggesting a conformational change in the peptide structure. In the case of the aminoisoflavones, these derivatives demonstrated very similar interaction patterns with only slight variances in CSP values. This result is interesting because our quinoline-triazole derivatives show significant differences in amino acid interactions in comparison to the structurally-similar aminoisoflavones.

Overall, the series of quinoline-triazoles interact with the hydrophobic region of the Aβ1-40, likely due to a combination of π – π stacking and hydrophobic interactions. Each derivative appears to interact with E3 and V18, while several other residues (D7, H13, F19, E22, G33, V36) interact to differing degrees with the three derivatives (Figure 3.9). Due to the interactions shown by the 2D-NMR experiments, these derivatives may be able to limit hydrophobic peptide-peptide interactions and therefore modify Aβ aggregation processes.
Figure 3.6. (Left) 2D SOFAST-HMQC NMR spectra of 80 µM Aβ1-40 and 0-10 eq. QTMorph (Red = 0 eq. QTMorph, Blue = 10 eq. QTMorph). (Top Right) Chemical shift changes in relation to specific Aβ1-40 residues. The dotted line represents the average CSP for the entire experiment and the long dashed line represents one standard deviation above the average CSP. Residues denoted with * indicates the inability to resolve that specific residue. (Bottom Right) Solution NMR structure of Aβ1-40 (PDB 2LFM) with residues possessing a CSP > 0.02ppm highlighted in red and residues with a CSP between 0.01 – 0.02 are highlighted in yellow.
Figure 3.7. (Left) 2D SOFAST-HMQC NMR spectra of 80 µM Aβ1-40 and 0-10 eq. QOH (Red = 0 eq. QOH, Blue = 10 eq. QOH). (Top Right) Chemical shift changes in relation to specific Aβ1-40 residues. The dotted line represents the average CSP for the entire experiment and the long dashed line represents one standard deviation above the average CSP. Residues denoted with * indicates the inability to resolve that specific residue. (Bottom Right) Solution NMR structure of Aβ1-40 (PDB 2LFM)\textsuperscript{305} with residues possessing a CSP > 0.02ppm highlighted in red and residues with a CSP between 0.01 – 0.02 are highlighted in yellow.
Figure 3.8. Chemical scaffolds that have been used in 2D SOFAST-HMQC NMR experiments to determine specific amino acid residue interactions with the Aβ1-40 peptide.

Figure 3.9. Comparison of all three quinoline derivatives using 2D SOFAST-HMQC NMR to determine which amino acid residues each quinoline-triazole interacts with. E3 and V18 appear to be shifted in all three cases.
3.3.6. Molecular Docking Simulations

To further understand the interaction of the quinoline-triazole derivatives with the Aβ1-40 peptide, molecular docking experiments were performed using Schrödinger Suite 2014 and the non-SDS solution NMR structure of Aβ1-40, PDB: 2LFM.\textsuperscript{305} In comparison to the docking studies performed in Chapter 2, this chapter focuses on a more physiologically relevant peptide structure where no detergents are present. 2LFM (Protein Data Bank ID) is an ensemble of 20 solution NMR structures. Each quinoline-triazole ligand was docked with each of the 20 solution NMR structures over the entire peptide molecule, and based on the lowest energy docking poses obtained, were compared to the experimental 2-D SOFAST-HMQC NMR studies to describe the interaction of each ligand with the Aβ1-40 peptide. For each pose, highlighted amino acid interactions were < 3 Å.

A docking pose that describes the interactions observed in the experimental 2D NMR experiments for QMorph include a cis-\(N\), \(N\) orientation of the quinoline-triazole rings where the morpholine ring is oriented towards the V18 and F19 side chains (Figure 3.10). The quinoline ring system was found to reside within the van der Waals radius of the E3 backbone. These three residues possessed the largest CSP values when interacting with QMorph (vide supra).
Figure 3.10. Molecular docking pose of QMorph with the NMR solution structure of Aβ1-40 (PDB: 2LFM). (Left) The quinoline ring interacts with the side chain of V18, while the triazole ring is within the van der Waals radius of the E3 side chain. (Right) QMorph has a favourable pose that resides in a hydrophobic region on Aβ1-40. Red = positively charged region; Blue = negatively charged region; Yellow = neutral region.

A docking pose involving QTMorph that best describes the 2D NMR interactions includes the quinoline ring positioned towards the V18 side chain, while the triazole ring was found to be within the van der Waals radius of the E3 side chain (Figure 3.11). E3 and V18 were found to have the largest CSP values during the 2D NMR experiment (vide supra).

The pose that best describes the QOH-Aβ1-40 interaction involves the quinoline ring situated within the van der Waals radius of both H13 and F20 side chains with the benzene ring oriented towards the imidazole of H13 and the pyridine ring towards the phenyl side chain of F20 (Figure 3.12). The propanol side chain of QOH was found to also reside within the van der Waals radius of the D23 side chain.
Figure 3.11. Molecular docking pose of QTMorph with the NMR solution structure of Aβ1-40 (PDB: 2LFM). (Left) The quinoline ring interacts with the side chain of V18, while the triazole ring is within the van der Waals radius of the E3 side chain. (Right) QTMorph has a favourable pose that resides in a hydrophobic region of the Aβ1-40 peptide. Red = positively charged region; Blue = negatively charged region; Yellow = neutral region.

Overall, each ligand exhibited a confirmation that described the experimental interactions observed during the 2D SOFAST-HMQC NMR experiment. In comparison to Chapter 2, using a peptide model in the absence of SDS is more physiologically relevant. Each peptide pose is different for each ligand as all 20 solution NMR structures were used and only the lowest energy poses were compared to the experimental 2D NMR results. In each case, E3 had a significant CSP and each ligand was able to interact with hydrophobic residues on the peptide.
Figure 3.12. Molecular docking pose for QOH with the NMR solution structure of the Aβ40 peptide (PDB: 2LFM). The quinoline ring is found to be within the van der Waals radius of the H13 side chain along with the phenyl side chain of F20. The OH group of QOH is also within the van der Waals radius of the D23 side chain (left). Overall, QOH resides in a hydrophobic region of the Aβ40 peptide (right). Red = positively charged region; Blue = negatively charged region; Brown = neutral region.

3.3.7. Native Gel Electrophoresis, Western Blotting, and TEM

To gain further insight and understanding into if the hydrophobic interactions of the quinoline-triazoles with the Aβ peptide affect aggregation, native gel electrophoresis using the anti-Aβ primary antibody 6E10 was employed. Two different experiments were performed to evaluate the interactions of these ligands in the absence and presence of Cu ions with the Aβ1-42 peptide. The Aβ1-42 peptide length was chosen for these studies due to the fact it is the most prone to aggregation. In the first experiment, 2 and 5 eq. ligand were incubated in the presence of Aβ1-42 in PBS pH 7.4. This provided information on the ability of the ligands to interfere with peptide aggregation (i.e. inhibition of hydrophobic self-interactions). The second experiment evaluated the ability for these ligands to influence peptide aggregation in the presence of stoichiometric and excess amounts of Cu²⁺. Recent studies suggested that in the presence of excess Cu, the Aβ peptide is able to bind to ca. 1.4 eq. Cu, suggesting that a second, lower-affinity Cu binding site exists on the Aβ peptide.¹⁴⁶ During these studies, 25 μM Aβ1-42 was incubated for 24 hours at 37 °C in the absence and presence of 1 eq. or 1.4 eq. Cu under constant agitation in the presence of 2 or 5 eq. ligand.
When 25 µM Aβ₁₋₄₂ was incubated alone, primarily high Mₙ species were observed in the native gel along with small amounts of oligomeric species in the 10 – 25 kDa region (Figure 3.13, lane 1). When 25 µM Aβ₁₋₄₂ was incubated in the presence of 2 or 5 eq. ligand, no changes in the aggregation profile were observed regardless of which ligand was used when compared to the Aβ₁₋₄₂ only lane (Figure 3.13, lanes 2-7). Based on the ligand-peptide interactions observed in the 2-D NMR and molecular docking studies, the observed hydrophobic interactions are likely too weak to influence the aggregation profile of the Aβ₁₋₄₂ peptide.

![Native gel electrophoresis](image)

<table>
<thead>
<tr>
<th>Lane</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aβ₁₋₄₂ only</td>
</tr>
<tr>
<td>2</td>
<td>Aβ₁₋₄₂ + 2 eq. QOH</td>
</tr>
<tr>
<td>3</td>
<td>Aβ₁₋₄₂ + 5 eq. QOH</td>
</tr>
<tr>
<td>4</td>
<td>Aβ₁₋₄₂ + 2 eq. QMorph</td>
</tr>
<tr>
<td>5</td>
<td>Aβ₁₋₄₂ + 5 eq. QMorph</td>
</tr>
<tr>
<td>6</td>
<td>Aβ₁₋₄₂ + 2 eq. QTMorph</td>
</tr>
<tr>
<td>7</td>
<td>Aβ₁₋₄₂ + 5 eq. QTMorph</td>
</tr>
</tbody>
</table>

**Figure 3.13.** Native gel electrophoresis of Aβ₁₋₄₂ in the absence and presence of varying equivalents of quinoline-triazole derivatives after incubation for 24 hours. No changes in the aggregation profile of Aβ₁₋₄₂ are observed by any of the quinoline-triazole derivatives.

Due to the significant presence of Cu found in Aβ plaque deposits, evaluation of these quinoline-triazole derivatives in the presence of stoichiometric and excess Cu + Aβ₁₋₄₂ were investigated. When Aβ₁₋₄₂ was incubated in the absence of Cu or ligands, significant amounts of high Mₙ species are observed along with definitive but minute amounts of low Mₙ species in the 10 – 25 kDa region (Figure 3.14, lane 1). When Aβ₁₋₄₂ was incubated in the presence of 1 eq. CuCl₂, primarily low Mₙ oligomeric species are observed, in line with previous literature reports (Figure 3.14, lane 2). When 25 µM Aβ₁₋₄₂ was incubated with 1.4 eq. CuCl₂, a large range of Mₙ were observed, including both low Mₙ oligomeric species (10 – 25 kDa range) and high Mₙ aggregates (100 – 250 kDa range) (Figure 3.14, lane 3). Upon incubation of 5 eq. QOH/QMorph/QTMorph with 1 eq. CuCl₂ in the presence of Aβ₁₋₄₂, a small increase in high Mₙ species is observed when compared to lane 2 containing no ligands (Fig. 3.14, lanes 4-6). Although an
increase in high \( M_w \) species is observed, very little difference in low \( M_w \) oligomeric species has occurred in comparison to lane 2. There is a possibility that a ligand-Cu-A\( \beta \) ternary complex is forming, which has been reported,\(^{325} \) resulting in the small amount of high \( M_w \) aggregates. This hypothesis has been proposed in the literature as a mechanism of action in dealing with metal-A\( \beta \) aggregates, leading them towards off-pathway, non-toxic species.\(^{123, 219, 322} \) When comparing lane 3 and lane 7, a significant decrease in high \( M_w \) species is observed with very little change in the amount of low \( M_w \) species in the 10 – 25 kDa range. Upon addition of 5 eq. Q\textit{Morph} to 1.4 eq. Cu\( Cl_2 \) + 1 eq. A\( \beta \)\(_{1-42}\) (lane 8), a comparable aggregation profile is observed to Q\textit{OH} (lane 7), although a larger amount of high \( M_w \) species was shown. Interestingly, when 5 eq. Q\textit{TMMorph} was incubated in the presence of 1.4 eq. Cu\( Cl_2 \) and 1 eq. A\( \beta \)\(_{1-42}\) (lane 9), a large range of \( M_w \) species was detected. This suggests that either Q\textit{TMMorph}, a Cu-Q\textit{TMMorph} complex, or the combination of both is reacting with the A\( \beta \)\(_{1-42}\) peptide causing the formation of a wide range of A\( \beta \) species. Intriguingly, such reactivity is not observed at lower Cu concentrations in the presence of 5 eq. Q\textit{TMMorph} (Figure 3.14 lane 6 vs. lane 9).

A number of reports suggest that the Cu\( ^{2+} \)-A\( \beta \) dissociation constant ranges from \( K_d = 10^{-11} – 10^{-7} \).\(^{27, 54} \) In order to influence Cu\( ^{2+} \)-A\( \beta \) interactions, a suggested ligand \( K_d \) in the range of \( 10^{-12} – 10^{-9} \) is necessary to observe reactivity.\(^{123} \) If the \( K_d > 10^{-5} \), no regulation of Cu\( ^{2+} \)-A\( \beta \) species is observed.\(^{326} \) Based on the limited changes to the aggregation profiles for 1:1 Cu\( ^{2+} \)-A\( \beta \) in the presence of the quinoline-triazole ligands (Figure 3.14, lanes 4-6), the quinoline-triazole derivatives likely exhibit \( K_d \) values at the high end of the required window (\( K_d = 10^{-12} – 10^{-9} \)), resulting in high nM/low \( \mu M \) affinity for Cu\( ^{2+} \).
Figure 3.14. Native gel electrophoresis of Aβ1-42 in the absence and presence of 1 eq. or 1.4 eq. Cu$^{2+}$ and 5 eq. quinoline-triazole derivatives. Conditions: 25 µM Aβ1-42, 25 (1 eq.) and 35 (1.4 eq.) µM Cu$^{2+}$, 5 eq. ligand, PBS pH 7.4, 24 hour incubation at 37 °C.

