titrations were carried out to obtain $pK_a$ values which were used to generate speciation diagrams (Figure 4.7). 6-8 like in previous studies with 1-3\textsuperscript{22} and L2\textsuperscript{24} are found to be predominately in their neutral (L) forms at physiological pH while 5 was mainly singly protonated [Figure 4.7; 5, $pK_{a1} = 4.9(5)$, $pK_{a2} = 8.2(6)$ (ca. 15% L at pH 7.4); 6, $pK_{a1} = n/a$, $pK_{a2} = 4.3(4)$ (ca. 100% L at pH 7.4); 7 $pK_{a1} = 4.5(8)$, $pK_{a2} = 6.7(5)$ (ca. 80% L at pH 7.4); 8, $pK_{a1} = 4.3(2)$, $pK_{a2} = 6.6(8)$ (ca. 85% pH 7.4)]. Note that 9 was unable to be tested due to its limited stability in solution.

Additionally, the stability of 5-9 and L2\textsuperscript{b} was assessed in the absence of metal ions by UV-Vis spectroscopy in HEPES buffer (Figure 4.8). Previous studies with 4 suggest that the hydrolysis of 4 to produce DMPD is responsible for its activity toward metal-free Aβ.\textsuperscript{22} 5-7, similar to 1-3, were found to be stable over the course of the experiment while 8, 9, and L2\textsuperscript{b} display an increase in the spectral features.\textsuperscript{22} The rates of the changes, however, are very slow with 8, 9, and L2\textsuperscript{b} having half-lives of > 300 min, ca. 230 min, and > 300 min, respectively, compared to the half-life of ca. 40 min.
Figure 4.8. Stability of 5-9 and L2-b in the absence of metal ions. (a-f) The UV-Vis spectra of 5-9 and L2-b were monitored over the course of 5 h at pH 7.4; blue: immediately after addition of the compound; orange: after 5 h incubation at 37 °C. (g) Rate of transformation and half-lives of 5-9 and L2-b in the presence and absence of Cu(II).<sup>a</sup>Rate of decay of the absorbance peak at 300 nm for 9. <sup>b</sup>Half life of the absorbance peak in minutes. <sup>c</sup>Spectral changes were too slow to accurately measure the rate during the duration of the experiment. Conditions: [Compound] = 50 µM; 25 µM HEPES, pH 7.4,150 µM NaCl; room temperature.

for 4 (Figure 4.8g).<sup>22</sup> ESI(+)MS studies were carried out to identify the species in solution with 8 and 9; however, no changes in mass were detected for both over 5 h supporting that the breakdown of 8 and 9 was too slow to result in detectable modulation of metal-free Aβ (Figure 4.9).

Finally, <sup>15</sup>N-labeled Aβ<sub>40</sub> monomer was titrated with 1-9 (0 to 10 equiv) and monitored by two dimensional (2D) <sup>1</sup>H-<sup>15</sup>N band-selective optimized flip-angle short-transient (SOFAST)-heteronuclear multiple quantum coherence (HMQC) nuclear
magnetic resonance (NMR) spectroscopy to observe the Aβ residues that are targeted by 1-9 (Figures 4.10 and 4.11). Overall, 1-9 showed relatively low chemical shift perturbations (CSP) similar to previous studies with L2-b31 suggesting weak nonspecific interactions (Figure 4.11). This is even true for 4, which did show reactivity toward metal-free Aβ, suggesting that the hydrolysis to produce DMPD is required before interaction and subsequent modulation of aggregation takes place.

4.2.4. Proposed mode of action (II) for control of Cu(II)–Aβ reactivity

UV-Vis and ESI(+)MS studies of 5-9 and L2-b in the presence of Cu(II) confirmed the design principles (Figure 4.12). 5, 8, and 9, which were made to oxidize in the presence of Cu(II), show the formation of the typical broad double peak spectral signature of the cationic radical form of substituted p-phenylenediamine compounds from ca. 500 nm to ca. 600 nm correlated to the decay of the initial peak formed ca. 400 nm previously suggested to be from metal-binding (Figure 4.12a, d, and e)22. This suggests that these compounds can modulate Cu(II)–Aβ aggregation through the
Figure 4.10. Interaction of compounds with $^{15}$N-labeled Aβ$_{40}$. SOFAST-HMQC NMR (900 MHz) spectra before (blue) and after (red) addition of 10 equiv of (a) 1, (b) 2, (c) 3, (d) 4, (e) 5, (f) 6, (g) 7, (h) 8, and (i) 9.

Figure 4.11. Normalized chemical shift perturbations (CSPs) of $^1$H and $^{15}$N amide atoms for Aβ$_{40}$ after addition of 10 equiv of compound from the SOFAST-HMQC NMR (900 MHz) spectra (Figure 4.10).

The proposed mechanism (a) (Figure 4.2). In the case of 6, there were no variations in the spectra over 5 h suggesting that this compound does not oxidize in the presence of Cu(II) which corresponds to the lack of observable affect on Cu(II)–Aβ aggregation in the gel/Western blot experiments (Figure 4.12b; vide supra).

7 is unique in that it initially forms the cationic radical at ca. 530 nm similar to 1, 2, 5, 8, 9, and L2-b but then undergoes another spectral change with the growth of two
Figure 4.12. Stability of 5-9 and L2-b in the presence of Cu(II). (a-f) The UV-Vis spectra of 5-9 and L2-b in the presence Cu(II) was monitored over the course of 5 h in 20 µM HEPES, pH 7.4, 150 µM NaCl; blue: immediately after addition of the compound; orange: after 5 h incubation at 37 °C. (g) Rate of transformation and half-lives of 5-9 and L2-b in the presence of Cu(II). aRate of decay of the absorbance peak at 473 nm, 400 nm, 420 nm, and 420 nm for 5, 8, 9, and L2-b, respectively. bHalf life of the absorbance peak in minutes. cNo spectral changes were observed during the duration of the experiment. dRate of growth of the peak at 400 nm for 7. Conditions: [Compound] = 50 µM; [Cu(II)] = 25 µM; 20 µM HEPES, pH 7.4, 150 µM NaCl.

show that this new species, which grows in with a half-life of ca. 40 min, is 4 Da less than 7 which could possibly result cyclization of the radical or quinoid form (Figure 4.13a-c). This transformation implies that the oxidized forms of 7 are not as stable as the strongly absorbing peaks at ca. 400 and 500 nm (Figure 4.12c). ESI(+)MS studies other compounds in this series. The formation of an unstable compound could possibly explain the different abilities to modulate Cu(II)–Aβ aggregation in the inhibition and
Figure 4.13. Electrospray ionization-mass spectrometry (ESI-MS) studies of the species in solution in the presence of Cu(II) and Zn(II). Mixtures of (a, b) Cu and 7 as well as Zn(II) and (e, f) 1, (h, i) 4, or (k, l) L2-b were analyzed (a, e, h, k) immediately after addition to ddH2O and (b, f, i, l) after 5 h incubation at 37°C and (c, g, j, m) their structures assigned.
Figure 4.14. Stability of 5-9 and L2-b in the presence of Zn(II). (a-f) The UV-Vis spectra of 5-9 and L2-b in the presence Zn(II) was monitored over the course of 5 h in 20 µM HEPES, pH 7.4, 150 µM NaCl; blue: immediately after addition of the compound; orange: after 5 h incubation at 37 °C. (g) Rate of

<table>
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</tr>
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<td>6</td>
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<tr>
<td>7</td>
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<tr>
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<tr>
<td>L2-b</td>
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transformation and half-lives of 5-9 and L2-b in the presence of Cu(II). Rate of growth of the absorbance peak at 255 nm, 500 nm, 500 nm, 290 nm, and 253 nm for 4, 7, 8, 9, and L2-b. Half-life of the absorbance peak in minutes. No measurable spectral changes were observed during the duration of the experiment. Conditions: [Compound] = 50 µM; [Cu(II)] = 25 µM; 20 µM HEPES, pH 7.4, 150 µM NaCl; room temperature.

disaggregation experiments. More detailed studies on 7 will be carried out in the future to fully understand its reactivity.

4.2.5. Proposed mode of action (III) for modulation of Zn(II)–Aβ reactivity

The species in solution in the presence of Zn(II) (0.5 equiv) was also studied (Figure 4.14). First, UV-Vis was used to observe the changes over 5 h. 1-3, 6, and showed limited spectral changes over the course of the experiment (Figure 14a-c, f, and i). While, 5, 7, 8, and L2-b in the presence of Zn(II) had a peak around ca. 480 nm which slowly grew in (Figure 4.14e, g, h, and j). The spectrum of 4 with Zn(II) has a broad flat peak at ca. 520 nm that grows in quickly with a doubling time of 10 min and is reminiscent of its previously reported spectra incubated in the absence of metal ions (Figure 4.14d and k).22

To identify the origin of these spectral features 1, 4, and L2-b (50 µM) were studied using ESI(+)/MS in the presence of Zn(II) (25 µM). Studies with 1 and L2-b (Figure 4.15e-g, k-m) present primarily unbound ligand with a small signal corresponding within error to complexes of Zn(II) and 1 or L2-b in a 1:2 M:L ratio suggesting both of these compounds form stable complexes with Zn(II). ESI(+)/MS experiments with 4 suggest, in the presence of Zn(II), 4 hydrolyses resulting in the formation of DMPD and likely polymers of the resulting pyrrole hydrolysis product as previously suggested in metal-free conditions (Figure 4.13k-l).22

Since 1 and L2-b are demonstrated to bind Zn(II) but have differing activities toward modeling Zn(II)–Aβ aggregation, the binding affinities (Kd) were considered. UV-Vis variable-pH spectrophotometric titration were carried out a mixture of Zn(II) and 1 (Figure 4.15). These experiments indicated the presence of a 1:1 complex under the experimental conditions having a stability constant (log β) of 5.6(3). Based on this value and the previously determined pKₐ values of 1, the pZn (pZn = −log[Zn unchelated]) was calculated to be 5.5 resulting in an approximate disassociation constant in the high
Figure 4.15. Solution speciation studies of the Zn(II)−1 complex. (a) Variable pH UV-Vis titration spectra for Zn(II)−1 were fit to determine stability constants (c, logβ) of Cu(II)−L species and used to generate speciation diagrams (b, FCu = Fraction of free Cu and Cu(II)−L). Parenthesis indicates that the error is in the last digit of the values. Conditions: Zn(II):L = 1:2; [1] = 100 µM; samples were incubated at room temperature for 24 h before titrations. Charges omitted for clarity.

