Assessment of an Initial Roadmap for Protein–Protein-Small Molecule Networks in the Brain of Alzheimer's Disease

by

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To my family with the utmost gratitude for their unconditional love and support.
With all my heart, respect and love always.
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**Figure 2.4.** Interaction of flavonoids with Cu(II) in both the absence and presence of Aβ_{40}, monitored by UV–Vis. (a) The spectra of flavonoids in the absence (black) and presence (gray and blue) of Cu(II) without Aβ_{40}. (b) The spectra of the samples containing Aβ_{40}, one equiv of CuCl\(_2\), and/or selected flavonoids. A solution containing Aβ (dark gray) was treated with CuCl\(_2\) for 2 min (light gray) followed by flavonoids (blue). The spectra of
flavonoids are presented in black. Conditions: \([A\beta_{40}] = 25 \text{ M}; [CuCl_2] = 12.5-25 \text{ M}; [\text{flavonoid}] = 25 \text{ M}; 20 \text{ mM HEPES, pH 7.4, 150 mM NaCl; room temperature; 10 min.} \)

**Figure 2.5.** The interaction of flavonoids with \(^{15}\text{N}\)-labeled A\(\beta_{40}\), monitored by 2D-SOFAST-HMQC NMR. Spectra were recorded as (a) morin, (b) quercetin, (c) galangin, and (d) luteolin was titrated into a solution of \(^{15}\text{N}\)-labeled A\(\beta_{40}\) from 0 (red spectra) and 10 (blue spectra) equiv of flavonoids. The chemical shift perturbation (CSP) was calculated for each residue upon the titration of (e) morin, (f) quercetin, (g) galangin, and (h) luteolin in order to investigate their potential interaction with A\(\beta\). The average chemical shift (dashed line) plus standard deviation (dotted line) were plotted for reference. CSP values which exceed the sum of the average CSP and the standard deviation are indicative of noticeable interactions. Conditions: \([A\beta_{40}] = 80 \text{ M}; [\text{flavonoid}] = 0-800 \text{ M}; 20 \text{ mM PO}_4, \text{ pH 7.4, 50 mM NaCl; 7% D}_2\text{O (v/v); 10 °C.} \)

**Figure 2.6.** Mass spectrometric analysis of flavonoid-bound A\(\beta_{40}\) monomers in both the absence and presence of Cu(II). Mass analysis of monomeric A\(\beta_{40}\) (20 \text{ M}) independently incubated with 120 \text{ M} of either (a) morin, (b) quercetin, (c) luteolin, or (d) galangin in the (i) absence and (ii) presence of a source of Cu(II) (Copper(II) acetate). Charge states are those that best represent the dominant ligand bound species observed. Dashed lines represent the expected binding locations of the noted species based on theoretical average \(m/z\) values. (e) Ion mobility arrival times extracted from the full width half maximum (FWHM) of the observed 4\(^+\) morin- and quercetin-bound A\(\beta_{40}\) species. Calculated collision cross section values for these data are summarized in Table 2.1.

**Figure 2.7.** Mass spectrometric analysis of luteolin- and quercetin-bound A\(\beta_{40}\) monomers in both the absence and presence of Cu(II). Whilst small molecules are expected to be observed in complex with both the 3\(^+\) and 4\(^+\) peptide species, luteolin and quercetin are shown to bind to only the 3\(^+\) peptide in the absence of Cu(II). 4\(^+\) species binding is indicated in the samples containing a source of Cu(II). Such differences may be explained by poor binding levels of these small molecules to A\(\beta\) peptides in the absence of Cu(II). The dashed lines represent the expected binding location of the noted species based on theoretical average \(m/z\) values.
Figure 2.8. Mass spectrometric analysis of flavonoid-bound Aβ_{40} dimers. Mass analysis of the dimeric metal free Aβ_{40} (5+) in the presence of each the natural products support that whilst quercetin and luteolin are capable of binding the monomeric species, morin binds to the peptide via a site comprised of a surface only present in oligomeric Aβ. The dashed lines represent the expected binding location of the noted species based on theoretical average m/z values.

Figure 2.9. Viability of flavonoids with or without metal ions in both the absence and presence of Aβ. Cytotoxicity was measured by the MTT assay after 24 h incubation of SH-SY5Y cells with and without Aβ, a metal chloride salt (CuCl_2 or ZnCl_2), or selected flavonoids. The cell viability (%) was calculated compared to cells treated with equivalent amounts of DMSO only (0–1%, v/v). Lanes: (1) Aβ ± [CuCl_2 or ZnCl_2]; (2) Aβ ± [CuCl_2 or ZnCl_2] + morin; (3) Aβ ± [CuCl_2 or ZnCl_2] + quercetin; (4) Aβ ± [CuCl_2 or ZnCl_2] + galangin; (5) Aβ ± [CuCl_2 or ZnCl_2] + luteolin. Conditions: [Aβ] = 10 µM; [CuCl_2 or ZnCl_2] = 10 µM; [flavonoid] = 10 µM. Values represent the mean of four independent experiments (± standard error).

Figure 3.1. Chemical structures of the compounds employed in this study. DMPD (N^1,N^1-dimethylbenzene-1,4-diamine; orange), L2-b (N^1,N^1-dimethyl-N^4-(pyridin-2-ylmethyl)benzene-1,4-diamine), L2-b1 (N,N-dimethyl-6-((phenylamino)methyl)pyridin-3-amine), L2-b2 (N^1-((5-(dimethylamino)pyridin-2-yl)methyl)-N^4,N^4-dimethylbenzene-1,4-diamine), DPA1 (bis(pyridin-2-ylmethyl)amine), DPA2 (6-(((5-(dimethyl-amino)pyridin-2-yl)methyl)amino)methyl)-N,N-dimethylpyridin-3-amine), PMA1 (pyridin-2-ylmethan-amine), and PMA2 (6-(aminomethyl)-N,N-dimethylpyridin-3-amine). Potential donor atoms for metal binding are highlighted in blue.

Figure 3.2. Effects of DMPD, L2-b, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 on the formation of metal-free Aβ_{40} and metal–Aβ_{40} aggregates. (a) Schemes of the inhibition (I) and disaggregation (II) experiments. (b) Visualization of molecular weight (MW) distributions of the resultant Aβ_{40} species by gel electrophoresis followed by Western blotting (gel/Western blot) with an anti-Aβ antibody (6E10). Conditions: [Aβ_{40}] = 25 µM; [CuCl_2 or ZnCl_2] = 25 µM; [compound] = 50 µM; pH 6.6 (for Cu(II) experiments) or pH 7.4 (for metal-free and Zn(II) experiments); 37 °C; constant agitation. Lanes: (C) Aβ ±
[CuCl$_2$ or ZnCl$_2$]; (1) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + DMPD; (2) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + L$_2$-b$_1$; (3) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + L$_2$-b$_2$; (4) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + DPA$_1$; (5) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + DPA$_2$; (6) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + PMA$_1$; (7) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + PMA$_2$; (8) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + L$_2$-b. (c) TEM images of the samples from (b). ............................... 56

**Figure 3.3.** Influence of DMPD, L$_2$-b, L$_2$-b$_1$, L$_2$-b$_2$, DPA$_1$, DPA$_2$, PMA$_1$, and PMA$_2$ on the metal-free Aβ$_{42}$ and metal–Aβ$_{42}$ aggregation pathways. (a) Schemes of the inhibition (I) and disaggregation (II) experiments. (b) Analysis of size distributions of the resultant Aβ species by gel/Western blot with an anti-Aβ antibody (6E10). Conditions: [Aβ$_{42}$] = 25 μM; [CuCl$_2$ or ZnCl$_2$] = 25 μM; [compound] = 50 μM; pH 6.6 (for Cu(II) experiments) or pH 7.4 (for metal-free and Zn(II) experiments); 37 °C; constant agitation. Lanes: (C) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$]; (1) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + DMPD; (2) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + L$_2$-b$_1$; (3) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + L$_2$-b$_2$; (4) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + DPA$_1$; (5) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + DPA$_2$; (6) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + PMA$_1$; (7) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + PMA$_2$; (8) Aβ $\pm$ [CuCl$_2$ or ZnCl$_2$] + L$_2$-b. (c) TEM images of the samples from (b). ............................... 58

**Figure 3.4.** Solution speciation studies of (a) L$_2$-b$_1$, (b, left) DPA$_1$, (b, right) DPA$_2$, (c, left) PMA$_1$, and (c, right) PMA$_2$. Left: Variable-pH spectrophotometric titration spectra in the range of pH 2–9. Right: Solution speciation diagrams in the range of pH 2–9 ($F_L$ = fraction of species at the given protonation). Bottom: Summary of the acidity constants ($pK_a$) of L (L = L$_2$-b$_1$, DPA$_1$, DPA$_2$, PMA$_1$, and PMA$_2$). Charges are omitted for clarity. aError in the parentheses is shown in the last digit. Conditions: [compound] = 30 μM; $I$ = 0.10 M NaCl; room temperature. .................................................. 60

**Figure 3.5.** Cu(II) binding studies of L$_2$-b$_1$, L$_2$-b$_2$, DPA$_1$, DPA$_2$, PMA$_1$, and PMA$_2$. UV–Vis spectra of (a) L$_2$-b$_1$ and L$_2$-b$_2$ with CuCl$_2$ (1–5 equiv and 1–2 equiv, respectively) in CH$_3$CN; (b) DPA$_1$ and DPA$_2$ with CuCl$_2$ (1–5 equiv) in EtOH; (c) PMA$_1$ and PMA$_2$ with CuCl$_2$ (1–5 equiv) in EtOH. Conditions: [L$_2$-b$_1$, DPA$_1$, DPA$_2$, PMA$_1$, and PMA$_2$] = 50 μM or [L$_2$-b$_2$] = 25 μM; room temperature; incubation for 30 min (for L$_2$-b$_1$ and L$_2$-b$_2$) and 10 min (for DPA$_1$, DPA$_2$, PMA$_1$, and PMA$_2$). .................................................. 62

**Figure 3.6.** Zn(II) binding of L$_2$-b$_1$, L$_2$-b$_2$, DPA$_1$, DPA$_2$, PMA$_1$, and PMA$_2$. UV–Vis spectra of (a) L$_2$-b$_2$ with ZnCl$_2$ (1–5 equiv) in CH$_3$CN; (b) DPA$_1$ and (c) DPA$_2$ with ZnCl$_2$ (1–5 equiv) in EtOH. Conditions: [L$_2$-b$_2$, DPA$_1$, and DPA$_2$] = 50 μM; room temperature;
incubation for 30 min (for \textbf{L2-b2}) and 10 min (for \textbf{DPA1} and \textbf{DPA2}). $^1$H NMR spectra of (d) \textbf{L2-b1} (black), (e) \textbf{PMA1} (black), and (f) \textbf{PMA2} (black) with ZnCl$_2$ (1 equiv, red) in CD$_3$CN. Conditions: [\textbf{L2-b1}, \textbf{PMA1}, and \textbf{PMA2}] = 4 mM; room temperature; incubation for 5 min. .............................................................. 63

**Figure 3.7.** Interactions of \textbf{L2-b}, \textbf{L2-b1} and \textbf{L2-b2} with monomeric A$_{\beta40}$. (a) 2D $^1$H–$^{15}$N SOFAST-HMQC NMR spectra of monomeric A$_{\beta40}$ upon addition of compound. Spectra were recorded as \textbf{L2-b} (left) \textbf{L2-b1} (middle) or \textbf{L2-b2} (right) [0 (red spectra) and 10 (blue spectra) equiv] was titrated into a solution of $^{15}$N-labeled A$_{\beta40}$. (b) Plots of the corresponding chemical shift perturbations (CSPs) of A$_{\beta40}$ residues upon the titration with \textbf{L2-b}, \textbf{L2-b1}, and \textbf{L2-b2}. The average chemical shifts (dashed line) plus standard deviation (dotted line) were presented. Conditions: [A$_{\beta40}$] = 80 $\mu$M; [\textbf{L2-b}, \textbf{L2-b1}, or \textbf{L2-b2}] = 0-800 $\mu$M; 20 mM PO$_4$, pH 7.4, 50 mM NaCl; 7% D$_2$O (v/v); 10 °C. ....................... 64

**Figure 3.8.** Interactions of \textbf{L2-b} and \textbf{L2-b2} with monomeric Zn(II)-bound A$_{\beta40}$ and metal-free A$_{\beta40}$ or Zn(II)-associated A$_{\beta40}$ fibrils. 2D $^1$H–$^{15}$N SOFAST-HMQC NMR spectra of (a and c) monomeric $^{15}$N-labeled with and without Zn(II); (b and d) $^{15}$N-labeled A$_{\beta40}$ pretreated with Zn(II) followed by addition of \textbf{L2-b} (left) or \textbf{L2-b2} (right). (e and f) Plots of the corresponding chemical shift perturbations (CSPs) of the spectra from $^{15}$N-labeled A$_{\beta40}$ with Zn(II) (blue) and Zn(II)-treated-$^{15}$N-labeled A$_{\beta40}$ with \textbf{L2-b} or \textbf{L2-b2} (black). Conditions: [A$_{\beta40}$] = 80 $\mu$M; [ZnCl$_2$] = 80 $\mu$M; [\textbf{L2-b} and \textbf{L2-b2}] = 80-800 $\mu$M; 20 mM PO$_4$, pH 7.4, 50 mM NaCl; 7% v/v D$_2$O (g and h) Normalized STD intensities mapped to compound’s structure [(g) \textbf{L2-b} or (h) \textbf{L2-b2}] against metal-free A$\beta$ fibrils (left) or Zn(II)–A$_{\beta42}$ fibrils (right). Yellow, orange, and blue circles indicate the STD effects of >75%, 50-75%, and <50%, respectively. Gray circles indicate the absence of the STD effect. ... 65

**Figure 3.9.** Docking studies of \textbf{DMPD}, \textbf{L2-b}, \textbf{L2-b1}, \textbf{L2-b2}, \textbf{DPA1}, \textbf{DPA2}, \textbf{PMA1}, and \textbf{PMA2} with (a) A$_{\beta40}$ monomer or (b) Zn(II)-bound A$_{\beta16}$ monomer. The lowest energy conformation of \textbf{DMPD} (orange), \textbf{L2-b} (blue), \textbf{L2-b1} (purple), \textbf{L2-b2} (green), \textbf{DPA1} (light blue), \textbf{DPA2} (light purple), \textbf{PMA1} (yellow), and \textbf{PMA2} (pink) with cartoon (left) and surface (right) depictions of (a) A$_{\beta40}$ (PDB 2LFM$^{33}$) or (b) Zn(II)-bound A$_{\beta16}$ (PDB 1ZE9$^{34}$) monomer by AutoDock Vina. The helical region and metal binding sites of metal-free A$_{\beta40}$ and Zn(II)–A$_{\beta16}$ are illustrated in yellow and in light gray, respectively, in the surface.
representation. Potential hydrogen bonding is indicated with dashed lines (1.8-2.3 Å).

Bottom: Summaries of the calculated binding energies of DMPD, L2-b, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 to metal-free Aβ40 and Zn(II)-bound Aβ16.

Figure 3.10. Transfigurations of L2-b and L2-b2 in the absence and presence of metal ions and Aβ40, monitored by UV–Vis spectroscopy. Time-dependent changes in UV–Vis spectra of (a) L2-b and (b) L2-b2 were obtained. Blue, orange, red, black, and green lines correspond to the spectra recorded after incubation for 1, 10/30 min, 4, 12, and 24 h, respectively. Conditions: [Aβ40] = 25 μM; [CuCl2 or ZnCl2] = 25 μM; [L2-b and L2-b2] = 50 μM; pH 6.6 (for Cu(II) experiments) or pH 7.4 (for metal-free and Zn(II) experiments); room temperature; no agitation. Triangles, asterisks, and circles indicate optical bands for the expected transformation of the compound (oxidized L2-b, a cationic radical of DMPD, and oxidized L2-b2, respectively).

Figure 3.11. ESI-MS studies of the solutions containing L2-b or L2-b2 with and without a metal chloride salt (CuCl2 or ZnCl2, 25 μM) at various incubation times. (a) ESI-MS spectra of L2-b or L2-b2 without metal ions. (b and c) ESI-MS spectra of L2-b or L2-b2 with CuCl2 or ZnCl2. Conditions: [L2-b or L2-b2] = 50 μM; [CuCl2 or ZnCl2] = 25 μM; room temperature; incubation for 0, 4, 12, or 24 h.

Figure 3.12. Calculated ionization potentials (IP1 and IP2) of compounds. Isosurface plots of SOMOs of the compounds (blue, N; gray, C; white, H). Bottom: Summary of calculated values of IP1 and IP2 (kcal/mol). The IP1 and IP2 values of DMPD are previously reported.

Figure 3.13. Free radical scavenging capability of DMPD, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 was identified by the TEAC assay in EtOH or employing SK-N-BE(2)-M17 (M17) cell lysates (inset). The TEAC values are relative to a vitamin E analogue, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

Figure 3.14. Viability of cells treated with L2-b1, L2-b2, DPA1, DPA2, PMA1, or PMA2 in the absence and presence of Aβ40 with and without CuCl2 or ZnCl2. (a) Viability of M17 cells treated with metal-free Aβ40 and metal-treated Aβ40, followed by the addition of L2-b2. M17 cells were treated with (b) various concentrations of ligands (2.5-50 μM), CuCl2/ligands ((c) 1:1 or (d) 1:2), or ZnCl2/ligands ((e) 1:1 or (f) 1:2; 1% v/v DMSO). Cell viability (%) was determined by the MTT assay compared to cells treated with DMSO only.
Figure 4.1. Influence of HSA toward both metal-free Aβ40 and metal–Aβ40 aggregation pathways. (a) Scheme of the inhibition experiment. (b) Analysis of Aβ40 fibril formation from the samples incubated with metal-free Aβ40 (left) or Zn(II)–Aβ40 (right) in the absence and presence of HSA, monitored by the ThT assay. (c) TEM images of the 24 h incubated samples. Conditions: [Aβ40] = 20 μM; [ZnCl2 or CuCl2] = 20 μM; HSA = 20 μM; pH 6.6 (for Cu(II) experiment) or pH 7.4 (for metal-free and Zn(II) experiments); 37 °C; constant agitation.

Figure 4.2. Effect of HSA on metal-free Aβ40 and metal–Aβ40 aggregation pathways. (a) Scheme of the disaggregation experiment. (b) Analysis of the degree of metal-free Aβ40 (left) or Zn(II)–Aβ40 (right) fibrillation in the absence and presence of HSA from the disaggregation experiments, monitored by the ThT assay. (c) TEM images of the 24 h incubated samples. Conditions: [Aβ40] = 20 μM; [ZnCl2 or CuCl2] = 20 μM; HSA = 20 μM; pH 6.6 (for Cu(II) experiment) or pH 7.4 (for metal-free and Zn(II) experiments); 24 h incubation; 37 °C; constant agitation.

Figure 4.3. Gel analysis of the formation of HSA–Aβ40 complexes in both the absence and presence of metal ions. Resultant Aβ40 species after (a) 4, (b) 8, (c) 12, and (d) 24 h incubation with or without HSA were visualized by gel electrophoresis with coomassie blue staining (left) and Western blotting using an anti-Aβ antibody (6E10; right). Lanes: (1) Aβ40; (2) Aβ40 + HSA; (3) Aβ40 + ZnCl2; (4) Aβ40 + ZnCl2 + HSA; (5) Aβ40 + CuCl2; (6) Aβ40 + CuCl2 + HSA. Conditions: [Aβ40] = 20 μM; [ZnCl2 or CuCl2] = 20 μM; HSA = 20 μM; pH 6.6 (for Cu(II) experiment) or pH 7.4 (for metal-free and Zn(II) experiments); 4, 8, 12, or 24 h incubation; 37 °C; constant agitation.

Figure 4.4. Effect of HSA on toxicity induced by metal-free Aβ and metal–Aβ species in human neuroblastoma SH-SY5Y (5Y) cells. The cell viability (%) of 5Y cells incubated with (a) metal-free Aβ40 as well as (b) Zn(II)- and (c) Cu(II)-associated Aβ40 with or without HSA for 4, 8, 12, and 24 h, monitored by the MTT assay. Cell viability was calculated in comparison to that treated with water only (1%, v/v). Error bars represent the standard error from four independent experiments. Samples: (M) [ZnCl2 or CuCl2]; (1) Aβ40 ± [ZnCl2 or CuCl2].
(2) HSA ± [ZnCl₂ or CuCl₂]; (3) Aβ₄₀ ± [ZnCl₂ or CuCl₂] + HSA. Conditions: [Aβ₄₀] = 10 μM; [ZnCl₂ or CuCl₂] = 10 μM; HSA = 10 μM.

**Figure 4.5.** Cellular membrane permeability of Hylite Fluor 488 conjugated Aβ₄₀ (HF₄₈₈Aβ₄₀) by HSA with or without Zn(II). Differential interference contrast (DIC) (left), fluorescence (middle), and overlayed (right) images of 5Y cells treated with metal-free HF₄₈₈Aβ₄₀ with or without HSA after 1 or 4 h incubation. 5Y cells were cultured under (a) FBS (10%)-containing or (b) FBS-free medium. Conditions: [HF₄₈₈Aβ₄₀] = 250 nM; [ZnCl₂] = 250 nM; HSA = 250 nM. Scale bar = 20 μm.