To further investigate the overall aggregation profile of each Cu-Aβ1-42 experiment, TEM analysis was used to evaluate bulk morphological. TEM grids were prepared by obtaining an aliquot from the native gel experiments prior to native gel electrophoresis separation and staining them using uranyl acetate. When Aβ1-42 was incubated in the absence of metals or ligands, significant amounts of fibrillar networks were observed, as has been previously reported (Figure 3.15A). Upon incorporation of 1 eq. CuCl$_2$ with Aβ1-42, fibrillar networks disappear and the formation of thick amorphous networks was observed (Figure 3.15B). Increasing the concentration to 1.4 eq. CuCl$_2$ results in slightly smaller, more defined, amorphous aggregates, but still a disappearance of any type of fibrillar structures (Figure 3.15C). Incubation of 1 eq. CuCl$_2$ + Aβ1-42 in the presence of 5 eq. QOH/QMorph/QTMorph does not change the morphology in comparison to 1 eq. CuCl$_2$ + Aβ1-42 alone. This suggests that the Cu-Aβ interactions have not been interrupted to a great extent. If QOH/QMorph/QTMorph were able to remove Cu from the peptide, a return to fibrillar structures would result, as is the case when the strong metal chelator DTPA is used. Each ligand was observed to have amorphous aggregates when incubated in the presence of 1 eq. Cu$^{2+}$ and Aβ1-42 (Figure 3.15D-F). When 5 eq. QOH/QMorph/QTMorph are exposed to 1.4 eq. CuCl$_2$ + 1 eq. Aβ1-42, no fibrillar structures are observed. Interestingly, only thick, undefined, amorphous networks are observed in contrast to the more defined aggregates observed in the 1.4 eq. CuCl$_2$ + 1 eq. Aβ1-42 case (Figure 3.15C and G-I).
Overall, these experiments demonstrate that the quinoline-triazole derivatives are not able to influence Aβ1-42 aggregation in the absence of metals, most likely due to weak hydrophobic interactions that were suggested in the 2-D NMR and molecular docking studies. Interestingly, when evaluating the ability of these ligands to regulate metal-mediated Aβ1-42 aggregation, all ligands showed the formation of a small amount of high M_w species when in the presence of 1 eq. CuCl_2 + Aβ1-42 instead of exclusive formation of oligomeric species, which was observed in the absence of ligands. This is a promising result due to the fact that oligomeric species are thought to be the most toxic aggregated form of the Aβ peptide. The ability to modulate the aggregation profile of the Aβ1-42 peptide towards off-pathway, non-toxic species via small molecular scaffolds is a promising therapeutic strategy that has been exploited. Due to the weak affinity of this series of quinoline-triazoles for Cu^{2+} binding (K_d(Cu) = high nM/low µM), the native gel result suggests primarily low M_w oligomeric species were observed and only small amounts of high M_w aggregates were imaged. TEM imaging also confirmed that the weak affinity of this series of ligands for Cu was not able to convert the amorphous aggregates back to a fibrillar state.
Figure 3.15. TEM analysis correlating the morphological changes of Aβ₁-42 in the absence and presence of Cu and ligands. A: Aβ₁-42; B: Aβ₁-42 + 1 eq. CuCl₂; C: Aβ₁-42 + 1.4 eq. CuCl₂; D: Aβ₁-42 + 1 eq. CuCl₂ + 5 eq. QOH; E: Aβ₁-42 + 1 eq. CuCl₂ + 5 eq. QMorph; F: Aβ₁-42 + 1 eq. CuCl₂ + 5 eq. QTMorph; G: Aβ₁-42 + 1.4 eq. CuCl₂ + 5 eq. QOH; H: Aβ₁-42 + 1.4 eq. CuCl₂ + 5 eq. QMorph; I: Aβ₁-42 + 1.4 eq. CuCl₂ + 5 eq. QTMorph.

3.4. Conclusion

A series of three quinoline-triazole derivatives have been synthesized and evaluated for their ability to influence the aggregation pathway of the Aβ peptide.
associated with AD. These three ligands are neutral at physiological pH, pass Lipinski’s rules for drug-likeness, and are predicted to have moderate BBB penetration. 2-D $^1$H – $^{15}$N SOFAST NMR was used to gain further insight into the potential interaction of these ligands with the Aβ$_{1-40}$ peptide in solution. QMorph and QTMorph were found to interact with specific amino acid residues in the hydrophobic region, along with the E3 residue in the hydrophilic region. Interestingly, QOH demonstrated non-specific amino acid interactions over the length of the peptide. Native gel electrophoresis provided insight into how these ligands change the aggregation profile of the peptide under different conditions. These ligands did not alter peptide aggregation when incubated with Aβ$_{1-42}$ alone, demonstrating that the ligand-peptide interactions were not sufficient to inhibit the more aggregation-prone peptide length. When the ligands were incubated in the presence of a 1:1 mixture of Cu$^{2+}$ + Aβ$_{1-42}$, oligomeric species were primarily observed while only small amounts of high M$_w$ species were imaged in comparison to the 1:1 Cu$^{2+}$ + Aβ$_{1-42}$ lane. Overall, this study demonstrated that these quinoline-triazole ligands possess relatively weak hydrophobic interactions with the Aβ peptide and likely moderate Cu$^{2+}$-binding affinity.
Chapter 4. Multifunctional Phenol-Triazole Ligands that Regulate Cu-induced Amyloid-β Aggregation Associated with Alzheimer’s Disease

M. R. Jones performed the syntheses, calculations for the drug-like properties and BBB-permeability, metal stability constant measurements, native gel electrophoresis and Western blotting, and TEM imaging. With assistance from M. R. Jones, E. Mathieu, aided in the syntheses, performed the acidity constant determinations, along with the Trolox and CCA assays. K. Korshavn and A. Ramamoorthy performed the 2-D SOFAST-HMQC NMR experiments. C. Dyrager completed the molecular modelling simulations.

4.1. Introduction

This chapter focuses on the development of a series of ligands that exhibit multifunctional properties towards treating AD. As previously described, regulation of metal-Aβ interactions is a viable therapeutic strategy. In chapters 2 and 3, I developed a series of pyridine- and quinoline-triazole ligands. These ligands contained neutral $N, N$ donor atoms, which resulted in ligands with weak-moderate binding affinity for Cu. The development of a ligand scaffold with an increased affinity for Cu was desired to better compete with the Aβ peptide for metal ions. To achieve this goal, a new series of phenol-triazole ligands was investigated (Figure 4.1). These ligands feature a $N$-triazole and an $O$-phenol donor atom framework. Due to the multifaceted nature of AD, a compound that is able to address multiple targets associated with the disease may provide an improved therapeutic response. In developing this series of phenol-triazoles, incorporation of a metal binding unit, anti-oxidant properties, Aβ peptide interactions, and alteration of the Aβ aggregation process in the absence and presence of Cu will be discussed.
Figure 4.1. Structure of the three phenol-triazole ligands, POH, PMorph, and PTMorph.

4.2. Experimental

4.2.1. Synthesis

1-azido-propanol\textsuperscript{253} and 2-((trimethylsilyl)ethynyl)phenol\textsuperscript{330} were synthesized according to previously reported procedures. Note: Compounds containing an azido moiety (-N\(_3\)) are potentially explosive and should be prepared in small quantities, kept in the dark, and at room temperature or lower. See Section 2.2.1. for the synthesis of 4-((2-azidoethyl)morpholine. See Section 3.2.1. for the synthesis of 4-((2-azidoethyl)thiomorpholine. For general methods and instrumentation, please see Section 2.2.
Scheme 4.1. Synthetic routes towards POH, PMorph, and PTMorph.

2-(1-(3-hydroxypropyl)-1H-1,2,3-triazol-4-yl)phenol (POH) To a solution of 2-((trimethylsilyl)ethynyl)phenol 4a (0.195 g, 1.02 mmol) and 3-azidopropanol (0.113 g, 1.11 mmol) in methanol (6 mL) was added a solution of CuSO₄•5H₂O (0.130 g, 0.52 mmol), L-ascorbic acid (0.072 g, 0.41 mmol), and K₂CO₃ (0.258 g, 1.87 mmol) in water (6 mL), while stirring vigorously. Pyridine (0.4 mL, 4.97 mmol) was added slowly to the resulting mixture and the solution was stirred at room temperature overnight. Chelex was added and the solution was stirred for an additional 2 hours. The solution was filtered, concentrated in vacuo and redissolved in H₂O. The aqueous layer was extracted three times with DCM, the organic layers were combined and concentrated in vacuo and purified by silica gel chromatography (100% EtOAc eluent) affording POH (0.054 g, 24% yield). ¹H NMR (CDCl₃, 400 MHz): δ 7.88 (s, 1H), 7.42 (dd, J = 7.8, 1.5 Hz, 1H), 7.24 (m, 1H), 7.06 (dd, J = 8.3, 1.0 Hz, 1H), 6.90 (td, J = 7.7, 1.2 Hz, 1H), 4.60 (t, J = 6.8 Hz, 2H), 3.68 (t, J = 5.8 Hz, 2H), 2.19 (quintet, J = 6.3 Hz, 2H). ¹³C NMR (CDCl₃, 151 MHz): δ 156.06, 147.74, 129.90, 125.95, 119.68, 119.62, 117.87, 114.06, 58.87, 47.45, 32.56. HR-ESI(+)–MS (m/z): [M + H]⁺ calculated for (C₁₁H₁₄N₃O₂), 220.1081; Found, 220.1103.
2-(1-(2-morpholinoethyl)-1H-1,2,3-triazol-4-yl)phenol (PMorph) To a solution of 2-((trimethylsilyl)ethynyl)phenol (4a) (0.195 g, 1.02 mmol) and 4-(2-azidoethyl)morpholine (0.176 g, 1.13 mmol) in methanol (6 mL) was added a solution of CuSO₄•5H₂O (0.130 g, 0.52 mmol), L-ascorbic acid (0.072 g, 0.4 mmol), and K₂CO₃ (0.258 g, 1.87 mmol) in water (6 mL), while stirring vigorously. Pyridine (0.4 mL, 4.97 mmol) was added slowly to the resulting mixture and the solution was stirred at room temperature using the same conditions to the synthesis of POH. The crude residue was purified by silica gel chromatography (DCM/MeOH 95:5 eluent) affording PMorph (0.106 g, 38% yield). \(^1\)H NMR (CDCl₃, 400 MHz): δ 10.84 (s, 1H), 7.99 (s, 1H), 7.42 (dd, J = 7.7, 1.6 Hz, 1H), 7.24 (m, 1H), 7.05 (dd, J = 8.3, 1.1 Hz, 1H), 6.91 (td, J = 7.5, 1.2 Hz, 1H), 4.55 (t, J = 6.4 Hz, 2H), 3.71 (t, J = 4.7 Hz, 4H), 2.89 (t, J = 6.3 Hz, 2H), 2.53 (t, J = 4.1 Hz, 4H). \(^13\)C NMR (CDCl₃, 151 MHz): δ 156.04, 147.8, 129.86, 125.92, 119.61, 119.57, 117.86, 114.16, 66.99, 57.95, 53.67, 47.92. HR-ESI(+)MS (m/z): [M + H]^+ calculated for (C₁₄H₁₉N₄O₂), 278.1413; Found, 278.1412.

2-(1-(2-thiomorpholinoethyl)-1H-1,2,3-triazol-4-yl)phenol (PTMorph) To a solution of 2-((trimethylsilyl)ethynyl)phenol 4a (0.195 g, 1.02 mmol) and 4-(2-azidoethyl)thiomorpholine (0.200 g, 1.16 mmol) in methanol (6 mL) was added a solution of CuSO₄•5H₂O (0.130 g, 0.52 mmol), L-ascorbic acid (0.072 g, 0.41 mmol), and K₂CO₃ (0.258 g, 1.87 mmol) in water (6 mL), while stirring vigorously. Pyridine (0.4 mL, 4.97 mmol) was added slowly to the resulting mixture and was stirred at room temperature using similar conditions to the synthesis of POH. The crude residue was purified by silica gel chromatography (DCM/MeOH 95:5 eluent) affording PTMorph (0.095 g, 42% yield). \(^1\)H NMR (CDCl₃, 400 MHz): δ 10.83 (s, 1H), 7.99 (s, 1H), 7.42 (dd, J = 7.7, 1.6 Hz, 1H), 7.28 (m, 1H), 7.06 (dd, J = 8.3, 1.0 Hz, 1H), 6.92 (td, J = 7.6, 1.2 Hz, 1H), 4.51 (t, J = 6.2, 2H), 2.90 (t, J = 6.3, 2H), 2.80 (t, J = 4.3, 4H), 2.67 (t, J = 4.4, 4H). \(^13\)C NMR (CDCl₃, 151 MHz): δ 155.95, 147.61, 129.79, 125.93, 119.63, 119.61, 117.87, 114.15, 58.20, 55.10, 48.18, 28.09. HR-ESI(+)MS (m/z): [M + H]^+ calculated for (C₁₄H₁₉N₄OS), 291.1274; Found, 291.1314.
General Procedure for synthesis of CuL₂ complexes:

To a stirred solution of ligand (2 eq.) and triethylamine (4 eq.) in MeCN (5 mL) was added slowly a solution of CuCl₂ (1 eq.) in MeCN (2 mL). The resulting solution turned green and was stirred overnight at room temperature. Diethyl ether was added in order to precipitate the complex. The solution was filtered and the residue obtained was washed with diethyl ether and dried in vacuo to afford a dark green powder.

Cu(POH)₂: 74% yield. ESI(+)-MS (m/z): [Cu(POH)₂ + H]⁺ calculated for (C₂₂H₂₅N₆O₄Cu), 501.02; Found, 501.13.

Cu(PMorph)₂: 54% yield. ESI(+)-MS (m/z): [Cu(PMorph)₂ + H]⁺ calculated for (C₂₈H₃₅N₈O₄Cu), 611.17; Found, 611.21.

Cu(PTMorph)₂: 44% yield. ESI(+)-MS (m/z): [Cu(PTMorph)₂ + H]⁺ calculated for (C₂₈H₃₅N₈O₂S₂), 643.31; Found, 643.17.

4.2.2. Predictability of Drug-Like/BBB Permeability Properties

See Section 3.2.3 for details.

4.2.3. Determination of Acidity Constants measured by UV-Vis

See Section 2.2.4. for details.