**Absorbance**

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<tr>
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</tr>
<tr>
<td>pZn (=−log[Zn(II)]free; pH 7.4)</td>
<td>5.54</td>
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**Figure 4.15.** Solution speciation studies of the Zn(II)−1 complex. (a) Variable pH UV-Vis titration spectra for Zn(II)−1 were fit to determine stability constants (c, logβ) of Cu(II)−L species and used to generate speciation diagrams (b, FCu = Fraction of free Cu and Cu(II)−L). Parenthesis indicates that the error is in the last digit of the values. Conditions: Zn(II):L = 1:2; [1] = 100 µM; samples were incubated at room temperature for 24 h before titrations. Charges omitted for clarity.

micromolar range (Figure 4.15, Kd = [Zn_{unchelated}]). This possibly explains the differences in reactivity. L2-b has previously been shown to have an apparent Kd of 10^{-6.1} M for Zn(II). The high micromolar binding affinity of 1 for Zn(II) is possibly too weak to compete with the micromolar to nanomolar Kd of Aβ for Zn(II),^2 while the slightly greater Zn(II) binding affinity for L2-b allows for it to compete for Zn(II) binding. This is further supported by previous studies with 3 which also does not modulate Zn(II)−Aβ aggregation where the Kd for Cu(II) is reported to be micromolar.^2,22 It is expected that 3 would have an even lower affinity for Zn(II) due to the trends observed in the Irving-Williams series.^32 Thus, also has a binding affinity for Zn(II) that is too low to interact
Figure 4.16. SOFAST-HMQC NMR (900 MHz) studies with $^{15}$N-labeled Aβ$_{40}$ in the presence of Zn(II). (a, d, g, and j) Spectra of Aβ$_{40}$ before (black) and after addition of Zn(II) (red). (b, e, h, and k) Spectra of Aβ$_{40}$ before (black) and after addition of Zn(II) and compound (red). (c, f, i, and l) Intensities of Aβ with Zn(II) before (black) and after addition of compound (red) normalized to the initial metal-free Aβ$_{40}$ signal.
with Zn(II)–Aβ. 2D SOFAST-HMQC NMR spectroscopy was also used to understand the interaction of 1, 2, 4, and L2-b with Zn(II)–Aβ40 ([15N]-labeled Aβ40 monomer; Figure 4.16). As expected by their limited reactivity toward Zn(II)–Aβ in the gel/Western blot experiments and low binding affinities for Zn(II), 1 and 2 did not demonstrate an ability to interact with Zn(II)–Aβ as evidenced by the limited changes in intensity upon their addition (Figure 4.16a-f). 4 also did not cause significant changes in the spectra (Figure 4.16g-i) this could be due to 4 having only a limited effect on the initial interaction of Zn(II) with Aβ. This is in contrast to L2-b which demonstrated larger changes in the spectra (Figure 4.16j-l) suggesting L2-b can directly interact with Zn(II)–Aβ in a way that increases the flexibility of the peptide possibly though the formation of a ternary complex as suggested by previous 2D 1H–15N transverse relaxation optimized spectroscopy (TROSY)-heteronuclear single quantum correlation (HSQC) NMR spectroscopy experiments in the presence of sodium dodecyl sulfate (SDS).24

Overall, these studies with Zn(II) suggest that compounds that do not modulate Zn(II)–Aβ reactivity (1-3 and 6) do not have significant interactions with Zn(II)–Aβ most likely due to low binding affinities for Zn(II). In contrast, those which have an effect on Zn(II)–Aβ aggregation (5-9 and L2-b) could have greater affinities for Zn(II) and possibly form stable ternary complexes with Zn(II)–Aβ [proposed mechanism (b); Figure 4.2]. The exception to this is 4 which undergoes hydrolysis to produce DMPD, a known Zn(II)–Aβ interacting compound [proposed mechanism (c); Figure 4.2].33

4.2.6. Exploration of structural features required for interacting with fibrils of Metal-free Aβ and Zn(II)–Aβ

Saturation transfer difference (STD) NMR studies were carried out in order to investigate what moieties were important for the interaction of 1, 2, and L2-b with fibrillar Aβ40 in the absence (Figure 4.17) and presence of Zn(II) (Figure 4.18). STD NMR allows for the atomic-level mapping of the ligand binding to Aβ as the proximity of the ligand atoms to Aβ is proportional to their signal intensity.34-36 In both the metal-free and Zn(II) samples, nearly the whole structure of 1, 2, and L2-b were found to bind to the fibril with the pyridine groups possibly being in closer contact. Studies with 4 were
Figure 4.17. Molecular level interaction with Aβ40 fibrils. 1H STD NMR spectra of (a) 1, (c) 2, (e) 4, and (g) L2-b with Aβ40 fibrils. Comparison of the STD signal intensity (red) with the STD reference (black) allows for the determination of the relative proximity of the corresponding proton to the fibrils. Normalized intensities of the STD signal mapped to the structures of (b) 1, (d) 2, (f) 4, and (h) L2-b. The STD effects are highlighted in color (green, > 75%; yellow, 50-75%; red, < 50%); no color represents that the STD
signal for the proton was not observed).

**Figure 4.18.** Molecular-level interaction with Aβ₄₀ fibrils treated with Zn(II). ¹H STD NMR spectra of (a) 1, (c) 2, (e) 4, and (g) L2-b with Zn(II)-treated Aβ₄₀ fibrils. Comparison of the STD signal intensity (red) with the STD reference (black) allows for the determination of the relative proximity of the corresponding proton to the fibrils. Normalized intensities of the STD signal mapped to the structures of (b) 1, (d) 2, (f) 4,
and (h) L2-b. The STD effects are highlighted in color (green, > 75%; yellow, 50-75%; and red, < 50%; no color represents that the STD signal for the proton was not observed).

also carried out but the STD effect was very weak. This could be due to either very strong binding or very weak binding as this technique first requires a binding event followed by the disassociation of the ligand to obtain signal.  

4.2.7. Mediation of oxidative stress

The activity of 5-9 to mediate oxidative stress was also studied (Figure 4.19). First, the ability to scavenge free organic radicals was examined using the Trolox Equivalence Antioxidant Capacity (TEAC) assay (Figure 4.19a).  

5, 7, 8, and 9 display a greater antioxidant capacity than Trolox, a known antioxidant and water-soluble analogue of vitamin E, with 5 and 9 having a slightly lower ability to quench radicals (ca. 1.5 and 1.7, respectively) than 7 and 8 (ca. 2.0). This is similar to the previous reported antioxidant capacities of 1-4 and L2-b. Conversely, 6 was found not to have an effect on the presence of radicals which can be attributed to it’s calculated high ionization potentials as shown in Table 4.1 (Figure 4.19a).

The ability of 5-9 and L2-b to reduce the production of ROS by Fenton-like chemistry promoted by Cu(I/II) was studied using the 2-deoxyribose assay (Figure 4.19b). 7, 8, and 9 were found to better control the production of hydroxyl radicals (•OH) than the previously studied 1-3 (ca. 50% production inhibited) with 9 being the best compound studied (ca. 85% production inhibited; Figure 4.19b). 5 and 6 have

Figure 4.19. Ability of 5-9 to mediate oxidative stress. (a) Antioxidant activity of 5-9 in the presence of cell lysates as evaluated by the TEAC assay. Values are relative to a vitamin E analog, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). (b) Ability of 5-9 (125 µM) to control Cu(I/II)-triggered ROS production by Fenton-like reactions, as measured by the 2-deoxyribose assay ([Cu(II)] = 10 µM).
comparably lower abilities to prevent the production of ROS being able to reduce the presence of •OH by only ca. 30% and 20%, respectively.

4.2.8. Regulation of toxicity induced by metal-free and metal-associated Aβ

Based on their different activities toward redirection of Aβ and metal–Aβ aggregation and ability to mediate oxidative stress, 6, 7, and 9 were chosen for further studies in their toxicity in human neuroblastoma SK-N-BE(2)-M17 (M17) cells (Figure 4.20). 20 µM of 6 is found to decrease cell viability by ca. 30% in the absence of metal ions as well as when Cu(II) (20 µM) or Zn(II) (20 µM) is added to the cultures (Figure 4.20a). Under the same conditions, 7 and 9 are shown to be relatively nontoxic with 9 slightly decreasing cell viability to ca. 85% in the presence of Cu(II). Furthermore, 7 and 9 are able to mediate the toxicity of Aβ40 (Figure 4.20b) and Aβ42 (Figure 4.20c) in the absence of externally introduced metal ions (left) and in the presence of externally added Cu(II) (middle) and Zn(II) (right). Thus, 7 and 9 are able to reduce the toxicity of metal–Aβ complexes.

4.3. Conclusions

Inspired by initial studies regarding molecular modes of action of structurally similar compounds, a chemical library of small molecules was designed to target
multiple factors (i.e., Aβ, metals, metal-bound Aβ, and ROS) involved in AD pathogenesis and mediate their reactivities to different degrees. The differentiated activity of the small molecules toward targets was confirmed to result from their chemical properties and molecular level interactions with Aβ and metal–Aβ as demonstrated by investigations into the solution species, computational calculations, and 2D NMR experiments. These studies demonstrated specificity toward tempering the reactivity of Cu(II)–Aβ over Zn(II)–Aβ and metal-free Aβ for 1-3, metal–Aβ versus metal-free for 5, 7, 8, 9, and L2-b, no specificity for 4 (modulating with Aβ reactivity regardless if metal ions are or are not present), or having no detectable effects for 6. These properties can be explained through three structure-dependent proposed mechanisms: (1) radical mediated peptide cleavage, (2) ternary complex formation, and (3) fragmentation followed by covalent peptide adduct formation (Figure 4.2). Overall, we demonstrate the value of understanding the molecular level mechanisms of small molecules in the development of potential chemical tools employed to better understand the pathogenesis of neurodegenerative diseases. A clear knowledge of the molecular interactions of these compounds with their biological targets can lead to the production of successful diagnostics and treatments for these devastating diseases.