**Figure A.1.** Effects of the proteins on both metal-free Aβ₄₀/Aβ₄₂ and Zn(II)–Aβ₄₀/Aβ₄₂ aggregation pathways. (a) Scheme of the inhibition experiment. Analysis of (b and c) Aβ₄₀ and (d and e) Aβ₄₂ fibril formation from the samples after 24 h incubation with (b and d) CP-Ser and CP-SerΔΔ; (c and e) S100B and S100BΔ, monitored by the ThT assay. Samples: (1) Aβ (2) Aβ ± protein; (3) Aβ ± 0.1 equiv of ZnCl₂; (4) Aβ ± 0.1 equiv of ZnCl₂ + protein; (5) Aβ ± 1.0 equiv of ZnCl₂; (6) Aβ ± 1.0 equiv of ZnCl₂ + protein; (7) Aβ ± 2.0 equiv of ZnCl₂; (8) Aβ ± 2.0 equiv of ZnCl₂ + protein; (9) Aβ ± 5.0 equiv of ZnCl₂; (10) Aβ ± 5.0 equiv of ZnCl₂ + protein; (11) Aβ ± 10.0 equiv of ZnCl₂; (12) Aβ ± 10.0 equiv of ZnCl₂ + protein. Conditions: [Aβ] = 20 μM; [ZnCl₂] = 2, 20, 40, 100, or 200 μM; [protein] = 20 μM; pH 7.4; 24 h incubation; 37 °C; constant agitation.

**Figure A.2.** Influence of CP-Ser and CP-SerΔΔ on Aβ₄₀/Aβ₄₂ aggregation with or without 0.1 and 1 equiv of metal ions. Fibrillization of (a) Aβ₄₀ and (b) Aβ₄₂ with or without Cu(II) upon treatment of CP-Ser and CP-SerΔΔ, monitored by the ThT assay. Samples: (1) Aβ (2) Aβ ± protein; (3) Aβ ± 0.1 equiv of CuCl₂; (4) Aβ ± 0.1 equiv of CuCl₂ + protein; (5) Aβ ± 1.0 equiv of CuCl₂; (6) Aβ ± 1.0 equiv of CuCl₂ + protein. (c and d) TEM images of 24 h incubated samples with 1 equiv of ZnCl₂ or CuCl₂. Conditions: [Aβ] = 20 μM; [ZnCl₂ or CuCl₂] = 2 or 20 μM; [CP-Ser or CP-SerΔΔ] = 20 μM; pH 7.4 (for metal-free and Zn(II) experiments) or pH 6.6 (for Cu(II) experiments); 24 h incubation; 37 °C; constant agitation. Scale bar = 500 nm.

**Figure A.3.** Gel analysis of the formation of CP-Ser/CP-SerΔΔ– and S100B/S100BΔΔ–Aβ₄₀ complexes in the absence and presence of metal ions. Resultant Aβ₄₀ species after 24 h
incubation with or without (a) CP-Ser or (b) CP-Ser, were visualized by gel/Western blot using an anti-Aβ antibody (upper row) and anti-S100A9 antibody (lower row); Aβ samples treated with or without (c) S100B or (d) S100B, were also visualized by gel/Western blot using an anti-Aβ antibody (upper row) and coomassie blue staining. Lanes: (1) Aβ (2) Aβ ± protein; (3) Aβ ± 0.1 equiv of [ZnCl₂ or CuCl₂]; (4) Aβ ± 0.1 equiv of [ZnCl₂ or CuCl₂] + protein; (5) Aβ ± 1.0 equiv of [ZnCl₂ or CuCl₂]; (6) Aβ ± 1.0 equiv of [ZnCl₂ or CuCl₂] + protein. Conditions: [Aβ₄₀] = 20 μM; [ZnCl₂ or CuCl₂] = 2 or 20 μM; [protein] = 20 μM; pH pH 6.6 (for Cu(II) experiment) or 7.4 (for metal-free and Zn(II) experiments); 24 h incubation; 37 °C; constant agitation. .......................................................... 115

**Figure A.4.** Interaction of CP-Ser with monomeric Aβ₄₀. 2D ¹H–¹⁵N SOFAST-HMQC NMR spectra of monomeric ¹⁵N-labeled Aβ₄₀ upon addition of CP-Ser. Spectra were recorded as CP-Ser was titrated into a solution of ¹⁵N-labeled Aβ₄₀, and the plot of the corresponding chemical shift perturbations (CSPs). The average chemical shifts (dashed line) plus standard deviation (dotted line) were presented. Conditions: [Aβ₄₀] = 80 μM; [CP-Ser] = 80 μM; 20 mM PO₄, pH 7.4, 50 mM NaCl; 7% D₂O (v/v); 10 °C. ................. 117

**Figure A.5.** The free radical scavenging capability of CP-Ser, CP-Ser, S100B, and S100B, determined by the TEAC assay in SK-N-BE(2)-M17 (M17) cell lysates. The TEAC values are relative to that of a vitamin E analogue, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)............................................................... 118
## List of Abbreviations

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<td>Double-distilled water</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>EGCG</td>
<td>(-)-Epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>Et$_2$O</td>
<td>Diethylether</td>
</tr>
<tr>
<td>Et$_3$N</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethylacetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>F12K</td>
<td>Ham’s F12K Kaighn’s modification media</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>F$_L$</td>
<td>Fraction of species at the given protonation</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width half maximum</td>
</tr>
<tr>
<td>GABA</td>
<td>3-aminobutyric acid</td>
</tr>
<tr>
<td>Gel/Western blot</td>
<td>Gel electrophoresis followed by Western blotting</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HBA</td>
<td>Hydrogen bond acceptor</td>
</tr>
<tr>
<td>HBD</td>
<td>Hydrogen bond donor</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrocholric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>(2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid)</td>
</tr>
<tr>
<td>$^{HF488}$A$_{40}$</td>
<td>Hylite Fluor 488 conjugated A$_{40}$</td>
</tr>
<tr>
<td>HO</td>
<td>Hemeoxigenase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>Hx</td>
<td>Hexane</td>
</tr>
<tr>
<td>IDN 5706</td>
<td>Tetrahydrohyperforin</td>
</tr>
<tr>
<td>IM-MS</td>
<td>Ion mobility-mass spectrometry</td>
</tr>
<tr>
<td>IP</td>
<td>Ionization potential</td>
</tr>
<tr>
<td>$K_a$</td>
<td>Acidity constant</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>$m/z$</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule-associated protein</td>
</tr>
<tr>
<td>M17</td>
<td>SK-N-BE(2)-M17</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential media</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Meaning</td>
</tr>
<tr>
<td>--------------</td>
<td>---------</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>Metal–Aβ</td>
<td>Metal-associated Aβ</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NaBH₄</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>nESI</td>
<td>Nanospray ESI</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
</tr>
<tr>
<td>NHE</td>
<td>Normal hydrogen electrode</td>
</tr>
<tr>
<td>NH₄OH</td>
<td>Ammonium hydroxide</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>O₂</td>
<td>Dioxygen</td>
</tr>
<tr>
<td>O₂⁻⁻</td>
<td>Superoxide radical anion</td>
</tr>
<tr>
<td>Pd/C</td>
<td>Palladium on carbon</td>
</tr>
<tr>
<td>PAMPA</td>
<td>Parallel artificial membrane permeability assay</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAS</td>
<td>Peripheral anionic site</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Pd/C</td>
<td>Palladium on carbon</td>
</tr>
<tr>
<td>PHF</td>
<td>Paired helical filament</td>
</tr>
<tr>
<td>pl</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PSA</td>
<td>Polar surface area</td>
</tr>
<tr>
<td>ptau</td>
<td>Hyperphosphorylated tau</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-exclusion chromatography</td>
</tr>
<tr>
<td>SiO₂</td>
<td>Silicon dioxide</td>
</tr>
<tr>
<td>SOD1</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SOMO</td>
<td>Singly occupied molecular orbital</td>
</tr>
<tr>
<td>STD</td>
<td>Saturation transfer difference</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline containing Tween-20</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>ThS</td>
<td>Thioflavin-S</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin-T</td>
</tr>
<tr>
<td>UV–vis</td>
<td>UV–Visible spectroscopy</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume ratio</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume ratio</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>Zinc(II) chloride</td>
</tr>
<tr>
<td>ZnT</td>
<td>Zinc transporter</td>
</tr>
</tbody>
</table>
Abstract

Alzheimer’s disease (AD) is one of the fatal and leading causes of death. Amyloid-β (Aβ) and transition metal ions have been suggested to be involved in AD pathogenesis. These two pathological factors can bind each other and may influence peptide aggregation, producing toxic oligomeric Aβ species, as well as generation of reactive oxygen species (ROS) leading to oxidative stress. In addition, there may be interactions of metal-free and -associated Aβ (metal–Aβ) with other proteins [i.e., human serum albumin (HSA), S100 proteins]. Unfortunately, a clear relationship between Aβ, metal ions, and other proteins in AD etiology has not been identified.

In order to understand the role of metal–Aβ species in AD, several chemical tools have been studied and presented their abilities to modulate metal-free Aβ and/or metal–Aβ aggregation, control metal–Aβ-mediated ROS formation, scavenge free radicals, as well as alleviate metal-free Aβ-/metal–Aβ-associated cytotoxicity. The structural moieties responsible for such reactivities, however, are not completely elucidated. Therefore, in this thesis, to gain a better understanding of structure-interaction-reactivity relationships between small molecules and metal-free Aβ or metal–Aβ, naturally occurring flavonoids (morin, quercetin, galangin, and luteolin) with structural variations (i.e., the number and position of hydroxyl functionality on a flavonoid backbone) and two series of rationally designed chemical reagents were investigated. Four flavonoids in this study could significantly modulate aggregation pathways of metal–Aβ over metal-free Aβ and mediate metal-free Aβ-/metal–Aβ-linked cytotoxicity to different extents. Our studies demonstrate that the variations of hydroxyl groups within a flavonoid backbone can modify their reactivity toward metal-free Aβ and metal–Aβ species. In addition, the motif of \( N',N'' \)-dimethylbenzene-1,4-diamine could be considered to be essential for targeting and modulating Aβ aggregation pathways and scavenging free radicals. Our findings and understandings about structure-interaction-reactivity relationships between small molecules and metal-free Aβ or metal–Aβ can help optimize activities of small molecules as chemical tools toward amyloidogenic peptides. Moreover, we have examined the interactions of Aβ with other proteins (i.e., HSA, S100 proteins) with and without metal.
ions and their influence on the peptide aggregation. HSA and S100 proteins could alter the Aβ aggregation pathways by directly binding to metal-free Aβ and metal–Aβ followed by formation of complexes which possibly are less toxic than protein-free Aβ aggregates.

Taken together, the studies described in this thesis demonstrate the pivotal functionalities of chemical tools for their reactivity toward metal-free and metal-bound Aβ, as well as present the initial information on a link of protein–protein–small molecule (metal) networks to AD etiology. Our overall results and observations will be able to provide insight into new discovery of chemical tools and therapeutics toward AD.
1.1. Alzheimer’s disease (AD)

Alzheimer’s disease (AD) is the most common neurodegenerative disease and currently afflicts more than 28.5 million people worldwide.\textsuperscript{1-5} Patients suffering from AD experience a gradual impairment of both short-term and long-term memory due to neuronal loss.\textsuperscript{2,6} Multiple features, including metal ion dyshomeostasis and disruption of membrane structures by oligomeric formation of aggregated proteins [\textit{i.e.}, amyloid-\(\beta\) (\(A\beta\)), hyperphosphorylated tau (ptau) aggregates], have been implicated in the onset and progression of AD.\textsuperscript{4,6-14} Recent researches have suggested that some of these characteristics could be intertwined with other facets (\textit{i.e.}, metal–protein interactions, protein–protein interactions, ROS) of the disease.\textsuperscript{3,4}

AD is commonly associated with shrinkage of both the neocortex and hippocampus, as well as deposits of senile plaques (mainly composed of aggregated \(A\beta\)) and neurofibrillary tangles (NFTs; containing ptau aggregates).\textsuperscript{6,15,16} Along with the protein aggregation, metal ion dyshomeostasis is observed within the AD-affected brain.\textsuperscript{2} In the samples obtained from the brains of AD patients, plaques are shown to contain highly concentrated Cu, Zn, and Fe as high as \textit{ca.} 0.4, 1.0 and 0.9 mM, respectively. In contrast, the age-matched, healthy brain tissues contain concentrations of \textit{ca.} 0.07, 0.3, and 0.3 mM for Cu, Zn and Fe, respectively.\textsuperscript{2,6} As a complicated condition in the brain, there may be interactions between pathological factors, such as metal–protein or protein–protein interactions [\textit{e.g.}, interaction between \(A\beta\) and biomolecules with or without metal
ions], could affect the pathogenesis of AD. Particularly, tau and acetylcholinesterase (AChE) have been reported to be interacting with Aβ and metal ions directly and indirectly leading to AD. Herein, we discuss the current knowledge of interrelationships among Aβ, tau, AChE, and/or metal ions toward AD etiology.

1.2. Proteins in AD pathology

1.2.1. Amyloid-β (Aβ)

Aβ is an aggregation-prone peptide and its aggregates are the major component of the senile plaques, one of the AD hallmarks, which are located in various regions of the brain. Thus, the amyloid cascade hypothesis, suggested to be linked to the onset and progression of AD which may be caused by the imbalance of generation, aggregation, and clearance of Aβ. The Aβ peptide is produced via the proteolytic cleavage reaction of the amyloid precursor protein (APP) by β- and γ-secretases producing 38-43 amino acids in length (Figure 1.1a). The cleaved and released Aβ peptides are unfolded as mostly random coils with some α-helical and β-sheet structures. Two most common isoforms of Aβ are Aβ40 and Aβ42. Aβ42 has been observed to be predominant in senile plaques as well as considered more toxic than Aβ40. Two more hydrophobic amino acids in the C-terminus region of Aβ42 may lead the peptide more aggregation-prone than Aβ40. The sequential addition of Aβ peptides to aggregate from monomers to oligomers, protofibrils, or fibrils, occurs via hydrophobic interaction and/or hydrogen bonding through both the central self-recognition region (residues from L17 to A21; Figure 1.1b) and C-terminus hydrophobic region. Moreover, Aβ has been shown to coordinate with metal ions, including Cu(I/II), Zn(II), Fe(II/III), and these metal ions can facilitate the peptide aggregation and stabilize toxic conformations of Aβ (vide infra). Upon the aggregation process (Figure 1.1c), soluble oligomeric Aβ aggregates have been observed to be toxic by disrupting the membranes (resulting in ion channels and subsequently disturbing cation and anion homeostasis) as well as binding to synaptic protein receptors with decreasing neuronal plasticity, which could lead to memory impairment and consequently neurodegeneration.
1.2.2. Tau

The other hallmark of AD is the NFT that is mainly composed of aggregates of ptau.\textsuperscript{6,15,16} It has been proposed that the toxicity and physiological changes induced by ptau can be a critical cause of neurotoxicity and subsequently lead to neurodegeneration and AD.\textsuperscript{5,6,10,12,25,30} In addition, tau and ptau also can interact with Aβ directly and
indirectly (*vide infra*).\(^{19,31-33}\)

Tau protein has 352-441 amino acid residues in length and been classified as a microtubule-associated protein (MAP) for structural integrity and stability to microtubules which help maintaining the shape of neurons for signal transduction.\(^ {6,10,12,13,25,30,34}\)

Moreover, tau has been suggested to be involved in transport nutrients, neurotransmitters and organelles.\(^ {10,12,25,30}\)

Six isoforms of tau exist and these isoforms have different numbers of microtubule-binding repeat domains (R; R1, R2, R3, and R4); three or four R domains (3R or 4R) of 31-32 amino acids in the C-terminal region; 0, 1, or 2 inserted acid repeats (N; N1 or N2) in the N-terminal region.\(^ {6,10,12,13,30,34-37}\)

Furthermore, tau is water-soluble because of its numerous charged residues and its net isoelectric point (pI) for the protein is from 6.5 to 9.5 depending on isoforms.\(^ {30,34,38}\)

The N-terminal region (pI = 3.8) is negatively charged; microtubule-binding domains in C-terminus (pI = 10.8) and proline-rich domains (pI = 11.4) in the middle of the protein are positively charged at a physiologically relevant pH (pH 7.4).\(^ {30,34,38}\)

To interact with microtubules, positively charged microtubule-binding and proline-rich domains of tau are binding to negatively charged residues of microtubules and \(\beta\)-tubulin on the inner microtubule surface, respectively.\(^ {12,34}\)

These bindings are controlled by kinases and phosphatases. Once tau is phosphorylated, it disassembles from microtubules and makes them to be depolymerized, resulting in the disruption of axonal transport of vesicles by kinesin and dendrite structures. Tau can obtain its function back upon dephosphorylated, however.\(^ {6,10,12,13,30,34,35}\)

Although normal healthy tau is less aggregation-prone,\(^ {37}\) the misregulated activity of kinases and phosphatase can lead the formation of ptau to aggregate and form paired helical filaments (PHF) and NFT.\(^ {10,12,30,34,38}\)

Moreover, oligomeric forms of ptau may bind and disrupt membranes similar to A\(\beta\) oligomers (*vide*...
1.2.3. Acetylcholinesterase (AChE)

The synaptic dysfunction of cholinergic systems has been suggested to be involved in neurodegeneration and possibly cause AD.\textsuperscript{39-41} Since the loss of cholinergic neurons, which have a function of releasing acetylcholine (ACh, a neurotransmitter released by nerve cells to send signals to other neurons) in basal forebrain is observed in the AD environment, this cholinergic hypothesis for AD pathogenesis is proposed.\textsuperscript{2,25,39-41} Misregulation between ACh release and choline uptake for recycle and synthesis of ACh has been also observed in the AD-afflicted brain.\textsuperscript{42,43} Since ACh plays important roles in neurological signaling pathways, the misregulated level of ACh is critical.\textsuperscript{44,45} To maintain the appropriate amount of AChs in nerve systems, current therapeutics have been focused on inhibiting the activity of AChE, involved in the cleavage reaction of neurotransmitters, including ACh.\textsuperscript{46-48} AChE is a serine hydrolase which hydrolyzes the neurotransmitter, ACh, to terminate the stimulation in neurosynaptic clefts (Figure 1.3).\textsuperscript{49-52} The catalytic triad S200, H440, and E327; \textit{Torpedo californica}; electric eel AChE) in the esteric site is located at the bottom of ca. 20 Å deep cavity with aromatic amino acids.\textsuperscript{50-52} Moreover, the anionic site is responsible for proper orientation of substrates toward the catalytic triad.\textsuperscript{49,53}

![Figure 1.3](image1.png)

\textbf{Figure 1.3.} A schematic description of the active site and enzymatic reaction of AChE. Aromatic amino acids in anionic sites interact with ACh via electrostatic interaction, which leads ACh to the esteric site with a proper orientation for the enzymatic reaction. AChE cleaves ACh to produce choline and acetic acid to terminate the stimulation. Choline will be reused for generating ACh.
1.3. Metal ions in AD

1.3.1. Metal ions in the brain

Metal ion [i.e., Cu(I/II), Zn(II), Fe(II/III)] dyshomeostasis and oxidative stress by misregulated, redox-active metal ions [i.e., Cu(I/II), Fe(II/III)] in the brain could be causes of the onset and progression of AD.\textsuperscript{2,3,5,6,15,54-56} To uncover the effects of metal ions on AD pathology, gaining a better understanding about the functions of metal ions in the brain is important. Metal ions serve many vital roles, including secondary messengers in cellular signaling as well as stabilizing the proteins’ structures to function properly.\textsuperscript{2,3,54}

Copper is the third most abundant transition metal in the body; this metal in the brain accounts for ca. 9% of total Cu in the body.\textsuperscript{3,54} The major oxidation states of Cu are +1 and +2. Cu(I) is typically found in the intracellular environment while Cu(II) is more common in the extracellular environment.\textsuperscript{54} Cu in the brain is categorized into two main groups, either bound to proteins, including cytochrome c oxidase (CcO) for adenosine triphosphate (ATP) production, Cu/Zn superoxide dismutase (SOD1) for ROS regulation, or unbound in labile, synaptic pools.\textsuperscript{2,3,54}

In addition, zinc is the second most abundant metal in the body; it is most concentrated in the brain.\textsuperscript{54} Like Cu, Zn exists either protein-bound or labile forms.\textsuperscript{54} The majority of Zn in biology is protein-bound, where it is regulated [i.e., metallothioneins (MTs)] and transported [i.e., Zn transporters (ZnTs)] to play a role in other biological processes or aid in the conformational stability of proteins (i.e., SOD1).\textsuperscript{2,54} Moreover, the labile Zn pool is also necessary for neurotransmission; Zn(II) is released into the synaptic cleft, along with glutamate, following the excitation of glutamatergic neurons.\textsuperscript{2,3,54} Although the role of Zn(II) in neurotransmission is not fully understood, it is suggested to be involved in the modulation of neurotransmission through interactions with other biomolecules, such as N-methyl-D-aspartate (NMDA) and \( \gamma \)-aminobutyric acid (GABA) receptors, to regulate their activity.\textsuperscript{2,3,54}

Iron is the most abundant transition metal in the brain, likely related to the high demand of dioxygen (O\_2).\textsuperscript{54} Fe is required for many biological processes, including neurotransmitter production and O\_2 transport/storage.\textsuperscript{2,54} Fe is physiologically found
mainly in two oxidation states (+2 and +3); approximately 25% of Fe in the body is bound to transferrin and ferritin in the Fe(III) state.\textsuperscript{2,54} Ferritin, a globular protein consisting of 24 subunits, binds up to 4,500 atoms of Fe in order to protect cells from Fe-mediated oxidative damage and allow for the release of Fe(III) when needed.\textsuperscript{54,57} Additionally, Fe is indispensable for critical functions in the body and a product generated upon the breakdown of heme, an Fe containing cofactor, has physiological roles as antioxidants and anti-inflammatory agents.\textsuperscript{58} Heme is degraded by hemeoxygenase (HO) enzymes.\textsuperscript{58} Among HO isoforms, HO1 is used in a defense mechanism against cellular oxidative stress in concert with SOD1.\textsuperscript{58} HO2 is implicated in the maintenance of heme homeostasis and the prevention of nitric oxide (NO)-mediated damage, such as inflammation and glutamate excitotoxicity.\textsuperscript{59,60}