4.2.4. Determination of Acidity Constants measured by NMR

See Section 2.2.5. for details.

4.2.5. Metal Stability Constant Measurements

Metal stability constant measurements were performed by running spectra of 75 µM ligand + 37.5 µM CuCl₂. The solution was subjected to variable pH measurements using HCl and NaOH in the range pH 3-11. Approximately 30 UV-vis spectra were
obtained at several pH points. The UV-vis spectra were tabulated and HypSpec was used to analyze the data. A model of the potential species was developed and simulated to obtain the best fit to the experimental data.

4.2.6. Job’s Plot Analysis

To determine the ligand:Cu stoichiometry for the phenol-triazole series of ligands, a stock solution (0.7 mM) of PMorph and CuCl₂ (1 mM) were prepared in PBS pH 7.4 buffer and spectra were measured using a Cary UV-Vis-NIR instrument. Each phenol-triazole ligand has the same metal binding unit, therefore each ligand is assumed to bind to Cu in a similar manner. Solutions containing different ratios of ligand and Cu ions were recorded from 0 to 100 mol % Cu (total concentration = 100 µM). Appropriate amounts of the stock solutions were dissolved into 1 mL of PBS buffer and allowed to equilibrate for 5 minutes before recording the spectra. The absorption at 320 nm was plotted as a function of the mole fraction Cu (Cuₓ).

4.2.7. 2-D SOFAST HMQC NMR Spectroscopy

See Section 3.2.5. for details.

4.2.8. Molecular Modeling

See Section 3.2.6. for details.

4.2.9. Native Gel Electrophoresis and Western Blotting

The ability of POH, PMorph, and PTMorph to influence the aggregation of Aβ₁₋₄₂ was further evaluated by molecular weight separation on a 10 – 20% gradient tris-tricine gel and visualized using western blotting techniques. Each sample was incubated for 24 hours under constant agitation at 37 °C in a 96-well plate, covered with a lid and sealed with parafilm. Final concentrations, diluted in PBS pH 7.4, were as follows: 25 µM Aβ₁₋₄₂, 25 µM CuCl₂, 75 µM (3 eq.) ligand. Samples were loaded onto a 10-20 % gradient tris-tricine gel (Bio-Rad #456-3114) and run at 100 V for 100 minutes in a tricine running
buffer, followed by transferring to a nitrocellulose membrane for 3 hours at 60 V in a 4 °C cold room. The membrane was blocked in 3% BSA solution in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T buffer) for 1 hour at room temperature, followed by incubation with a primary anti-Aβ antibody (6E10, Covance) overnight at room temperature under constant agitation. The membrane was washed 4 x 15 minutes with TBS buffer and then incubated at room temperature under constant agitation for 2 hours with a horseradish peroxidase conjugated goat anti-mouse secondary antibody in 2% BSA solution. The membrane was washed with TBS buffer for 4 x 15 minutes, incubated with the Thermo Scientific Supersignal West Pico Chemiluminescent Substrate kit (ThermoScientific #34087) for 5 minutes and visualized with a Fujifilm Luminescent imager.

4.2.10. Transmission Electron Microscopy

See Section 2.2.9. for details.

4.2.11. CCA Anti-Oxidant Assay

Aqueous Cu ions can react with O₂ to produce reactive oxygen species, such as the hydroxyl radical (OH•), and in the presence of a reducing agent (ie: ascorbic acid), this process can become catalytic. The non-fluorescent coumarin-3-carboxylic acid (CCA) reacts with hydroxyl radicals to produce the fluorescent 7-hydroxycoumarin-3-carboxylic acid. The production of the fluorophore can be monitored to evaluate the efficiency of ligands to bind Cu, and therefore limit hydroxyl radical formation. This assay was adapted to be performed in a 96-well plate. Stock solutions (10X concentrations) were prepared in H₂O and added to each well in this order with the following final concentrations: CuSO₄ (40 µM), CCA (100 µM), ligands (80 µM), and L-ascorbic acid (400 µM). Final volume in each well was 200 µL, which was constituted up using pH 7.4 PBS buffer. Fluorophore formation was monitored by λₑₓ: 395 nm and λₑᵐ: 450 nm. Each experiment was performed in quadruplicate and allowed to react for 75 minutes. Blanks consisted of CCA, ligand, L-ascorbic acid, and pH 7.4 PBS buffer.
Figure 4.2. Coumarin-3-carboxylic acid (left) in the presence of Cu under aerobic aqueous conditions can react with hydroxyl radicals to produce the fluorescent 7-hydroxycoumarin-3-carboxylic acid (right).

4.2.12. Trolox-Equivalent Anti-Oxidant Capacity (TEAC) Assay

The method described by Rice-Evans et al. was adapted to a plate reader in order to perform several experiments simultaneously. First, ABTS (0.0082 g, 2.7 eq.) and K$_2$S$_2$O$_8$ (0.0016 g, 1 eq.) were dissolved in deionized water (2 mL) to create ABTS$.^{333}$ The solution was allowed to react for 16 hours in the dark at room temperature. An ABTS$.^*$ concentration exhibiting an absorbance value around 0.7 was achieved by aliquoting 3-42 µL ABTS$^*$ solution into a transparent 96-well plate dissolved in MeOH up to a final volume of 300 µL. Each volume was measured in triplicate to determine which volume had an absorbance at 0.7. The absorbance at 470 nm was recorded using a Synergy 4 Fluorometer plate reader from BioTek, and plotted as a function of ABTS concentration. The absorbance increased linearly in this range and a volume of 30 µL was chosen for the next step as this corresponded to an absorbance measurement of 0.7 at 470 nm.

The TEAC values of the ligands, Trolox, PBT2 and glutathione were determined. Stock solutions of the compounds in MeOH (1.5 mM) were prepared to final concentrations of 25, 50, 75, 100, and 125 µM and added into a transparent 96-well plate, then diluted with MeOH up to a volume of 270 µL. Just before the measurement, 30 µL of the ABTS$^{**}$ solution was added into each well with a multichannel pipette. The plate was shaken for 30 seconds, and the absorbance at 470 nm was recorded every minute for 6 minutes using a Synergy 4 Fluorometer plate reader from BioTek. Each measurement was performed in triplicate. The value of the absorbance was plotted as a function of the concentration of compound at a set time and the slopes obtained were then compared to that of Trolox, which was normalized to 1, to give the TEAC value at 1, 3 and 6 minutes.
4.3. Results and Discussion

4.3.1. Design and Synthesis

The series of phenol-triazole derivatives were proposed to have certain advantages over their pyridine and quinoline counterparts discussed in Chapters 2 and 3. The pyridine and quinoline series possess two neutral N-donor atoms in the ligand scaffold that weakly interact with Cu and Zn metal ions. These ligands, however, were able to influence the metal interactions that occurred with the Aβ peptide. In order to increase the metal binding affinity, the pyridine and quinoline moieties were replaced with a phenol group. This provides increased metal binding via an anionic phenolate. Since oxidative stress plays a major role in AD, the incorporation of a phenol function was hypothesized to provide additional antioxidant activity to the compounds with respect to the pyridine and quinoline derivatives. Finally, increasing hydrophobic interactions between the peptide and the ligands is desirable in order to regulate peptide aggregation; this process is thought to initiate from hydrophobic interactions of adjacent peptide chains.

The synthesis of these phenol-triazole derivatives was achieved by using Huisgen’s 1,3-dipolar cycloaddition. 2-((Trimethylsilyl)ethynyl)phenol was prepared according to literature precedence from 2-iodophenol using a Sonogashira cross-coupling reaction. Some complications arose during the deprotection and isolation of 2-ethynylphenol, therefore the protected phenol was used in a one-pot reaction where the phenol was deprotected in situ and coupled to the corresponding azide. The production of each derivative was achieved over 3-4 steps.

4.3.2. Drug-Like Properties and BBB Permeability

In order to predict the drug-like properties of a specific compound, several physicochemical parameters can be easily calculated in order to identify any potential in vivo side reactions. Functional groups involved in H-bonding, the topological potential surface area of the compound, and lipophilic/hydrophilic properties are all essential characteristics of a drug molecule. Using these fundamental properties, it is also
possible to predict whether a compound is BBB-permeable using Clark’s equation (Eq. 3.1). LogBB is used to predict compound permeability across the BBB with values > 0.3 indicating a compound readily crosses the BBB while values < -1.0 indicate a compound poorly crosses the BBB. Clark’s equation is governed solely by the total potential surface area (TPSA) and cLogP values. Compounds that have a small TPSA and are highly lipophilic (ie: high cLogP values) are predicted to have greater BBB permeability. The three phenol-triazole ligands satisfied each of the parameters associated with Lipinski’s rule of 5 with a $M_w < 500$, appropriate H-bonding donors and acceptors, and a TPSA < 90 Å$^2$ (Table 4.1). Using these parameters, the LogBB values were calculated using Clark’s equation (Eq. 3.1). LogBB values of -0.78 to -0.41 were obtained, indicating that this series of ligands is within the predicted window for permeation across the BBB. Overall, this series of phenol-triazole compounds exhibit promising physicochemical properties that are required for bioavailability.

<table>
<thead>
<tr>
<th></th>
<th>POH</th>
<th>PMorph</th>
<th>PTMorph</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_w$</td>
<td>219.244</td>
<td>274.324</td>
<td>290.392</td>
</tr>
<tr>
<td>cLogP</td>
<td>0.897</td>
<td>1.135</td>
<td>1.676</td>
</tr>
<tr>
<td>TPSA</td>
<td>71.174</td>
<td>63.418</td>
<td>54.184</td>
</tr>
<tr>
<td>HBA</td>
<td>5</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>HBD</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lipinski’s rules</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
</tr>
<tr>
<td>LogBB</td>
<td>-0.78</td>
<td>-0.63</td>
<td>-0.41</td>
</tr>
</tbody>
</table>

4.3.3. Job’s Plot

Each Cu complex was synthesized by reacting 2 eq. ligand with 1 eq. CuCl$_2$ in the presence of 4 eq. NEt$_3$ as a base to deprotonate the phenol moiety. Cu complexes were isolated by dissolving the product in MeOH and precipitating out the complex by adding Et$_2$O to the solution. To confirm that each complex had a 2:1 ligand:Cu ratio, mass spectrometry and Job’s plot analysis were performed. Each Cu complex mass
matched the calculated mass for a 2:1 ligand:Cu complex as determined by mass spectrometry. Further to confirm this ratio, a Job’s plot analysis was performed by monitoring the absorbance at 320 nm, which is related to the deprotonation of the phenol residue to a phenolate. Due to each phenol-triazole ligand possessing the same metal binding site, the PMorph ligand was used as a representative ligand. Various mole fractions of Cu and ligand were evaluated to determine at which stoichiometry was most favourable for the Cu complexes (Figure 4.3). The point at which the slopes of the two lines intersect dictates what mole fraction of Cu is the dominant species. In the general case of PMorph, the slopes of the two lines crossed at ca. 32% mole fraction Cu. The remaining 68% mole fraction therefore, belongs to the ligand. The mole fractions are approximately in a 2:1 ratio of ligand:Cu, suggesting the presence of a 2:1 ligand:Cu complex for each phenol-triazole ligand. The Job plot along with MS data (vide supra) suggest the formation of a 2:1 ligand:Cu complex in solution.

Figure 4.3. Job plot analysis of PMorph + CuCl₂. Various mole fractions of CuCl₂ and PMorph were combined in solution (PBS pH 7.4) and their absorbance at 320 nm measured to determine the most favourable stoichiometry between the metal and ligand. The intersection of the slopes is at 32% mole fraction Cu, suggesting a 2:1 ligand:Cu complex is present.

4.3.4. Ligand Speciation Measurements

The development of ligands that are neutral at physiological pH is optimal in order to penetrate the BBB. To determine this information, variable pH UV-Vis and ¹H NMR spectra were obtained. Using HypSpec and HypNMR to fit the variable pH data,
acidity constant values were determined for each ligand and their speciation at physiological pH was determined using HySS (Figure 4.4, Table 4.2). Fitting the variable pH UV-Vis data afforded pKₐ values of 9.54 – 9.55. The major transition is attributed to the deprotonation of the phenol group (280 nm, red spectra, Figure 4.4) to a phenolate (318 nm, blue spectra, Figure 4.4). These values are comparable to the deprotonation of free phenol, which has a pKₐ = 9.98. In order to determine the complete speciation of each ligand, the pKₐ of each peripheral R-group was completed using ¹H NMR spectroscopy. POH does not have a pKₐ value for its R-group in the evaluated pH range. PMorph and PTMorph both possess a heterocyclic amine located on the morpholine and thiomorpholine rings, respectively. pKₐ values of 5.5 and 5.6 were obtained for the morpholine and thiomorpholine rings, respectively. Once again, these values are significantly lower than those for free morpholine and thiomorpholine (pKₐ Morpholine = 8.36, Thiomorpholine = 9.0) as previously reported in Chapters 2 and 3. Overall, each ligand was found to be neutral at physiological pH 7.4. Using these acidity constants, determination of metal stability constants for each ligand is possible.
**Figure 4.4.** (Top Row) Variable pH UV-Vis titration of PMorph (Left), POH (Middle), and PTMorph (Right) demonstrating a clear transition from the protonated phenol form (red) to a deprotonated phenolate (blue). (Middle Row) Using HypSpec, HypNMR and HySS, variable pH data was fit to determine the acidity constants and the overall speciation of each ligand. Each ligand is neutral at physiological pH. (Bottom Row) Chemical structures of PMorph (Left), POH (Middle), PTMorph (Right).

**Table 4.2** Phenol-triazole pKₐ values and speciation at physiological pH as determined by variable pH UV-Vis and ¹H NMR spectroscopy data fit using HypSpec, HypNMR, and HySS.