4.4. Experimental section

4.4.1. Materials and methods

All reagents were purchased from commercial suppliers and used as received unless otherwise noted. Aβ₄₀ and Aβ₄₂ (the sequence of Aβ₄₂: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVIA) were purchased from Anaspec Inc. (Fremont, CA, USA). Trace metals were removed from buffers and solutions used in Aβ experiments by treating with Chelex overnight (Sigma-Aldrich, St. Louis, MO, USA). Optical spectra were recorded on an Agilent 8453 UV-visible (UV-Vis) spectrophotometer. Absorbance values for biological assays, including cell viability and antioxidant assays, were measured on a Molecular Devices SpectraMax 190 microplate reader (Sunnyvale, CA, USA). ¹H and ¹³C 1D spectra were recorded using a 400 MHz Agilent NMR spectrometer.
4.4.2. Preparation of 1-4, 6, and L2-b

1-4, 6, and L2-b were obtained as previously reported.\textsuperscript{22,24}

4.4.3. Preparation of 5

The compound was purchased from Ryan Scientific (Mt. Pleasant, SC) and was purified by column chromatography ($\text{Al}_2\text{O}_3$, 3:1 Hx:EtOAc, $R_f = 0.51$). Yellow oil. $^1\text{H}$ NMR [400 MHz; (CD$_3$)$_2$SO] $\delta$ (ppm): 8.51 (1H, dd, $J = 4$ Hz), 7.72 (1H, t, $J = 8$ Hz), 7.37 (1H, dd, $J = 8$ Hz), 7.23 (1H, t, $J = 8$ Hz), 6.54 (4H, q, $J = 12$ Hz), 5.67 (1H, t, $J = 8$ Hz), 4.28 (2H, d, $J = 4$ Hz), 3.10 (4H, q, $J = 8$ Hz), 0.96 (6H, t, $J = 8$ Hz). $^{13}\text{C}$ NMR (100 MHz; (CD$_3$)$_2$SO) $\delta$ (ppm): 160.3, 148.7, 140.5, 139.8, 136.5, 121.8, 121.1, 116.5, 113.6, 49.47, 44.81, 12.37. HRMS Calcd for $\text{C}_{16}\text{H}_{22}\text{N}_3$ [M+H]$^+$: 256.1814; found 256.1808.

4.4.4. Preparation of 7

The compound was purchased from Ryan Scientific and was recrystallized from 1:1 DCM:hexanes 1x and washed 2x with hexanes. Light brown powder. $^1\text{H}$ NMR (400 MHz; CD$_2$Cl$_2$) $\delta$ (ppm): 8.55 (d, 1H, $J = 4$ Hz), 7.61 (t, 1H, $J = 8$ Hz), 7.33 (d, 1H, 4 Hz), 6.63 (d, 2H, $J = 8$ Hz), 6.53 (d, 2H, $J = 8$ Hz), 4.52 (m, 1H), 4.15 (s, 1H), 2.76 (s, 6H), 1.49 (d, 3H, $J = 4$ Hz). $^{13}\text{C}$ NMR (100 MHz, CD$_2$Cl$_2$) $\delta$ (ppm): 165.1, 149.7, 144.7, 140.1, 137.0, 122.3, 120.9, 115.8, 115.4, 56.0, 42.3, 23.6. HRMS Calcd for $\text{C}_{15}\text{H}_{20}\text{N}_3$ [M+H]$^+$: 242.1652; found 242.1649.

4.4.5. Preparation of 8

The compound was purchased from Ryan Scientific and was recrystallized from hot hexanes and washed 5x with cold hexanes. Yellow powder. $^1\text{H}$ NMR [400 MHz, (CD$_3$)$_2$SO] $\delta$ (ppm): 8.29 (d, 1H, $J = 8$ Hz), 8.00 (d, 1H, $J = 8$ Hz), 7.93 (d, 1H, $J = 8$ Hz), 7.74 (t, 1H, $J = 8$ Hz), 7.56 (m, 2H), 6.57 (m, 4H), 5.91 (t, 1H, $J = 4$ Hz), 4.47 (d, 2H, $J = 8$ Hz), 2.67 (s, 6H). $^{13}\text{C}$ NMR (100 MHz; CD$_2$Cl$_2$) $\delta$ (ppm): 159.9, 148.2, 144.8, 141.0, 136.9, 130.0, 129.4, 128.2, 127.9, 126.6, 120.5, 116.1, 114.7, 51.2, 42.39. HRMS Calcd for $\text{C}_{18}\text{H}_{19}\text{N}_3$ [M]$^+$: 277.1573; found 277.1574.
4.4.6. Preparation of 9

The compound was purchased from Ryan Scientific (Mt. Pleasant, SC) and was recrystallized from ethyl acetate, DCM/hexanes, and washed 2x with hexanes or dissolved in ethyl acetate and filtered over an neutral aluminum oxide plug. $^1$H NMR [400 MHz; (CD$_3$)$_2$SO] $\delta$ (ppm): 7.04 (d, 1H, $J = 4$ Hz), 6.76 (d, 1H, 4 Hz), 6.63 (m, 4H), 5.36 (t, 1H, 4 Hz), 4.18 (d, 2H, 4 Hz), 3.62 (s, 3H), 2.70 (s, 6H). $^{13}$C NMR (100 MHz; CD$_2$Cl$_2$) $\delta$ (ppm): 146.2, 145.2, 140.7, 127.5, 121.9, 115.8, 151.2, 42.5, 42.3, 30.0. HRMS Calcd for C$_{13}$H$_{19}$N$_4$ [M+H]$^+$: 231.1604; found 231.1603.

4.4.7. Calculation of ionization potentials

First-principles calculations using Gaussian09$^{38}$ were performed using previously reported procedures.$^{22}$ Oxidation potentials were calculated using only thermodynamic parameters due to the difficulty of computing the kinetics of electron transfer steps. All the relevant chemical species were optimized at M06/6-31G(d) level, and their thermodynamic parameters were calculated at M06/6-311+G(2df,2p) level.

4.4.8. Parallel artificial membrane permeability assay adapted for blood-brain barrier (PAMPA-BBB)

PAMPA-BBB experiments were carried out using the PAMPA Explorer kit (pION Inc., Billerica, MA, USA) using previously reported protocols.$^{22,24,37,39}$ UV–Vis absorbance of the solutions in the reference, acceptor, and donor plates were measured using a microplate reader. The PAMPA Explorer software v. 3.5 (pION) was used to calculate the $-\log P_e$ values for the compounds. CNS± designations were assigned by comparison to compounds that were identified in previous reports.$^{24,40-42}$

4.4.9. Aβ aggregation experiments

Aβ experiments were performed according to previously published methods.$^{23,24,31,37,39,43,44}$ Prior to experiments, Aβ$_{40}$ or Aβ$_{42}$ was dissolved in ammonium hydroxide (NH$_4$OH; 1% v/v, aq). The resulting solution was aliquoted, lyophilized overnight, and stored at $-80$ °C. A stock solution of Aβ was then prepared by dissolving lyophilized peptide in 1% NH$_4$OH (10 μL) and diluting with ddH$_2$O. The concentration of
the solution was determined by measuring the absorbance of the solution at 280 nm ($\varepsilon = 1450 \text{ M}^{-1}\text{cm}^{-1}$ for A$\beta_{40}$; $\varepsilon = 1490 \text{ M}^{-1}\text{cm}^{-1}$ for A$\beta_{42}$). The peptide stock solution was diluted to a final concentration of 25 $\mu$M in Chelex-treated buffered solution containing HEPES (20 $\mu$M, pH 6.6 for Cu(II) samples; pH 7.4 for metal-free and Zn(II) samples) and NaCl (150 $\mu$M). For the inhibition studies, compounds (final concentration 50 $\mu$M, 1% v/v DMSO) were added to the sample of A$\beta$ (25 $\mu$M) in the absence and presence of a metal chloride salt (CuCl$_2$ or ZnCl$_2$; 25, 50, 100 or 125 $\mu$M) followed by incubation at 37 °C with constant agitation for 24 h. For the disaggregation studies, A$\beta$ with and without a metal chloride salt was incubated for 24 h at 37 °C with constant agitation to generate preformed A$\beta$ aggregates. The resulting samples were then treated with compounds (50 $\mu$M) and incubated with constant agitation for additional 24 h.

4.4.10 Gel electrophoresis and Western blotting

The samples from the inhibition and disaggregation experiments were analyzed by gel electrophoresis followed by Western blotting using an anti-A$\beta$ antibody (6E10) using previously established procedures. Samples (10 $\mu$L) were separated on a 10-20% Tris-tricine gel (Invitrogen, Grand Island, NY, USA). Following separation, the proteins were transferred onto nitrocellulose membranes and blocked with bovine serum albumin (BSA, 3% w/v, Sigma-Aldrich, St. Louis, MO, USA) in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature or overnight at 4 °C. The membranes were incubated with an anti-A$\beta$ antibody (6E10, 1:2000, Covance, Princeton, NJ, USA) in a solution of 2% BSA (w/v in TBS-T) for 4 h at room temperature or overnight at 4 °C. After washing with TBS-T (3x, 10 min), a horseradish peroxidase-conjugated goat antimouse secondary antibody (1:5000 in 2% BSA w/v in TBS-T; Cayman Chemical Company, Ann Arbor, MI, USA) was added for 1 h at room temperature. The Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA), Biosesang ECL Plus kit (Biosesang, Gyeonggi-do, Republic of Korea), or a homemade ECL kit was used to visualize the results on a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) or film.
4.4.11. Transmission electron microscopy (TEM)

Samples for TEM were prepared according to a previously reported method using glow-discharged grids (Formar/Carbon 300-mesh, Electron Microscopy Sciences, Hatfield, PA, USA). Images for each sample were taken on a JEOL JEM-2100 transmission electron microscope (UNIST Central Research Facilities, Ulsan National Institute of Science and Technology, Ulsan, Republic of Korea).