1.3.2. Reactive oxygen species (ROS) induced by metal ions in AD

The AD environment (\textit{i.e.}, miscompartmentalization of metal ions, oxidative stress) may lead to the disruption of proteins' activities,\textsuperscript{2,54} which may promote the conditions observed in the diseased state.\textsuperscript{2,54,61} Dysregulated metals could cause oxidative stress, which is believed to be correlated to AD development. Labile redox-active metal ions could generate ROS via Fenton-like reactions (eqs 1 and 2).\textsuperscript{2,3,6,62}

\[
\begin{align*}
\text{Cu(II)/Fe(III)} & + \text{H}_2\text{O}_2 \rightarrow \text{Cu(I)/Fe(II)} + \text{HO}_2^- + \text{H}^+ \quad (\text{eq 1}) \\
\text{Cu(I)/Fe(II)} & + \text{H}_2\text{O}_2 \rightarrow \text{Cu(II)/Fe(III)} + \text{HO}^- + \text{OH}^- \quad (\text{eq 2})
\end{align*}
\]

Organisms have natural antioxidant defense mechanisms that regulate the amount of ROS present in the body; however, in the diseased state, the system in place for ROS elimination and production (\textit{e.g.}, SOD1) may be malfunctional, leading to ROS accumulation and oxidative stress.\textsuperscript{2,54} High levels of ROS can oxidize lipids on the membranes and proteins, rendering them unstable and/or nonfunctional which can cause neurodegeneration and subsequently AD.\textsuperscript{2}

Along with the overproduction of ROS by various metal ions, the loss of ROS detoxification machinery could overwhelm endogenous antioxidant systems.\textsuperscript{2,54} Current research has found one of the key antioxidant enzymes, SOD1, a homodimeric Cu- and
Zn-containing enzyme to be a major target of oxidation.\textsuperscript{54,63} Through a two-step redox reaction on the Cu center at the catalytic site, SOD1 converts harmful superoxide radical anion (O$_2$•$^-$) that escapes from the mitochondria into O$_2$ and hydrogen peroxide (H$_2$O$_2$).\textsuperscript{2,63} The loss of Cu results in complete inactivation of the enzyme.\textsuperscript{63} SOD1 binds Zn to stabilize the active structure, facilitating Cu-catalyzed antioxidant activities.\textsuperscript{64} The malfunction of SOD1 due to loss of Cu and Zn can be a factor for ROS accumulation, ultimately leading to oxidative stress and neurodegeneration.\textsuperscript{54,65}

1.4. Metal–protein interactions in AD

1.4.1. Interaction of metals with A\textbeta

\textit{In vitro} studies have demonstrated that Cu(I/II), Zn(II) and Fe(II/III) can coordinate with A\textbeta generating metal–A\textbeta complexes. Cu(I/II)–A\textbeta or Zn(II)–A\textbeta are shown to alter the aggregation of the peptide; Cu(I/II)–A\textbeta or Fe(II/III)–A\textbeta can overproduce ROS causing oxidative stress (eqs 3-8).\textsuperscript{2,3,6,66}

The coordination of Cu–A\textbeta is very dynamic and pH dependent with $K_d$ ranging from 10$^{-11}$ to 10$^{-7}$ M for 1:1 complexes of Cu(II)–A\textbeta and Cu(I)–A\textbeta$\_40$/Cu(I)–A\textbeta$\_42$ (10$^{-14}$ and 10$^{-7}$ M, respectively).\textsuperscript{2,3,6,67-70} In addition, Zn(II) binding of A\textbeta has $K_d$ values (ca. 10$^{-9}$–10$^{-6}$ M)\textsuperscript{2,3,6,67-69} and the $K_d$ value is shown to be ca. 10$^{-4}$ M for Fe(II) binding to A\textbeta$\_16$,\textsuperscript{71,72} which suggests weaker binding of these metal ions to A\textbeta than Cu(I/II). The predominant coordination at a physiologically relevant pH (e.g., 7.4) for Cu(II) or Cu(I) is suggested to occur \textit{via} 3N1O (three nitrogen donor atoms and one oxygen donor atom; proposed to be H6, H13/H14, N-terminal amine, and D1 carboxylate) or 2N (two nitrogen donor atoms; suggested to be H13 and H14), respectively.\textsuperscript{2,3,6,66} Furthermore, all three histidine residues and additional candidates (D1, R5, Y10, E11 or water molecules) are involved in the coordination of Zn(II)–A\textbeta.\textsuperscript{16,67-69} These binding of Cu(I/II) and Zn(II) to A\textbeta could facilitate the peptide aggregation and increase neurotoxicity.\textsuperscript{67,68} Depending on the conditions, Cu(II) could promote the formation of A\textbeta fibrillar or amorphous aggregates facilitating the rate of the nucleated aggregate formation compare to metal-free conditions.\textsuperscript{68,73} At a physiological pH, a supra-stoichiometric amount of Zn(II) could cause the generation of
amorphous Aβ aggregates while fibrils were formed under sub-stoichiometric conditions.⁷³,⁷⁴

Redox-active transition metal ions bound to Aβ, Cu(I/II)–Aβ and Fe(II/III)–Aβ species, could lead to oxidative stress and eventually the disease due to their potential involvement in ROS overproduction (Fenton-like reactions are described in eqs 3-8). Cu–Aβ species have a redox potential, 0.30 V, versus normal hydrogen electrode (NHE).²,³,⁶⁶

\[
\begin{align*}
\text{Cu(II)}-\text{Aβ} + \text{reductant} & \rightarrow \text{Cu(I)}-\text{Aβ} + \text{reductant}^+ \quad (\text{eq } 3) \\
\text{Cu(I)}-\text{Aβ} + \text{O}_2 & \rightarrow \text{Cu(II)}-\text{Aβ}-\text{O}_2^- \quad (\text{eq } 4) \\
\text{Cu(II)}-\text{Aβ}-\text{O}_2^- + e^- + \text{H}^+ & \rightarrow \text{Cu(II)}-\text{Aβ}-\text{HO}_2^- \quad (\text{eq } 5) \\
\text{Cu(II)}-\text{Aβ}-\text{HO}_2^- + \text{H}^+ & \rightarrow \text{Cu(II)}-\text{Aβ} + \text{H}_2\text{O}_2 \quad (\text{eq } 6) \\
\text{Cu(II)}-\text{Aβ} + \text{H}_2\text{O}_2 & \rightarrow \text{Cu(I)}-\text{Aβ} + \text{HO}_2^- + \text{H}^+ \quad (\text{eq } 7) \\
\text{Cu(I)}-\text{Aβ} + \text{H}_2\text{O}_2 & \rightarrow \text{Cu(II)}-\text{Aβ} + \text{HO}^- + \text{OH}^- \quad (\text{eq } 8)
\end{align*}
\]

Few structural studies have been reported for Fe coordination to Aβ due to the propensity of Fe(II) to be oxidized to Fe(III) and precipitated as Fe(OH)₃ (s) and other complexes.⁶⁶ Preliminary results proposed D1, E3, and three H₆/H₁₃/H₁₄ may be involved in Fe(II) coordination to Aβ.⁶⁶ The redox potential of Fe(II)/Fe(III) in the Fe–Aβ-nitriloacetic acid complex was determined to be 0.23 V versus NHE.⁷⁵

### 1.4.2. Relations of metal ions and tau

Unlike Aβ with metal ions, tau with metal ions has been studied relatively limited.¹⁷,¹⁸ Although the full length of tau has not been applied to metal binding studies, metal binding of smaller fragments with different R domains was investigated by biochemical and biophysical analyses.¹⁷,¹⁸,⁷⁶-⁷⁹ From the Cu(II) binding studies with R1, R2, and R3 domains, the fragment could bind more than one equiv of Cu(II) with two histidine residues and modulate the secondary structure of the protein once Cu(II) was added.⁷⁶-⁷⁸ In addition, the longer fragment which has 198 amino acids (called as K32; containing all four R domains with two flanking regions to mimic the full length tau) was applied to Cu(II) binding studies and demonstrated 1:1 binding.¹⁷ Two cysteine and two
histidine residues (C291 and C322; two of H299, H329, or H330) were suggested to be involved in Cu(II) binding.\textsuperscript{17} Zn(II) binding also affects the neurotoxicity and aggregation of tau.\textsuperscript{18,79} Tau\textsubscript{244-372} fragment has been applied for Zn(II) binding studies and a tetrahedral geometry was observed through C291, C322 and two histidine residues.\textsuperscript{79} The fibrillization of the peptide was accelerated upon addition of sub-stoichiometric amounts of Zn(II) while less fibrils were formed in the excess of Zn(II) which was similar to Zn(II)–A\textsubscript{β} aggregation.\textsuperscript{79} Additionally, Zn(II) could induce the phosphorylation of tau by activating kinases and inhibiting phosphatases,\textsuperscript{18,80,81} which may enhance the neurotoxicity.\textsuperscript{18} Trivalent cations \textit{i.e.}, Fe(III), Al(III) could induce ptau aggregation as well.\textsuperscript{6,82,83} Tau is observed to interact with the metal ions; however, it is required to perform metal binding studies with full-length tau, instead of its fragments, for a better understanding of the relationship between metal ions and tau.

1.4.3. Metal ions and AChE

The metal interactions of AChE has been rarely studied.\textsuperscript{49} When AChE was surrounded by metal ions \textit{i.e.}, Hg(II), As(III), Cu(II), Zn(II)], its activity was observed to be decreased while alkali metal ions \textit{i.e.}, Na(I), K(I)] may not affect its activity.\textsuperscript{49,84} The amino acids with aromatic substituents [W84, Y121, W279, F330, and Y334], located at the anionic site near the esteric site (Figure 1.3), may interact with Cu(II) and Al(III); however, it has not been clearly studied.\textsuperscript{51,53} Interestingly, Li(I) may have the neuroprotective activity toward A\textsubscript{β}–AChE complexes.\textsuperscript{22,23} Li(I) has been reported that it can robustly protect individual brain mitochondria loaded with Rhodamine 123 (mitochondrial potential dye) against Ca(II)-induced depolarization indicating this metal ion could prevent the cytoplasmic Ca(II) influx induced by the protein complex.\textsuperscript{22,85}

1.5. Protein–protein interactions found in AD

1.5.1. A\textsubscript{β}–tau interactions

Since either the amyloid cascade hypothesis or tau hypothesis cannot explain completely the AD etiology, there has been suggested the main proteins in these hypotheses are related to each other directly or indirectly.\textsuperscript{6,17,19,21,25,86,87} A\textsubscript{β}\textsubscript{42} and ptau
were colocalized in dystrophic neurites near Aβ plaques; monomeric and oligomeric Aβ species were also detected with hyperphosphorylated tau in the neurons from the postmortem brain sections of AD patients and the AD transgenic 3xTg mouse model. The direct interaction between Aβ and tau can modulate their actions (i.e., aggregation, toxicity, phosphorylation). The toxicity of Aβ in hippocampal neurons has also been observed to be increased with tau. Tau phosphorylation has been shown in both the hippocampal and cholinergic neurons with Aβ. Once Aβ25-35 presents excess to tau273-284, amyloid fibrils were formed, while granular aggregates were generated under tau273-284-abundant conditions to Aβ25-35. Although the binding of two proteins has been studied, it has not been clearly revealed the binding sites and modes in the full length proteins. The previous studies demonstrate that the hydrophobic region of Aβ may bind to the R2 domain of tau to form stable complexes; furthermore, experimental results with Aβ25-35 and tau273-284 also suggest their hydrophobic and hydrogen bonding interactions. Moreover, Aβ could indirectly modulate tau phosphorylation and ptau degradation by upregulating kinases and pro-inflammatory cytokines. These findings indicate that Aβ and tau are interacting each other directly and indirectly, which can damage neuronal structures and functions, resulting in facilitation of AD progression. Unfortunately, there are limited studies about the Aβ–tau interactions under metal-present conditions.

### 1.5.2. Aβ–AChE interactions

In the AD-afflicted brain, AChE has been found to colocalize with Aβ deposits in senile plaques. AChE can interact with Aβ directly via hydrophobic interaction, through its peripheral anionic binding site (PAS). The Aβ fibrillization process was facilitated by forming Aβ–AChE complexes. In addition, the aggregates of Aβ–AChE complexes may be more toxic than Aβ aggregates without AChE. Similar to Aβ-associated cytotoxicity, the disruption of intracellular Ca(II) levels by Aβ–AChE complexes could lead to the loss of mitochondrial membrane potential and malfunction, subsequently linked to neurotoxicity.
1. Usage of chemical tools to understand the relationships between Aβ, tau, and AChE with or without metal ions in AD

1.6. Aβ-related chemical reagents

In order to design small molecules to investigate and alter the interactions between Aβ and metal ions, we must consider multiple properties (i.e., Aβ interaction, metal chelation, ROS scavenging capability). Several compounds such as natural products (i.e., EGCG and myricetin) or synthetic chemicals [i.e., N1,N1-dimethyl-N4-(pyridin-2-ylmethyl)benzene-1,4-diamine (L2-b), N1,N1-dimethylbenzene-1,4-diamine (DMPD), 4-(dimethylamino)-2-(((2-(hydroxymethyl)quinolin-8-yl)amino)methyl)phenol (ML)], have been screened or designed, respectively, to present the following properties (Figure 1.4). First, to target and redirect the actions of metal-free Aβ and metal–Aβ (i.e., aggregation, ROS generation), small molecules should have Aβ interacting properties through specific structural moieties (e.g., a dimethylamino group, the DMPD motif) and metal interacting sites. For targeting metal–Aβ, the geometry and coordination number at the metal center of potential metal-ligand complexes should be considered. Since metal binding affinity and reactivity toward metal–Aβ species are related, the reactivity of small molecules can be affected by the metal binding affinity. If the Kd values for metal ions of small molecules are similar or lower compared to those of Aβ, these compounds may not be able to chelate out the metal ions from metal–Aβ complexes and could generate ternary complexes with metal–Aβ, showing modulation of metal–Aβ aggregation. Secondly, to control the generation of ROS, redox cycle of redox-active metals should be inhibited through consideration of the preferred geometry at the metal center depending on the oxidation states of metal ions. Thirdly, the compounds also need to have ability to remove the existing ROS regulating-oxidative stress. Lastly, as therapeutics, compounds should (i) not be toxic with or without metal ions and to perform the desired reactivity in vivo; (ii) pass into the blood-brain barrier (BBB) for their efficacy in the brain.
1.6.2. Tau-related chemical tools

In order to modulate the peptide aggregation pathways and alleviate the toxicity induced by tau and ptau with metal ions, microtubule stabilizers (Paclitaxel and Epothilone D) and Clioquinol (CQ; an effective chemical reagents for redirecting Aβ...
aggregation) were applied (Figure 1.4, right).\textsuperscript{18,56,110,111} Paclitaxel is a natural product extracted from Western yew and has a function as a microtubule stabilizer.\textsuperscript{110,111} Depending on its concentration, Paclitaxel shows its neuroprotective effects. The lower concentration of Paclitaxel could attenuate the toxicity, while excess of the compound causes several side effects (\textit{e.g.}, neutropenia, peripheral neuropathy).\textsuperscript{110,111} Another compound, Epothilone D, displays its BBB permeability and restores the axonal transport \textit{in vivo}.\textsuperscript{111} Furthermore, CQ was examined its ability to mediate the toxicity induced by tau and ptau proteins in fly models.\textsuperscript{18} CQ could cause conformational changes of ptau to normal tau under Zn(II)-present conditions diminishing tau-related toxicity.\textsuperscript{18} Overall, some chemical tools have been applied to study the interaction between tau and ptau with or without metal ions, modulate their aggregation pathways and reduce the associated toxicity. Unfortunately, multiple research groups have not been conducting to accumulate the knowledge of metal–tau interactions. Once sufficient information and knowledge are obtained about the structure-interaction-reactivity of multi-targeting chemical tools toward both Aβ and tau with and without metal ions, small molecules will be developed as future multi-targeting therapeutics for AD with being nontoxic and BBB permeable.

1.6.3. Current and potential AD therapeutics related to AChE

Several AD therapeutics targeting AChE (\textit{i.e.}, donepezil, rivastigmine, galantamine, tarcrine; Figure 1.4, bottom) toward inhibition of AChE’s activity are currently available; however, they are not the fundamental cure for the disease.\textsuperscript{2,23,42,51,87} These drugs are interacting with different binding sites of AChE. For example, donepezil forms a complex with AChE through PAS; rivastigmine targets the esteric site (Figure 1.3); galantamine and tarcrine bind to the anonic site (Figure 1.3).\textsuperscript{42,50}

Recently, chemical reagents which target Aβ, metals, and AChE have been screened or designed and developed.\textsuperscript{112-116} A natural product (a flavonoid), Silibinin, has been reported to inhibit the Aβ aggregation and AChE’s activity \textit{in vitro} and \textit{in vivo}, as well as improve the spatial learning ability of APP/PS1 Tg mice.\textsuperscript{113} Another small molecule, tetrahydrohyperforin (IDN 5706), has been suggested to be a next potential therapeutic for AD.\textsuperscript{23,114-116} This compound has high stability and oral bioavailability and presents less memory impairments.\textsuperscript{114,115} Furthermore, it can diminish the size of
thioflavin-S (ThS)-positive plaques which may be caused from its ability to release AChE from Aβ–AChE complexes or inhibit the interaction between Aβ and AChE in vitro and in vivo.\textsuperscript{114,116} Moreover, \textbf{IDN 5706} could improve the memory of APP/PS1 mice.\textsuperscript{115} In addition, a synthetic chemical reagent, \textbf{Hybrid 5}, which is designed to target both Aβ and AChE presented its ability to redirect the peptide aggregation pathways and inhibition of AChE 's activity.\textsuperscript{112}

\section*{1.7. Methods to study the relations among Aβ, other proteins, and/or metal ions by small molecules}

In order to understand and analyze the interaction between Aβ, other proteins [\textit{i.e.}, human serum albumin (HSA), S100 protein], and/or metal ions, chemical tools can be utilized to many biochemical and biophysical methods. The techniques in this thesis to evaluate the interaction and influence of small molecules and proteins with metal-free Aβ and metal–Aβ to modulate peptide aggregation and alleviate peptide associated cytotoxicity, as well as the free radical scavenging capability, metal binding, and BBB permeability of compounds listed with brief explanation.

\subsection*{1.7.1. The examination of the protein aggregation}

The thioflavin-T (ThT) assay has been applied to determine the progression of amyloidogenic proteins aggregation, especially the formation of fibrils (Figure 1.5).\textsuperscript{117-120} Since ThT assay may be significantly interfered if the absorbance of small molecules and their metal complexes overlap with the assay ($\lambda_{ex}$ = ca. 440 nm, $\lambda_{em}$ = ca. 490 nm for ThT), this assay can be only employed to evaluate the influence of proteins (\textit{i.e.}, HSA, S100 proteins) on Aβ aggregation.\textsuperscript{117-120} Moreover, many metal ions, including Cu ions, are known to quench the fluorescence of ThT,\textsuperscript{121} mostly employed for measuring the metal-free or Zn(II)-induced Aβ aggregations.

To determine the effect of proteins and small molecules on Aβ aggregation pathways with or without metal ions [\textit{i.e.}, Cu(II), Zn(II)] by other than ThT assay, gel electrophoresis can be applied to separate and detect the Aβ aggregates based on size, charge, and molecular weight.\textsuperscript{97-100,103,122} The protein bands can be visualized by Western
 blotting (gel/Western blotting) with proper anti-bodies or coomassie blue staining; however, it only presents the Aβ species which can enter into the gel matrix and cannot provide the morphologies of resulting Aβ aggregates (e.g., amorphous, fibrils).\textsuperscript{122,123}

Figure 1.5. ThT assay mechanism. The single bond in ThT (red arrow) can rotate freely once it is bound to non-fibrillar Aβ species. In contrast, less rotation of the bond is allowed with presenting fluorescence ($\lambda_{ex} = \text{ca.} ~ 440$ nm and $\lambda_{em} = \text{ca.} ~ 490$ nm) when ThT is bound to amyloid fibrils.

Thus, the additional method for investigating the morphologies of Aβ aggregates in resulting solution is required and transmission electron microscopy (TEM) can be used.\textsuperscript{97-100,103,124} Using this technique, structures of resulting Aβ aggregates can be classified as fibrils, protofibrils, or amorphous aggregates.\textsuperscript{125} By comparison the morphological differences from the small molecules-treated or other proteins-treated and -untreated Aβ, their influences and interactions toward Aβ aggregation pathways and interactions can be understood better.