<table>
<thead>
<tr>
<th></th>
<th>UV-vis</th>
<th>NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKₐ (Phenol)</td>
<td>pKₐ (R-group)</td>
</tr>
<tr>
<td>PMorph</td>
<td>9.559(6)</td>
<td>5.5(9)</td>
</tr>
<tr>
<td>POH</td>
<td>9.549(2)</td>
<td>n/a</td>
</tr>
<tr>
<td>PTMorph</td>
<td>9.54(1)</td>
<td>5.6(6)</td>
</tr>
</tbody>
</table>
4.3.5. Metal Stability Constants

To understand how these ligands further interact with metal ions, variable pH UV-Vis titrations in the presence of Cu$^{2+}$ were performed. These data were further analyzed using HypSpec and speciation diagrams were produced using HySS. In comparison to chapters 2 (pyridine) and 3 (quinoline), the incorporation of a mixed O/N donor atom system was pursued to increase metal ion affinity.

Upon fitting the variable pH data for PMorph + Cu, stability constants ($\log\beta_1$, $\log\beta_2$, etc.) for a 1:1 and 2:1 ligand:Cu ratio were determined to be 8.90 and 15.65, respectively (Figure 4.5, Table 4.3). In order to estimate the dissociation constant ($K_d$) for PMorph + Cu, the pCu at pH 7.4 was determined using the program HySS. The pCu (pCu = $-\log([\text{Cu}]_{\text{unbound}})$ at pH 7.4 is an approximate method to determine the $K_d$ at physiological pH. The pCu value is obtained from the speciation curves produced by the program HySS. At pH 7.4, [Cu]$_{\text{unbound}}$ is calculated based on the stability constants determined using HypSpec and the initial [Cu]. At pH 7.4, the pCu value for PMorph was 6.8, indicating a high nM affinity for Cu. For POH + Cu, similar stability constants (8.971 and 15.459) were obtained for 1:1 and 2:1 ligand:Cu ratios, respectively (Figure 4.6, Table 4.3). A slightly higher pCu value was obtained for POH (pCu = 6.9), which indicates that POH has an affinity in the high nM range for Cu. Finally, PTMorph had comparable stability constant values to the other two derivatives for both the 1:1 and 2:1 ligand:Cu species (Figure 4.7, Table 4.3), which also resulted in a similar pCu value (pCu = 6.6), indicating a high nM affinity for Cu. Unfortunately at high pH, an increase in the baseline resulted from precipitation of the PTMorph + Cu species. As a result, these pH values (pH 10-12) were not included in the fitting for the PTMorph + Cu stability constant measurements.
Figure 4.5. (Left) Variable pH UV-Vis titration of PMorph (75 µM) and CuCl$_2$ (37.5 µM) where the red spectra represents pH 2 and the blue spectra pH 12. (Right) Using HypSpec and HySS, the variable pH data was fit to a model including a 1:1 and 2:1 ligand:Cu species along with free Cu and a Cu(PMorph)$_2$OH component. At physiological pH 7.4, very little free Cu exists and a combination of 1:1 and 2:1 ligand:Cu species exist.

Figure 4.6. (Left) Variable pH UV-Vis titration of POH (75 µM) and CuCl$_2$ (37.5 µM) where the red spectra represents pH 2 and the blue spectra at pH 12. (Right) Using HypSpec and HySS, the variable pH data was fit to a model including a 1:1 and 2:1 ligand:Cu species along with free Cu and a Cu(POH)$_2$OH component. At physiological pH 7.4, very little free Cu exists and a combination of 1:1 and 2:1 ligand:Cu species exist.
**Figure 4.7.** *(Left)* Variable pH UV-Vis titration of **PTMorph** (75 µM) and CuCl₂ (37.5 µM) where the red spectra represents pH 2 and the blue spectra at pH 12. *(Right)* Using HypSpec and HySS, the variable pH data was fit to a model incorporating a 1:1 and 2:1 Ligand:Cu ratio along with free Cu and a Cu(PTMorph)₂OH component. At physiological pH 7.4, a small amount of free Cu exists and a combination of 1:1 and 2:1 Ligand:Cu species are primarily observed.

**Table 4.3** Summary of the stability constants determined via variable pH UV-vis titrations and fit using the programs HypSpec and HySS.

<table>
<thead>
<tr>
<th>L</th>
<th>PMorph</th>
<th>POH</th>
<th>PTMorph</th>
</tr>
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<tbody>
<tr>
<td>CuL</td>
<td>8.90(1)</td>
<td>8.971(2)</td>
<td>8.765(7)</td>
</tr>
<tr>
<td>CuL₂</td>
<td>15.65(2)</td>
<td>15.459(3)</td>
<td>15.162(9)</td>
</tr>
<tr>
<td>CuL₂OH</td>
<td>13.976(9)</td>
<td>14.355(3)</td>
<td>12.96(2)</td>
</tr>
<tr>
<td>pCu (pH 7.4)</td>
<td>6.8</td>
<td>6.9</td>
<td>6.6</td>
</tr>
</tbody>
</table>

In comparison to other phenol-based ligands reported in the literature, the phenol-triazoles demonstrate slightly lower pCu values. Mirica and co-workers reported two separate phenol-based derivatives with appended benzothiazole groups (Figure 4.8).\(^{210, 215}\) **BzOH** is a bidentate ligand that is comparable to the phenol-triazole series and has a slightly higher pCu value (pCu (**BzOH**) = 8.2 compared to 6.6 – 6.9 for the phenol-triazole series). **BzPyOH** (R = H) is a tridentate ligand and, as such, has a higher pCu value (pCu = 7.9) in comparison to the phenol-triazole series. When **BzPyOH** has an additional pyridine unit (R = CH₂Py), this produces a tetradebate ligand, which results in a significant increase in pCu (Figure 4.8).
4.3.6. **Ligand-Peptide Interactions via 2-D $^1$H-$^{15}$N SOFAST-HMQC NMR**

In order to investigate the interaction between the phenol-triazole series of ligands and the Aβ$_{1-40}$ peptide, 2-D $^1$H-$^{15}$N SOFAST-HMQC NMR was employed (introduced in Section 3.3.4) in order to provide information on which amino acid residues interacted with each ligand. In collaboration with the Ramamoorthy group at the University of Michigan, each ligand (0 – 10 eq.) was incubated with $^{15}$N-labeled Aβ$_{1-40}$ peptide and measured by NMR to determine which residues exhibited new chemical shifts. Our goal was to determine if the phenol-triazole series has an affinity for a specific region of the Aβ peptide, and to compare these amino acid shifts to those of the analogous quinoline-triazole series.

**P**Morph exhibited interactions with several amino acids including aspartic acid–7 (D7), phenylalanine–19 (F19), aspartic acid-23 (D23), and asparagine–27 (N27) (Figure 4.7). These residues were determined to be statistically-relevant based on their chemical shift perturbation (CSP) being greater than one standard deviation above the average (Figure 4.9, Top Right, dashed line). **P**Morph interacts with residues in the hydrophobic pocket, residues 15-20 of the peptide, which mediate Aβ aggregation. As discussed in Chapter 3, hydrophobic interactions are integral factors associated with Aβ aggregation. Ligands that regulate these hydrophobic interactions could play a role in
changing the aggregation profile of the Aβ peptide. Other moderate interactions were also observed with residues in the C-terminus region including glycine–33 (G33), methionine–35 (M35), and valine–36 (V36).

PTMorph demonstrates similar interactions to those observed for PMorph but with different intensities. The most significant CSP’s were with residues D7, F19, phenylalanine–20 (F20), N27, and G33 (Figure 4.10). These residues exhibited CSP’s
above the average + one standard deviation (Figure 4.10, Top Right, dashed line). The similarity in peptide interactions between PMorph and PTMorph is not surprising due to the fact that these two molecules only differ by one atom in the morpholine ring. Once again, several of the residues exhibiting the largest CSP’s were located in the hydrophobic region of the peptide. Other moderate intensity interactions were observed with residues near the C-terminus including D23, M35, and V36.

Figure 4.10. 2-D $^1$H-$^{15}$N SOFAST-HMQC NMR experiments using $^{15}$N-labeled Aβ$_{1-40}$ and 0-10 eq. PTMorph. (Top) Chemical structure of PTMorph. (Left) 2-D $^1$H-$^{15}$N SOFAST NMR spectra showing the assignment of specific amino acid residues in the Aβ$_{1-40}$ peptide. (Top Right) Summary of the specific amino acid residues that have shifted at 10 eq. PTMorph. The dotted line represents the average CSP while the dashed line is the average + one standard deviation. (Bottom Right) Aβ$_{1-40}$ solution NMR structure highlighting specific amino acid residues that have significant CSP shifts (Red, > 0.03 ppm shift) and moderate CSP shifts (Orange, between 0.015 and 0.03 ppm).
Interestingly, **POH** was observed to have interactions over the entire peptide sequence. The most significantly shifted residues were glutamic acid – 3 (E3), D7, tyrosine – 10 (Y10), valine – 18 (V18), F20, G33, V36, and glycine – 38 (G38) (Figure 4.11). These residues reside in both the hydrophilic and hydrophobic regions of the peptide, indicating that **POH** demonstrates non-specific interactions over the length of Aβ peptide. Moderate affinity interactions were also observed with residues phenylalanine – 4 (F4), F19, D23, M35, and valine – 40 (V40).

Overall, the three phenol-triazole series interacted with several common amino acid residues including D7, F19, D23, G33, M35, and V36 (Figure 4.12). Many of these interactions are found in the hydrophobic region of the Aβ peptide, suggesting that the phenol-triazole series may be able to regulate hydrophobic peptide interactions involved in the aggregation process. Interestingly, **PMorph** and **PTMorph** caused CSP's to occur with very similar amino acid residues, while **POH** was observed to interact with several residues, non-specifically, along the length of the peptide. Due to the contrast between specific and non-specific amino acid interactions, further investigation to determine if any differences in the peptide aggregation profile will occur in the presence of these ligands was investigated (*vide infra*). In comparing the phenol derivatives to those of the quinoline series, several amino acid residues were shown to have similar interactions. When comparing **QMorph** (Figure 3.9) and **PMorph** (Figure 4.9), F19 and G33 were observed to have significant CSP values in both ligand spectra. **QTMorph** (Figure 3.10) and **PTMorph** (Figure 4.10) were observed to both interact with residues N27 and G33. **QOH** (Figure 3.11) and **POH** (Figure 4.11) were both observed to interact with a wide range of amino acid residues including E3, F20, D23, G33, M35, V36, and G38. In general, both of these series of ligands have an affinity for residues in the hydrophobic region of the Aβ peptide and more specifically, the phenol-triazole series interact with several phenylalanine residues, suggesting the presence of π-π stacking interactions.
Figure 4.11.  2-D $^1$H-$^{15}$N SOFAST-HMQC NMR experiments using $^{15}$N-labeled Aβ$_{1-40}$ and 0-10 eq. POH. (Top) Chemical structure of POH. (Left) 2-D $^1$H-$^{15}$N SOFAST NMR spectra showing the assignment of specific amino acid residues in the Aβ$_{1-40}$ peptide. (Top Right) Summary of the specific amino acid residues that have shifted at 10 eq. POH. The dotted line represents the average CSP while the dashed line is the average + one standard deviation, which was used to identify which CSP were statistically relevant. (Bottom Right) Aβ$_{1-40}$ solution NMR structure highlighting specific amino acid residues that have significant CSP shifts (Red, > 0.03 ppm shift) and moderate CSP shifts (Orange, between 0.02 and 0.03 ppm).
Summary of the 2D $^1$H-$^{15}$N SOFAST NMR experiment for the phenol-triazole series of ligands, demonstrating which amino acid residues have CSP's associated with each ligand. D7, F19, D23, G33, M35, and V36 were found to have statistically relevant CSP's across all three investigated ligands.

4.3.7. Molecular Docking Simulations

Molecular docking simulations using Schrödinger Suite were used to visualize the orientation in which these ligands interact with the Aβ peptide, complementing the 2-D NMR experiments. An NMR solution structure (PDB: 2LFM) containing an ensemble of 20 different structural poses of the Aβ$_{1-40}$ peptide was used. Each ligand was docked with each NMR solution structure pose and each of the lowest energy confirmations was compared to the results obtained from the 2D SOFAST NMR experiments (see Section 3.3.7 for further details).

PMorph was found to have a conformation that interacted with three residues that were found to have significant CSP’s during the 2D SOFAST NMR experiment. Specifically, PMorph exhibited a H-bonding interaction (1.9 Å) derived from the phenol-O on PMorph to the H-N amide of the D7 residue (Figure 4.13). This conformation also positions the morpholine group on PMorph towards the phenyl ring on F19 where a van der Waals interaction is predicted. This simulation also highlights a secondary effect.
between the residues F19 and D23. Here, a H-bonding interaction (1.9 Å) was observed between the carbonyl C=O of F19 and the H-N amide group on D23. It is possible that upon interaction of PMorph, the resulting orientation of the Aβ peptide favours this H-bonding interaction.

**Figure 4.13.** Docking pose of PMorph with the NMR solution structure of Aβ1-40 (PDB: 2LFM).

PTMorph also exhibits a H-bonding interaction with D7 although the interaction stems from the phenol O-H and the O=C carbonyl of D7 (Figure 4.14). The phenol ring is also predicted to have a van der Waals interaction with the R-group on the valine-18 residue (not shown). The thiomorpholine group is pointed directly towards the phenyl ring located on F19 and a van der Waals interaction is predicted.

The NMR results suggested that POH interacted non-specifically with several different amino acid residues across the Aβ1-40 peptide. One pose that describes these interactions is presented (Figure 4.15). Here, POH is positioned in a hydrophobic pocket with the phenol-O pointing towards the C-terminus while the alcohol moiety of POH is oriented towards H13. Overall, the three ligands appear to have poses that favour hydrophobic interactions along the central region of the Aβ peptide.
**Figure 4.14.** Docking pose of PTMorph with the NMR solution structure of Aβ₁-₄₀ (PDB: 2LFM).