4.4.12. Determination of solution speciation for 5-8 and Zn(II)–1 complex

The pKa values for 5-8 were determined by UV−Vis variable-pH titrations as previously reported. To establish the pKa values, a solution (100 mM NaCl, 10 mM NaOH, pH 12) of 5 (100 µM), 6 (25 µM), 7 (100 µM), and 8 (20 µM) was titrated with small amounts of HCl. At least 30 spectra were recorded in the range of pH 2–10. Similarly, a solution containing ZnCl2 and 1 (100 µM) in a metal to ligand ratio of 1:2 was titrated with small additions of HCl and at least 30 spectra were recorded over the range pH 2–8. The acidity and stability constants were calculated by using the HypSpec program (Protonic Software, UK). Speciation diagrams were modeled in the HySS2009 program (Protonic Software).

4.4.13. Stability studies

The stability of the compounds (50 µM) in the absence and presence of CuCl2 (25 µM) or ZnCl2 (25 µM) were monitored every 10 min using UV-Vis spectroscopy for 5 h in buffer (20 µM HEPES, 150 µM NaCl, 1% DMSO, pH 7.4) at 37 °C. The resulting spectra were corrected for baseline shifts at 800 nm and the half-life or doubling time and rate of decay or growth of the absorbance was calculated using the first order exponential decay function as implemented in Origin 9.1 (8 = 300 nm; 9 = 300 nm; L2-b = 300 nm; Cu + 5 = 473 nm; Cu + 7 = 505 nm; Cu + 8 = 400 nm; Cu + 9 = 420 nm; Cu + L2-b = 420 nm; Zn + 4 = 255 nm; Zn + 7 = 255 nm; Zn + 8 = 500 nm; Zn + 9 = 500 nm; Zn + 9 = 290 nm; L2-b = 253 nm; OrginLab Corp., Northampton, MA, USA). Additionally, selected compounds (50 µM) were studied by ESI(+)MS on a Bruker HCT basic system mass spectrometer equipped with an ESI ion source. Triplicate samples determined the
mass accuracy of the instrument to be approximately ±1 Da. Samples with or without CuCl₂ (25 μM) or ZnCl₂ (25 μM) were incubated in ddH₂O (1% DMSO) at 37 °C for the selected time points before being flash frozen using liquid nitrogen and stored at −80 °C until thawed immediately before measurement.

4.4.14. 2D NMR spectroscopy

The interaction of Aβ₄₀ with ligands in the L2D series was monitored by 2D band-Selective Optimized Flip-Angle Short Transient Heteronuclear Multiple Quantum Coherence (SOFAST-HMQC) at 8 °C. Uniformly-¹⁵N-labeled Aβ₄₀ (rPeptide, Bogart, GA, USA) was first dissolved in 1% NH₄OH and lyophilized. The peptide was re-dissolved in 3 μL of DMSO-d₆ (Cambridge Isotope, Tewksbury, MA, USA) and diluted with phosphate buffer, NaCl, D₂O, and ddH₂O to a final peptide concentration of 80 μM (20 mM PO₄³⁻, pH 7.4, 50 mM NaCl, 7% v/v D₂O). Ligands were dissolved in DMSO-d₆ (50 mM) and titrated into the solution of Aβ₄₀. Each spectrum was obtained using 64 complex t₁ points and a 0.1 sec recycle delay.

The interaction of L2D series ligands with Zn(II)−Aβ₄₀ was also explored by SOFAST-HMQC at 8 °C. Uniformly-¹⁵N-labeled Aβ₄₀ was treated as described above, but diluted into buffer containing 20 mM Tris, rather than PO₄³⁻, to avoid complex formation with the Zn(II). To the solution of Aβ₄₀ was first added 1 equiv ZnCl₂; 1 equiv ligand was subsequently added to this solution. Each spectrum was obtained using 96 complex t₁ points and a 0.1 sec recycle delay.

All data were acquired on a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe. The 2D data were processed using TOPSPIN 2.1 (Bruker). Resonance assignment was performed with SPARKY 3.1134 using published assignments for Aβ₄₀ as a guide. Chemical shift perturbation (CSP) was calculated using the following equation:

\[
\Delta \delta_{NH} = \sqrt{(\Delta \delta H^2 + \left(\frac{\Delta \delta N}{5}\right)^2)}
\]

4.4.15. Saturation transfer difference (STD) NMR spectroscopy

For the STD NMR experiments, an 100 μM solution of fibrillar Aβ₄₀ was prepared.
by incubating Aβ<sub>40</sub> for 48 h at 37 °C with constant agitation in 10 mM deuterated Tris–DCl, 95% D<sub>2</sub>O at pD 7.4 (corrected for the isotope effect). The samples for STD experiments were prepared by diluting fiber to 2 µM (effective monomer concentration) into 10 mM deuterated Tris–DCl to which was added 200 µM of ligand (0.5% DMSO-<em>d</em><sub>6</sub>). STD experiments were acquired with a train of 50 dB Gaussian-shaped pulses of 0.049 sec with an interval of 0.001 sec at either -1.0 ppm (on resonance) or 40 ppm (off resonance) with a total saturation time of 2 sec on a Bruker 600 MHz NMR spectrometer. A total of 2048 scans were recorded for the STD spectrum and 1024 scans were recorded for the reference spectrum at 25 °C. An inter-scan delay of 1 sec was used for both the STD and the reference experiments.

4.4.16. Antioxidant assay

The antioxidant activity of the compounds was determined by the TEAC assay employing N2a cell line (ATCC) lysates following the previous reported procedures. The percent inhibition [% inhibition = (A<sub>0</sub> – A)/A<sub>0</sub>] was calculated using the absorbance of the samples with compound (A) compared to the absorbance of control samples lacking compound (A<sub>0</sub>) and was plotted as a function of compound concentration. The TEAC value of ligands were calculated as a ratio of the slope of the standard curve of the compound to the slope of Trolox (Sigma-Aldrich; Trolox = 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; dissolved in DMSO). Duplicate measurements were conducted in three different experiments.

4.4.17. 2-Deoxyribose assay

The ability of compounds to control free radical formation from Fenton-like chemistry by Cu(I/II) was determined using previously reported procedures.

4.4.18. Cell viability studies

The human neuroblastoma M17 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell line was maintained in media containing 50% minimum essential medium (MEM) and 50% F12 (GIBCO, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Sigma), 100 U/mL
penicillin, and 100 mg/mL streptomycin (GIBCO). The cells were grown and maintained at 37 °C in a humidified atmosphere with 5% CO₂. Cell viability upon treatment of compounds was determined using the MTT assay (Sigma). M17 cells were seeded in a 96 well plate (15,000 cells in 100 µL per well). The cells were treated with Aβ (20 µM) with or without CuCl₂ or ZnCl₂ (20 µM), followed by the addition of compound (20 µM, 1% v/v final DMSO concentration) and incubated for 24 h with the cells. After incubation, 25 µL MTT (5 mg/mL in phosphate buffered saline (PBS, pH 7.4, GIBCO) was added to each well and the plate was incubated for 4 h at 37 °C. Formazan produced by the cells was solubilized using an acidic solution of N,N-dimethylformamide (DMF, 50%, v/v aq) and sodium dodecyl sulfate (SDS, 20%, w/v) overnight at room temperature in the dark. The absorbance was measured at 600 nm using a microplate reader. Cell viability was calculated relative to cells containing an equivalent amount of DMSO.

4.5. Acknowledgements

This work was supported by the 2013 Research Fund (Project Number 1.130068.01) of Ulsan National Institute of Science and Technology (UNIST) and the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (NRF-2014R1A2A2A01004877) (to M.H.L.); the National Research Foundation of Korea (NRF) Grant funded by the Korean Government (NRF-2014S1A2A2028270) (to M.H.L. and A.R.); the University of Michigan Protein Folding Disease Initiative (to A.R., B.T.R., and M.H.L.).
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Chapter 5
Concluding remarks and perspective

Aberrant interactions between metal ions and proteins have been implicated in the pathogenesis of multiple neurodegenerative diseases.\textsuperscript{1,2} The most prevalent of these is Alzheimer’s disease (AD), which involves, among other pathological features, the abnormal aggregation of amyloid-\(\beta\) (A\(\beta\)), metal ion dyshomeostasis, and oxidative stress.\textsuperscript{1,3-6} In vitro studies have suggested the formation of complexes of metal ions and A\(\beta\) (metal–A\(\beta\)) as being central to the interrelationship of these factors.\textsuperscript{1,3-8} To understand the role of these species, multiple chemical tools have been developed that can target and mediate the interaction of metal ions with A\(\beta\) and their reactivity (i.e., aggregation, production of reactive oxygen species, and toxicity).\textsuperscript{1,9-11}

In this thesis, such small molecules were designed and selected utilizing a rational development approach. Studies are carried out to characterize the activity of these compounds and understand how they interact with and mediate the metal-free and metal-induced reactivity of A\(\beta\) \textit{in vitro} and, in the case of one stilbene derivative, L2-b, \textit{in vivo}. In Chapter 2, L2-b was demonstrated to be specific for controlling metal-induced A\(\beta\) reactivity over metal-free \textit{in vitro} and, when applied to 5XFAD AD model mice, L2-b could target and modulate metal–A\(\beta\) complexes in the brain resulting in reduced amyloid pathology and alleviation of AD-associated cognitive defects. To better understand this activity, in Chapter 3, a new series of stilbene-based compounds with minor structural variations were explored and were shown to adjust metal-free A\(\beta\) and metal–A\(\beta\) reactivity to different degrees. The in-depth investigations in Chapter 3 reveal that this was due to the molecules having different modes of action. The molecular-level comprehension of the activity of these molecules was then exploited in Chapter 4 to generate a new library of chemical tools that can mediate the metal-free and metal-induced reactivity of A\(\beta\).