1.7.2. Strategies for studying the interactions among Aβ, proteins, metal ions and small molecules

The interaction between the factors, Aβ, other proteins with or without metal ions and/or small molecules can be studied by mass spectrometry (MS). With nano electrospray ionization (nESI; can use small sample volumes, 1-3 $\mu$L of nM-μM of samples, is less sensitive to salts in the buffer, and ionizes the samples very softly to

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allow observe relative weak and biologically relevant non-covalent interactions\textsuperscript{126}, MS can be applied to detect and observe various A\beta species from monomeric to soluble oligomeric forms of complexes with metal ions, other proteins, and/or small molecules.\textsuperscript{100,103,126-128} Additionally, the exact species and stoichiometry of the factors in the complexes can be investigated by ion mobility-MS (IM-MS) technique.\textsuperscript{100,103,126,128} IM-MS, when used in tandem MS/MS, can measure the arrival time of each species based on its \textit{m}/\textit{z} values passing through the inert gas that depends on the 3D structural volume of each species,\textsuperscript{126,128,129} thus, the structural changes by the interaction with other bio-/small molecules can be detected as well.\textsuperscript{100,128}

NMR can also be employed to study the interactions between factors at the molecular level.\textsuperscript{103,130} 2D \textit{^1H-}\textit{^15N} band-selective optimized flip-angle short transient heteronuclear multiple quantum coherence (SOFAST-HMQC) NMR can be used to observe changes in the spectra of a \textit{^15N}-backbone-labeled A\beta upon titration of a metal ion, protein or small molecule.\textsuperscript{100,103,130} The titration induced spectral changes with specific residues indicating the binding site of the protein. Moreover, reappearance, partial reappearance, or change in signal corresponding to residues involved in metal binding can also give information on the compound’s interaction with the metal ion component of the metal–protein complex.\textsuperscript{100,131}

In addition, the interaction of small molecules with proteins in the absence and presence of metal ions can be studied by another NMR method, saturation transfer difference (STD).\textsuperscript{103,132} This technique can produce an atomic-level map of the small molecules’ atoms are in contact or interact with the protein species indicating the important moieties for the interaction by exploiting the dynamic of ligand binding.\textsuperscript{100,103,132-134} Since the non-covalent binding of a compound with a protein and metal–protein species is labile, the protein is magnetically saturated. Upon the ligand binds to protein, the saturation is transferred from the protein to the compound. If the saturated molecule disassociates before relaxing, signal is produced proportional to the previous proximity of the ligand atoms to the protein or metal–protein complex.\textsuperscript{132-134} Furthermore, unlike SOFAST-HMQC NMR, STD method can be used to study the interaction of compounds with aggregated forms (\textit{e.g.}, fibrils).\textsuperscript{103}
1.7.3. Approaches for investigating chemical and biological properties of small molecules

The characterization of each small molecule needs to be carried out, especially, metal bindings, anti-oxidant capacity, and BBB permeability of the compounds are important for our studies. The metal binding properties of small molecules can be determined by monitoring the changes in the UV–Visible (UV–Vis) spectroscopic or $^1$H NMR spectra of the compound upon the addition of metal ions.\textsuperscript{99,106,108} Additionally, p$K_a$ and p$M$ values can be obtained through observing UV–Vis spectra with pH titrations and employed special software for the calculation.\textsuperscript{99,102,135}

In addition, in order to evaluate the ability of compounds to modulate oxidative stress, their anti-oxidant property, Trolox equivalent antioxidant capacity (TEAC) assay has been performed. This assay measures the organic free radical scavenging capability of compounds by comparing that of Trolox, a water soluble vitamin E analogue.\textsuperscript{103,136,137} Through the assay, the small molecules undergo electron transfer mechanisms to scavenge free radicals and it can be monitored at a wavelength which does not have any interference with the compounds at ca. 734 nm.\textsuperscript{136,137} Moreover, this assay can be carried out in more biological relevant condition, in cell lysate.\textsuperscript{103,138}

Furthermore, the BBB permeability of each small molecule is an important feature to be considered once designing and developing them. The parallel artificial membrane permeability assay adapted for the blood-brain barrier (PAMPA-BBB; common assay for estimating the ability to passively diffuse across the BBB) can be applied to calculate logBB values and by following Lipinski’s rules, the BBB permeability of compounds can be evaluated.\textsuperscript{139-141} In this assay, the porcine brain lipid-soaked membrane is used for simulating the blood-brain barrier. The diffusion of each compound across this membrane from the donor well to acceptor well (Figure 1.6) is measured and log$P_e$ values are calculated by ‘two-way flux’ equation: $C_A(t) = M/(V_D + V_A) + (C_A(0) - M/(V_D + V_A))e^{-P_eA/(V_D + 1/V_A)} + C_A(t)$, the concentration of the compound in the acceptor well at time $t$; $M$, the total amount of the compound in the system; $V_D$, the volume of donor well; $V_A$, the volume of the acceptor well; $C_A(0)$, the concentration of the compound in the acceptor well at the beginning of the experiment; $P_e$, the effective artificial-membrane permeability; $A$, the area of the membrane.\textsuperscript{139-141} Additionally, since neutral form of molecules typically
passively diffuse across the BBB more readily, the charge of the compounds need to be estimated at a physiological relevant pH (pH 7.4),\textsuperscript{142} by obtaining pK\textsubscript{a} values of compounds (\textit{vide supra}).

![Diagram of PAMPA-BBB assay](image)

\textbf{Figure 1.6.} The PAMPA-BBB assay. Small molecules in the donor wells diffuse across the lipid membrane which is simulated as BBB into the acceptor wells. After 4 h incubation in this study, the concentration of the compounds in the both donor and acceptor wells is measured and calculated the permeability value, log$P_e$.

\textbf{1.7.4. Determination of the ability of small molecules to regulate the toxicity induced by metal–protein and protein–protein complexes in cells}

The toxicity of metal–protein or protein–protein complexes in cells and the recovery of this cytotoxicity by small molecules can be estimated by measuring cell viability. The studies in this thesis used human neuroblastoma cell lines for cell viability assays, MTT assay. This MTT assay monitors the produced purple-colored formazan from the reduction of a yellow MTT. This reduction reaction only can occur in living cells by NADPH-dependent cellular reductase.\textsuperscript{143-145} The absorbance values at ca. 600 nm are compared to control cells that were not treated by any complexes and compounds to determine the cell viability.\textsuperscript{143}

\textbf{1.8. Conclusion}

The correlations of multiple factors (\textit{i.e.}, Aβ, tau, AChE, metal ions, ROS) cause the complication of AD pathogenesis and lack of the accurate information about the etiology of the disease limiting the development of effective drugs for fundamental cure. In order to reveal the mechanisms of the onset and progression of the disease, the interactions between the factors have to be understood and studies have been focused
on interpreting the relationships between the proteins (e.g., Aβ, tau, AChE) and metal ions by employing small molecules. These investigations can be the first-stepping stone to establish protein–protein–small molecules networks in the disease pathology and discover treatments for AD, and to achieve this successfully, continuous studies for understanding the relationships between these multiple pathological factors are necessary.

![Figure 1.7](image)

**Figure 1.7.** MTT reduction reaction by NADPH-dependent cellular reductase in living cells. Once yellow colored MTT reduces by cellular reductase in living cells, purple colored MTT formazan is produced. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; MTT Formazan, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan.

1.9. **Scope of this thesis**

Either screened natural products or designed chemical tools presented their ability to target and redirect metal-free Aβ and/or metal–Aβ aggregation. Although small molecules have been examined for their reactivity (i.e., modulation of metal-free Aβ and/or metal–Aβ aggregation pathways, regulation of Aβ/metal–Aβ-associated cytotoxicity), along with antioxidant capacity and BBB permeability, the structural moieties responsible for such reactivity have not been clearly understood. In addition, the protein–protein interactions between Aβ and Aβ-interacting proteins in the absence and presence of metal ions, which may be related to AD pathology, have not been fully revealed. Therefore, the studies presented in this thesis demonstrate the critical structural motifs of small molecules for their reactivity toward metal-free Aβ and metal–Aβ aggregation, ROS levels, cytotoxicity, as well as Aβ–protein interactions. In Chapter 2, four naturally occurred flavonoids, morin, quercetin, galangin, and luteolin, which have slightly different structures from myricetin (that presents its anti-amyloidogenic and anti-oxidant properties, and mediation of cytotoxicity), have been examined for their inhibitory abilities toward
metal-free Aβ and metal–Aβ aggregation and toxicity, as well as their free radical scavenging capacity \textit{in vitro} by biochemical and biophysical methods. Chapter 3 describes the chemical library of L2-b and DMPD derivatives for identifying the structural moieties essential for regulating activities of metal-free Aβ, metal–Aβ, and free radicals. From Chapters 2 and 3, an understanding about the structure-interaction-reactivity relationship of small molecules toward metal-free Aβ and metal–Aβ could be obtained and applied this knowledge for future screening of natural products and designing synthetic chemicals. Chapter 4 and Appendix A outline the information of the interaction between Aβ and Aβ-interacting proteins (\textit{i.e.}, HSA, S100 proteins) with and without metal ions. HSA and S100 proteins could influence on AD etiology by themselves and interacting with other pathological features. Taken together, the investigations from these studies have revealed a part of relations between multiple AD pathological facets, and can be a milestone to understand the complicated AD etiology.

1.10. References

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Chapter 2: Effects of Hydroxyl Group Variations on a Flavonoid Backbone toward Modulation of Metal-free and Metal-induced Amyloid-β Aggregation

This chapter is based on work that will be submitted for publication. We thank Professor Ayyalusamy Ramamoorthy and Kyle J. Korshavn for NMR experiments; Professor Brandon T. Ruotolo and Dr. Richard A. Kerr for IM-MS analysis; Jeeyeon Lee for studies of metal binding and cell experiments with Juhye Kang. I was involved in metal binding studies, gel and TEM analyses for Aβ aggregation, cell viability measurements as well as manuscript writing.

2.1. Introduction

Alzheimer’s disease (AD) has been grown as one of the most severe incurable neurodegenerative diseases.1-8 AD patients have symptoms of memory loss and being unable to conduct daily activities and eventually, the disease can lead patients to death.2-8 This fatal disease can be characterized by shrinkage of the brain size and the presence of abnormally folded protein aggregates, such as amyloid-β (Aβ) peptides aggregates and neurofibrillary tangles composed with hyperphosphorylated tau proteins in the brain.2-10 In addition to that, it has been suggested that dyshomeostasis of metals (i.e., Cu and Zn) is linked to the onset and progression of AD pathology as well.2-14 Cu(II) and Zn(II) are observed to bind to Aβ facilitating peptide aggregation, and generating oligomeric species, suggested to be toxic; Cu(II)-bound Aβ could generate reactive oxygen species (ROS) causing oxidative stress.2-14 Although toxic Aβ conformations and oxidative stress induced by metal-associated Aβ (metal–Aβ) species have been proposed to be involved in AD pathogenesis,2-14 the interrelationship between metal ions and Aβ peptides in AD development has not been fully elucidated.

To gain a better understanding of the relationship between metal–Aβ and AD pathogenesis, chemical tools capable of interacting directly with both metal ions and Aβ species and subsequently modulating Aβ aggregation pathways have been developed
and examined their reactivity (i.e., redirection of metal–Aβ aggregation pathways, regulation of metal-free Aβ and metal–Aβ triggered cytotoxicity).\textsuperscript{15-27} Recently, these chemical tools have been found through a rational structure-based design strategy and screening of natural products, including flavonoids and curcumin.\textsuperscript{15-27}

Among natural products, flavonoids have been of interest due to their anti-oxidant and anti-inflammation properties and potential usage for cancer, cardiovascular diseases, and dementia cares.\textsuperscript{18,20,28-32} Flavonoids are polyphenolic compounds which are abundant in vegetables, fruits, or berries.\textsuperscript{18,20,28-32} Recently, myricetin (Figure 2.1) and (−)-epigallocatechin-3-gallate (EGCG) have been presented to interact with both metal ions and Aβ peptides, confirmed by biochemical and biophysical techniques, as well as have their abilities to modulate metal–Aβ aggregation in vitro and alleviate toxicity triggered by metal–Aβ in living cells.\textsuperscript{18,20} Although some flavonoids present their anti-amyloidogenic property, the structural moieties of flavonoids, responsible for such reactivity, are not identified. Multiple previous studies have probed potential metal chelation sites of numerous flavonoids and their influence on metal-free Aβ aggregation.\textsuperscript{18,20,30-40} Detailed investigations of the interaction between Aβ and flavonoids, and their effects on metal–

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{flavonoid_structures.png}
\caption{Structures of flavonoids. Myricetin: 3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-4H-chromen-4-one; morin: 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one; quercetin: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one; galangin: 3,5,7-trihydroxy-2-phenyl-4H-chromen-4-one; luteolin: 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one. Groups highlighted in blue and red indicate the structural variations from the B and C rings of myricetin. Potential donor atoms for metal interaction are in bold.}
\end{figure}
Aβ aggregation have been rarely reported, however.\textsuperscript{18,20} In particular, a structure-interaction-reactivity relationship between flavonoids and metal–Aβ has not been clearly described and presented.

Herein, we present the interaction and reactivity with metal-free Aβ and metal–Aβ of four rationally selected flavonoids (\textit{i.e.}, morin, quercetin, galangin, luteolin; Figure 2.1) along with their metal chelation property. These flavonoids contain structural variations (\textit{i.e.}, number and position of hydroxyl groups) on the structure of myricetin (Figure 2.1) which is indicated to control metal–Aβ\textsubscript{40} aggregation pathways and mitigate cytotoxicity induced by metal–Aβ.\textsuperscript{20} Morin, quercetin, and galangin (Figure 2.1) have different numbers of hydroxyl groups on the B ring while A and C rings have same structure as myricetin. Luteolin has the same catechol group on the B ring as quercetin, while it does not have a hydroxyl group on the C ring (C3 position; 3-OH). Through our studies, the selected flavonoids are observed to display the influence on both aggregation and toxicity of metal-free Aβ and metal–Aβ with different degrees. Hydroxyl groups on the B ring might have a significant effect on modulation of metal–Aβ aggregation pathways; the 3-OH group might also play a role to interact with metal ions and Aβ peptides. Moreover, our biophysical analyses employing 2D nuclear magnetic resonance (NMR) and ion mobility-mass spectrometry (IM-MS) also demonstrate interactions of our selected flavonoids, composed of different numbers and positions of hydroxyl substituents, with metal-free Aβ and/or metal-bound Aβ to distinct extents. Taken together, our studies provide insights into a structure-interaction-reactivity relationship between flavonoids and metal–Aβ (over metal-free Aβ), which could advance our knowledge on the development of chemical tools for elucidating the role of metal–Aβ in AD.

\section*{2.2. Results and discussion}

\subsection*{2.2.1. Rational selection of flavonoids toward metal-free Aβ and metal-induced Aβ aggregation}

Some naturally occurring flavonoids (\textit{i.e.}, myricetin) are shown to target metal–Aβ\textsubscript{40} species and modulate their aggregation pathways \textit{in vitro};\textsuperscript{20} however, the structural
moieties responsible for interacting with metal–Aβ40 species and affecting their aggregation are not fully identified. To obtain a better understanding of a structure-interaction-reactivity relationship between flavonoids and metal-free Aβ or metal–Aβ, four different flavonoids (morin, quercetin, galangin, and luteolin; Figure 2.1) were selected with the structural variations of hydroxyl groups on a flavonoid backbone of myricetin. Investigations of flavonoids, composed of the different number or position of hydroxyl groups on the B and C rings (Figure 2.1), toward metal-free Aβ and metal–Aβ [two major isoforms of Aβ (Aβ40 and Aβ42) found in the AD-affected brain] \(^2\), \(^6\) could be valuable to determine which structural portions are important for their influences on Aβ aggregation pathways. Thus, morin, quercetin, and galangin were chosen for our studies, which have the different number of the hydroxyl groups on the B ring while they have the same structure of the A and C rings (Figure 2.1). A hydroxyl group on the C ring at the 3C position (3-OH group) with the most acidic proton is shown to be involved in metal binding; \(^{39,41}\) hence, for our studies, we also included luteolin, which has a catechol group on the B ring as quercetin but does not contain the 3-OH moiety.

### 2.2.2. Effects of flavonoids, morin, quercetin, galangin, and luteolin on metal-free Aβ and metal-induced Aβ aggregation in vitro

To identify how the structural variations of flavonoids affect metal-free Aβ and metal–Aβ aggregation, two different experiments, inhibition and disaggregation experiments (Figures 2.2. and 2.3) were conducted. For inhibition experiments (Figures 2.2. and 2.3, left), freshly dissolved Aβ (25 μM) with or without CuCl₂ or ZnCl₂ (25 μM) was treated with flavonoids (50 μM) for 24 h. In the case of disaggregation experiments (Figures 2.2 and 2.3, right), fresh Aβ (25 μM) was dissolved and incubated for 24 h with or without CuCl₂ or ZnCl₂ (25 μM), then flavonoids (50 μM) were added to the resulting solution and incubated for additional 24 h. Size distributions and morphological changes of resulting Aβ species from both experiments were observed by gel electrophoresis followed by Western blotting (gel/Western blot) with an anti-Aβ antibody (6E10) and TEM, respectively.
From both inhibition and disaggregation experiments, flavonoids might not be able to significantly modulate metal-free Aβ40/Aβ42 aggregation (Figures 2.2 and 2.3). Relatively similar molecular weight (MW) distribution of resulting metal-free Aβ species from both inhibition and disaggregation experiments with or without flavonoids were detected by gel/Western blot (Figures 2.2 and 2.3). Myricetin is not observed to noticeably present its regulatory property toward metal-free Aβ40 aggregation, and simple variations on its structural backbone (i.e., number and position of hydroxyl groups) could not help the molecules have ability to redirect metal-free Aβ40/Aβ42 aggregation. On the other hand, in both inhibition and disaggregation experiments, various MW distributions was shown upon treatment of flavonoids to metal–Aβ with different degrees (Figures 2.2 and 2.3). From inhibition studies, influence of flavonoids on the formation of diverse MW distributed metal–Aβ40 aggregates was indicative and visualized by the gel/Western blot (Figure 2.2a). Noticeably, as depicted in Figure 2.2a, the samples containing Cu(II)–Aβ40 and flavonoids showed more various-sized peptide species, compared to Zn(II)–Aβ40 with flavonoids. In the case of Zn(II)–Aβ40, higher sized Aβ40 species (above 100 kDa MW) and smaller sized Aβ40 species (lower than 50 kDa) were detected by gel/Western blot (Figure 2.2a). Besides, upon treatment with flavonoids, smaller and more amorphous Cu(II)–Aβ40 species and less structured Zn(II)–Aβ40 species were observed by TEM, relative to flavonoid-untreated Aβ samples (Figure 2.2b).

These results are similar to experimental data of previous studies using myricetin that presents its noticeable modulation ability toward metal–Aβ40 aggregation over metal-free Aβ40 aggregation.20 Although morin and quercetin have less hydroxyl groups on the B ring than myricetin, they still exhibited their ability to redirect metal–Aβ40 aggregation to different extents. A catechol moiety on the B ring (shown in quercetin) may have other influence on modulation of metal–Aβ aggregation pathways than hydroxyl groups on the B ring located apart from each other (shown in morin). Moreover, luteolin, which lacks an additional hydroxyl group on the C ring from quercetin, may have a slight effect on metal–Aβ40 aggregation.
Figure 2.2. Influence of flavonoids on metal-free and metal-induced Aβ40 aggregation. Top: Scheme of inhibition (left) and disaggregation (right) experiments. For the inhibition experiment (a and b), Aβ40 was first treated with or without CuCl₂ or ZnCl₂ followed by addition of flavonoids. The resulting samples were incubated at 37 °C for 24 h with constant agitation. For the disaggregation experiment (c and d), Aβ40 in the absence and presence of CuCl₂ or ZnCl₂ was initially incubated for 24 h with steady agitation. Flavonoids were then added into the resulting solution which was followed by an additional incubation for 24 h at 37 °C with constant agitation. Resultant Aβ40 species were visualized by gel/Western blot with an anti-Aβ antibody (6E10) and TEM. Conditions: [Aβ40] = 25 μM; [CuCl₂ or ZnCl₂] = 25 μM; [flavonoid] = 50 μM; pH 6.6 (for Cu(II) experiments) or pH 7.4 (for metal-free and Zn(II) experiments); 37 °C; 24 h; constant agitation. (more noticeable for Cu(II)–Aβ40 than Zn(II)–Aβ40). The only structural variation between quercetin and luteolin is the presence and absence of 3-OH, respectively, and this small substituent change can cause different oxidized forms which could alter the interaction between flavonoids and metal–Aβ40, and modulation of metal–Aβ40 aggregation to different extents.42-44 Besides, as 3-OH along with 4-oxo is suggested to be involved in metal chelation by quercetin,33,34,39,45,46 the presence of 3-OH may help quercetin interact with metal–Aβ species. In addition, galangin does not have a hydroxyl group on the B ring, but it has 3-OH and 4-oxo; thus, it might maintain its reactivity toward metal–Aβ40. Overall, metal–Aβ40 aggregation could be altered by treatments of the flavonoids with different degrees when the structure variations on the backbone occur.
Furthermore, from inhibition experiments with metal–Aβ42, morin and quercetin presented greater influence on the peptide aggregation than luteolin, while galangin might not have an effect on metal–Aβ42 aggregation (Figure 2.3a). Various-sized Cu(II)–Aβ42 species treated with morin or quercetin were indicated showing the smearing throughout the lanes from gel/Western blot results (Figure 2.3a). Luteolin may redirect Cu(II)–Aβ42 aggregation slightly, while galangin may not be able to modulate Cu(II)–Aβ42 aggregation pathways. When Zn(II)–Aβ42 was treated with morin and quercetin, different MW distribution patterns of resulting Aβ42 species from the samples without flavonoids were observed (Figure 2.3a). Luteolin also displayed a very slight difference MW distribution of Zn(II)–Aβ42 over the flavonoid-untreated Zn(II)–Aβ42 sample. The morphologies of metal–Aβ42

**Figure 2.3.** Effect of flavonoids on metal-free and metal-induced Aβ42 aggregation. Top: Schemes of inhibition (left) and disaggregation (right) experiments. For the inhibition experiment (a and b), Aβ42 was first treated with CuCl2 or ZnCl2 and followed by addition of flavonoids. The resulting samples were incubated at 37 °C for 24 h with constant agitation. For the disaggregation experiment (c and d), Aβ42 in the presence of CuCl2 or ZnCl2 were initially incubated for 24 h with steady agitation. Flavonoids were then introduced into the resulting solution, followed by an additional incubation for 24 h at 37 °C with constant agitation. The resultant Aβ42 species were visualized by gel/Western blot with an anti-Aβ antibody (6E10) and TEM. Conditions: [Aβ42] = 25 μM; [CuCl2 or ZnCl2] = 25 μM; [flavonoid] = 50 μM; pH 6.6 (for Cu(II) experiments) or pH 7.4 (for metal-free and Zn(II) experiments); 37 °C; 24 h; constant agitation.
incubated with morin, quercetin, and luteolin showed smaller and more amorphous than samples without flavonoids (Figure 2.3b), observed by TEM. The results of inhibition experiments with Aβ42, flavonoids with hydroxyl groups on the B ring (morin, quercetin, and luteolin) may be important to target and modulate metal–Aβ42 aggregation pathways.