**Figure 4.15.** Docking pose of POH with the NMR solution structure of Aβ₁-₄₀ (PDB: 2LFM).
4.3.8. Native Gel Electrophoresis, Western Blotting, and TEM

Native gel electrophoresis followed by visualization using Western blotting was employed to correlate the hydrophobic interactions of the phenol-triazole series determined by 2D SOFAST NMR spectroscopy and their effect on regulating Aβ peptide aggregation. Two types of experiments were studied by evaluating this ligand series in the absence and presence of Cu. The interactions of Cu + Aβ have been shown to lead to an increase in the formation of oligomeric Aβ species and oxidative stress. Herein, 25 µM Aβ1-42 was incubated in the absence and presence of 1 eq. CuCl2 and 3 eq. ligand for 24 hours at 37 °C under constant agitation. Aliquots were obtained from each experiment and separated by molecular weight using native gel electrophoresis and imaged using Western blotting techniques (Figure 4.16). When Aβ1-42 only is incubated under these conditions, a wide range of species is observed from oligomers to high molecular weight aggregates (Figure 4.16, Lane 1).

Upon incubating Aβ1-42 with 1 eq. CuCl2, a distinct decrease in high molecular weight species is observed and primarily oligomeric species are visualized (Figure 4.16, Lane 2). When 3 eq. PMorph, PTMorph, or POH are incubated with Aβ1-42 in the absence of Cu, very few oligomeric species are observed and speciation shifts towards high molecular weight species (Figure 4.16, Lanes 3-5). When 3 eq. PMorph, PTMorph, or POH are incubated with Aβ1-42 in the presence of 1 eq. Cu, different results are observed for each ligand. POH demonstrates a small reduction in oligomeric species in comparison to the control (Figure 4.16, lane 2) but a significant shift towards high molecular weight species is also observed (Figure 4.16, Lane 6). The shift towards high molecular weight species is very similar to what is observed in the absence of Cu with POH (Figure 4.16, lane 3). This provides significant evidence that POH is able to regulate the Cu-Aβ interactions that lead to peptide aggregation. For PMorph and PTMorph, similar aggregation profiles are observed in the presence of Cu (Figure 4.16, lanes 7 and 8), which differs significantly from the Cu + Aβ1-42 experiment (Figure 4.16, lane 2) and more interestingly from the Cu + POH + Aβ1-42 experiment (Figure 4.16, lane 6). Interestingly, PMorph and PTMorph shifted Aβ aggregation towards distinctly different high molecular weight species (100 kDa vs. 250 kDa) in comparison to POH. A potential rationale for the difference in aggregation profile of POH vs. PMorph/PTMorph.
relates back to the amino acid interactions determined in the 2D $^1$H-$^{15}$N SOFAST NMR experiment (Section 4.3.6). POH exhibited interactions non-specifically along the length of the Aβ peptide, while both PMorph and PTMorph exhibited interactions with fewer amino acid residues. The increased number of interactions for POH, in comparison to both PMorph and PTMorph, may result in the preferential formation of high molecular weight aggregates thus limiting Aβ$_{1-42}$ oligomer formation. All ligands have similar stability constants for Cu, therefore it is assumed that each ligand competes with Aβ$_{1-42}$ for Cu. In combination, these results suggest that the peripheral R-group extending from the 4-position of the triazole plays a role in the regulation of Cu- Aβ$_{1-42}$ aggregation.

![Figure 4.16](image)

Figure 4.16. Native gel electrophoresis of Aβ$_{1-42}$ in the absence and presence of 1 eq. CuCl$_2$ and the absence and presence of 3 eq. ligand. Conditions: 25 µM Aβ$_{1-42}$, 25 µM CuCl$_2$, 75 µM ligand, 0.1 M PBS pH 7.4 buffer, 37 °C incubation for 24 hours.

To further probe the Aβ$_{1-42}$ species that were visualized in the native gel electrophoresis and Western blotting experiment, TEM images have been obtained to identify specific morphologies. When Aβ$_{1-42}$ is incubated alone for 24 hours, significant fibrillization is observed (Figure 4.17, Image 1). Incubation of Aβ$_{1-42}$ with 1 eq. CuCl$_2$ causes a significant shift in morphology from fibrillar to larger amorphous aggregates (Figure 4.17, Image 2). This morphology was found throughout the TEM grid. The lack of uniformity has been commonly observed in Cu + Aβ species that have been incubated for 24 hours. In the presence of 3 eq. POH, PMorph, or PTMorph, Aβ$_{1-42}$ species were observed to be smaller and primarily amorphous in comparison to Aβ$_{1-42}$ alone, which is a significant change in morphology (Figure 4.17, Images 3-5). When Aβ$_{1-42}$ + 1 eq. CuCl$_2$ + 3 eq. POH are incubated together, primarily fibrillar species are observed (Figure 4.17, Image 6). This type of result was observed when, instead of using POH,
DTPA, a known high affinity ligand for Cu, was used to bind Cu. In the native gel, POH produced primarily high molecular weight Aβ species, therefore this result suggests that inhibition of Cu-Aβ interactions has occurred and that the peptide self-aggregated. A similar result was observed for Aβ_{1-42} + 1 eq. CuCl₂ + 3 eq. PMorph where significant amounts of fibrillar structures were imaged (Figure 4.17, Image 7). Interestingly, when 3 eq. PTMorph is incubated with Aβ_{1-42} + Cu, a combination of amorphous and fibrillar structures are observed by TEM (Figure 4.17, Image 8). These images correlate well with the species that were observed in the native gel experiment for PMorph and PTMorph as a combination of species (oligomeric and high molecular weight) were also present in the native gel as were observed in the TEM image. For PTMorph, a similar aggregation profile was observed in the native gel experiment in comparison to PMorph, but slightly different morphologies were observed by TEM. This highlights the importance of not only studying the degree of aggregation via molecular weight separation but also the size and morphology of the aggregates by TEM in order to obtain a more complete picture of the resulting Aβ species.

This set of experiments shows that the phenol-triazole series is capable of regulating Cu-Aβ_{1-42} interactions that lead to aggregate formation and aggregates that form in the absence of Cu. POH exhibits interactions with several amino acid residues throughout the length of the Aβ_{1-42} peptide in comparison to PMorph and PTMorph, which were found to have fewer statistically-relevant interactions. This may contribute to the modulation of the Aβ aggregation profile in the absence of Cu. Each ligand was determined to have a similar stability constant for Cu (vide supra), therefore each ligand demonstrates the ability to compete with the Aβ_{1-42} peptide for Cu. Differences in both the types of molecular weight species and morphologies in experiments of both Cu and ligand suggest that that the peripheral R-group has a significant effect on the aggregation process.
4.3.9. Antioxidant Assays

To fully characterize the multifunctional nature of these ligands, determination of their antioxidant capability was probed using two separate methods. The trolox-equivalent antioxidant capacity (TEAC) assay is a method that evaluates a compound’s ability to convert the 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical
cation (ABTS\(^{+}\)) back to the neutral form (Figure 4.18). The reduction of ABTS\(^{+}\) to its neutral form can be measured by its absorbance at 470 nm, therefore the increase in absorbance at this wavelength can be correlated to the disappearance of the ABTS\(^{+}\). Trolox, a water-soluble vitamin-E analog, is used as a standard to which each ligand is compared.

![Chemical structure of ABTS](image)

**Figure 4.18.** Chemical structure of ABTS used in the TEAC assay.

The ABTS\(^{+}\) is produced by reaction with sodium persulfate (Na\(_2\)S\(_2\)O\(_8\)), which is a strong oxidizing agent. The ABTS concentration was standardized by obtaining a linear relationship of absorbance vs. ABTS concentration. Determining the specific volume that was required to obtain an absorbance value of 0.7 was achieved by extrapolating from this linear plot. This volume was subsequently used for each experiment in triplicate. Each phenol-triazole ligand was compared against glutathione, a known reducing agent found naturally in biological environments, and PBT2, an 8-hydroxyquinoline derivative, that shows promise as an Alzheimer’s disease therapeutic. Each ligand was found to have a TEAC similar to Trolox and slightly enhanced in comparison to PBT2 (Figure 4.18). The antioxidant capability stems from the ability for phenolic compounds to scavenge and stabilize radical species.
Figure 4.19. Trolox-equivalent antioxidant capacity (TEAC) values at 1, 3, and 6 minutes for Trolox, PBT2, glutathione, and each phenol-triazole ligand. Each experiment was completed in triplicate and error bars represent the ± SD for the average TEAC values.

To evaluate the ability for POH, PMorph, and PTMorph to sequester free Cu and limit its ability to undergo the Fenton reaction, a fluorescent coumarin carboxylic acid (CCA) assay was performed. In this assay, free Cu, under reducing conditions, is able to produce hydroxyl radical (Figure 4.20). The hydroxyl radical is extremely reactive and upon formation, will hydroxylate at the 7-position on coumarin-3-carboxylic acid. The hydroxylated product is fluorescent, therefore monitoring the production of this fluorophore in the presence and absence of ligands allows for an indirect measurement of hydroxyl radical formation under these test conditions. Limiting Cu-based reactivity by saturating the metal’s coordination sphere or by shifting the redox potential outside the biologically accessible range will modulate this catalytic process. When CCA is in the presence of Cu and no exogenous ligands, significant fluorescence is observed due to the hydroxylation of CCA (Figure 4.21). When POH, PMorph, or PTMorph are added to Cu and CCA under reducing conditions, a limited increase in fluorescence is observed (Figure 4.21). Based on the fact that each ligand has a significant affinity for Cu (vide infra), these results suggest that chelation of free Cu by
the ligands limits the ability for Cu to undergo Fenton chemistry and therefore, inhibits hydroxyl radical production.

**Figure 4.20.** Under reducing conditions, O\(_2\)(g) is reduced by Cu\(^+\) through several steps to the highly reactive hydroxyl radical. The hydroxyl radical will specifically react at the 7-position on coumarin-3-carboxylic acid to produce a fluorescent product.

One of the many facets of Alzheimer’s disease involves oxidative stress and the brain’s inability to deal with reactive oxygen species (ROS). These two assays further demonstrate the multifunctional ability of this series of ligands. In the absence of metals, all three phenol-triazole ligands quenched the ABTS\(^-\) species. In the presence of Cu, each ligand limits the ability of free Cu to catalyze the formation of the fluorescent 7-hydroxyCCA. Overall, these two studies demonstrate the antioxidant capability of each ligand and their ability to regulate Fenton-like chemistry.
A fluorogenic assay monitoring the production \(7\)-hydroxyCCA over time. In the absence of any exogenous ligands, free Cu exhibits Fenton-like chemistry to produce hydroxyl radicals, which react with CCA to form a fluorescent product. When POH, PMorph, or PTMorph are introduced into a solution containing CCA, Cu, and ascorbate, a distinct inhibition in fluorescent product is observed.

4.4. Conclusions

A series of phenol-triazole ligands were synthesized, characterized and tested in a number of peptide aggregation and antioxidant assays. The incorporation of a phenol group into the triazole scaffold has afforded a ligand with increased Cu binding affinity in comparison to the quinoline series, maintenance of the hydrophobic interactions as determined by 2D SOFAST NMR experiments, and anti-oxidant capabilities. Due to the increased Cu binding affinity of these ligands, regulation of Cu-Aβ interactions that lead to toxic oligomeric Aβ species was confirmed by native gel electrophoresis. Overall, the multifunctional nature of these ligands has been investigated and warrants further study.
to determine their ability to permeate the BBB and demonstrate efficacy in an animal model of Alzheimer's disease.
Chapter 5. Modulation of the Aβ peptide aggregation pathway by KP1019 limits Aβ-associated neurotoxicity


M. R. Jones performed the thioflavin-T assays, TEM preparation, EPR experimental preparation, dot blot assay, native gel electrophoresis and western blotting, and cell culture and viability studies. C. Mu and M. I. Webb collected and analyzed EPR data. M. C. P. Wang collected TEM images.

5.1. Introduction

Many therapeutic strategies for AD attempt to inhibit the aggregation of Aβ, break down fibrillar aggregates using monoclonal antibodies or small molecule derivatives, or modulate aggregate formation by producing non-toxic off-pathway species. The first strategy is difficult to implement since AD symptoms are usually manifested following significant plaque deposition. Breaking down pre-formed amyloid plaques has shown promise, but this approach could lead to the formation of smaller molecular weight species such as toxic soluble oligomers. Thus, the development of a therapeutic compound that can regulate the Aβ aggregation pathway, leading to off-pathway non-toxic species, is of current interest.

Metal-based therapeutics have recently been identified as potential treatments for AD (Figure 5.1). The success of cisplatin for the treatment of ovarian and testicular cancer has highlighted the promise of metal complexes in medicine. In the context of AD, Barnham et al. reported in 2008 a series of Pt(II)
phenanthroline derivatives that bind to the Aβ peptide, limiting its aggregation and toxicity. More recently, the same group demonstrated that a Pt(IV) complex with improved bioavailability is able to decrease plaque formation in the brains of APP/PS1 mice. Emissive cyclo-metalated Ir and Rh complexes were recently shown to bind to the Aβ peptide, and alter its aggregation profile. A small number of Ru-based complexes have also been evaluated for their ability to bind to the Aβ peptide, inhibit aggregation, and limit Aβ-associated toxicity in cultured rat primary cortical neurons.