The work presented in this thesis focuses on AD and metal–A\(\beta\) complexes as a
model for metal–protein interactions in neurodegenerative diseases; however, other metal–protein interactions are known both in AD and other neurodegenerative diseases that have not been well studied even in vitro.\textsuperscript{1,10,11} Metal–tau complexes in AD\textsuperscript{1,5} could be a good target as several tau imaging agents are known\textsuperscript{12,13} and could be modified using the techniques described in Chapter 1 to generate chemical tools to study these species. Furthermore, chemical tools that target metal–protein interactions in other neurodegenerative diseases could also yield valuable information on their role in the pathogenesis of these diseases. For example, the interaction between TDP-43 and metal ions in ALS, frontotemporal lobar degeneration (FTLD), and chronic traumatic encephalopathy (CTE) has only been recently suggested with only limited studies utilizing untargeted metal chelators having been carried out resulting in a great need for more targeted compounds.\textsuperscript{1,14-19}

In the future, as the relationships between metal ions and proteins in neurodegenerative diseases become more established, the need will eventually shift to requiring targeted chemical tools towards specific metal ions. Some examples of Cu(II)-specific chemical tools have been reported here and elsewhere\textsuperscript{1,20} for AD. Compounds specific for other metal ions have remained unavailable; however, the studies presented here suggest that detailed mode of action studies could result in metal ion specific probes. The application of such molecules to in vivo systems will allow for the elucidation of the role of a specific metal ion’s protein interaction.

Additionally, the development of metal ion specific metal–protein complex imaging agents based on these chemical tools, similar to the recently developed PET probe for metal–Aβ species based on L2-b,\textsuperscript{21} could lead to the monitoring of the distribution of metal–protein complexes in the brain of disease-afflicted humans and in animal models as the disease progresses. This could lead to a better understanding of the role these interactions play in neurodegenerative disease and determine the optimal intervention point for therapeutics.

Overall, our investigations have validated the hypothesis that metal–protein interactions could be involved in neurodegenerative diseases and have served as a good starting point for future development of chemical tools that can control the reactivity of aggregation-prone proteins and their metallated counterparts. The
knowledge gained from the application of such molecules could lead to a better understanding of these devastating diseases, which could result in the discovery of effective diagnostics and therapeutics.
References


I thank Dr. Alaina DeToma and Emma Garst for initial investigations of L3-b and the PAMPA-BBB studies, as well as, Jeffrey Derrick and Milim Jang for the TEAC assay in the absence and presence of cell lysates, respectively. Milim Jang also conducted the living cell toxicity experiments. Additionally, I appreciate Dr. Masha Savelieff’s instruction and guidance when obtaining the TEM images. I carried out all remaining experiments (gel/Western blots, pKₐ titrations, Kₐ determination, metal binding studies, and TEM studies).
A.1. Introduction

In order to expand the chemical library of small molecules that can interact with and modulate the aggregation, production of oxidative stress, and toxicity (reactivity) of aberrant metal–protein interactions developed in the previous chapters, 2-(4-dimethylaminophenylazo)pyridine (L3-b) was selected considering the incorporation approach as the structure integrates metal binding capabilities of 1,10-phenanthroline (phen) into a known amyloid-β (Aβ) interacting stilbene framework (Figure A.1). This framework differs from the previously reported chemical name N1,N1-dimethyl-N4-pyridin-2-ylmethylene-benzene-1,4-diamine (L1-b) and the previously discussed N1,N1-dimethyl-N4-(pyridin-2-ylmethyl)benzene-1,4-diamine (L2-b) by replacing the bridging carbon with a nitrogen to form a diazo group in order to increase solution stability (Figure A.1). L3-b and similar diazo compounds are known dye molecules and have been reported to be able to bind some metal ions. Thus based on these designed and inherent properties of this molecule, the ability of L3-b to mediate metal-free and metal-induced Aβ reactivity was assessed.

A.2. Results and Discussion

A.2.1. Blood-brain barrier permeability

In order for L3-b to be suitable for studying the role of aberrant metal–protein interactions,
interactions in neurodegenerative diseases, it should be able to cross the blood brain barrier (BBB).\textsuperscript{16} This was first evaluated using \( pK_a \) titrations as compounds that are neutral at physiological pH (7.4) can pass the BBB more easily (Figure A.2).\textsuperscript{17,18} Using UV-Vis variable-pH titrations, \textit{L3-b} was determined to be completely neutral at pH 7.4 (\( pK_{a1} = 1.4(1) \), \( pK_{a2} = 4.4(3) \); Figure A.2). Additionally, Lipinski’s rules, calculated logBB values, and the \textit{in vitro} parallel artificial membrane permeability assay adapted for the BBB (PAMPA-BBB assay) were used to analyze the ability of \textit{L3-b} to passively diffuse across the BBB.\textsuperscript{16,17,19,20} As shown in Table A.1, all methods predicted that \textit{L3-b} could readily cross the BBB, suggesting that \textit{L3-b} is a good candidate for studying the role of abnormal metal-induced protein reactivity in neurodegenerative diseases.

### A.2.2 Metal-binding properties

The ability of \textit{L3-b} to interact with metal ions was first explored by observing the optical changes in the UV-Vis spectroscopy (UV-Vis). Upon addition of CuCl\(_2\), the characteristic absorption band of \textit{L3-b} at ca. 450 nm decreased with a new band arising at ca. 590 nm in aqueous buffer (20 mM HEPES, 150 mM NaCl, 1% DMSO, pH 6.6; Figure A.3a). Additionally, since compounds similar to \textit{L3-b} have been found to bind Cu(I),\textsuperscript{12} 10 equivalents of sodium ascorbate (Asc) was added to the buffer solution to

<table>
<thead>
<tr>
<th>Calculation</th>
<th>\textit{L3-b}</th>
<th>Lipinski’s rules &amp; others</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW\textsuperscript{a}</td>
<td>226.28</td>
<td>( \leq 450 )</td>
</tr>
<tr>
<td>clogP\textsuperscript{a}</td>
<td>3.03</td>
<td>( \leq 5.0 )</td>
</tr>
<tr>
<td>HBA\textsuperscript{c}</td>
<td>4</td>
<td>( \leq 10 )</td>
</tr>
<tr>
<td>HBD\textsuperscript{d}</td>
<td>0</td>
<td>( \leq 5 )</td>
</tr>
<tr>
<td>PSA\textsuperscript{e}</td>
<td>40.9</td>
<td>( \leq 90 )</td>
</tr>
<tr>
<td>logBB\textsuperscript{f}</td>
<td>-0.014</td>
<td>(&lt; -1.0 ) (poorly distributed in the brain)</td>
</tr>
<tr>
<td>-logP\textsuperscript{g}</td>
<td>4.24 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>CNS +/− prediction</td>
<td>CNS+</td>
<td>-logP&lt;sub&gt;e&lt;/sub&gt; &lt; 5.4 (CNS+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-logP&lt;sub&gt;e&lt;/sub&gt; &gt; 5.7 (CNS-)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}MW, molecular weight; \textsuperscript{b}clogP, calculated log of water–octanol partition coefficient.; \textsuperscript{c}HBA, hydrogen bond acceptor; \textsuperscript{d}HBD, hydrogen bond donor; \textsuperscript{e}PSA, polar surface area; \textsuperscript{f}logBB = -0.0148 \times PSA + 0.152 \times clogP \times 0.130. \textsuperscript{g}Determined using the Parallel Artificial Membrane Permeability Assay adapted for BBB (PAMPA-BBB).
reduce the Cu(II) to Cu(I) (Figure A.3b). This had a bathochromic effect on the spectra resulting in the peak to shift to ca. 500 nm and not changing after 0.5 equivalents of CuCl$_2$ suggesting the formation of 1:2 M:L complexes (Figure A.3b). Binding of Cu(I) in CH$_3$CN was also explored in a nitrogen atmosphere, the presence of [Cu(CH$_3$CN)$_4$(BF$_4$)$_2$] (tetraakis(acetonitrile)copper(I) tetrafluoroborate) resulted in the decrease of the free ligand absorption band of L3-b at ca. 420 and the rise of a new band at ca. 500 nm (Figure A.3c). The ability of L3-b to interact with Zn(II) was also explored, the addition of ZnCl$_2$ to L3-b in buffer (20 mM HEPES, 150 mM NaCl, 1% DMSO, pH 7.4) resulted in a slight decrease of the band at ca. 450 nm with a corresponding small increase at ca. 590 nm (Figure A.3d). Together, these studies suggest that L3-b can bind Cu(II), Cu(I), and Zn(II).

Furthermore, the binding affinity (K$_d$) for Cu(II) was determined using UV-Vis variable-pH titrations (Figure A.4). The resulting spectra from these studies and the pK$_a$ values from the previous experiments were used to model a speciation diagram of the formation of a 1:1 complex modeled and the pCu (pCu = −log[Cu$_{unchelated}$]) and K$_d$ values were calculated determined from the stability constant (log$\beta$; Figure A.4). This apparent

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**Figure A.2.** Solution speciation studies of L3-b. Variable pH titrations of L3-b (15 µM) in solution (100 mM NaCl, 10 mM NaOH) were monitored by UV-Vis. The resulting (a) spectra were fit to obtain (c) the pK$_a$ values and plot (b) a speciation diagram. $F_L$ = Fraction of ligand with at the specified protonation state. Charges omitted for clarity.
Figure A.3. Metal binding studies of L3-b. CuCl₂ binding of L3-b in the (a) absence and (b) presence of 10 equiv of sodium ascorbate (Asc) in 20 mM HEPES, pH 6.6, 150 mM NaCl (20 µM, 1% v/v DMSO). (c) Cu(I) binding of L3-b in CH₃CN (20 µM, 1% v/v DMSO); (d) Zn(II) binding of L3-b in 20 mM HEPES, pH 7.4, 150 mM NaCl (40 µM, 1% v/v DMSO), as monitored by UV-Vis spectroscopy.