From the disaggregation experiments (Figures. 2.2c,d and 2.3c,d), morin, quercetin, galangin and luteolin could disassemble both Cu(II)– and Zn(II)–Aβ40 aggregates or alter their further aggregation to different extents. The resultant Cu(II)–Aβ40 species treated with flavonoids, except luteolin, presented various distributed MW by gel/Western blot (Figure 2.2c). Relative to other flavonoids, luteolin displayed very slight modulation of Cu(II)–Aβ40 aggregation. Upon addition of flavonoids to Zn(II)–Aβ40, higher-sized (above 100 kDa MW) and smaller sized (lower than 50 kDa MW) Aβ40 species were detected (Figure 2.2c) as similar to the results of inhibition experiments. By TEM, the morphologies of metal–Aβ40 incubated with flavonoids were observed smaller and more amorphous metal–Aβ40 than compound-free metal–Aβ40 samples (Figure 2.2d).

Moreover, Cu(II)– or Zn(II)–Aβ42 aggregates incubated with morin and quercetin displayed various distributions of MW, while luteolin- or galangin-treated samples presented slight different MW distribution and did not show difference compared to the samples without flavonoids, respectively (Figure 2.3c). Furthermore, the morphologies of resultant metal–Aβ42 aggregates incubated with flavonoids were detected as thinner fibrils or smaller and amorphous metal–Aβ42 species than metal–Aβ42 samples without compounds. As mentioned from the inhibition studies, the results from disaggregation experiments, the presence of hydroxyl groups on the B ring of flavonoids may have their influence on the interaction with metal–Aβ42 and subsequent ability to control the aggregation pathways.

Taken together, the overall gel/Western blot and TEM results, the flavonoids (morin, quercetin, galangin, and luteolin; Figure 2.1) suggest that they could alter metal-induced Aβ aggregation over metal-free Aβ aggregation. The structural difference, such
as the number and position of hydroxyl groups on the B and C rings of flavonoid backbone, could affect their ability to alter metal–Aβ aggregation pathway to different extents. Morin and quercetin containing hydroxyl groups on both B and C rings are observed to redirect metal–Aβ_{40} and Aβ_{42} aggregation pathways significantly; galangin, which does not have a hydroxyl group on the B ring, may prefer to modulate metal–Aβ_{40} aggregation over metal–Aβ_{42} aggregation; luteolin, which has a catechol moiety on the B ring and does not have 3-OH, could slightly influence on metal–Aβ_{40}/Aβ_{42} aggregation pathways, compare to morin and quercetin.

2.2.3. Cu(II) binding of morin, quercetin, galangin, and luteolin

Since the selected flavonoids have shown more noticeable ability to modulate Cu(II)–Aβ_{40} aggregation over both metal-free Aβ_{40} and Zn(II)–Aβ_{40} aggregation, Cu(II) binding of flavonoids in both the absence and presence of Aβ_{40} were investigated by UV–Visible spectroscopy (UV–Vis; Figure 2.4). As expected from the previously reported studies (O donor atoms for metal binding), optical spectra changes were observed upon the addition of CuCl_{2} into the solution containing flavonoids [20 mM HEPES, pH 7.4, 150 mM NaCl].

![Figure 2.4](image.png)

**Figure 2.4.** Interaction of flavonoids with Cu(II) in both the absence and presence of Aβ_{40}, monitored by UV–Vis. (a) The spectra of flavonoids in the absence (black) and presence (gray and blue) of Cu(II) without Aβ_{40}. (b) The spectra of the samples containing Aβ_{40}, one equiv of CuCl_{2}, and/or selected flavonoids. A solution containing Aβ (dark gray) was treated with CuCl_{2} for 2 min (light gray) followed by flavonoids (blue). The spectra of flavonoids are presented in black. Conditions: [Aβ_{40}] = 25 μM; [CuCl_{2}] = 12.5-25 μM; [flavonoid] = 25 μM; 20 mM HEPES, pH 7.4, 150 mM NaCl; room temperature; 10 min.
Variations in UV–Vis spectra, such as new optical bands and changes in absorbance intensity, were indicated. New optical bands were detected after the addition of CuCl$_2$ at ca. 325 and 405 nm (for morin), 448 nm (for quercetin), 417 nm (for galangin), and 413 nm (for luteolin) (Figure 2.4a). These spectral changes were similar to previous metal binding studies, indicating the potential involvement of hydroxyl groups on the B and C rings in Cu(II) binding.$^{33,34,39,45,46}$ Based on the previous studies, the groups of 3-OH/4-oxo, 5-OH/4-oxo, or a catechol (Figure 2.1) have been proposed as metal chelation sites.$^{39,45,46}$

In addition, Cu(II) binding of these flavonoids in the presence of Aβ$_{40}$, which could help understand their reactivity toward Cu(II)–Aβ aggregation, was studied by UV–Vis. To determine if flavonoids could interact with Cu(II) surrounded by Aβ$_{40}$, flavonoids were introduced to the solution containing Cu(II) pre-treated with Aβ$_{40}$. After morin, quercetin, galangin, and luteolin were added to Cu(II)–Aβ$_{40}$ solution [Cu(II):Aβ:flavonoids, 1:1:1], optical spectra indicative of Cu(II) binding were obtained (Figure 2.4b), which were slightly different from those of Cu(II)–ligand complexes [Cu(II):flavonoids, 1:1] without Aβ$_{40}$ in the solution (Figure 2.4a), suggesting that Cu(II) binding to these flavonoids could be interfered by Aβ peptides. Overall, from our UV–Vis experiments, morin, quercetin, galangin, and luteolin in this study can interact with Cu(II) even in the presence of Aβ$_{40}$.

### 2.2.4. Interaction of flavonoids with soluble Aβ species

The interaction of morin, quercetin, galangin, and luteolin with Aβ$_{40}$ was investigated with 2D band-Selective Optimized Flip-Angle Short Transient Heteronuclear Multiple Quantum Correlation (SOFAST-HMQC) NMR.$^{47}$ Previously, this has been applied to identify which chemical alterations to a framework can direct the binding of ligand to Aβ$_{40}$ and can elucidate the structural basis for distinct reactivity.$^{18,19,21,22,27}$ The chemical shift perturbation (CSP) induced by the addition of compound to the peptide was monitored to determine potentially preferred binding modes (Figure 2.5).

All four flavonoids caused modest (0.02-0.04 ppm) chemical shifts in different regions of the Aβ$_{40}$ sequence. Among them, morin induced the most chemical shifts of amino acid residues in Aβ and may primarily target the self-recognition site (residues from L17 to A21)$^{5,8,14}$ and C-terminus hydrophobic region (Figure 2.5). Quercetin relatively
noticeably interacts with F20, in the central hydrophobic region of the peptide,\textsuperscript{5-8,14} and V12 and Q15 which are forming a groove between $\alpha$-helix and N-terminus tail (Figure 2.5f). Galangin may be able to interact with V18 and F20 from the self-recognition region\textsuperscript{5-8,14} and V12 (Figure 2.5g). Luteolin, which lacks 3-OH, preferentially perturbs E11 and L17 above all other residues (Figure 2.5h). Furthermore, morin, quercetin, and galangin which have 3-OH could cause higher CSP on F20 than other A$\beta$\textsubscript{40} amino acid residues, the presence of 3-OH may lead the interaction of the flavonoid framework with F20 in the self-recognition region of the peptide. Although these three flavonoids could target F20, they have interacted with different regions of A$\beta$\textsubscript{40} peptides; thus, the position of the hydroxyl groups on the B ring also may have an effect on the interaction between A$\beta$\textsubscript{40} and flavonoids. This suggests that the flavonoids showing small different substitution patterns around the B ring interact with soluble A$\beta$\textsubscript{40} in a slight different manner. Taken together, the variations of hydroxyl groups on both B and C rings could alter the interaction of flavonoids toward A$\beta$\textsubscript{40} peptides.

**Figure 2.5.** The interaction of flavonoids with $^{15}$N-labeled A$\beta$\textsubscript{40}, monitored by 2D-SOFAST-HMQC NMR. Spectra were recorded as (a) morin, (b) quercetin, (c) galangin, and (d) luteolin was titrated into a solution of $^{15}$N-labeled A$\beta$\textsubscript{40} from 0 (red spectra) and 10 (blue spectra) equiv of flavonoids. The chemical shift perturbation (CSP) was calculated for each residue upon the titration of (e) morin, (f) quercetin, (g) galangin, and (h) luteolin in order to investigate their potential interaction with A$\beta$. The average chemical shift (dashed line) plus standard deviation (dotted line) were plotted for reference. CSP values which exceed the sum of...
the average CSP and the standard deviation are indicative of noticeable interactions. Conditions: [Aβ40] = 80 μM; [flavonoid] = 0-800 μM; 20 mM PO₄, pH 7.4, 50 mM NaCl; 7% D₂O (v/v); 10 °C.

2.2.5. Direct binding properties of flavonoids to Aβ species and conformational changes

The interactions between monomeric and dimeric Aβ40 species and the flavonoids studied herein were further monitored in the absence and presence of Cu(II) by nano-electrospray ionization MS (nESI-MS) combined with ion mobility-mass spectrometry (IM-MS), optimized for the detection of non-covalent protein complexes. Data presented in Figures 2.6 and 2.7 supports that both quercetin and luteolin are capable of binding Aβ40 in the absence of any metal ions. Contrary to expectations, monomeric Aβ40 binding to quercetin and luteolin was only observed for the 3⁺ charge state, highlighting the weak binding of these molecules to the peptide. Expanding our analyses to incorporate Aβ40 dimers (Figure 2.8), the results reveal that morin is shown to interact with metal-free Aβ40, pointing to a likely binding site comprised of a surface only presented in oligomeric Aβ. The analyses of dissociation constants (Kₐ; Table 2.1) on all metal-free data sets indicate that this oligomeric binding surface results in the complex generation of Aβ with morin, quercetin, and luteolin; however, their binding with metal-free Aβ monomer and dimer is relatively weak (Kₐ ≥ 400 μM). The absence of any observable Aβ–galangin complexes in our data suggests no noticeable and possible interactions of this flavonoid with metal-free Aβ or larger and higher-order oligomers, too transient for IM-MS detection.

In order to investigate the redirecting activity of these small molecules toward metal-induced Aβ aggregation in more detail, we performed MS experiments on Aβ complexes in samples incubated in the presence of Cu(II) (Figure 2.6). Interestingly, our data supports that each sequential copy of morin bound to Aβ40 requires an stoichiometric equivalents of Cu(II). These observations, when compared to our metal-free analyses, highlight the metal dependence of Aβ–morin binding. Quercetin, in contrast, is not shown to have such stoichiometric dependencies. Consistent with other data presented here, our results support the greater ability of morin and quercetin to target metal-bound Aβ40 than luteolin and galangin. Note that, while the analysis of flavonoid
binding to Cu(II)-bound Aβ40 dimers was attempted, data proved to be inconclusive due to poor signal to noise levels associated with increased metal adduct formation and propensity for Aβ aggregation.

To gain further insight into the mode of action at molecular level for the flavonoids studied herein, in the context of Aβ40, we applied IM-MS in order to capture the size distributions of the metal–Aβ–flavonoid complexes discussed above. Data for all observed 4+ ligated Aβ40 monomer species are presented in Figure 2.6, with collision cross section data (Table 2.2). Our overall IM-MS results indicate, in all instances, that
Figure 2.7. Mass spectrometric analysis of luteolin- and quercetin-bound Aβ₄₀ monomers in both the absence and presence of Cu(II). Whilst small molecules are expected to be observed in complex with both the 3⁺ and 4⁺ peptide species,¹⁷,¹⁸ luteolin and quercetin are shown to bind to only the 3⁺ peptide in the absence of Cu(II).¹⁷,¹⁸ 4⁺ species binding is indicated in the samples containing a source of Cu(II). Such differences may be explained by poor binding levels of these small molecules to Aβ peptides in the absence of Cu(II). The dashed lines represent the expected binding location of the noted species based on theoretical average m/z values.

Flavonoid binding leads to the formation of conformationally distinct species compared to the metal-free/-bound states. As such, these results are consistent with other IM-MS results for small molecules, previously reported to alter Aβ aggregation pathways.¹⁷,¹⁸
Table 2.1. $K_d$ analysis of flavonoid-bound Aβ40 in the absence of Cu(II).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dissociation Constant ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Aβ₄₀][Luteolin]</td>
<td>9128.58 (+/- 6526.54)</td>
</tr>
<tr>
<td>[Aβ₄₀]₂[Luteolin]</td>
<td>429.99 (+/- 127.48)</td>
</tr>
<tr>
<td>[Aβ₄₀][Morin]</td>
<td>360.17 (+/- 146.57)</td>
</tr>
<tr>
<td>[Aβ₄₀][Quercetin]</td>
<td>8755.75 (+/- 1368.39)</td>
</tr>
<tr>
<td>[Aβ₄₀]₂[Quercetin]</td>
<td>558.97 (+/- 298.35)</td>
</tr>
<tr>
<td>[Aβ₄₀][Galangin]</td>
<td>385.71 (+/- 195.13)</td>
</tr>
</tbody>
</table>

Values were calculated using previously published methods. Conditions: [Aβ] = 20 μM; [Cu(II)] = 20 μM; [compound] = 120 μM. Errors shown represent single standard deviations.

Figure 2.8. Mass spectrometric analysis of flavonoid-bound Aβ₄₀ dimers. Mass analysis of the dimeric metal free Aβ₄₀ (5') in the presence of each the natural products support that whilst quercetin and luteolin are capable of binding the monomeric species, morin binds to the peptide via a site comprised of a surface only present in oligomeric Aβ. The dashed lines represent the expected binding location of the noted species based on theoretical average m/z values.
Table 2.2. Collision cross section data of all extracted ion mobility arrival times for the observed 4+ monomer species.

<table>
<thead>
<tr>
<th>Conformational Species (Å²)</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Aβ&lt;sub&gt;40&lt;/sub&gt;]</td>
<td>667.33 +/- 29.05</td>
<td>726.42* +/- 29.05</td>
<td>-</td>
</tr>
<tr>
<td>[Aβ&lt;sub&gt;40&lt;/sub&gt;][Cu]</td>
<td>669.80* +/- 27.44</td>
<td>735.95 +/- 27.59</td>
<td>801.50 +/- 31.17</td>
</tr>
<tr>
<td>[Aβ&lt;sub&gt;40&lt;/sub&gt;][Morin][Cu]</td>
<td>692.08* +/- 30.12</td>
<td>751.54 +/- 26.67</td>
<td>-</td>
</tr>
<tr>
<td>[Aβ&lt;sub&gt;40&lt;/sub&gt;][Morin][Cu]&lt;sub&gt;2&lt;/sub&gt;</td>
<td>693.51* +/- 27.15</td>
<td>770.23 +/- 28.03</td>
<td>811.85 +/- 30.47</td>
</tr>
<tr>
<td>[Aβ&lt;sub&gt;40&lt;/sub&gt;][Quercetin][Cu]</td>
<td>675.98* +/- 31.07</td>
<td>768.68 +/- 29.96</td>
<td>812.63 +/- 27.66</td>
</tr>
<tr>
<td>[Aβ&lt;sub&gt;40&lt;/sub&gt;][Quercetin][Cu]&lt;sub&gt;2&lt;/sub&gt;</td>
<td>681.41* +/- 32.82</td>
<td>745.36 +/- 29.33</td>
<td>779.71 +/- 34.79</td>
</tr>
</tbody>
</table>

Collision Cross Section values calculated for 4+ morin- and quercetin-bound Aβ<sub>40</sub> species arrival times extracted from the full width half maximum (FWHM). Errors represent least square analysis encapsulating inherent calibrant error from drift tube measurements (3%), calibration curve error, and two times the replicate standard deviation error. The dominant conformational species for each extracted data set is denoted with the suffix *.

2.2.6. Regulation of toxicity induced by metal-free Aβ and metal–Aβ by morin, quercetin, galangin, and luteolin in living cells

The ability of morin, quercetin, galangin, and luteolin to recover toxicity induced by metal-free Aβ and metal–Aβ was investigated in human neuroblastoma SH-SY5Y (5Y) cells. The cytotoxicity was determined by the MTT assay following previously published methods (Figure 2.9).<sup>18,20-22,27</sup> 5Y cells were incubated with metal ions (10 μM) and flavonoids (10 μM) with or without Aβ<sub>40</sub> or Aβ<sub>42</sub> (10 μM) for 24 h.

In the absence of Aβ, flavonoids may not significantly affect cell viability with and without metal ions. On the other hand, when Aβ<sub>40</sub> or Aβ<sub>42</sub> was introduced to 5Y cells, it presented cell survival by 85(±3)% and 86(±1)%, respectively (Figure 2.9). Moreover, cells which were treated with Aβ<sub>40</sub> or Aβ<sub>42</sub> and Cu(II) lowered viability [73(±1)% and 68(±2)%, respectively, while cells added with Aβ<sub>40</sub> or Aβ<sub>42</sub> and Zn(II) showed similar survival to metal-free Aβ-treated cells [87(±2)% and 83(±2)%], respectively] (Figure 2.9). Both morin and quercetin could reduce the toxicity induced by metal–Aβ more than 10% while galangin and luteolin could recover very slightly cell viability by ca. 3-5% from toxicity triggered by metal–Aβ (Figure 2.9). These results may be related to their ability to redirect metal–Aβ aggregation pathways to less toxic pathways, along with their known antioxidant activity.<sup>55-57</sup> Morin and quercetin, which have hydroxyl functionality on both B and C rings [possibly important for modulation of Aβ aggregation (<i>vide supra</i>) and anti-oxidant
property\textsuperscript{56} may be able to alleviate the toxicity triggered by A\(\beta\) and metal–A\(\beta\) in living cells more significantly than galangin or luteolin.

Figure 2.9. Viability of flavonoids with or without metal ions in both the absence and presence of A\(\beta\). Cytotoxicity was measured by the MTT assay after 24 h incubation of SH-SY5Y cells with and without A\(\beta\), a metal chloride salt (CuCl\(_2\) or ZnCl\(_2\)), or selected flavonoids. The cell viability (%) was calculated compared to cells treated with equivalent amounts of DMSO only (0–1%, v/v). Lanes: (1) A\(\beta\) ± [CuCl\(_2\) or ZnCl\(_2\)]; (2) A\(\beta\) ± [CuCl\(_2\) or ZnCl\(_2\)] + morin; (3) A\(\beta\) ± [CuCl\(_2\) or ZnCl\(_2\)] + quercetin; (4) A\(\beta\) ± [CuCl\(_2\) or ZnCl\(_2\)] + galangin; (5) A\(\beta\) ± [CuCl\(_2\) or ZnCl\(_2\)] + luteolin. Conditions: [A\(\beta\)] = 10 \(\mu\)M; [CuCl\(_2\) or ZnCl\(_2\)] = 10 \(\mu\)M; [flavonoid] = 10 \(\mu\)M. Values represent the mean of four independent experiments (± standard error).

2.3. Conclusion

Morin, quercetin, galangin, and luteolin are shown to have their ability to redirect metal–A\(\beta\)\textsubscript{40}/A\(\beta\)\textsubscript{42} aggregation pathways more noticeably than metal-free A\(\beta\)\textsubscript{40}/A\(\beta\)\textsubscript{42} aggregation. Each flavonoid shows different degrees on metal binding, A\(\beta\) interaction, and modulation of metal–A\(\beta\) aggregation. Such different properties are observed to be dependent on structural variations. The flavonoids with hydroxyl groups on both B and C rings (morin and quercetin) are indicated to significantly present their reactivity toward metal–A\(\beta\) aggregation and metal binding properties. Furthermore, these flavonoids could distinguishably attenuate toxicity induced by metal–A\(\beta\) in living cells. On the other hands, the flavonoids with lack of hydroxyl groups on the B or C ring (galangin and luteolin) are observed to have less reactivity toward metal–A\(\beta\) species. Such reactivity differences between the flavonoids with different number and location of hydroxyl groups are also demonstrated through biophysical investigations on direct interactions with metal-free A\(\beta\) and/or metal-bound A\(\beta\) using 2D NMR and IM-MS. The data indicate weak interactions
of these flavonoids with metal-free Aβ monomer and dimer to distinct extents. Given the low affinity for metal-free Aβ binding, it is unsurprising that significant effect on metal-free Aβ aggregation is not observed in our inhibition and disaggregation studies using the relatively low concentration of the flavonoids when compared to the 2D NMR and IM-MS results. Toward targeting and interacting metal-bound Aβ40, morin and quercetin are shown to have the greater ability than luteolin and galangin. Moreover, upon binding with morin and quercetin, the formation of conformationally distinct peptide species occurs compared to the metal-free/-bound states. Taken together, our studies demonstrate that such slight structure modifications of flavonoids (i.e., the variation of hydroxyl groups on their B and C rings) could tune their interactions with metal ions, Aβ species, and metal–Aβ, which could subsequently alter their abilities to metal–Aβ aggregation pathways and recover Aβ/metal–Aβ related cytotoxicity.

2.4. Experimental section

2.4.1. Materials and methods

All reagents were purchased from commercial suppliers and used as received unless otherwise stated. Morin and quercetin were purchased from Abcam (Cambridge, MA, USA); galangin and luteolin were acquired from Santa Cruz Biotechnology (Dallas, TX, USA) and Alfa Aesar (Ward Hill, MA, USA), respectively. The flavonoids were used without further purification. Trace metal contamination was removed from buffers and solutions used for Aβ experiments by treating with Chelex (Sigma-Aldrich, St Louis, MO, USA). Aβ40 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV) and Aβ42 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV) were obtained from Anaspec (Fremont, CA, USA) and Anygen (Nam-myun, Jangseong-gun, Korea). Double distilled H2O (ddH2O) was obtained from a Milli-Q Direct 16 system (Merck KGaA, Darmstadt, Germany). Optical spectra for metal binding studies were recorded on an Agilent 8453 UV–Visible (UV–Vis) spectrophotometer. Transmission electron microscopy (TEM) images were collected on a JEOL JEM-2100 transmission electron microscope (UNIST Central Research Facilities, Ulsan, Korea). A SpectraMax M5 microplate reader
(Molecular Devices, Sunnyvale, CA, USA) was used to measure the absorbance for the MTT assay [MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide].