![Chemical structures of metal complexes](image)

**Figure 5.1.** Examples of metal-based Aβ aggregation inhibitors.

Ru(III) complexes are leading candidates for the next generation of metal-based anti-cancer compounds. Central to this development has been the Ru(III) complex indazolium [trans-RuCl₄(1H-indazole)₂] (KP1019, Figure 5.2), which has shown activity against a wide array of cancer cell lines and has also performed well in clinical trials. A notable property of KP1019 is its low toxicity, particularly in comparison to Pt chemotherapeutics. The mechanism of action of KP1019 and other related Ru(III) complexes remains under investigation. However, favourable ligand exchange rates, typical of Ru(III) complexes, and interactions with biomolecules have been implicated in the transport, uptake and anti-cancer activity of KP1019. Ru(III) complexes exhibit a high affinity for accessible histidine residues on biomolecules, and KP1019 has
been shown to coordinate to human serum albumin (hsA), human serum transferrin (hsTf) and other proteins. Based on these results, and the potential for hydrophobic interactions of the axial indazole moieties with the Aβ peptide, we have investigated the interaction of KP1019 with the Aβ peptide and the effect of this Ru(III) complex on the Aβ aggregation profile and resulting in vitro toxicity. Herein, we describe how KP1019 regulates the Aβ aggregation pathway towards non-toxic, off-pathway aggregates using a combination of physical and biochemical methods.

Figure 5.2. Chemical structure of KP1019.

5.2. Experimental

5.2.1. Thioflavin-T Assay

In a 96-well plate, the inhibitory effect of KP1019 (0.25 – 2 eq.) on Aβ1-42 aggregation was investigated. Aβ1-42 was prepared in 1:1 DMSO/H2O to a final volume of 200 µL. Peptide concentration was measured using a Thermo Nicolet UV nanodrop and an ε = 1450 M⁻¹ cm⁻¹ for Aβ1-42. Final Aβ concentration in the assay was 5 µM. KP1019 was prepared as a 50 µM stock solution in 1:1 DMSO/H2O and diluted with PBS pH 7.4 buffer to the desired concentration. ThT was prepared as a 50 µM stock solution in 10% DMSO/H2O. Final concentration of ThT was 5 µM in all wells with a final DMSO concentration of 1%. CR was used as positive control since this compound is a potent inhibitor of Aβ peptide aggregation. Congo Red (CR) was prepared as a 50 µM stock solution in 1:1 ethanol/PBS pH 7.4 and had a final concentration of 10 µM. All wells were
brought to a final volume of 200 µL using PBS pH 7.4. ThT fluorescence was measured by excitation at 404 nm and monitoring of the emission at 477 nm. Measurements were performed in quadruplicate at 24 and 48 hours.

5.2.2. Transmission Electron Microscopy

See Section 2.2.9 for further details.

5.2.3. EPR Measurements and Simulations

X-band (9.4 GHz) EPR spectra were collected using a Bruker EMXplus spectrometer equipped with a PremiumX microwave bridge and HS resonator. All measurements were conducted at 20 K, using a Bruker ER 4112HV helium temperature-control system and continuous-flow cryostat. Final concentrations of KP1019 and Aβ1-28 were 1.5 mM and 0.5 mM, respectively. Peptide and KP1019 were incubated at 37 °C under constant agitation. Samples (250 µL) were obtained after 2 and 24 hour incubation periods, mixed with 50 µL glycerol and frozen in liquid N2 in 4 mm outer-diameter quartz tubes. A weak background signal was subtracted from each spectrum. Bruker WinEPR Simfonia simulations were performed to deconvolute the spectra into separate spectral components of KP1019 alone, and protein-coordinated KP1019 (KP1019-Aβ1-28).

5.2.4. Dot Blot Assay

To further evaluate the specific region of the Aβ peptide KP1019 interacts with, a dot blot assay was performed at varying KP1019:Aβ1-42 ratios and time periods. A 96-well plate was incubated at 37 °C for a total of 72 hours. Aβ1-42 was reconstituted in 1:1 DMSO:H2O in a total volume of 200 µL. The final Aβ1-42 concentration was 10 µM in the 96-well plate. KP1019 was prepared as a 250 µM stock solution in 1:1 DMSO:H2O. Final KP1019 concentrations ranged from 10 – 100 µM in the 96-well plate. PBS pH 7.4 was then added to the wells to a final volume of 100 µL. 10 µL aliquots were obtained immediately following mixing (time 0), and at each 24 hour timepoint thereafter up to 72 hours. From these aliquots, a 2 µL drop was absorbed onto a sheet of nitrocellulose. The
nitrocellulose membrane was immediately blocked for 1 hour at room temperature using 3% BSA in TBS-T under constant agitation, followed by incubation with a primary antibody (6E10) overnight at room temperature. The membrane was washed 4 x 15 minutes with TBS buffer then incubated with a horseradish peroxidase conjugated goat anti-mouse secondary antibody in 2% BSA solution for 2-3 hours at room temperature. The membrane was washed with TBS buffer for 4 x 15 minutes and then incubated at room temperature with the Thermo Scientific Supersignal West Pico Chemiluminescent Substrate kit for 5 minutes and visualized with a Fujifilm Luminescent imager.

5.2.5. Native Gel Electrophoresis and Western Blotting

The ability of KP1019 to regulate the aggregation of Aβ1-42 was further evaluated by molecular weight separation on a 10 – 20% gradient tris-tricine gel and visualized using western blotting techniques. For the inhibition experiment, each sample was incubated for 24 hours under constant agitation at 37 °C in a 96-well plate, covered with a lid and sealed with parafilm. Final concentrations, diluted in PBS pH 7.4, were as follows: 25 µM Aβ1-42, 0 – 2 eq. KP1019. Disaggregation experiments commenced by incubating a 96-well plate with 25 µM Aβ1-42 for 24 hours, followed by incubation for 24 hours with 0.25 – 2 eq. KP1019. Samples were loaded onto a 10-20 % gradient tris-tricine gel (Bio-Rad) and run at 100 V for 100 minutes in a tricine running buffer, followed by transferring to a nitrocellulose membrane for 3 hours at 40 V in a 4 °C cold room. The membrane was immediately blocked in 3 % BSA solution in tris-buffered saline containing 0.1 % Tween-20 (TBS-T) for 1 hour at room temperature, followed by incubation with a primary anti-Aβ antibody (6E10) overnight at room temperature under constant agitation. The membrane was washed 4 x 15 minutes with TBS buffer and then incubated at room temperature with a horseradish peroxidase conjugated goat anti-mouse secondary antibody in 2 % BSA solution. The membrane was washed with TBS buffer for 4 x 15 minutes, incubated with the Thermo Scientific Supersignal West Pico Chemiluminescent Substrate kit for 5 minutes and visualized with a Fujifilm Luminescent imager.
5.2.6. **Alamar Blue Cell Viability Assay**

SH-SY5Y cells were a gift from Prof. Frank Lee in the Department of Health Sciences at Simon Fraser University. Cells were grown in T-75 tissue culture flasks (Greiner) until 80-90% confluency. Media consisted of 1:1 DMEM/Ham’s F-12 nutrient mixture (Sigma-Aldrich), 1% Penicillin/Streptomycin solution (Life Technologies), 1% MEM non-essential amino acid solution (Gibco), and 10% Fetal Bovine Serum (ATCC). Media was replaced every 3-4 days. Trypsinized cells were pelleted, re-suspended in DMEM/Ham’s F-12 media, and seeded into a 96-well plate at a density of ca. 20,000 cells/well using a haemocytometer. Cells were placed 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. Media was changed every 3-4 days. Cells were allowed to differentiate for 5-7 days before incubation experiments were initiated. Cells were treated with varying KP1019 concentrations to determine if the concentration range used in the incubation studies (20 – 100 µM) demonstrated any toxicity (Appendix, Figure S2). A 5 mM KP1019 stock solution was prepared in 20% DMSO/PBS buffer at pH 7.4. The toxicity of KP1019 was evaluated using concentrations ranging from 500 nM – 1 mM (Appendix, Figure S2). The experimental concentration range used for KP1019 was outside of its toxicity range (>300 µM). Aβ$_{1-42}$ was also evaluated for toxicity using concentrations ranging from 10 – 40 µM (Appendix, Figure S3). Significant Aβ toxicity at 20 µM was observed and used as the concentration for all cell studies. In all toxicity experiments, differentiated cells were incubated at 37 °C for 24 hours at a final volume of 100 µL with pre-mixed KP1019:Aβ$_{1-42}$ species incubated for 5 minutes, 2 hours, and 24 hours. To measure the cell viability, Alamar Blue reagent (10 µL, Life Technologies) was added to each well and allowed to incubate for 2 hours at 37 °C. Fluorescence measurements were performed by monitoring the emission at 600 nm after excitation at 570 nm. % Cell Viability was determined by comparing all measurements to wells containing cells only. All experiments were completed in quadruplicate.
5.3. Results and Discussion

5.3.1. Effect of KP1019 on Aβ Peptide Aggregation

The aggregation of the amyloid-beta (Aβ) peptide leading to the formation of soluble toxic oligomers and eventually plaque deposits has been linked to the pathology of AD. The fluorogenic dye Thioflavin-T (ThT) is commonly used to monitor Aβ fibril formation, exhibiting a marked fluorescence response (excitation wavelength ($\lambda_{ex}$) = 404 nm, emission wavelength ($\lambda_{em}$) = 477 nm) upon binding to fibrils. We evaluated the concentration-dependent effect of KP1019 (0.25 – 2 eq. KP1019/Aβ) on Aβ fibril formation over a 48-hour period using ThT. Both the Aβ1-40 (Appendix, Figure S1) and Aβ1-42 (Figure 5.3) isoforms were evaluated to assess KP1019’s ability to disrupt peptide fibrillization. We focus our discussion on the more aggregation prone Aβ1-42 isoform, however KP1019 exhibits the same concentration-dependent anti-aggregation effect on both peptides. When Aβ is incubated alone significant fibrillization occurs as indicated by the increase in ThT fluorescence (Figure 5.3 and Appendix, Figure S1). At 0.25 eq. KP1019/Aβ1-42, Aβ1-42 fibril formation is significantly retarded even after 24 hours and continues after 48 hours (Figure 5.3). At 0.5-2 eq. KP1019/Aβ1-42, no significant ThT fluorescence was observed. KP1019 at ≥ 0.5 eq. limits ThT fluorescence in a similar manner to Congo red, a known inhibitor of Aβ fibril formation.
Figure 5.3  (Left) 24 (red) and 48 (blue) hour aggregation profiles of Aβ₁₋₄₂ (5 µM) analyzed by monitoring the ThT fluorescence (5 µM). Varying equivalents of KP1019/Aβ₁₋₄₂ (0.25 – 2 eq.) were compared against a known inhibitor (Congo red, 2 eq. as a control) of Aβ peptide aggregation. (Right) TEM images of Aβ₁₋₄₂ morphology sampled from the ThT assay after 48 hours of incubation. A: Aβ₁₋₄₂ only; B: Aβ₁₋₄₂ + 2 eq. Congo red; C: Aβ₁₋₄₂ + 0.25 eq. KP1019; D: Aβ₁₋₄₂ + 0.5 eq. KP1019; E: Aβ₁₋₄₂ + 1 eq. KP1019. Conditions: PBS pH 7.4 buffer, 37 °C, constant agitation for 48 hours. Scale bars are 500 nm.

Interestingly, addition of KP1019 to ThT-stained pre-formed Aβ₁₋₄₂ aggregates leads to a ca. 50% decrease in fluorescence (Appendix, Figure S2), indicating possible interference of the Ru(III) complex with this assay. The cause of the decreased ThT fluorescence upon addition of KP1019 could be due to an immediate change in aggregate morphology, KP1019-ThT interactions, or displacement of ThT from the pre-formed aggregates. We thus turned to TEM analysis to further understand the effect of KP1019 on Aβ₁₋₄₂ aggregation (Figure 5.3, bottom A-E). As expected, Aβ₁₋₄₂ alone displays large heterogeneous aggregates consisting of a combination of amorphous and fibrillar structures (Figure 5.3A), while treatment with Congo red demonstrates a significant reduction in aggregate size and no fibrillar structures were observed (Figure 5.3B). Incubation in the presence of 0.25 eq. KP1019/Aβ₁₋₄₂ results in smaller and less dense heterogeneous aggregates in comparison to peptide alone (Figure 5.3C). Incubation in the presence of 0.5 eq. KP1019/Aβ₁₋₄₂ results in smaller aggregate sizes in comparison to both Aβ₁₋₄₂ alone and 0.25 eq. KP1019/Aβ₁₋₄₂ (Figure 5.3D). At 1 eq. KP1019/Aβ₁₋₄₂, morphologies and aggregate sizes resemble those observed in the presence of the aggregation inhibitor Congo red (Figure 5.3E). Based upon results
obtained from the ThT assay and TEM analysis, KP1019 demonstrates a concentration-dependent effect on Aβ1-42 aggregation, and at ≥ 0.5 eq. KP1019 significant regulation of Aβ1-42 peptide aggregation is observed.

5.3.2. Aβ Peptide Coordination to KP1019

In order to delineate specific interactions of KP1019 with the Aβ peptide of relevance to aggregation, we investigated the time-dependent interaction of KP1019 with the Aβ1-28 peptide, a water soluble non-aggregating fragment containing the metal binding N-terminus. Ru(III) \((d^5\) low spin, \(S = \frac{1}{2}\)) is amenable to electron paramagnetic resonance (EPR) analysis and this technique has been used in previous studies to probe interactions of Ru(III) complexes with biomolecules such as hsA.\(^{367, 375, 376}\) In the presence of serum proteins, KP1019 has been proposed to bind to the imidazole nitrogen of accessible histidine amino acid residues.\(^{367, 369, 375}\) The three histidine residues (H6, H13, and H14) at the N-terminus of the Aβ peptide are known to bind to biologically-relevant metal ions (Fe, Cu, Zn) with moderate to high affinity.\(^{28}\) To investigate the binding of KP1019 to Aβ peptide histidine residues, we incubated KP1019 with the shorter-length Aβ1-28 peptide and evaluated the EPR spectra at 2 and 24 hour time points (Figure 5.4). Free KP1019 in solution exhibits a characteristic spectrum of overlapping axial \((g = 2.64, g_{||} = 1.20, \text{linewidth}_{||} = 120, \text{linewidth}_{\perp} = 500 \text{ Gauss})\) and rhombic signals \((g = [2.94, 2.31, 0.95], \text{linewidths} = [100, 200, 600] \text{ Gauss})\), which have been reported previously.\(^{367, 375}\) Changes to the Ru(III) coordination environment via ligand exchange are reflected in the EPR spectra, providing clear evidence for peptide binding. KP1019, when in the presence of hsA or hsTf, exhibits a coordinate protein interaction detected by the appearance of a broad signal with \(g = [2.44, 2.24, 1.79], \text{Linewidths} = [200, 220, 300] \text{ Gauss}\), assigned to histidine coordination.\(^{367}\)

The EPR spectra from KP1019 incubated with Aβ1-28 for 2 and 24 hours were simulated using the previously reported parameters for free KP1019 and protein-coordinated KP1019 (Figure 5.4).\(^{367}\) After 2 hours, a significant amount of KP1019 is bound to the peptide, which is essentially unchanged after 24 hours. Excess free KP1019 undergoes ligand exchange of chloride for H₂O, and the resulting neutral
species displays low solubility, leading to a loss of EPR signal intensity, correlating with the decrease in free KP1019 signal from 2 hours to 24 hours in the experimental data (Figure 5.4). Thus, the EPR data are consistent with binding of KP1019 to an N-terminus histidine of the Aβ peptide, of which there are 3 possible residues: H6, H13, or H14.