Figure A.4. Solution speciation study of the Cu(II)–L3-b complex. (a) UV–vis spectra of the Cu(II)–L3-b complex ([Cu(II)]/[L] = 1:2; [Cu(II)]total = 7.5 µM; 3 h incubation with L3-b prior to pH titration, L = L3-b; room temperature). (b) Solution speciation diagram (F₃ = fraction of free Cu and Cu complexes). (c) Stability constant (log/l) and pCu values determined in these investigations. Charges are omitted for clarity. Error in the parentheses is shown in the last digit.
Figure A.5. Effect of the L3-b on metal-free and metal-induced Aβ aggregation. (a) Scheme of inhibition experiments: freshly prepared Aβ40 or Aβ42 (25 µM) in the presence or absence of Cu(II) (blue, 25 µM) or Zn(II) (green, 25 µM) was mixed without (−) or with L3-b (+, 50 µM) and incubated at 37 °C with constant agitation for 4 h (left) or 24 h (right). Gel/Western blot analysis of the molecular weight distribution of the resulting (b,c) Aβ40 and (d,e) Aβ42 species using anti-Aβ antibody (6E10).

low micromolar (ca. $10^{-5.6}$) $K_d$ of L3-b for Cu(II) is possibly too weak to compete with Aβ for metal binding.16

A.2.3. Modulation of Aβ40 and Aβ42 aggregation in the absence and presence of metal ions
Studies toward the ability of L3-b to control metal-free and metal-induced aggregation of Aβ began by using gel electrophoresis followed by Western blotting (gel/Western blot) using the anti-Aβ antibody 6E10. Both inhibition and disaggregation experiments were carried out at 4 h and 24 h time points with the two most prevalent Aβ isoforms, Aβ40 and Aβ42, in the presence and absence of Cu(II) or Zn(II) (Figures A.5 and A.6). L3-b was found to have no noticeable effect on metal-free and Zn(II)-induced aggregation.

**Figure A.6.** Ability of L3-b to disrupt preformed metal-free and metal–Aβ aggregates. (a) Disaggregation experiment scheme: Metal-free and metal induced aggregates of Aβ were generated by incubating mixtures of freshly prepared Aβ40 or Aβ42 (25 µM) in the presence or absence of Cu(II) (blue, 25 µM) or Zn(II) (green, 25 µM) at 37 °C with agitation. After 24 h, samples were treated with L3-b (+; 50 µM) and incubated for an additional 4 h (left) or 24 h (right). Gel electrophoresis and Western blot analysis of the molecular weight distribution of the resulting (b,c) Aβ40 and (d,e) Aβ42 species using anti-Aβ antibody (6E10).
aggregation across all time points for Aβ40 and Aβ42 samples in both inhibition and disaggregation experiments (Figures A.5 and A.6). For Cu(II) containing samples, there were slight changes to the distribution of the molecular weights of the species present after 24 h treatment with L3-b in the inhibition blots for Aβ42 and in the disaggregation blots for Aβ40 and Aβ42.

In order to explore the differences in aggregation in more detail, images of the resulting aggregates from Aβ42 from the inhibition experiments (24 h incubation) were obtained using transmission electron microscopy (TEM; Figure A.7). Similar to the gel/Western blot results, no morphological changes were observed for metal-free and Zn(II) experiments with metal-free samples having clusters of short-fibrils with some longer more mature fibrils dispersed throughout the images and Zn(II) specimens having primarily amorphous morphologies. In the TEM images of aggregates formed in the presence of Cu(II), short and long fibrillar structures were observed. L3-b-treated Cu(II)–Aβ samples had similar structures were seen but tangles of thin fibers were also present, suggesting the ability of L3-b to slightly change the aggregation pathway of Cu(II)–Aβ. The limited control of metal-induced Aβ aggregation is likely due to the weak metal binding affinity of L3-b for metal ions (vide supra) preventing the interaction with metal–Aβ.
A.2.4. Mediation of oxidative stress

The ability of L3-b to mediate oxidative stress was determined using the Trolox antioxidant capacity (TEAC) assay in the absence and presence of cell lysates (Figure A.8). In both conditions, L3-b was determined to have negligible activity toward quenching organic radicals, suggesting that L3-b would be unable to control the presence reactive oxygen species (ROS) produced by metal – Aβ species.

A.2.5. Cytotoxicity of L3-b

The cytotoxicity L3-b was studied in Neuro-2a (N2a) murine neuroblastoma cells in the absence (Figure A.9a) and presence of Cu(II) and Zn(II) (Figure A.9b,c). In the metal-free and Zn(II) containing samples, L3-b was determined to be relatively nontoxic.

Figure A.8. Trolox antioxidant capacity (TEAC) of L3-b. The ability of L3-b to quench organic ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radicals was measured in ethanol (middle) and cell lysates (right) and compared to the activity of known antioxidant trolox (left).

Figure A.9. Cytotoxicity of L3-b. The toxicity of L3-b (10 µM) was assessed in the (a) absence and presence of (b) CuCl₂ (10 µM) or (c) ZnCl₂ (10 µM) was studied with N2a cells. Viability of cells (%) was calculated relative to that of cells incubated only with 1% v/v DMSO. Error bars represent the standard deviation from three independent experiments.
up to 50 μM (Figure A.9a,c). This is in contrast to studies in the presence of Cu(II), where \( \text{L3-b} \) was shown to be cytotoxic even at low concentrations (Figure A.9b). This high cytotoxicity in the presence of Cu(II) precludes the further study of \( \text{L3-b} \) in biological systems.

A.3. Conclusions

The studies presented here demonstrate \( \text{L3-b} \) has limited effect on metal-free and metal-induced \( \text{A}_\beta \) aggregation and oxidative stress possibly due to the low metal binding affinity of \( \text{L3-b} \) for Cu(II). Additionally, the high cytotoxicity of the molecule itself in the presence of Cu(II) further limits its application as a chemical tool. Overall, these studies suggest that this diazo framework is not a viable scaffold for future development of chemical tools.

A.4. Experimental section

A.4.1. Materials and methods

All reagents including \( \text{L3-b} \) were purchased from commercial suppliers and used as received unless otherwise noted. \( \text{A}_\beta_{40} \) and \( \text{A}_\beta_{42} \) (the sequence of \( \text{A}_\beta_{42} \): DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) were purchased from Anaspec Inc. (Fremont, CA, USA). Trace metals were removed from buffers and solutions used in \( \text{A}_\beta \) experiments by treating with Chelex overnight (Sigma-Aldrich, St. Louis, MO, USA). Optical spectra were recorded on an Agilent 8453 UV-visible spectrophotometer. Absorbance values for biological assays, including cell viability and antioxidant assays, were measured on a Molecular Devices SpectraMax 190 microplate reader (Sunnyvale, CA, USA).

A.4.2. Parallel artificial membrane permeability assay adapted for blood-brain barrier (PAMPA-BBB)

PAMPA-BBB experiments were carried out using the PAMPA Explorer kit (pION Inc., Billerica, MA, USA) using previously reported protocols.\(^{21,22}\) The PAMPA Explorer software v. 3.5 (pION) was used to calculate the \( -\log P_e \) values for the compounds.
CNS± designations were assigned by comparison to compounds that were identified in previous reports.\textsuperscript{6,23-25}

A.4.3. Determination of solution speciation for L3-b and the Cu(II)–L3-b complex

The pK\textsubscript{a} values for L3-b were determined by UV–Vis variable-pH titrations as previously reported.\textsuperscript{6,7,21,26-29} To establish the pK\textsubscript{a} values, a solution (100 mM NaCl, 10 mM NaOH, pH 12) of L3-b (15 \(\mu\)M) was titrated with small amounts of HCl. At least 30 spectra were recorded in the range of pH 1–10. Similarly, a solution containing CuCl\textsubscript{2} and L3-b (15 \(\mu\)M) in a metal to ligand ratio of 1:2 was titrated with small additions of HCl and at least 30 spectra were recorded over the range pH 2–7. The acidity and stability constants were calculated by using the HypSpec program (Protonic Software, UK).\textsuperscript{30} Speciation diagrams were modeled in the HySS2009 program (Protonic Software).\textsuperscript{31}

A.4.4. Metal binding studies

To study Cu(II) and Zn(II) binding, aqueous solutions of CuCl\textsubscript{2} or ZnCl\textsubscript{2} were added to 20 \(\mu\)M solutions of L3-b (20 mM HEPES, 150 mM NaCl, 1% DMSO, pH 6.6 for CuCl\textsubscript{2} or pH 7.4 for ZnCl\textsubscript{2}). The resulting UV-Vis spectra were recorded after 10 min of incubation at room temperature. Cu(I) binding was measured in two different experiments. First, Cu(I) was generated \textit{in situ} by creating 5 \(\mu\)M, 10 \(\mu\)M, and 20\(\mu\)M solutions of CuCl\textsubscript{2} and adding 200 \(\mu\)M sodium acetate to the buffer solutions (20 mM HEPES, 150 mM NaCl, 1% DMSO). L3-b was added to these solutions to generate a final concentration of ligand of 20 \(\mu\)M and the UV-Vis spectra were obtained after 10 min of incubation at room temperature. Additionally, in a nitrogen-filled glovebox solutions (CH\textsubscript{3}CN, 1% DMSO) containing 10 \(\mu\)M, 20 \(\mu\)M, and 40 \(\mu\)M [Cu(MeCN)\textsubscript{4}](BF\textsubscript{4}) \textit{(tetrakis(acetonitrile)copper(I) tetrafluoroborate)} and 20 \(\mu\)M of L3-b were prepared in cuvettes with airtight seals. After incubating for 10 min at room temperature, the cuvettes were removed from the glovebox and the UV-Vis spectra were obtained.

A.4.5. A\textsubscript{β} aggregation experiments

A\textsubscript{β} experiments were performed according to previously published
Stock peptide solutions were diluted to a final concentration of 25 µM in Chelex-treated buffered solution containing HEPES (20 µM, pH 6.6 for Cu(II) samples; pH 7.4 for metal-free and Zn(II) samples) and NaCl (150 µM). For the inhibition studies, L3-b (final concentration 50 µM, 1% v/v DMSO) was added to the sample of Aβ (25 µM) in the absence and presence of a metal chloride salt (CuCl₂ or ZnCl₂; 25 µM) followed by incubation at 37 °C with constant agitation for 4 or 24 h. For the disaggregation studies, Aβ with and without a metal chloride salt was incubated for 24 h at 37 °C with constant agitation to generate preformed Aβ aggregates. The resulting samples were then treated with L3-b (50 µM) and incubated with constant agitation for additional 4 or 24 h.