2.4.2. Amyloid-β (Aβ) peptide experiments

Aβ experiments were conducted as previously published methods.\textsuperscript{16-27} Aβ peptides were dissolved with ammonium hydroxide (NH\textsubscript{4}OH, 1% v/v, aq), aliquoted, lyophilized, and stored at −80 °C. A stock solution (ca. 200 μM) was prepared by re-dissolving Aβ with NH\textsubscript{4}OH (1% w/v, aq, 10 μL) followed by dilution with ddH\textsubscript{2}O. The concentration of the solution was determined by measuring the absorbance of the solution at 280 nm (ε = 1450 M\textsuperscript{-1}cm\textsuperscript{-1} for Aβ\textsubscript{40}; ε = 1490 M\textsuperscript{-1}cm\textsuperscript{-1} for Aβ\textsubscript{42}). Buffered solutions [20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 6.6 or 7.4, 150 mM NaCl] were used for both inhibition and disaggregation studies [pH 6.6 for Cu(II) samples; pH 7.4 for metal-free and Zn(II) samples]. For the inhibition experiment, Aβ (25 μM) was first treated with or without a metal chloride salt (CuCl\textsubscript{2} or ZnCl\textsubscript{2}, 25 μM) for 2 min followed by addition of morin, quercetin, galangin, or luteolin (50 μM, 1% v/v final DMSO concentration). The resulting samples were incubated at 37 °C for 24 h with constant agitation. For the disaggregation experiment, Aβ in the absence or presence of a metal chloride salt (CuCl\textsubscript{2} or ZnCl\textsubscript{2}) was initially incubated at 37 °C for 24 h with steady agitation. The compound was added afterward, followed by an additional 24 h of incubation at 37 °C with constant agitation.

2.4.3. Gel electrophoresis with Western blotting

The Aβ peptide experiments described above were analyzed by gel electrophoresis followed by Western blotting (gel/Western blot) using an anti-Aβ antibody (6E10).\textsuperscript{16-27} Each sample (10 μL, [Aβ] = 25 μM) was separated using a 10-20% gradient Tris-tricine gel (Invitrogen, Grand Island, NY, USA). The gel was transferred to a nitrocellulose membrane and blocked with a bovine serum albumin (BSA) solution (3% w/v; Sigma, St. Louis, MO, USA) in Tris-buffered saline (TBS; Fisher, Pittsburgh, PA, USA) containing 0.1% Tween-20 (TBS-T; Sigma-Aldrich) for 3 h at room temperature. The membrane was treated with the Aβ monoclonal antibody (6E10; Covance, Princeton,
NJ, USA; 1:2,000; BSA, 2% w/v, in TBS-T) for overnight at 4 °C and then probed with a horseradish peroxidase conjugated goat anti-mouse secondary antibody (1:5,000; Cayman Chemical, Ann Arbor, MI, USA) in 2% BSA in TBS-T solution for 1 h at room temperature. Aβ species were visualized using Thermo Scientific Supersignal West Pico Chemiluminescent (ECL) Substrate (Rockford, IL, USA) or self-made ECL solution (2.5 mM luminol, 0.20 mM p-coumaric scid, 0.018% H2O2 in 100 mM Tris, pH 8.6). Note that the gel analysis presented herein is qualitative due to properties of resulting Aβ species.

2.4.4. Transmission electron microscopy (TEM)

Samples for TEM were prepared following previously reported methods.16-27 Glow discharged grids (Formar/Carbon 300-mesh; Electron Microscopy Sciences, Hatfield, PA, USA) were treated with samples from either inhibition or disaggregation experiments (5 μL) for 2 min at room temperature. Excess sample was removed with filter paper and the grids were washed with ddH2O three times. Each grid was stained with uranyl acetate (1% w/v ddH2O, 5 μL) for 1 min. Uranyl acetate was blotted off and grids were dried for 20 min at room temperature. Images of samples were taken by a JEOL JEM-2100 transmission electron microscope (200 kV, 25 000× magnification).

2.4.5. Cu(II) binding studies

The interaction of flavonoids with Cu(II) was determined by UV−Vis based on previously reported procedures.18-20,23 A solution of ligand (25 μM in 20 mM HEPES, pH 7.4, 150 mM NaCl) was prepared, treated with 0.5 and 1 equiv of CuCl2, and incubated at room temperature for 10 min. In order to verify if Cu(II) binding to ligand occurs in the presence of Aβ40, optical studies were performed on samples of Aβ40 (25 μM) preincubated with 0 or 1 equiv of CuCl2 in the absence and presence of flavonoids (25 μM). The optical spectra of the resulting solutions were measured after 10 min incubation.

2.4.6. 2D nuclear magnetic resonance (NMR) spectroscopy

The interaction between ligands and 15N-labeled Aβ40 was monitored by 2D band-Selective Optimized Flip-Angle Short Transient Heteronuclear Multiple Quantum Coherence (SOFAST-HMQC) at 10 °C.47 Uniformly 15N-labeled Aβ40 (rPeptide, Bogart,
GA, USA) was dissolved in 1% NH$_4$OH, aliquoted, and lyophilized. The peptide (80 µM peptide) was re-dissolved in 3 µL of DMSO-$d_6$ (Cambridge Isotope, Tewksbury, MA, USA) and diluted into PBS (20 mM PO$_4$, pH 7.4, 50 mM NaCl; 7% v/v D$_2$O). Compounds were titrated into the peptide solution from a 50 mM stock solution in DMSO-$d_6$ up to 10 equiv (800 µM). Spectra were acquired using 64 complex $t_1$ points and a 0.1 sec recycle delay on a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe. 2D data were processed using TOPSPIN 2.1 (from Bruker) and assignment was performed using SPARKY 3.1134 using published assignments for A$\beta_{40}$ as a guide.$^{48,58,59}$ Chemical shift perturbation (CSP) was calculated by the following equation (eq 1):

$$\Delta \delta_{NH} = \sqrt{\Delta \delta H^2 + \left(\frac{\Delta \delta N}{5}\right)^2} \quad \text{(eq 1)}$$

2.4.7. Ion mobility-mass spectrometry (IM-MS)

All Ion Mobility-Mass Spectrometry (IM-MS) experiments were carried out on a Synapt G2 (Waters, Milford, MA).$^{60,61}$ Samples were ionized using a nano-electrospray source operated in positive ion mode. MS instrumentation was operated at a backing pressure of 2.7 mbar and sample cone voltage of 40 V. For peptide-derivative-metal ligation studies aliquots of A$\beta_{40}$ peptides (final concentration 20 µM) were sonicated for 5 sec prior to preincubation with or without a source of Cu(II) (Copper(II) acetate, 20 µM) at 37 ºC for 10 min. After preincubation, samples were titrated with or without the flavonoid of interest (final concentrations: 20, 40, 80, and 120 µM) and incubated at 37 ºC for 30 min prior to analysis. Solution conditions were 100 mM ammonium acetate (pH 7.5) with 1% v/v DMSO. For control purposes, all data are compared against incubations of A$\beta_{40}$ peptides with EGCG under the same conditions. Collision cross-section (CCS) measurements were externally calibrated using a database of known values in helium, using values for proteins that bracket the likely CCS and ion mobility values of the unknown ions.$^{50,54}$ CCS values are the mean average of five replicates, with errors reported as the least square product. This least square analysis combines inherent calibrant error from drift tube measurements (3%),$^{54}$ calibration curve error, and twice the replicate standard deviation error. Measurements ($K_d$) for A$\beta$–flavonoid binding were calculated using the total ion count extracted from the peak of interest at its full width half
maximum using methods previously described. All other conditions are consistent with previously published methods.

### 2.4.8. Cytotoxicity studies

The human neuroblastoma SH-SY5Y cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in media containing 1:1 Minimum Essential Media (MEM; GIBCO, Grand Island, NY, USA) and Ham’s F12K Kaisyn’s Modification Media (F12K; GIBCO), 10% (v/v) fetal bovine serum (FBS; Sigma), and 1% (v/v) penicillin (GIBCO). The cells were grown and maintained at 37 °C in a humidified atmosphere with 5% CO₂. Cell viability with treatment of Aβ and/or flavonoids was determined using the MTT assay (Sigma) as previously reported. SH-SY5Y cells were seeded in a 96 well plate (15,000 cells in 100 μL per well) and treated with Aβ (10 μM) and/or flavonoids (10 μM; final 1% v/v DMSO). After 24 h incubation at 37 °C, MTT (25 μL of 5 mg/mL in phosphate buffered saline, PBS, pH 7.4; GIBCO) was added to each well and the plates were incubated for 4 h at 37 °C. Formazan produced by the cells was dissolved overnight at room temperature by the addition of a solubilization buffer (100 μL) containing N,N-dimethylformamide (DMF; 50% v/v, aq, pH 4.5) and sodium dodecyl sulfate (SDS; 20% w/v). The absorbance (A₆₀₀) was measured on a microplate reader. Cell viability was determined relative to cells containing an equivalent amount of DMSO (1% v/v). Error bars were calculated as standard errors (SEs) from four independent experiments.

### 2.5. Acknowledgment

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2.6. References

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Chapter 3: Identification of Structural Moieties Essential for Regulating Metal-free and Metal-associated Amyloid-β and Free Radicals

This chapter is based on work that will be submitted for publication. We thank Professor Ayyalusamy Ramamoorthy and Kyle J. Korshavn for NMR experiments; Professor Kwang S. Kim, Dr. Han M. Lee, Dr. Woo J. Cho, and Dr. Zahra A. Tehrani for calculation studies; Dr. Akiko Kochi for in vitro PAMPA assay; Younwoo Nam for synthesis of compounds, studies of metal binding, solution speciation, docking, ESI-MS, gel analysis for Aβ aggregation and cell experiments. I was involved in copper binding, docking studies, gel and TEM analyses for Aβ aggregation, determination of antioxidant properties, as well as manuscript writing with Younwoo Nam.

3.1. Introduction

Alzheimer’s disease (AD) is one of the most fatal neurodegenerative disorders featured by a progressive neuronal loss and cognitive impairment.\textsuperscript{1-6} The cause and mechanism of AD onset and progression remain unclear resulting in lack of fundamental cures for the disease.\textsuperscript{1-6} Neurotoxicity associated with amyloid-β (Aβ) peptide has been suggested as one of the hypotheses for AD pathogenesis since misfolded Aβ aggregates are primary components of senile plaques in the AD-afflicted brain.\textsuperscript{1-9} Aβ is produced upon the proteolytic cleavage of amyloid precursor protein (APP) [two major isoforms, Aβ\textsubscript{40} and Aβ\textsubscript{42} (ca. 90 % and 9 % in the brain, respectively), that are aggregation-prone to form aggregates from various oligomers to mature fibrils].\textsuperscript{1-9} Soluble Aβ oligomers are observed to be toxic from recent studies; however, the relationship between specific Aβ conformations and neurotoxicity is still unidentified.\textsuperscript{1-9}

In addition, the AD-affected brain exhibits highly concentrated metal ions within senile plaques [e.g., ca. 0.4 mM for Cu(I/II), 1 mM for Zn(II), 0.9 mM for Fe(II/III)].\textsuperscript{1-13} Previous studies in vitro demonstrated that these metal ions, particularly Cu(I/II) and Zn(II), can interact with Aβ peptides and promote the peptide aggregation.\textsuperscript{3-16} Furthermore, complexes of Aβ with redox-active metal ions, such as Cu(I/II) and Fe(II/III), are shown to generate reactive oxygen species (ROS) via Fenton-like chemistry leading to oxidative stress.\textsuperscript{1-16} Thus, it has been proposed that the inter-relation between metal
ions, Aβ, metal-bound Aβ (metal–Aβ), and ROS is linked to AD pathogenesis. Given the limited efficacy of current anti-AD agents only targeting individual pathogenic factors (e.g., metal-free Aβ, metal ions, ROS), as well as such the interconnection, the development of single molecules which are able to regulate multiple pathogenic features could be more effective for toward the discovery of AD treatment.

Hence, some chemical tools have been designed to control metal-free Aβ and metal–Aβ aggregation and associated toxicity.\(^{17-32}\) Toward this effort, the derivatization of amyloid imaging agents, such as \(p\)-stilbene and thioflavin-T (ThT) has been utilized as a strategy for the design and synthesis of chemical reagents which can target and interact with metal ions, metal-free Aβ, and metal–Aβ.\(^{17-32}\) Although several compounds showed their inhibitory reactivity toward Aβ aggregation\(^{17-30}\) structural motifs responsible for such reactivity are not understood clearly. Herein, we report the determination of structural groups essential for reactivities toward metal-free Aβ, metal–Aβ, and free radicals employing a new series of small molecules (Figure 3.1). Our newly generated molecules are derived from the systematic structural variation of \(N^1,N^1\)-dimethyl-\(N^4\)-(pyridin-2-ylmethyl)benzene-1,4-diamine (L2-b) and \(N^1,N^1\)-dimethylbenzene-1,4-diamine (DMPD).\(^{20-23}\) L2-b displayed that it could interact with metal–Aβ species and modulate metal–Aβ aggregation pathways to form less toxic species both \(\textit{in vitro}\) and \(\textit{in vivo}\).\(^{21-23}\) In addition, a recent report presents that a small and simple monodentate ligand, DMPD (Figure 3.1), a moiety of L2-b, affords the desired anti-amyloidogenic activity.\(^{20}\) DMPD is also able to interact with both metal-free Aβ and metal–Aβ and redirect their aggregation into off-pathway and form less toxic species both \(\textit{in vitro}\) and \(\textit{in vivo}\).\(^{20}\) Moreover, L2-b and DMPD are shown to be antioxidants.\(^{20,23}\) Thus, structural moieties of both DMPD and L2-b may be important for modulating amyloidogenesis and scavenging free radicals.\(^{20}\)

In our chemical library, the compounds which contain a DMPD motif (DMPD, L2-b, and L2-b2; Figure 3.1) exhibit noticeable modulation of metal-free Aβ and/or metal–Aβ aggregation along with antioxidant capacity. Similar to DMPD and L2-b, L2-b2 is observed to attenuate metal-free Aβ and metal–Aβ induced toxicity in living cells. Particularly, biophysical analyses on the interaction of L2-b\(^{21-23}\) and L2-b2 with both metal-free Aβ and metal–Aβ suggest as follows: (i) their activity with metal-free Aβ or
Figure 3.1. Chemical structures of the compounds employed in this study. **DMPD** ($N^1,N^1$-dimethylbenzene-1,4-diamine; orange), **L2-b** ($N^1,N^1$-dimethyl-$N^4$-(pyridin-2-ylmethyl)benzene-1,4-diamine), **L2-b1** ($N,N$-dimethyl-6-((phenylimino)methyl)pyridin-3-amine), **L2-b2** ($N^1$-((5-(dimethylamino)pyridin-2-yl)methyl)-$N^4,N^4$-dimethylbenzene-1,4-diamine), **DPA1** (bis(pyridin-2-ylmethyl)amine), **DPA2** (6-(((5-(dimethylamino)pyridin-2-yl)methyl)amino)methyl)-$N,N$-dimethylpyridin-3-amine), **PMA1** (pyridin-2-ylmethanamine), and **PMA2** (6-(aminomethyl)-$N,N$-dimethylpyridin-3-amine). Potential donor atoms for metal binding are highlighted in blue.

metal–$\text{A}\beta$ could occur via the complex formation between the ligand and metal-free $\text{A}\beta$ or metal–$\text{A}\beta$; (ii) potential chemical reaction and observed antioxidant capability of the ligands might be associated with the redox-active **DMPD** moiety. Overall, our studies present that **DMPD**-containing compounds can interact with metal-free $\text{A}\beta$ and/or metal–$\text{A}\beta$, control their aggregation pathways into forming less toxic aggregates and noticeably scavenge free radicals. Therefore, our present work demonstrates that the **DMPD** functionality is essential for the interaction and regulation of multiple causative factors (i.e., metal-free $\text{A}\beta$, metal–$\text{A}\beta$, ROS) in AD, which proposes its promising utilization for the development of chemical tools and therapeutics for misfolded-protein-related diseases.

3.2. Results and discussion
3.2.1. Rational design, selection and preparation of small molecules

Some stilbene-based molecules were previously examined to modulate metal-free Aβ and/or metal–Aβ aggregation in vitro as well as reduce peptide-associated toxicity in living cells.21-24 Mechanistic understanding of their structure-related anti-amyloidogenic activity, however, has not been established and thus, the development of molecular frameworks has been limited. A recent report indicates that DMPD (Figure 3.1), a small chemical tool, is capable of controlling both metal-free Aβ and metal–Aβ aggregation pathways.20 Identification of pivotal structures for the reactivity (i.e., redirection of Aβ aggregation pathways, regulation of free radicals, attenuation of cytotoxicity induced by metal-free Aβ and metal–Aβ) is critical for invention of new, effective chemical reagents for such targets. In our present studies, a chemical series of the DMPD and L2-b derivatives (Figure 3.1) was rationally selected to determine essential structural moieties (e.g., DMPD, the dimethylamino functionality, the metal chelation site of L2-b) for their desired reactivity.

Scheme 3.1. Synthetic routes to (a) L2-b1, L2-b2, and (b) DPA2.

Based on the structures of DMPD and L2-b, L2-b1 and L2-b2 (Figure 3.1) were first chosen to investigate the influence of the dimethylamino functionality on the reactivity toward aggregation and toxicity of metal-free Aβ, metal–Aβ, and free radicals. Since the dimethylamino group was known to be important for the interaction with metal-free Aβ and metal–Aβ,23-28 the position of this moiety has been altered from L2-b. PMA1 and PMA2 (Figure 3.1), composed of the metal chelation sites of L2-b and L2-b2, respectively, were included for the structure-reactivity investigation in our chemical library.
Figure 3.2. Effects of DMPD, L2-b, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 on the formation of metal-free Aβ40 and metal–Aβ40 aggregates. (a) Schemes of the inhibition (I) and disaggregation (II) experiments. (b) Visualization of molecular weight (MW) distributions of the resultant Aβ40 species by gel electrophoresis followed by Western blotting (gel/Western blot) with an anti-Aβ antibody (6E10). Conditions: [Aβ40] = 25 μM; [CuCl2 or ZnCl2] = 25 μM; [compound] = 50 μM; pH 6.6 (for Cu(II) experiments) or pH 7.4 (for metal-free and Zn(II) experiments); 37 °C; constant agitation. Lanes: (C) Aβ ± [CuCl2 or ZnCl2]; (1) Aβ ± [CuCl2 or ZnCl2] + DMPD; (2) Aβ ± [CuCl2 or ZnCl2] + L2-b1; (3) Aβ ± [CuCl2 or ZnCl2] + L2-b2; (4) Aβ ± [CuCl2 or ZnCl2] + DPA1; (5) Aβ ± [CuCl2 or ZnCl2] + DPA2; (6) Aβ ± [CuCl2 or ZnCl2] + PMA1; (7) Aβ ± [CuCl2 or ZnCl2] + PMA2; (8) Aβ ± [CuCl2 or ZnCl2] + L2-b. (c) TEM images of the samples from (b).

Through comparison between PMA1 and DMPD, the structural components responsible for the reactivity of L2-b can be determined. Moreover, DPA1 and DPA2
(Figure 3.1) were selected to elucidate whether increased denticity and flexibility of L2-b could tune the inhibitory effect of small molecules on metal-free Aβ and metal–Aβ aggregation as well as their antioxidant activity. The impact of the dimethylamino group on the overall structural frameworks (DPA1 versus DPA2, PMA1 versus PMA2) on the compounds’ reactivities with the targets can be further probed. Taken together, a structure-interaction-reactivity relationship determined within the selected compounds is able to address structural moieties needed for anti-amyloidogenic and antioxidant activities. For the preparation of new compounds, L2-b1, L2-b2, and DPA2 were synthesized based on previously reported methods with slight modifications (Scheme 3.1).23,27

3.2. Modulation of Aβ aggregation pathways

To evaluate the ability of compounds to modulate Aβ aggregation pathways, two different experiments, inhibition and disaggregation experiments (Figures 3.2a and 3.3a), were carried out with Aβ40 and Aβ42, two major Aβ isoforms found in the brain of AD.1-9 The molecular weight (MW) distributions and morphological changes of the resultant Aβ species were visualized and monitored by gel electrophoresis followed by Western blot (gel/Western blot) and transmission electron microscopy (TEM), respectively (Figures 3.2 and 3.3).20-30

From the inhibition experiment with Aβ40, as shown in Figure 3.2b (left), metal-free, various-sized Aβ40 aggregates were indicated from the samples containing DMPD and L2-b2. In addition, noticeable changes in the size distribution of metal–Aβ40 aggregates were observed upon treatment with DMPD, L2-b2, or L2-b to different extents (Figure 3.2b, left; lane 2, 4, and 9). On the other hand, much less significant influence on metal-free Aβ40 and metal–Aβ40 aggregation was observed upon incubation with L2-b1, DPA1, DPA2, PMA1, or PMA2 (Figure 3.2b, left). Unlike compound-free samples (lane C), DPA1, PMA1, and PMA2 may stabilize higher MW Cu(II)–Aβ40 aggregates (above 150 kDa); DPA1 and DPA2 may be able to generate larger-sized Zn(II)–Aβ40 aggregates (above 240 kDa). The DMPD-containing compounds (i.e., DMPD, L2-b, L2-b2) could effectively modulate metal-free Aβ40 and/or metal–Aβ40 aggregation. L2-b1, DPA1, DPA2, PMA1, and PMA2, lack of the DMPD moiety, presented no or very slight and
Figure 3.3. Influence of DMPD, L2-b, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 on the metal-free Aβ_{42} and metal-Aβ_{42} aggregation pathways. (a) Schemes of the inhibition (I) and disaggregation (II) experiments. (b) Analysis of size distributions of the resultant Aβ species by gel/Western blot with an anti-Aβ antibody (6E10). Conditions: [Aβ_{42}] = 25 μM; [CuCl_2 or ZnCl_2] = 25 μM; [compound] = 50 μM; pH 6.6 (for Cu(II) experiments) or pH 7.4 (for metal-free and Zn(II) experiments); 37 °C; constant agitation. Lanes: (C) Aβ ± [CuCl_2 or ZnCl_2]; (1) Aβ ± [CuCl_2 or ZnCl_2] + DMPD; (2) Aβ ± [CuCl_2 or ZnCl_2] + L2-b1; (3) Aβ ± [CuCl_2 or ZnCl_2] + L2-b2; (4) Aβ ± [CuCl_2 or ZnCl_2] + DPA1; (5) Aβ ± [CuCl_2 or ZnCl_2] + DPA2; (6) Aβ ± [CuCl_2 or ZnCl_2] + PMA1; (7) Aβ ± [CuCl_2 or ZnCl_2] + PMA2; (8) Aβ ± [CuCl_2 or ZnCl_2] + L2-b. (c) TEM images of the samples from (b).

different effect on metal–Aβ_{40} aggregation. These results may indicate that the dimethylamino moiety itself may not be enough to redirect metal-free Aβ_{40} and/or metal–Aβ_{40} aggregation pathways. From L2-b-, L2-b1- and L2-b2-treated samples, the position
of the dimethylamino functionality onto the whole structure of L2-b is shown to play a role in modulation of the peptide aggregation. In addition, based on the results obtained upon treatment of metal–Aβ with L2-b, L2-b2, PMA2, and DPA2, the entire structure of molecules with the dimethylamino group(s) (not dimethylamino group itself) is observed to determine the reactivity toward metal-free Aβ and metal–Aβ. In the inhibition experiment with Aβ42 (Figure 3.3b, left), similar to the results with Aβ40, both metal-free Aβ42 and metal–Aβ42 aggregation pathways were altered by DMPD and L2-b2 (Figure 3.3b, left; lanes 2 and 4). L2-b can only redirect metal–Aβ42 aggregation as previously reported (Figure 3.3b, left; lane 9).21,23 The compounds without the DMPD motif did not present noticeable effect on Aβ42 aggregation compared to compound-untreated samples.