**Figure 5.4** Frozen-solution EPR spectra of KP1019 (1500 μM) and Aβ1-28 (500 μM) incubated for 2 hours (left) and 24 hours (right) in PBS pH 7.4 buffer. The experimental spectra were deconvoluted into KP1019, and Aβ1-28 coordinated KP1019 (KP1019-Aβ1-28) spectral components via simulation. Experimental conditions: frequency 9.38 GHz microwave power 2.0 mW, time constant 40.96 ms, modulation amplitude 6 G, average of 10 scans of 2 minutes, temperature 20 K.

Time-course dot-blot assays were also performed to determine if increased KP1019 binding to the Aβ peptide would have an effect on antibody recognition at specific sites (i.e.: hydrophilic vs. hydrophobic regions) along the peptide chain (Appendix, Figure S3). Two different primary antibodies were used that specifically recognize the hydrophilic N-terminus region (6E10; amino acids 4-9) and the hydrophobic region (4G8; amino acids 18-22) of the Aβ peptide. At a 1:1 ratio of KP1019:Aβ1-42 the 6E10 antibody recognizes the N-terminus over the 24, 48, and 72 hour incubation period (Appendix, Figure S1). An increased KP1019:Aβ1-42 ratio (2:1, 4:1, 10:1), however, results in decreased recognition by 6E10, likely due to saturation of the N-terminus with KP1019 (multiple binding events to His residues). In comparison to the results for 6E10, KP1019 exhibits little effect on 4G8 recognition in the dot blot assay.
over time. The dot blot assays thus provide further evidence for KP1019 binding to the N-terminus region of the Aβ peptide, specifically targeting histidine residues (H6, H13, and H14) of the Aβ peptide.

5.3.3. Native Gel Electrophoresis/Western Blotting

To further evaluate the Aβ1-42 peptide aggregation process in the presence of KP1019, molecular weight separation by native gel electrophoresis followed by imaging using western blotting techniques was performed. The Aβ1-42 peptide with or without KP1019 (0 – 2 eq.) was incubated for 24 hours and the soluble fraction was then subjected to native gel electrophoresis and western blotting to determine relative abundance and the molecular weight of aggregates. In the absence of KP1019, primarily low molecular weight oligomeric species (ca. 10-20 kDa) are observed in the soluble fraction (Figure 5.5, lane 5). Upon addition of 0.25 eq. KP1019 (Figure 5.5, lane 1), little change in the amount of oligomeric species is observed in comparison to Aβ1-42 alone, but soluble high molecular weight (> 100 kDa) species are visible. At 0.5 - 2 eq. KP1019 (Figure 5.5, lanes 2-4), less oligomer formation is observed when compared to Aβ1-42 alone, and a concentration-dependent increase in soluble high molecular weight species is evident. Further analysis of these species was also completed by TEM (Figure 5.5, right). Aβ1-42 alone displays large heterogeneous aggregates matching the ThT fluorescence assay (vide supra) (Figure 5.2A). Incubation of Aβ1-42 with 0.25 – 1 eq. KP1019 results in a decrease in population and density of Aβ1-42 aggregates (Figure 5.5B-D), also matching the results of the ThT experiment. Based on the native gel results, increased concentrations of KP1019 drive the formation of soluble high molecular weight Aβ1-42 species, while the TEM images demonstrate that the overall size and abundance of insoluble aggregates decrease with increased KP1019 concentration. Morphologies observed by TEM correlate with how aggregates assemble and therefore it is important to describe the overall state of aggregation using TEM in combination with native gel results of the soluble fraction. These results demonstrate the ability of KP1019 to drive the formation of soluble high molecular weight aggregates while potentially limiting the formation of toxic oligomeric Aβ1-42 species.
Figure 5.5 24 hour inhibition experiment of Aβ₁₋₄₂ (25 µM) aggregation probed by native gel electrophoresis/western blotting using the 6E10 primary antibody and TEM imaging. (Left) Lane 1 0.25 eq. KP1019/Aβ₁₋₄₂; 2: 0.5 eq. KP1019/Aβ₁₋₄₂; 3: 1 eq. KP1019/Aβ₁₋₄₂; 4: 2 eq. KP1019/Aβ₁₋₄₂; 5: Aβ₁₋₄₂ only (Right) A: Aβ₁₋₄₂ only; B: 0.25 eq. KP1019/Aβ₁₋₄₂; C: 0.5 eq. KP1019/Aβ₁₋₄₂; D: 1 eq. KP1019/Aβ₁₋₄₂. Scale bar = 500 nm.

To determine the effect of KP1019 on pre-formed Aβ₁₋₄₂ aggregates, Aβ₁₋₄₂ was incubated for 24 hours under constant agitation in PBS pH 7.4 buffer, followed by a 24 hour treatment with 0 – 2 eq. KP1019 (Figure 5.6). In the absence of KP1019, Aβ₁₋₄₂ incubation affords primarily oligomeric species in the soluble fraction (Figure 5.6, lane 5). Interestingly, in all cases where Aβ₁₋₄₂ is treated with KP1019, a general decrease in low molecular weight oligomeric species (ca. 10-25 kDa) is observed along with an increase in the formation of soluble high molecular weight aggregates (> 100 kDa, Figure 5.6, lanes 2-4). When comparing the native gel results with images obtained from TEM, an increase in KP1019 concentration leads to the formation of smaller, more diffuse aggregates (Figure 5.6, right). In combination, the inhibition and disaggregation results demonstrate that KP1019 is not only able to modulate the Aβ₁₋₄₂ aggregation pathway, but can also alter the assembly of pre-formed Aβ₁₋₄₂ aggregates.
124

Figure 5.6 Native gel electrophoresis/western blotting of the Aβ_{1-42} disaggregation experiment. Aβ_{1-42} (25 µM) was incubated for 24 hours, followed by treatment with 0.25 – 2 eq. KP1019 for a further 24 hours. (Left) Lane 1: 0.25 eq. KP1019/Aβ_{1-42}; 2: 0.5 eq. KP1019/Aβ_{1-42}; 3: 1 eq. KP1019/Aβ_{1-42}; 4: 2 eq. KP1019/Aβ_{1-42}; 5: Aβ_{1-42} only (Right) A: Aβ_{1-42} only; B: 0.25 eq. KP1019/Aβ_{1-42}; C: 0.5 eq. KP1019/Aβ_{1-42}; D: 1 eq. KP1019/Aβ_{1-42}. Scale bar = 500 nm.

5.3.4. KP1019 Rescues Differentiated SH-SY5Y Cells from Aβ_{1-42}– induced Toxicity

Aβ_{1-42} neurotoxicity has been reported to stem from multiple factors including disrupted synaptic plasticity,\(^\text{46, 372, 379}\) increased inflammation\(^\text{380}\) and oxidative stress,\(^\text{379}\) dysregulation of ion homeostasis,\(^\text{381, 382}\) and disrupted kinase/phosphatase activity.\(^\text{383}\) To probe if KP1019 could reduce the neurotoxicity of Aβ_{1-42}, SH-SY5Y human neuroblastoma cells were cultured and differentiated for use as a neuronal cell model.\(^\text{344, 384-386}\) Pre-formed Aβ_{1-42} oligomers, or those formed \textit{in situ}, show concentration-dependent toxicity to SH-SY5Y cells,\(^\text{344}\) and recent work has correlated this toxicity to membrane pore formation.\(^\text{387-389}\) Varying equivalents of KP1019 (0.1, 0.5, 1 or 2 eq.) were combined with Aβ_{1-42} and incubated for a set period prior to administration to differentiated SH-SY5Y cells (Figure 5.7). In line with previous reports,\(^\text{390, 391}\) exposure of SH-SY5Y cells to Aβ_{1-42} for 24 hours results in a significant decrease in cell viability at >10 µM (Appendix, Figure S4). The toxicity at 20 µM Aβ_{1-42} (52 ± 7% viability, Figure 5.6).
was found to be appropriate for the current work. When SH-SY5Y cells have been treated with incubated KP1019-Aβ1-42 species, a significant reversal of cell viability is observed at ≥ 1 eq. KP1019, even at post 5 minute incubation (88 ± 4%) when compared to cells treated with Aβ1-42 only. Our results show that a 1:1 ratio of KP1019 to Aβ1-42 peptide is needed to rescue cell viability in this assay.

KP1019 alone exhibits slight toxicity at 1 mM in the SH-SY5Y cell line, outside the concentration range that was investigated in this work (Appendix, Figure S5). Our *in vitro* results demonstrate that the interaction of KP1019 with Aβ1-42 reverses the neurotoxicity of the Aβ1-42 peptide in the SH-SY5Y cell line, likely by promoting the formation of non-toxic high molecular weight aggregates (*vide supra*). While outside the scope of this work, we speculate that the different morphology and/or charge of the KP1019-Aβ1-42 species that form limit cell membrane interactions and pore formation in the SH-SY5Y cell line.
Figure 5.7. Toxicity studies using differentiated SH-SY5Y human neuroblastoma cells. Cells were treated with Aβ1-42 (20 µM) or pre-incubated KP1019-Aβ1-42 for 24 hours and then the cell viability measured using an Alamar Blue assay. Varying equivalents of KP1019-Aβ1-42 were incubated for 5 minutes (yellow), 2 hours (orange), or 24 hours (red) and then exposed to cells for a further 24 hours. Alamar Blue reagent was then incubated with cells for 2 hours at 37 °C, followed by fluorescence measurements to determine cell viability. Data are means ± SEM; n = 4. The t-test analysis of experimental data compared to Aβ1-42 identifies values of p < 0.0001 except for *p < 0.1.

5.4. Conclusions

This report highlights the ability of the well-known Ru(III) anti-cancer compound KP1019 to modulate the aggregation profile of the Aβ peptide associated with AD. KP1019 covalently binds to the Aβ peptide within 2 hours and remains bound for at least 24 hours, demonstrating promising kinetic and thermodynamic properties for regulating Aβ aggregation. Regardless of whether KP1019 is exposed to monomeric or pre-aggregated Aβ species, this Ru(III) complex facilitates the formation of soluble high molecular weight Aβ aggregates. In addition, KP1019 was shown to limit the cell toxicity of the Aβ1-42 peptide in the SH-SY5Y human neuroblastoma cell line. Our results suggest
that Ru(III) complexes may be promising candidates for modulating the aggregation pathway, and associated toxicity of the Aβ peptide in AD.
Chapter 6.
Future Directions

This thesis outlines several strategies to regulate the aggregation of the Aβ peptide associated with AD. A significant body of literature has identified dysregulated metal ions as a causal factor in the aggregation of Aβ.\textsuperscript{15, 243, 392} This thesis presents three scaffolds that contain a triazole unit to produce ligand frameworks in a modular fashion. Along with developing ligands that regulate metal-Aβ interactions, a Ru(III)-based scaffold has also been evaluated that binds to Aβ and modulates Aβ aggregation and neurotoxicity. While pursuing this research new ideas and directions have emerged that are further discussed in this chapter.

6.1. Cell Viability and BBB-Permeability Studies

In order to further understand the mechanism of action of both quinoline- and phenol-triazole ligands under \textit{in vitro} conditions, evaluation of cell viability using differentiated human neuroblastoma cells (SH-SY5Y) in conjunction with an Alamar Blue cell viability assay is proposed. This cell line has already been evaluated in Chapter 5 using KP1019 and Aβ species. These studies should be expanded to include assessment of the toxicity of both ligand sets and 20 µM Aβ\textsubscript{1-42} in the absence and presence of Cu\textsuperscript{2+}. These new experiments can be used to gauge whether either ligand series is able to limit Aβ\textsubscript{1-42} neurotoxicity, and correlate with previous experiments on Aβ\textsubscript{1-42} aggregation and morphology discussed in this thesis. Finally, one of the biggest obstacles to development that has not been addressed is evaluating whether these ligand scaffolds are able access the brain via passive diffusion. Several \textit{in vitro} assays exist in order to evaluate BBB permeability, including the parallel artificial membrane permeability assay (PAMPA). This assay demonstrates excellent correlation with agents that are known to access the brain.\textsuperscript{393} Alternatively, the use of a transgenic animal model that exhibits Aβ plaque deposition and neuronal impairment (eg: Tg2576 or 5xFAD)
could be used. Ligands could be administered over a set period of time, followed by animal sacrifice and evaluation of brain homogenates by mass spectrometric analysis to determine if the ligand is BBB permeable.