A.4.6. Gel electrophoresis and Western blotting

The samples from the inhibition and disaggregation experiments were analyzed by gel electrophoresis followed by Western blotting using an anti-Aβ antibody (6E10) using established procedures. Samples (10 µL), following separation on a 10-20% Tris-tricine gel (Invitrogen, Grand Island, NY, USA), were transferred onto nitrocellulose membranes and blocked with bovine serum albumin (BSA, 3% w/v, Sigma-Aldrich, St. Louis, MO, USA) in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature or overnight at 4 °C. The membranes were incubated with an anti-Aβ antibody (6E10, 1:2000, Covance, Princeton, NJ, USA) in a solution of 2% BSA (w/v in TBS-T) for 4 h at room temperature or overnight at 4 °C. After washing with TBS-T (3x, 10 min), a horseradish peroxidase-conjugated goat antimouse secondary antibody (1:5000 in 2% BSA w/v in TBS-T; Cayman Chemical Company, Ann Arbor, MI, USA) was added for 1 h at room temperature. The ThermoScientific SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) was used to visualize the results on film.

A.4.7. Transmission electron microscopy (TEM) images

TEM images were obtained using previously reported methods utilizing glow discharged formar/Carbon 300-mesh grids (Electron Microscopy Sciences, Grand Island, NY, USA).
Hatfield, PA, USA) on a Philips CM-100 transmission electron microscope (magnification x25,000; 80 kV).

A.4.8. Antioxidant assay

The antioxidant activity of 1-4 was determined by the Trolox equivalent antioxidant capacity (TEAC) assay in ethanol as well as N2a cell line (ATCC) lysates following published procedures.16,29,32

A.4.9. Cytotoxicity studies

Murine neuroblastoma N2a cell line was purchased from the American Type Cell Collection (ATCC, Manassas, VA, USA). The cell line was maintained in the same conditions as previous reports.4 For the MTT assay, N2a cells were seeded in a 96 well plate (15,000 cells per 100 μL). The N2a cells were incubated with a metal chloride salt (CuCl₂ or ZnCl₂; 10 μM), L3-b (10 μM), or metal–L3-b (1:1 metal:ligand ratio). After 24 h incubation, 25 μL of MTT (Sigma-Aldrich; 5 mg/mL in PBS, pH 7.4; Gibco) was added to each well, and the plate was incubated for 4 h at 37 °C. Formazan produced by the cells was solubilized by addition of an acidic solution of N,N-dimethylformamide (50% v/v) and sodium dodecyl sulfate (SDS; 20% w/v, aq) overnight at room temperature in the dark. The absorbance was measured at 600 nm by a microplate reader. Cell viability was calculated relative to that of cells containing an equivalent amount of DMSO. Error bars were calculated as standard errors from three independent experiments.
A.5. References


I thank Dr. Alaina DeToma for running the PAMPA-BBB assay for these compounds, as well as, Heidi Phillips-Hendrickson and Professor Eitan Geva for their training and assistance with the DFT calculations. I designed and synthesized the compounds, in addition to performing the gel/Western blot experiments, DFT calculations, and overseeing the metal binding experiments carried out by Jillian Charon.
B.1. Introduction

The development of new frameworks for bifunctional molecules can lead to a diverse library of chemical tools that can modulate the interaction and reactivity of metal–protein complexes involved in neurodegenerative diseases. Thus, presented in this appendix, a new class of bifunctional small molecules based on a 1,4-substituted 1,2,3-triazole framework. The 1,2,3-triazole foundation for this series was chosen for several reasons. First, in addition to being able to target Aβ aggregates, the structure of these compounds are similar to triazole derivatives of resveratrol, a naturally occurring stilbene, which has been shown to have a variety of biological properties, including the ability to modulate Aβ aggregation. Furthermore, the reactivity of a few 1,2,3-triazole bifunctional molecules with Cu(II) and Zn(II), Aβ species, and metal-Aβ species has been previously demonstrated; however, they were not based on a structure with known Aβ interaction. Additionally, 1,4-diphenyltriazoles are readily synthesized through a modular, stereospecific, and high yielding Cu(I)-catalyzed azide-alkyne cycloaddition “click” reaction. Thus, by utilizing a triazole framework, a facile method can be employed to develop novel bifunctional small molecules as chemical tools to target

![Diagram]

**Figure B.1.** Design approach of cLick2 series. Metal chelation proprieties were incorporated into a known Aβ interaction structure to generate small molecules with the potential to interact with metal–Aβ complexes. cLick2-a, 2-(4-phenyl-1H-1,2,3-triazol-1-yl)pyridine; cLick2-b, N,N-dimethyl-4-(1-(pyridin-2-yl)-1H-1,2,3-triazol-4-yl)aniline; cLick2-c, 2-(4-(3,5-dimethoxyphenyl)-1H-1,2,3-triazol-1-yl)pyridine.
metal-Aβ species, modulate the interaction between metal and Aβ, subsequently regulating their reactivity.

**B.2. Results and discussion**

**B.2.1. Design of the cLick2 series**

The cLick2 series was designed using the incorporation approach of generating molecules that can interact with metal–protein complexes install a metal binding site (N,N) into a 1,2,3-triazole-based proposed single-photon emission computed tomography (SPECT) imaging agent for Aβ aggregates (Figure B.1). In order to vary the activity of the molecules, the base framework (cLick2-a) was altered by installing either a dimethylamino moiety (cLick2-b) or a 3,5-dimethoxy moiety (cLick2-c; Figure B.1). These substitutions were studied due to their reported Aβ interaction properties.

![Figure B.2. Synthetic scheme for the cLick2 series.](image)

![Figure B.3. DFT calculations of the cLick2 series structures.](image)
other frameworks.\textsuperscript{9-11} To synthesize these compounds, a modified previously reported atypical 1,2,3-triazole “click” reaction was employed which produced cLick2-a, cLick2-b, and cLick2-c in two steps with moderate yields (28\% for cLick2-a; 17\% for cLick2-b; 42\% for cLick2-c; Figure B.2).\textsuperscript{12,13}

Since the planarity of molecules has been suggested to be important for interacting with A\textsubscript{β} fibrils,\textsuperscript{14,15} DFT calculations were carried out to predict the three dimensional (3D) structure of these molecules in water (Figure B.3). The computed dihedral angles formed by the pyridine and triazole rings as well as the triazole and phenyl rings predict that the molecules in their lowest energy state are relatively planar suggesting that it is possible for these molecules to intercalate into structured metal-free A\textsubscript{β} and metal−A\textsubscript{β} aggregates (Figure B.3d).

In addition to interacting with A\textsubscript{β} and metal−A\textsubscript{β}, these molecules should be able to penetrate the blood-brain barrier (BBB) where these aggregates are present.\textsuperscript{5} Thus, their adherence to the criteria of restricted Lipinski’s rules and calculated logBB values were confirmed and an \textit{in vitro} parallel artificial membrane permeability assay adapted

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
Calculation\textsuperscript{a} & cLick2-a & cLick2-b & cLick2-c & Lipinski’s rules & others \\
\hline
MW\textsuperscript{a} & 222 & 265 & 282 & \leq 450 \\
clogP\textsuperscript{b} & 3.00 & 3.18 & 3.01 & \leq 5.0 \\
HBA\textsuperscript{c} & 4 & 5 & 6 & \leq 10 \\
HBD\textsuperscript{d} & 0 & 0 & 0 & \leq 5 \\
PSA\textsuperscript{e} & 43.1 & 46.3 & 61.5 & \leq 90 \\
logBB\textsuperscript{f} & -0.0516 & -0.0722 & -0.323 & < -1.0 (poorly distributed in the brain) \\
-logP\textsubscript{e}\textsuperscript{g} & 4.24 ± 0.01 & 4.36 ± 0.01 & 4.16 ± 0.01 & \\
CNS +/− prediction & CNS+ & CNS+ & CNS+ & -logP\textsubscript{e} < 5.4 (CNS+) \\
& & & & -logP\textsubscript{e} > 5.7 (CNS-) \\
\hline
\end{tabular}
\caption{Calculated and measured BBB permeability parameters for the cLick2 series.}
\end{table}

\textsuperscript{a}MW, molecular weight; \textsuperscript{b}clogP, calculated log of water−octanol partition coefficient.; \textsuperscript{c}HBA, hydrogen bond acceptor; \textsuperscript{d}HBD, hydrogen bond donor; \textsuperscript{e}PSA, polar surface area; \textsuperscript{f}logBB = -0.0148 × PSA + 0.152 × clogP × 0.130. \textsuperscript{g}Determined using the parallel artificial membrane permeability assay adapted for BBB (PAMPA-BBB).
for the BBB (PAMPA-BBB) was performed (Table B.1). Both the calculated values and in vitro assay predict that the cLick2 series could be BBB permeable (Table B.1). Taken together, based on the designed, calculated, and in vitro tested properties of the cLick2 series, the compounds presented herein should be able to penetrate the BBB.

B.2.2. Interaction with metal ions

In order to study the ability of the cLick2 series to interact with metal ions, the UV-Vis spectral changes upon titration with CuCl$_2$ were monitored (Figure B.4). The addition of Cu(II) to ethanolic solutions of the cLick2 series caused an increase and blue-shift in the absorption band at ca. 290 nm. This suggests that the cLick2 series can bind metal ions.

B.2.3. Modulation of A$\beta_{40}$ and A$\beta_{42}$ aggregation in the absence and presence of metal ions

To explore the ability to redirect the metal-free and metal-induced aggregation of A$\beta_{40}$ and A$\beta_{42}$, inhibition (Figure B.5) and disaggregation (Figure B.6) experiments were then performed with A$\beta_{40}$ and A$\beta_{42}$. In all tested conditions, there was very little change in the molecular weight distribution of the A$\beta$ species detected. These results suggest that the cLick2 series has limited ability to modulate A$\beta$ aggregation.

B.3. Conclusions

The results presented herein demonstrate that while rationally designed the cLick2 series cannot modulate the aggregation of metal-free A$\beta$ and metal–A$\beta$. This is
likely due to the limited metal binding abilities of the compounds as it has been recently reported that a pyridine-N2 triazole metal binding site is significantly weaker than the pyridine-N3 triazole isomer.\textsuperscript{8,16} Overall, this appendix highlights the importance of using well-characterized metal binding moieties when developing potential chemical tools that can mediate the aggregation, oxidative stress, and toxicity caused by aberrant metal–protein interactions in neurodegenerative diseases in order to ensure sufficient metal binding capabilities.