Furthermore, the morphologies of L2-b1- or L2-b2-treated metal-free Aβ and metal–Aβ aggregates were observed by TEM. The resultant Aβ40 species incubated with L2-b2 are shown to be smaller and more amorphous than those from compound-free or L2-b1-treated samples (Figure 3.2c, left). Metal-free Aβ42 and metal–Aβ42 aggregates generated with L2-b2 have less structured or shorter fibrils than compound-free or L2-b1-incubated Aβ42 aggregates (Figure 3.3c, left).

The results from the disaggregation experiments were similar to those from inhibition experiments except those from the samples of PMA2 with Cu(II)–Aβ40 and DPA1- and DPA2-treated Zn(II)–Aβ40 indicating similar MW distributions to the compound-free samples (Figures 3.2b, right). The compounds containing the DMPD motif, DMPD, L2-b and L2-b2, displayed noticeable various sized metal-free Aβ40 and/or metal–Aβ40 aggregates to different degrees (Figures 3.2b, right). Among the small molecules without the DMPD moiety, DPA1 and PMA1, may generate Cu(II)–Aβ40 aggregates with high MWs (Figure 3.2b, right) similar to the results from the inhibition experiments (Figure 3.2b, left). DPA2 and PMA2 could not influence preformed Aβ aggregates under both metal-free and metal present environments (Figure 3.2b, right). Moreover, the compounds containing the DMPD group could also affect preformed metal-free Aβ42 and/or metal–Aβ42 aggregates (Figure 3.3b, right). The compounds without the DMPD motif could not alter the aggregation process (Figure 3.3b, right). Besides, the
morphological changes upon addition of L2-b2 to both metal-free Aβ40/Aβ42 and metal–Aβ40/Aβ42 aggregates were more significantly occurred than L2-b1-treated samples (Figures 3.2c and 3.3c). The Aβ samples incubated with L2-b2 presented more

![Graphs showing solution speciation studies](image)

![Table showing acidity constants](image)

**Figure 3.4.** Solution speciation studies of (a) L2-b1, (b, left) DPA1, (b, right) DPA2, (c, left) PMA1, and (c, right) PMA2. Left: Variable-pH spectrophotometric titration spectra in the range of pH 2–9. Right: Solution speciation diagrams in the range of pH 2–9 (F_l = fraction of species at the given protonation). Bottom: Summary of the acidity constants (pK_a) of L (L = L2-b1, DPA1, DPA2, PMA1, and PMA2). Charges are omitted for clarity. *Error in the parentheses is shown in the last digit. Conditions: [compound] = 30 μM; I = 0.10 M NaCl; room temperature.
amorphous or thinner fibrils than compound-free and L2-b1-contained samples (Figures 3.2c and 3.3c).

Taken together, our results and observations from the inhibition and disaggregation studies of Aβ aggregation employing our chemical library suggest that the overall framework containing the DMPD moiety at proper structural positions, instead of the dimethylamino functionality itself, is essential for the ability of small molecules to control metal-free Aβ and/or metal-induced Aβ aggregation pathways.

3.2.3. Solution speciation studies

Acidity constants (pKa) for L2-b1, DPA1, DPA2, PMA1, and PMA2, were determined by variable-pH spectrophotometric titrations following previous procedures. It is noted that the pKa values of DMPD and L2-b2 were not able to be experimentally obtained due to their instability (vide infra). As summarized in Figure 3.4, single pKa values were obtained for L2-b1 [pKa1 = 6.1(7)], PMA1 [pKa1 = 2.0(0)], and PMA2 [pKa1 = 3.7(2)]; two pKa values for DPA1 [pKa1 = 2.2(9) and pKa2 = 7.4(8)]; three pKa values for DPA2 [pKa1 = 2.2(6), pKa2 = 4.0(3), and pKa3 = 8.2(2)] in the pH range 2–9. These pKa values suggest the presence of monoprotonated (LH) and neutral (L) forms of L2-b1, PMA1, and PMA2; L, LH, and diprotonated (LH2) forms for DPA1; and L, LH, LH2, and triprotonated (LH3) forms for DPA2 in the pH range of 2–9 (Figure 3.4). Based on these pKa values, speciation diagrams were drawn to illustrate the fraction of ligand protonation states in the pH range from 2 to 9. The associated solution speciation diagrams indicate that the neutral form (L) of L2-b1 mainly presents at a physiological pH (i.e., pH 7.4) while the monoprotonated form (LH) is the major species of DPA1, DPA2, PMA1, and PMA2 (Figure 3.4).

3.2.4. Metal binding properties

Cu(II) or Zn(II) binding of L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 was monitored by UV–Visible (UV–Vis) and 1H nuclear magnetic resonance (NMR) spectroscopy. As depicted in Figure 3.5, the changes of UV–Vis spectra (e.g., change in absorbance intensity, new optical bands) were observed upon addition of CuCl2 to L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 in CH3CN or EtOH, indicative of Cu(II)
Figure 3.5. Cu(II) binding studies of L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2. UV–Vis spectra of (a) L2-b1 and L2-b2 with CuCl$_2$ (1–5 equiv and 1–2 equiv, respectively) in CH$_3$CN; (b) DPA1 and DPA2 with CuCl$_2$ (1–5 equiv) in EtOH; (c) PMA1 and PMA2 with CuCl$_2$ (1–5 equiv) in EtOH. Conditions: [L2-b1, DPA1, DPA2, PMA1, and PMA2] = 50 μM or [L2-b2] = 25 μM; room temperature; incubation for 30 min (for L2-b1 and L2-b2) and 10 min (for DPA1, DPA2, PMA1, and PMA2).

binding to the compounds (Figure 3.5). New optical bands at 400 nm and 483 nm from L2-b1 with Cu(II) and 392 nm and 496 nm from L2-b2 with Cu(II) were detected. In the case of DPA1 and PMA1, the intensity of absorption spectra was increased when Cu(II) was added. The optical spectral shifts from 323 nm to 337 nm and 334 nm were observed when DPA2 or PMA2 was treated with Cu(II), respectively. Furthermore, Zn(II) binding of L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 were investigated by UV–Vis and $^1$H NMR spectroscopy. The introduction of Zn(II) to the solution of L2-b2, DPA1, and DPA2 (in CH$_3$CN or EtOH) resulted in changes of optical spectra. As shown in Figures 3.6a–c, a
new optical band at 472 nm for L2-b2, the enhanced intensity of spectra for DPA1, as well as the shifted optical spectrum from 323 nm to 336 nm for DPA2 were indicated. The addition of Zn(II) (1 equiv) to the CD₃CN solutions of L2-b1, PMA1, and PMA2 caused the alteration of chemical shifts of the pyridyl protons (Figures 3.6d-f), suggesting the involvement of the N donor atoms from the pyridine ring in Zn(II) binding. Overall, our optical and NMR studies demonstrate that L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 can interact with Cu(II) and Zn(II).

Figure 3.6. Zn(II) binding of L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2. UV–Vis spectra of (a) L2-b2 with ZnCl₂ (1–5 equiv) in CH₃CN; (b) DPA1 and (c) DPA2 with ZnCl₂ (1–5 equiv) in EtOH. Conditions: [L2-b2, DPA1, and DPA2] = 50 μM; room temperature; incubation for 30 min (for L2-b2) and 10 min (for DPA1 and DPA2). ¹H NMR spectra of (d) L2-b1 (black), (e) PMA1 (black), and (f) PMA2 (black) with ZnCl₂ (1 equiv, red) in CD₃CN. Conditions: [L2-b1, PMA1, and PMA2] = 4 mM; room temperature; incubation for 5 min.
3.2.5. NMR studies for the interaction of compounds with metal-free Aβ and Zn(II)-bound Aβ monomers and fibrils

Direct interaction of L2-b, L2-b1, and L2-b2 with monomeric Aβ_{40} was investigated by 2D band-selective optimized flip-angle short transient heteronuclear multiple quantum correlation (SOFAST-HMQC) NMR spectroscopy. As depicted in Figure 3.7, small but detectable chemical shifts were presented upon the titration of 10...
equiv of compounds to soluble A\(\beta\)\(_{40}\) peptides. The chemical shift perturbation (CSP) was monitored to determine the potential binding site and modes of compounds to the peptide.

**Figure 3.8.** Interactions of L2-b and L2-b2 with monomeric Zn(II)-bound A\(\beta\)\(_{40}\) and metal-free A\(\beta\)\(_{40}\) or Zn(II)-associated A\(\beta\)\(_{40}\) fibrils. 2D \(^1\)H–\(^{15}\)N SOFAST-HMQC NMR spectra of (a and c) monomeric \(^{15}\)N-labeled with and without Zn(II); (b and d) \(^{15}\)N-labeled A\(\beta\)\(_{40}\) pretreated with Zn(II) followed by addition of L2-b (left) or L2-b2 (right). (e and f) Plots of the corresponding chemical shift perturbations (CSPs) of the spectra from \(^{15}\)N-labeled A\(\beta\)\(_{40}\) with Zn(II) (blue) and Zn(II)-treated-\(^{15}\)N-labeled A\(\beta\)\(_{40}\) with L2-b or L2-b2 (black). Conditions: [A\(\beta\)\(_{40}\)] = 80 \(\mu\)M; [ZnCl\(_2\)] = 80 \(\mu\)M; [L2-b and L2-b2] = 80-800 \(\mu\)M; 20 mM PO\(_4\), pH 7.4, 50 mM NaCl; 7% v/v \(D_2O\) (g and h) Normalized STD intensities mapped to compound’s structure [(g) L2-b or (h) L2-b2] against metal-free A\(\beta\) fibrils (left) or Zn(II)–A\(\beta\)\(_{42}\) fibrils (right). Yellow, orange, and blue circles indicate the STD effects of > 75%, 50-75%, and < 50%, respectively. Gray circles indicate the absence of the STD effect.

All L2-b\(_1\), L2-b1, and L2-b2 triggered moderate chemical shifts (lower than 0.02 ppm) from the different regions of A\(\beta\)\(_{40}\). These ligands could interact with the self-recognition region of A\(\beta\)\(_{40}\) peptides (residues from L17 to A21)\(^{2-5}\) and C-terminal regions.
of Aβ40 which are shown to be critical for the peptide aggregation.2–5 L2-b and L2-b1 were observed to interact with more than two residues from the self-recognition site (L17 and F20 for L2-b; V18, F19 and F20 for L2-b1) while L2-b2 was indicated to affect the chemical shift of only F19. In addition, all of these compounds induced the chemical shift of V40 at the C-terminus which may reflect the rearrangement of the disordered C-terminus to pack against the compounds instead of interaction or binding between the ligand and peptide.22 These NMR results are consistent to docking studies employing Aβ40 monomer (PDB 2LFM33) which visualize the interaction between compounds and Aβ40 peptide. L2-b, L2-b1, and L2-b2 are resided in the groove between the α-helix and N-terminal random coil of Aβ peptides (Figure 3.9a). L2-b, L2-b1, and L2-b2 showed weak interaction with Aβ40 monomer and this interaction may not have noticeable effect on modulation of peptide aggregation pathways. There may be other mechanisms to redirect the peptide aggregation that require more than the interaction between compounds and Aβ peptides (vide infra).

In addition, the interaction between L2-b or L2-b2 and Zn(II)–15N-labeled Aβ40 monomer was further analyzed by 2D NMR. As shown in Figure 3.8, L2-b strongly interacted with R5 and H13 close to a metal binding site in Aβ40.17 L2-b2 caused chemical shifts on the residues suggested to be associated with metal binding as well. These observations from NMR studies are also visualized by docking studies using Zn(II)-bound Aβ16 (PDB 1ZE934). From our docking investigations, both L2-b and L2-b2 might have contacts with the amino acid residues close to metal binding region (Figure 3.9b). Overall, our NMR and docking studies between the compounds and Zn(II)–Aβ suggest their direct interaction with Zn(II)–Aβ (particularly with the metal binding site of Aβ17), which could be related to their activity to modulate Zn(II)–Aβ40 aggregation pathways, observed in Figure 3.2.

Moving forward, the interactions between L2-b or L2-b2 and metal-free Aβ42 or Zn(II)–Aβ42 fibers were studied by STD. Signals in STD NMR are proportional to each atoms of ligand to its macromolecular binding partner, allowing atomic-level mapping of ligand binding to be made.35 Similar saturation patterns were monitored from both metal-free Aβ42 fibrils and Zn(II)–Aβ42 fibrils with L2-b. On the pyridine ring of L2-b, relatively
strong saturation effects were detected indicating that the pyridine ring portion of L2-b could play an important role in the interactions with both metal-free Aβ42 and Zn(II)–Aβ42 fibrils (Figure 3.8g). Different from L2-b, dimethylamino groups of L2-b2 presented stronger saturation effects than other moieties with both metal-free Aβ42 and Zn(II)–Aβ42 fibrils. When Zn(II) was introduced to Aβ42 fibrils, the saturation effects on the pyridine

<table>
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<th>Compound</th>
<th>DMPD</th>
<th>L2-b</th>
<th>L2-b1</th>
<th>L2-b2</th>
<th>DPA1</th>
<th>DPA2</th>
<th>PMA1</th>
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<tbody>
<tr>
<td>Binding energy (kcal/mol)</td>
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<td>-5.8</td>
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<td>-5.6</td>
<td>-5.6</td>
<td>-3.9</td>
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</table>
Figure 3.9. Docking studies of DMPD, L2-b, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 with (a) Aβ_{40} monomer or (b) Zn(II)-bound Aβ_{16} monomer. The lowest energy conformation of DMPD (orange), L2-b (blue), L2-b1 (purple), L2-b2 (green), DPA1 (light blue), DPA2 (light purple), PMA1 (yellow), and PMA2 (pink) with cartoon (left) and surface (right) depictions of (a) Aβ_{40} (PDB 2LFM) or (b) Zn(II)-bound Aβ_{16} (PDB 1ZE9) monomer by AutoDock Vina. The helical region and metal binding sites of metal-free Aβ_{40} and Zn(II)-Aβ_{16} are illustrated in yellow and in light gray, respectively, in the surface representation. Potential hydrogen bonding is indicated with dashed lines (1.8-2.3 Å). Bottom: Summaries of the calculated binding energies of DMPD, L2-b, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 to metal-free Aβ_{40} and Zn(II)-bound Aβ_{16}.  

<table>
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<th>Compound</th>
<th>Binding energy (kcal/mol)</th>
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<td>DMPD</td>
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<td>PMA2</td>
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ring of L2-b2 were more noticeable. Additionally, since the STD intensities were relatively distributed through the whole molecule of L2-b2 against Zn(II)–Aβ42 fibrils, its framework could be packed into the fibrillar conformation of Zn(II)–Aβ42. Taken together, the dimethylamino group from the DMPD motif of L2-b2 is shown to be involved in the interactions between metal-free Aβ fibrils, which could direct L2-b2 possibly to control the peptide aggregation. In addition, the pyridine ring of L2-b and L2-b2, associated with the metal chelation, is observed to be more related to the contact with Zn(II)–Aβ42 fibrils than metal-free fibrils, indicating the compounds’ modulating ability toward the Zn(II)–Aβ42 aggregation. Overall, our STD NMR results help understand the results from the disaggregation experiments treating L2-b and L2-b2 to preformed metal-free Aβ aggregates and/or Zn(II)–Aβ aggregates (vide supra, Figure 3.3b, right).

3.2.6. Transformation of compounds

Previous study suggests that the oxidative transformation of DMPD is linked to its inhibitory activity toward both metal-free Aβ and metal–Aβ. To elucidate the noticeable inhibitory reactivity of L2-b and L2-b2 with metal-free Aβ and/or metal–Aβ, their transfiguration was optically monitored following reported methods. Absorption spectra of L2-b or L2-b2 were obtained in a time-dependent manner upon incubation with or without Aβ40 in the absence and presence of Cu(II) and Zn(II) (Figures 3.10). Under metal-free and Zn(II) present conditions, characteristic optical changes of L2-b or L2-b2 (i.e., increase in the intensity, appearance of new bands) were indicated in both the absence and presence of Aβ40 (Figure 3.10). Moreover, the optical variations of L2-b or L2-b2 in the presence of Aβ are much slower than those of Aβ40-free conditions affording fewer amounts of new species (Figure 3.10). The absorption bands at 510 nm (for L2-b) and 496 nm (for L2-b2) upon transformation are possibly resulted from the oxidized forms of L2-b and L2-b2 in the both absence and presence of Aβ40. These results are consistent with those measured by electrospray ionization mass spectrometry (ESI-MS) (vide infra; Figure 3.11). Unlike metal-free conditions, in the presence of Cu(II), different optical changes were observed for both L2-b and L2-b2. As discussed in the section of metal binding above, after the addition of Cu(II) to the Aβ40-free ligand solutions distinct
Figure 3.10. Transfigurations of L2-b and L2-b2 in the absence and presence of metal ions and Aβ40, monitored by UV–Vis spectroscopy. Time-dependent changes in UV–Vis spectra of (a) L2-b and (b) L2-b2 were obtained. Blue, orange, red, black, and green lines correspond to the spectra recorded after incubation for 1, 10/30 min, 4, 12, and 24 h, respectively. Conditions: [Aβ40] = 25 μM; [CuCl2 or ZnCl2] = 25 μM; [L2-b and L2-b2] = 50 μM; pH 6.6 (for Cu(II) experiments) or pH 7.4 (for metal-free and Zn(II) experiments); room temperature; no agitation. Triangles, asterisks, and circles indicate optical bands for the expected transformation of the compound (oxidized L2-b, a cationic radical of DMPD, and oxidized L2-b2, respectively).
Figure 3.11. ESI-MS studies of the solutions containing L2-b or L2-b2 with and without a metal chloride salt (CuCl$_2$ or ZnCl$_2$, 25 μM) at various incubation times. (a) ESI-MS spectra of L2-b or L2-b2 without metal ions. (b and c) ESI-MS spectra of L2-b or L2-b2 with CuCl$_2$ or ZnCl$_2$. Conditions: [L2-b or L2-b2] = 50 μM; [CuCl$_2$ or ZnCl$_2$] = 25 μM; room temperature; incubation for 0, 4, 12, or 24 h.
optical features for \( \text{L2-b} \) and \( \text{L2-b2} \) were indicated (Figure 3.10) suggesting the formation of metal-ligand complexes. Over further incubation, the broad doublet peaks at ca. 550 nm were typically shown for both \( \text{L2-b} \) and \( \text{L2-b2} \) without A\( \beta \), maximized at 4 h incubation (Figure 3.10). These absorption bands are characteristic for the generation of a cationic radical of DMPD,\(^{20}\) which suggests that the Cu(II)-catalyzed hydrolytic cleavage of \( \text{L2-b} \) or \( \text{L2-b2} \) possibly produce DMPD or other structural analogs. In the presence of both Cu(II) and A\( \beta \), different optical variations occurred. Transformation of \( \text{L2-b} \) or \( \text{L2-b2} \) was further monitored by ESI-MS in both the absence and presence of Cu(II) or Zn(II) at various incubation times (0, 4, 12, and 24 h).