### 6.2. Ligand Design Strategies

Many research groups have focused efforts on developing agents that regulate metal-Aβ interactions. In reviewing this body of literature, several trends were identified that should be applied to the ligand frameworks in this thesis. The dimethylamino-moiety has been identified as a functional group that readily targets and interacts with the Aβ peptide. The modular synthetic approach that is possible via Huisgen’s 1,3-dipolar cycloaddition allows for the facile addition of a dimethylamino-moiety onto any of the ligand scaffolds presented in this thesis (Figure 6.1). An increase in Aβ interactions and potentially, regulation of peptide aggregation could occur with the installation of this functional group. The area of responsive or “turn-on” ligands is a fascinating approach to influencing metal-Aβ interactions. With the phenol-triazole scaffold, creating a pro-ligand that would mask the phenol moiety would be an interesting approach. Here, masking the phenol moiety to aid in BBB uptake would be the focus. Upon entering the brain, the protecting group could be removed using a disease hallmark (eg: oxidative stress, enzymatic processes, etc.) and a metal-binding site would be available to compete with Aβ species for dysregulated metal ions.

![Figure 6.1](image)

**Figure 6.1.** Incorporation of a dimethylamino-moiety will aid in targeting and interacting with Aβ species.

Enhancing the potential for hydrophobic interactions between the ligand and peptide could limit Aβ peptide aggregation. Applying this strategy for the development of peptide-targeting ligands bound to Ru(III) to afford bifunctional complexes could create...
an attractive therapeutic agent. The ligands could inhibit peptide self-recognition by interacting with the hydrophobic region (residues 17-21) along the Aβ peptide while the Ru(III) metal centre could bind to histidine residues near the N-terminus region of the peptide. If Ru(III) complexes bind to the N-terminus, this may influence the ability of endogenous Cu and Zn to bind to the peptide and may regulate metal-induced Aβ aggregation. By targeting both regions of the peptide, this may enhance the ability of these compounds to inhibit peptide aggregation. Several Ru(III) complexes with increased hydrophobic interactions have been prepared by our collaborator and are currently under investigation (Figure 6.2). 

Figure 6.2. Several examples of Ru(III) complexes that have demonstrated hydrophobic interactions with human serum albumin. 

Another functionality that can be embedded into the ligand that is bound to the Ru(III) complex is a targeting vector towards Aβ aggregates. Previous reports have identified a number of scaffolds that have an affinity for Aβ aggregates including benzothiazoles, stilbenes, and IMPY (Figure 6.3).
Several Ru(III) complexes that incorporate a ligand that has an affinity for Aβ species.

6.3. Cu-Aβ Catalysis

Elevated Cu concentrations have been found in Aβ plaque deposits, which have been shown to catalyze the formation of ROS. Due to this catalytic potential, I wanted to investigate whether Cu-Aβ species could catalyze other transformations, specifically Huisgen’s 1,3-dipolar cycloaddition reaction to produce triazolyl compounds (Figure 6.3). Cu-Aβ coordination also suggests that upon reduction to Cu⁺, a linear bis-histidine complex forms, which creates vacant coordination sites for substrates to bind and react with the metal centre (see Chapter 1).

General scheme of using Cu-Aβ species as a catalyst to produce active therapeutic/diagnostic tools.

Our first goal to demonstrate this concept was to use non-fluorescent alkyne and azide fragments that, upon triazole formation, will produce a fluorescent product. Here, a non-fluorescent azidocoumarin was coupled with propargyl alcohol to produce a fluorescent product (Figure 6.5). This fluorogenic assay has been previously reported.
using the same N$_3$/alkyne fragments. 253 20 µM Cu was used for catalysis where 100 µM N$_3$/alkyne were used in the presence of 20 µM ascorbate as a reducing agent. When aqueous Cu catalysis is compared to Cu in the presence of 1 eq. A$\beta_{1-40}$, a slight reduction in fluorescence intensity is observed (Figure 6.4). This suggests that Cu$^{2+}$ bound to A$\beta_{1-40}$ is maintained in a catalytic environment.

**Figure 6.5.** Fluorogenic assay evaluating Cu-A$\beta_{1-40}$ species as a catalyst for the production of a triazole-based fluorophore. Conditions: 100 µM N$_3$/Alkyne, 20 µM Cu$^{2+}$, 0 and 20 µM A$\beta_{1-40}$, 20 µM ascorbate, constant agitation in a 96-well plate reader at 37 °C. Each experiment was performed in quadruplicate.

The next step was to evaluate Cu-A$\beta$ catalysis to produce triazole-based ligands. As an example from Chapter 2, the fragments to synthesize L1 were used to evaluate if Cu-A$\beta$ species can catalytically produce L1. Using high-performance liquid chromatography (HPLC), L1 and 2-ethynylpyridine were used as standards to determine their respective retention times in order to monitor reaction progression (Figure 6.6). When the N$_3$/alkyne fragments (125 mM) were exposed to 1:1 Cu:A$\beta_{1-40}$ (50 µM) for 1
hour at room temperature, a significant amount of product was observed due to the peak at ca. 3 minutes. Starting material (2-ethynylpyridine) is still observed due to the peak at ca. 8 minutes, suggesting that the reaction has not gone to completion. These preliminary data suggest that Cu-Aβ species are able to catalyze the formation of triazole species, which may open a new avenue for therapeutic intervention.

Figure 6.6. (Black) HPLC trace of 2-ethynylpyridine with a retention time of 8.2 minutes. (Red) HPLC trace of reaction mixture of 1:1 N3/Alkyne (125 mM), 1:1 Cu²⁺:Aβ₁₄₀ (50 µM), and 1 mM ascorbate after incubation for 60 minutes. (Blue) HPLC trace of synthesized L1 product with a retention time of 2.8 minutes.

Further studies are needed, including a fluorogenic assay to evaluate Cu-Aβ catalysis using different peptide lengths such as Aβ₁₋₁₆, Aβ₁₋₂₈, and Aβ₁₋₄₂. Faller and co-workers have evaluated Cu redox potentials in the presence of different Aβ peptide lengths.¹⁵² Interestingly, Cu-Aβ₁₋₄₂ was able to produce hydroxyl radicals five times faster in comparison to Cu-Aβ₁₋₄₀, suggesting that the aggregation state may play an important role in ROS generation. In addition, other Cu:Aβ ratios should be evaluated (eg: 1.4 eq. Cu:Aβ) due to the fact that there is a 2nd Cu binding site on the Aβ peptide.¹⁴⁶ Cu-Aβ species should be screened to evaluate if other triazole-based ligands can also be synthesized, specifically the phenol-triazole series of ligands due to their promising in vitro properties (Chapter 4). Finally, it would be interesting to evaluate the catalytic efficiency by varying the pre-incubation period of Cu-Aβ (eg: 1, 4, 12, and 24 hours) in
order to determine if morphological and aggregation changes have an effect on catalysis.

6.4. Concluding Remarks

This thesis describes several multifunctional small molecule agents that are able to influence the Aβ aggregation process involved in AD. Specifically, three triazole-based ligands have been described that have a combination of properties including metal binding, peptide interactions, and antioxidant properties. Each series of ligands has been shown to influence Cu-Aβ interactions, as Cu ions have been shown to associate with the Aβ peptide and have profound effects on its aggregation and toxicity. From chapters 2 through 5, significant progress has been made in the study of the interactions of ligands, metals, and Aβ peptide. The potential to produce triazole-containing therapeutics in situ is a very attractive strategy to target Cu-Aβ species. Finally, the development of Ru(III) complexes with multifunctional ligands that can either target hydrophobic interactions along the peptide or aid in drug delivery are areas of research that require further exploration.
References


288. Given our unpublished 1H-15N TROSY-HSQC NMR data of HCl titrations against Aβ1-40 monomer in SDS solution, which shows similar chemical shift perturbations (E11, H13, Q15, F20, and E22) in both direction and, to a lesser extent, magnitude., M. H. Lim, 2012.


Appendix A. Supplementary Information for Chapter 2

Figure S1. Variable pH UV-vis spectra of L2.

Figure S2. Variable pH UV-vis spectra of L3.
Figure S3. Variable pH UV-vis spectra of L4.

Figure S4. Extended solid state packing of [CuL1Cl2] showing weak interactions of axially bound Cl ligand from another Cu centre and oxygen from a separate morpholine moiety.
Table S1. Summary of X-ray Crystallographic Data.

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Figure S5. Variable pH UV-vis spectra of L1.
Figure S6. Variable pH UV-vis spectra of L3.

Figure S7. Variable pH UV-vis spectra of L4.
**Figure S8a.** Variable pH $^1$H NMR spectra of L1 in the aromatic region.

**Figure S8b.** Variable pH $^1$H NMR spectra of L1 in the alkyl region.
Figure S9a. Variable pH $^1$H NMR spectra of L3 in the aromatic region.

Figure S9b. Variable pH $^1$H NMR spectra of L3 in the alkyl region.
**Figure S10a.** Variable pH $^1$H NMR spectra of L4 in the aromatic region.

**Figure S10b.** Variable pH $^1$H NMR spectra of L4 in the alkyl region.
Figure S11. 2D $^1$H-$^{15}$N TROSY-HSQC NMR spectra of (a) L3, (b) L2, and (c) L4 against $^{15}$N-labeled Aβ1-40 in the SDS condition (200 mM SDS-$d_{25}$, 20 mM NaPi, pH 7.3, 7% v/v D2O). Chemical shifts are depicted with increasing equivalents of corresponding ligands, indicated by: black (0 eq.), blue (1 eq.), red (5 eq.), and green (10 eq.). Plots of calculated $^1$H and $^{15}$N chemical shifts of Aβ1-40 in the presence of 10 equivalents of the corresponding ligand are shown. Peaks that could not be resolved due to overlap or absence are indicated by an asterisk (*).
Figure S12. NMR studies of L2 with \( ^{15}\text{N} \)-labeled A\( \beta \)\(_{1-40} \) in the SDS-free condition. (a) 2D \(^1\text{H}-^{15}\text{N} \) TROSY-HSQC NMR spectra of L2 against \( ^{15}\text{N} \)-labeled A\( \beta \)\(_{1-40} \) (20 mM HEPES, pH 7.3, 50 mM NaCl, and 7% v/v D\(_2\)O; 0, 1, 5, and 10 eq. of L2, indicated by black, blue, red, and green, respectively). (b) Calculated \(^1\text{H} \) and \(^{15}\text{N} \) chemical shifts of A\( \beta \)\(_{1-40} \) in the presence of 10 eq. of L2. Peaks that could not be resolved due to overlap or absence are indicated by an asterisk (*). (c) Plot of the normalized peak intensity in the presence of 10 eq. of L2 A\( \beta \) only.
Figure S13. Docking of small molecules with Aβ1-40 monomer in the presence of SDS (PDB 1BA4) predicted by AutoDock Vina. Surface (left) and cartoon (right) depictions of L3 (carboxylate form, magenta), L1 (light blue), L2 (yellow), and L4 (protonated form, light pink) are shown. a) Conformation B; b) Conformation C; c) Conformation D. Potential hydrogen bonding contacts between the ligands and receptors are indicated with a dashed line (1.9 – 2.3 Å).
Figure S14. Docking of small molecules with Aβ₁₋₄₀ monomer in the absence of SDS (PDB 2LFM) predicted by AutoDock Vina. Surface (left) and cartoon (right) depictions of L₃ (carboxylate form, magenta), L₁ (light blue), L₂ (yellow), and L₄ (protonated form, light pink) are shown. a) Conformation B; b) Conformation C; c) Conformation D; d) Conformation E. Potential hydrogen bonding contacts between the ligands and receptors are indicated with a dashed line (2.3 – 2.7 Å).
Appendix B. Supplementary Information for Chapter 5

Figure S1. (Top) Aβ_{1-40} (5 µM) was incubated for 48 hours in the absence and presence of varying concentrations of KP1019 (0.5 – 2 eq.) and the known aggregation inhibitor Congo Red. (Bottom) Even at 0.5 eq. KP1019/Aβ_{1-40}, KP1019 is still able to limit the fibrillization process. TEM images were obtained after 48 hours of incubation. When Aβ_{1-40} was incubated in the absence of KP1019, significant fibrillar aggregates are observed (bottom, A). Upon addition of 2 eq. Congo Red, an apparent decrease in aggregate size is clear (bottom, B). When 0.5 and 1 eq. KP1019 are incubated with Aβ_{1-40}, a concentration-dependent decrease in aggregate size was characterized (bottom, C and D).
Figure S2. \(\beta_{1-42}\) (5 \(\mu\text{M}\)) was incubated for 48 hours in the presence of ThT (5 \(\mu\text{M}\)) causing a significant increase in fluorescence indicative of peptide aggregation. Upon addition of either 1 or 2 eq. KP1019, an immediate decrease in fluorescence was observed and did not change even after 10 mins.

Figure S3. 24, 48, and 72 hour time course dot blot assay using 6E10 (left) and 4G8 (right) primary anti-A\(\beta\) antibodies. KP1019 (10 – 100 \(\mu\text{M}\)) binding to \(\beta_{1-42}\) (10 \(\mu\text{M}\)) reduced the immunoreactivity of the N-terminus primary antibody 6E10, but did not inhibit the immunoreactivity of the 4G8 primary antibody for the hydrophobic region of the \(\beta_{1-42}\) peptide. Interestingly, when the peptide is incubated in the absence of KP1019, 6E10 immunoreactivity decreased over time, likely due to slow aggregation and precipitation of the peptide.
Differentiated SH-SY5Y cells were treated with variable [Aβ1-42] in quadruplicate measurements for 24 hours. To assess cell viability, an Alamar Blue assay was used to evaluate Aβ1-42-associated neurotoxicity. 20 µM Aβ1-42 was used in all other measurements as this was determined to be an optimal concentration to evaluate the rescuing of cell viability with KP1019.
Figure S5. Differentiated SH-SY5Y cells were treated with variable [KP1019] in quadruplicate measurements and evaluated for toxicity using an Alamar Blue cell viability assay. KP1019 is relatively non-toxic up to 250 µM under these conditions.