**Figure B.5.** Effect of the cLick2 series on metal-free and metal-induced Aβ aggregation. (a) Scheme of inhibition experiments: freshly prepared Aβ\textsubscript{40} or Aβ\textsubscript{42} (25 μM) in the presence or absence of Cu(II) (blue, 25 μM) or Zn(II) (green, 25 μM) was mixed without (lane 1) or with the compounds (lane 2, cLick2-a; lane 3, cLick2-b; lane 4, cLick2-c; 50 μM) and incubated at 37 °C with constant agitation for 4 h (left) or 24 h (right). Gel/Western blot analysis of the molecular weight distribution of the resulting (b,c) Aβ\textsubscript{40} and (d,e) Aβ\textsubscript{42} species using anti-Aβ antibody (6E10).
B.4. Experimental section

B.4.1 Materials and methods

All reagents were purchased from commercial suppliers and used as received unless otherwise noted. $\text{A}_\text{40}$ and $\text{A}_\text{42}$ (the sequence of $\text{A}_\text{42}$: DAEFRHDSGYE-VHHQKLVFFAEDVGSNKGAIIGLMVGGVIA) were purchased from Anaspec Inc.
(Fremont, CA, USA). Trace metals were removed from buffers and solutions used in Aβ experiments by treating with Chelex overnight (Sigma-Aldrich, St. Louis, MO, USA). Optical spectra were recorded on an Agilent 8453 UV-visible (UV-Vis) spectrophotometer. Absorbance values for biological assays, including cell viability and antioxidant assays, were measured on a Molecular Devices SpectraMax 190 microplate reader (Sunnyvale, CA, USA).

B.4.2. General synthesis of cLick2 series

The cLick2 series was synthesized using a modified reported method. In a glovebox under nitrogen atmosphere, 1 equiv. of fused pyridine tetrazole previously prepared using techniques reported by Boyer et al., copper(I) trifluoromethanesulfonate benzene complex \([\text{CuOTf}]_2\cdot\text{C}_6\text{H}_6; 10 \text{ mol\%}\), and 1.1 equiv phenylacetylene (for cLick2-a), 4-ethynyl-N,N-dimethylaniline (for cLick2-b), or 1-ethynyl-3,5-dimethoxybenzene (for cLick2-c) were added to a 50 mL round bottom flask charged with a stirbar and 25 mL of toluene. The flask was then connected to a reflux condenser, sealed with Teflon tape, and then removed from the glovebox and immediately purged with nitrogen for 5 minutes. The solution was then heated at 100 °C. After 24 hours, the solvent was removed by rotoevaporation and the remaining solids were dissolved in DCM (30 mL), washed with water (1x, 50 mL), washed with an aqueous ammonium hydroxide/ammonium chloride solution (pH 8, 1x, 50 mL) and brine (1x, 50 mL) before being dried with magnesium sulfate. The DCM was then removed by rotory evaporation yielding the crude product.

B.4.3. Purification of cLick2-a

The previously reported cLick2-a was purified by gradient silica column chromatography (1:3 EtOAc:hexanes to 100% EtOAc) and recrystallized from DCM:hexanes (1x) and washed with hexanes (3x) yielding a white powder (28%). TLC (1:2 EtOAc:hexanes v/v) \(R_t = 0.68\). \(^1\)H NMR (400 MHz; CDCl₃): \(\delta 8.82\) (s, 1H), 8.63 (d, 1H, \(J = 4\) Hz), 8.28 (d, 1H, \(J = 8\) Hz), 7.94 (m, 3H), 7.49 (t, 2H, \(J = 8\) Hz), 7.38 (m, 2H) ppm. \(^13\)C NMR (100 MHz; CDCl₃): \(\delta 149.4, 148.7, 148.2, 139.3, 130.4, 129.1, 128.6,\)
126.1, 123.73, 116.9, 114.0 ppm. HRMS ($m/z$): [M+H]$^+$ calcd. for C$_{13}$H$_{11}$N$_4$, 223.0978; found, 223.0978.

B.4.4. Purification of cLick2-b

cLick2-b was purified by gradient silica column chromatography (1:3 EtOAc:hexanes to 100% EtOAc) and recrystallized from DCM:hexanes (1x) and washed with hexanes (3x) yielding a white powder (28%). TLC (1:2 EtOAc:hexanes v/v) $R_f$ = 0.65. $^1$H NMR [400 MHz; (CD$_3$)$_2$SO]: δ 9.12 (s, 1H), 8.63 (d, 1H, $J = 8$ Hz), 8.16-8.10 (m, 2H), 7.85 (d, 2H, $J = 12$ Hz), 7.56-7.53 (m, 1H), 6.81 (d, 2H, $J = 8$), 2.95 (s, 6H) ppm. $^{13}$C NMR [100 MHz; (CD$_3$)$_2$SO]: δ 150.5, 148.9, 148.6, 147.9, 140.0, 126.5, 124.1, 117.6, 116.1, 113.6, 112.3, 40.4 ppm. HRMS ($m/z$): [M+H]$^+$ calcd. for C$_{15}$H$_{16}$N$_5$, 266.1400; found, 266.1419.

B.4.5. Purification of cLick2-c

cLick2-c was purified by silica column chromatography (1:3 EtOAc:hexanes) and recrystallized from DCM:hexanes (1x) and washed with hexanes (3x) yielding a white powder (42%). TLC (1:2 EtOAc:hexanes v/v): $R_f$ = 0.50. $^1$H NMR [400 MHz; (CD$_3$)$_2$CO]: δ 9.14 (s, 1H), 8.61 (d, 1H, $J = 8$ Hz), 8.23 (d, 1H, $J = 8$ Hz), 8.15-8.10 (m, 1H), 7.54-7.51 (m, 1H), 7.27 (d, 2H, $J = 4$ Hz), 6.15 (t, 1H, $J = 8$ Hz), 3.87 (s, 6H) ppm. $^{13}$C NMR [100 MHz; (CD$_3$)$_2$CO]: δ 162.5, 150.2, 149.8, 148.6, 140.1, 133.4, 124.9, 118.7, 114.5, 104.5, 101.5, 55.9 ppm. HRMS ($m/z$): [M+H]$^+$ calcd. for C$_{15}$H$_{15}$N$_4$O$_2$, 283.1190; found, 283.1193.

B.4.6. DFT geometry optimizations of cLick2 series structures

DFT calculations were carried out utilizing the commonly used B3LYP$^{17}$ exchange-correlation functional and the 6-31G(d) basis set for all atoms as implemented in the Q-Chem 4.0 quantum chemistry software (Q-Chem Inc., Pleasanton, CA, USA)$^{18}$ Additionally, in order to simulate aqueous conditions, geometry optimizations were carried out using the conductor-like screening model (COSMO) polarizable continuum model (PCM)$^{19}$ with all variables set at the default values for water. All calculations were performed on the Flux high performance computer cluster serviced by Advanced
Research Computing at the University of Michigan, Ann Arbor and results were analyzed using Avogadro molecule editor and visualizer. Before input into Q-Chem, the geometry of the molecules were estimated using molecular mechanics and the MMFF94 force field in Avogadro.

B.4.7. Parallel artificial membrane permeability assay adapted for blood-brain barrier (PAMPA-BBB)

PAMPA-BBB experiments were carried out using the PAMPA Explorer kit (pION Inc., Billerica, MA, USA) using previously reported protocols. The PAMPA Explorer software v. 3.5 (pION) was used to calculate the \(-\log P_e\) values for the compounds. CNS± designations were assigned by comparison to compounds that were identified in previous reports.

B.4.8. Metal binding studies

To study Cu(II) binding, aqueous solutions of CuCl$_2$ were added to cLick2-a (20 µM), cLick2-b (40 µM), and cLick2-c (20 µM) in ethanol (1% v/v DMSO). The resulting UV-Vis spectra were recorded after 15 min of incubation at room temperature.

B.4.9. Aβ aggregation experiments

Aβ experiments were performed according to previously published methods. Stock peptide solutions were diluted to a final concentration of 25 µM in Chelex-treated buffered solution containing HEPES (20 µM, pH 6.6 for Cu(II) samples; pH 7.4 for metal-free and Zn(II) samples) and NaCl (150 µM). For the inhibition studies, cLick2-a, cLick2-b, or cLick2-c (final concentration 50 µM, 1% v/v DMSO) were added to the sample of Aβ (25 µM) in the absence and presence of a metal chloride salt (CuCl$_2$ or ZnCl$_2$; 25 µM) followed by incubation at 37 °C with constant agitation for 4 or 24 h. For the disaggregation studies, Aβ with and without a metal chloride salt was incubated for 24 h at 37 °C with constant agitation to generate preformed Aβ aggregates. The resulting samples were then treated with cLick2-a, cLick2-b, or cLick2-c (50 µM) and incubated with constant agitation for additional 4 or 24 h.
B.4.10. Gel electrophoresis and Western blotting

The samples from the inhibition and disaggregation experiments were analyzed by gel/Western blot using an anti-Aβ antibody (6E10) using established procedures.\textsuperscript{22-24,28-30} Samples (10 µL), following separation on a 10-20% Tris-tricine gel (Invitrogen, Grand Island, NY, USA), were transferred onto nitrocellulose membranes and blocked with bovine serum albumin (BSA, 3% w/v, Sigma-Aldrich, St. Louis, MO, USA) in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature or overnight at 4 °C. The membranes were incubated with an anti-Aβ antibody (6E10, 1:2000, Covance, Princeton, NJ, USA) in a solution of 2% BSA (w/v in TBS-T) for 4 h at room temperature or overnight at 4 °C. After washing with TBS-T (3x, 10 min), a horseradish peroxidase-conjugated goat antimouse secondary antibody (1:5000 in 2% BSA w/v in TBS-T; Cayman Chemical Company, Ann Arbor, MI, USA) was added for 1 h at room temperature. The ThermoScientific SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) was used to visualize the results on film.
B.5. References