Under metal-free conditions, \( m/z \) signals (228/271, 227/270, 227/269, and 224/269; Figure 3.11a) of \( \text{L2-b} \) or \( \text{L2-b2} \) at different incubation times possibly correspond to the original ligand form (at 0 h), and an oxidized form (from 4, 12, and 24 h incubation).\(^{36-38}\) In the presence of Cu(II), different \( m/z \) values were indicated for both compounds, compared to those under metal-free and Zn(II)-added conditions (Figure 3.11b). When \( \text{L2-b} \) was incubated with CuCl\(_2\) for 4 h, a reproducible signal at \( m/z \) 138, possibly corresponding to 4-dimethylaminophenol (calculated \( m/z \) (138) of [M + H]\(^+\)) that might be generated by the hydrolytic cleavage of \( \text{L2-b} \), was indicated while various \( m/z \) signals were randomly detected from the samples incubated longer. For \( \text{L2-b2} \) treated with CuCl\(_2\), the signals of \( m/z \) 271 (the original form, at 0 h) and 267 (oxidized form, from 4, 12, and 24 h incubation) were dominantly observed in a time-dependent manner (Figure 3.12b). The signal of \( m/z \) 137 for \( \text{L2-b2} \) could correspond to DMPD (calculated \( m/z \) (137) of [M+H]\(^+\)) as supported by optical analysis (Figure 3.10; vide supra). Both compounds, \( \text{L2-b} \) and \( \text{L2-b2} \), can oxidize faster in the presence of Cu(II) than metal-free and Zn(II) present environments (Figure 3.11). These resultant oxidized forms of \( \text{L2-b} \) and \( \text{L2-b2} \) may be related to the interaction and modulation of A\( \beta \) peptide aggregation observed above.

\[\text{3.2.7. Computational studies}\]

The values of the first and second ionization potentials (IP\(_1\) and IP\(_2\)) for DMPD, \( \text{L2-b} \), \( \text{L2-b1} \), \( \text{L2-b2} \), DPA1, DPA2, PMA1, and PMA2 were calculated (Figure 3.12). The overall observations for these values indicate that the compounds containing the DMPD...
functionality are shown to have lower IP values than those which do not have a DMPD group. As depicted in Figure 3.12, DMPD, L2-b, and L2-b2 (Figure 3.12; top row) display the singly occupied molecular orbitals (SOMOs) on the DMPD moiety, indicating that this structural portion can be easily oxidized, and thus could afford to the formation of a cationic radical of DMPD. Based on the IP₁ and IP₂ values of our chemical series, possible
oxidative transformation of compounds could be involved in their reactions with metal-free Aβ or metal-bound Aβ.

3.2.8. Blood-brain barrier (BBB) permeability prediction and free radical scavenging capability

The potential BBB permeability of the compounds, predicted by Lipinski’s rules and calculated logBB values (Table 3.1), was evaluated by the in vitro parallel artificial membrane permeability assay adapted for BBB (PAMPA-BBB).\textsuperscript{23,28,39-41} As summarized in Table 3.1, these theoretical and empirical values indicate that all compounds in our chemical library are potentially BBB permeable.

Table 3.1. Values (MW, clogP, HBA, HBD, PSA, logBB, and \(-\log P_e\))\textsuperscript{a} for DMPD,\textsuperscript{20} L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2.

<table>
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<tr>
<th>Calculation</th>
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<th>L2-b1</th>
<th>L2-b2</th>
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<th>DPA2</th>
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<td>31.4</td>
<td>37.8</td>
<td>44.3</td>
<td>38.9</td>
<td>42.1</td>
</tr>
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</table>
| logBB       | -0.139| 0.064 | 0.041 | -0.327| -0.206| -0.507| -0.446| \(> 0.3\) (readily); \(< -1.0\) (poorly)
| \(-\log P_e\) | 5.0 \pm 0.02 | 4.1 \pm 0.01 | 4.5 \pm 0.01 | 5.18 \pm 0.05 | 4.47 \pm 0.01 | 5.02 \pm 0.06 | 4.68 \pm 0.03 |
| CNS\textsuperscript{+} prediction\textsuperscript{a} | CNS\textsuperscript{+} | CNS\textsuperscript{+} | CNS\textsuperscript{+} | CNS\textsuperscript{+} | CNS\textsuperscript{+} | CNS\textsuperscript{+} | CNS\textsuperscript{+} |

\textsuperscript{a}MW, molecular weight; clogP, calculated logarithm of the octanol water partition coefficient; HBA, hydrogen bond acceptor atoms; HBD, hydrogen bond donor atoms; PSA, polar surface area; logBB = \(-0.0148 \times \) PSA + 0.152 \times clogP + 0.139 (logBB > 0.3, readily crosses BBB; logBB < \(-1.0\), poorly distributed to the brain); \(-\log P_e\) values were determined using the Parallel Artificial Membrane Permeability Assay adapted for BBB (PAMPA-BBB) were then calculated by the PAMPA 9 Explorer software v. 3.8. \textsuperscript{b}Prediction of a compound’s ability to penetrate the central nervous system (CNS) on the basis of literature values. Compounds categorized as CNS\textsuperscript{+} have the possibility to penetrate the BBB and are available in the CNS. In the case of compounds assigned as CNS\textsuperscript{-} have poor permeability through the BBB, therefore, their bioavailability into the CNS is considered to be minimal.

The free radical scavenging capability of DMPD, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 was measured by the Trolox (vitamin E analogue) equivalent antioxidant capacity (TEAC) assay, which can evaluate their ability to quench ABTS...
cation radicals \([\text{ABTS}^+; \text{ABTS} = 2,2'\text{-azino-bis}(3\text{-ethylbenzothiazoline-6-sulfonic acid})]\) in an organic solution\(^{42,43}\) and in a more biological environment (e.g., cell lysates).\(^{28}\) As depicted in Figure 3.13, DMPD,\(^{20}\) L2-b1, and L2-b2 are shown to scavenge free organic radicals more significantly than DPA1, DPA2, PMA1, and PMA2. In addition, L2-b2 presents a two-fold greater ability to quench radicals than Trolox in an organic solution (Figure 3.13). The trend of compounds for their free radical scavenging ability is expected from their lower IP\(_1\) and IP\(_2\) values (\textit{vide supra}, Figure 3.12). Moreover, DMPD, L2-b1, and L2-b2 indicate their free radical scavenging capacity within human neuroblastoma SK-N-BE(2)-M17 (M17) cell lysates (Figure 3.13, inset). Overall, DMPD, L2-b1, and L2-b2 are shown to serve as free radical scavenging agents, possibly being BBB permeable.

![Figure 3.13](image-url) Free radical scavenging capability of DMPD,\(^{20}\) L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 was identified by the TEAC assay in EtOH or employing SK-N-BE(2)-M17 (M17) cell lysates (inset). The TEAC values are relative to a vitamin E analogue, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

3.2.9. Regulation of toxicity induced by metal-free A\(\beta\)\(_{40}\) and metal–A\(\beta\)\(_{40}\) in living cells

More than 85% cell survival was exhibited when L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 were treated with M17 cells in the absence and presence of metal ions (Figure 3.14). The neuroprotective capability of L2-b2, with greater activities toward A\(\beta\), metal–A\(\beta\), and free radicals, was further examined against metal-free A\(\beta\)\(_{40}\)- or metal–A\(\beta\)\(_{40}\)-induced toxicity in M17 cells.\(^{23}\) Cell viability [ca. 84% for metal-free A\(\beta\)\(_{40}\); ca. 79% for A\(\beta\)\(_{40}\) with Cu(II); ca. 80% for A\(\beta\)\(_{40}\) with Zn(II)] was indicated (Figure 3.14a). Upon treatment
of L2-b2 to M17 cells preincubated with metal-free Aβ40 or Zn(II)–Aβ40, ca. 100% cell survival was observed (Figure 3.14a). Additionally, L2-b2 was observed to slightly recover the toxicity induced by Cu(II)–Aβ40 (Figure 3.14a). Overall, these cell experiments indicate that the series of compounds in this study are relatively nontoxic. Moreover, L2-b2 is able to attenuate metal-free Aβ40- and metal–Aβ40-induced cytotoxicity similar to DMPD and L2-b.

Figure 3.14. Viability of cells treated with L2-b1, L2-b2, DPA1, DPA2, PMA1, or PMA2 in the absence and presence of Aβ40 with and without CuCl2 or ZnCl2. (a) Viability of M17 cells treated with metal-free Aβ40 and metal-treated Aβ40, followed by the addition of L2-b2. M17 cells were treated with (b) various concentrations of ligands (2.5–50 μM), CuCl2/ligands ((c) 1:1 or (d) 1:2), or ZnCl2/ligands ((e) 1:1 or (f) 1:2; 1% v/v DMSO). Cell viability (%) was determined by the MTT assay compared to cells treated with DMSO only (0–1%, v/v). Error bars represent the standard error for three independent experiments.
3.3. Conclusion

For the better understandings of AD pathogenesis, it is required to apply chemical tools which can target and interact with multiple pathological features involved in AD (i.e., metal ions, metal-free and -associated Aβ, free radicals). Although several small molecules have been developed to modulate metal-free Aβ and/or metal–Aβ aggregation pathways, reduce free radical levels, and attenuate associated cytotoxicity, it has not been clearly studied which structural moieties are responsible for such activities. Thus, we have examined the interaction and reactivity of a rationally selected series of compounds toward metal-free Aβ, metal–Aβ, and free radicals. In our chemical library, DMPD, L2-b, and L2-b2 have the DMPD functionality in their structure and are shown to perform better abilities to alter metal-free Aβ and/or metal–Aβ aggregation pathways, scavenge free radicals, and present neuroprotective properties in living cells than other small molecules (L2-b1, DPA1, DPA2, PMA1, and PMA2) that have no DMPD motif. L2-b and L2-b2 that contain a structural moiety of DMPD can interact with various Aβ species, from the monomer to fibrils in the absence and/or presence of metal ions. Moreover, the DMPD moiety is indicated to be related to the oxidization of these compounds, which could be applied to antioxidant properties and possibly linked to the redirecting activity toward Aβ aggregation pathways. In addition, a dimethylamino group, a structural portion of DMPD, is also observed to direct the reactivity of small molecules depending on its position at the overall structure. The dimethylamino functionality itself, however, may not be enough to present its reactivity toward the targets (particularly, metal-free Aβ and metal-bound Aβ). Taken together, our studies demonstrate that DMPD is a pivotal structural motif in the interaction with multiple pathological factors (e.g., metal-free Aβ and metal-bound Aβ, metal ions, free radicals) found in AD. Thus, such a structural group can be utilized for the development of better chemical tools and therapeutics for protein-misfolding diseases.

3.4. Experimental section

3.4.1. Materials and methods

All reagents and solvents were purchased from commercial suppliers and used as
received unless otherwise stated. Bis(pyridin-2-ylmethyl)amine (DPA1), pyridin-2-ylmethanamine (PMA1), 6-(aminomethyl)-N,N-dimethylpyridin-3-amine (PMA2), and N1,N1-dimethylbenzene-1,4-diamine (DMPD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N1,N1-dimethyl-N4-(pyridin-2-ylmethyl)benzene-1,4-diamine (L2-b) was synthesized as previously reported methods.23 N,N-dimethyl-6-((phenylamino)methyl)pyridin-3-amine (L2-b1), N1-((5-(dimethylamino)pyridin-2-yl)methyl)-N1,N1-dimethylbenzene-1,4-diamine (L2-b2), and 6-(((5-(dimethylamino)pyridin-2-yl)methyl)amino)methyl)-N,N-dimethylpyridin-3-amine (DPA2) were synthesized as previously reported procedures (vide infra).23,27 Aβ40 and Aβ42 were purchased from AnaSpec (Fremont, CA, USA) (Aβ40 = DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGGVV; Aβ42 = DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA). An Agilent 8453 UV–Visible (UV–Vis) spectrophotometer (Santa Clara, CA, USA) was used to measure the optical spectra. Transmission electron microscopic (TEM) images were taken using a Philips CM-100 transmission electron microscope (Microscopy and Image Analysis Laboratory, University of Michigan, Ann Arbor, MI, USA) or a JEOL JEM-2100 transmission electron microscope (UNIST Central Research Facilities, Ulsan, Republic of Korea). Absorbance values for biological assays, including cell viability assay, Parallel Artificial Membrane Permeability Assay adapted for Blood-Brain Barrier (PAMPA-BBB), Trolox Equivalent Antioxidant Capacity (TEAC) assay were measured on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Bruker HCT basic system mass spectrometer equipped with electrospray ionization (ESI) ion source was used to obtain time-dependent mass spectra of L2-b or L2-b2 with and without metal ions [Cu(II) or Zn(II)].

3.4.2. Preparation of N,N-dimethyl-6-((phenylamino)methyl)pyridin-3-amine (L2-b1)

L2-b1 was synthesized with slight modifications of the previously reported method.23 To a nitrogen purged flame dried flask, 150 mg (1.6 mmol) of aniline was added and dissolved in 15 mL of EtOH that was dried over molecular sieves. Then 285 mg (1.9 mmol) of 5-dimethylamino-2-pyridinecarboxaldehyde was added and the solution was allowed to stir at 45 °C for 10 min, and increased the temperature to 90 °C for 1 h. The
solution was allowed to cool to room temperature and was concentrated until precipitates were formed. The resulting solid precipitate was then dissolved in dry MeOH and cooled to 0 °C in a nitrogen-purged round-bottom flask. Then 307 mg (8.1 mmol) of sodium borohydride (NaBH₄) was slowly added and the solution was allowed to stir at 0 °C for 5 min. After warming up the solution to room temperature, the resulting solution was stirred for 45 min. The reaction mixture was then quenched with water, extracted three times with dichloromethane (CH₂Cl₂), washed once with brine, and concentrated. The crude products were purified by column chromatography (SiO₂; EtOAc:Et₃N 100:0.1; Rᵣ = 0.54) followed by recrystallization with Et₂O and Hexanes (Hx) (260 mg, 1.1 mmol, 71%).

\[
\begin{align*}
1^H \text{NMR} & \ (400 \text{ MHz, CD}_2\text{Cl}_2) / \delta (\text{ppm}): 8.10 \ (2H, d, J = 3.0 \text{ Hz}), \ 7.15 \ (3H, m), \ 7.00 \ (2H, dd, J = 8.6, 3.0 \text{ Hz}), \ 6.66 \ (3H, m), \ 4.75 \ (1H, \text{s(broad)}), \ 4.30 \ (2H, d, J = 5.3 \text{ Hz}), \ 2.95 \ (6H, s), \\
13^C \text{NMR} & \ (100 \text{ MHz; CD}_2\text{Cl}_2) / \delta (\text{ppm}): 149.0, \ 146.2, \ 146.0, \ 134.7, \ 129.6, \ 122.0, \ 120.0, \ 117.5, \ 113.4, \ 49.0, \ 40.6. \ \text{HRMS: Calcd for M}^+, \ 227.1422; \ \text{Found,} \ 227.1427.
\end{align*}
\]

3.4.3. Preparation of \(N^1\)-(5-(dimethylamino)pyridin-2-yl)methyl-\(N^4,N^4\)-dimethylbenzene-1,4-diamine (L2-b2)

L2-b2 was synthesized with slight modifications of the previously reported method.\(^23\) 50 mg (0.4 mmol) of DMPD was added into a flame-dried flask under nitrogen and then dissolved in 10 mL of ethanol that was treated with over molecular sieves. 70 mg (0.5 mmol) of 5-dimethylamino-2-pyridinecarboxaldehyde was added and stirred for 10 min at 45 °C, followed by stirring at 90 °C for 1 h. The resulting mixture was then allowed to cool to room temperature and the solvent was removed \textit{in vacuo}. Dry methanol (MeOH) was then added to the flask under nitrogen and cooled down to 0 °C. A portion of NaBH₄ (70 mg, 1.9 mmol) was slowly introduced with stirring at 0 °C for 5 min, followed by stirring for 45 min at room temperature. The reaction mixture was then quenched with water, extracted three times with CH₂Cl₂, washed once with brine, and concentrated. The crude products were purified by column chromatography (SiO₂, 100:1 EtOAc:Et₃N; \(Rᵣ = 0.29, \ 68 \text{ mg, 0.3 mmol, 70}\%\)).

\[
\begin{align*}
1^H \text{NMR} & \ (400 \text{ MHz, CD}_2\text{Cl}_2) / \delta (\text{ppm}): 8.09 \ (1H, d, J = 3.0 \text{ Hz}), \ 7.15 \ (1H, d, J = 8.6 \text{ Hz}), \ 6.99 \ (1H, dd, J = 8.6, 3.0 \text{ Hz}), \ 6.69 \ (2H, d, J = 8.9 \text{ Hz}), \ 6.62 \ (2H, d, J = 8.9 \text{ Hz}), \ 4.25 \ (1H, \text{s (broad)}), \ 2.95 \ (6H, s), \ 2.79 \ (6H, s). \ \text{HRMS: Calcd for M}^+, \ 227.1422; \ \text{Found,} \ 227.1427.
\end{align*}
\]
CD<sub>2</sub>Cl<sub>2</sub>/δ (ppm): 147.0, 145.8, 14.6, 141.4, 134.8, 122.1, 120.0, 116.0, 114.8, 50.1, 42.4, 40.6, HRMS: Calcd for M<sup>+</sup>, 270.1845; Found, 270.1846.

3.4.4. Preparation of 6-(((5-(dimethylamino)pyridin-2-yl)methyl)amino)methyl)-N,N-dimethyl pyridin-3-amine (DPA2)

DPA2 was synthesized with slight modifications of the previously reported method<sup>27</sup>. A dried 100 mL round-bottom flask, equipped with a magnetic stir bar, was charged with 5-(dimethylamino)picolinonitrile (100 mg, 0.7 mmol) in dry MeOH (20 mL). To the resulting mixture was added Pd/C (10 wt %; 150 mg, 1.6 mmol) at room temperature. The solution was stirred under N<sub>2</sub> for 10 min and then under H<sub>2</sub> for 5 or 6 h at room temperature. The Pd/C was filtered through the Celite and washed with cold MeOH (2 x 15 mL). To the combined MeOH was slowly added 4 M HCl (0.4 mL), which became light yellow, with gentle vortexing. The mixture was concentrated in vacuo, generating light yellow products, and purified by recrystallization (MeOH/Et<sub>2</sub>O). The final product (yellow powder) was washed out with two solvents (CH<sub>2</sub>Cl<sub>2</sub> and Et<sub>2</sub>O) via vacuum filtering (SiO<sub>2</sub>, MeOH:DCM = 1:10; R<sub>f</sub> = 0.70; 66%). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD) /δ (ppm): 8.25 (2H, d, J = 3.1 Hz), 8.07 (2H, d, J = 9.2 Hz), 7.83 (2H, dd, J = 4.6, 1.6 Hz), 4.68 (4H, s), 3.18 (12H, s). <sup>13</sup>C NMR (100 MHz; CD<sub>3</sub>OD) /δ (ppm): 149.7, 130.7, 130.4, 127.4, 126.4, 47.8, 40.3, HRMS: Calcd for [M + H]<sup>+</sup>, 286.2026; Found, 286.2024.

3.4.5. Aβ aggregation experiments

Experiments with Aβ were conducted according to previously published methods<sup>20-30</sup>. To prepare Aβ peptides, either Aβ<sub>40</sub> or Aβ<sub>42</sub> was dissolved in ammonium hydroxide (NH<sub>4</sub>OH, 1% v/v, aq), aliquoted, lyophilized overnight, and stored at -80 °C. For the experiments, a stock solution of Aβ was prepared by dissolving lyophilized peptide in 1% NH<sub>4</sub>OH (10 μL) and diluting with ddH<sub>2</sub>O. The concentration of Aβ peptides in the solution was determined by measuring the absorbance of the solution at 280 nm (ε = 1450 M<sup>-1</sup> cm<sup>-1</sup> for Aβ<sub>40</sub>; ε = 1490 M<sup>-1</sup> cm<sup>-1</sup> for Aβ<sub>42</sub>). The peptide stock solution was diluted to a final concentration of 25 μM in chelex-treated buffered solution containing HEPES [4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 20 μM, pH 6.6 for Cu(II) samples;
pH 7.4 for metal-free and Zn(II) samples] and 150 µM NaCl. For the inhibition studies, DMPD, L2-b, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 [50 µM; 1% v/v dimethyl sulfoxide (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 24 h. For the disaggregation studies, Aβ with and without metal ions was incubated for 24 h at 37 °C with constant agitation. DMPD, L2-b, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 were added afterward to the solution containing Aβ aggregates, and incubated for additional 24 h at 37 °C.

3.4.6. Gel electrophoresis and Western blot

Molecular weight (MW) distribution of samples from the inhibition and disaggregation experiments were analyzed by gel electrophoresis and followed by Western blot (Gel/Western blot) using an anti-Aβ antibody (6E10). Each sample (10 µL) was separated on a 10–20% Tris-tricine gel (Invitrogen, Grand Island, NY, USA) and transferred onto nitrocellulose membrane which was blocked with bovine serum albumin (BSA) solution (3% w/v; Sigma Aldrich) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature. Then, the membranes were incubated with a primary antibody (6E10, Covance, Princeton, NJ, USA; 1:2,000) in a solution of 2% w/v BSA (in TBS-T) overnight at 4 °C. After washing with TBS-T (3x, 10 min each), the horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000; Cayman Chemical Company, Ann Arbor, MI, USA) in 2% BSA (in TBS-T) was added to the membrane and incubated for 1 h at room temperature. SuperSiganl West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) was used to visualize protein bands.

3.4.7. Transmission electron microscopy (TEM)

Samples for transmission electron microscopy (TEM) were prepared according to previously reported methods. Glow-discharged grids (Formar/Carbon 300-mesh, Electron Microscopy Sciences, Hatfield, PA, USA) were treated with samples from the inhibition and disaggregation experiments (5 µL) for 2 min at room temperature. Excess sample was removed by blotting carefully with filter paper then washed twice with ddH₂O. Each grid was treated with uranyl acetate (1% w/v ddH₂O, 5 µL) for 1 min. Excess stain
was blotted off and the grids were air dried for at least 20 min at room temperature. Images from each sample were taken on a Philips CM-100 (80 kV) or a JEOL JEM-2100 TEM (200 kV) at 25,000x magnification.

3.4.8. Determination of acidity constants for ligands

The acidity constants (pK\textsubscript{a}) for L\textsubscript{2-b1}, DPA\textsubscript{1}, DPA\textsubscript{2}, PMA\textsubscript{1}, and PMA\textsubscript{2} were determined by UV–Vis variable-pH titrations based on a previously published procedure.\textsuperscript{26-29} To obtain pK\textsubscript{a} values for a ligand, a solution (100 mM NaCl, 10 mM NaOH, pH 12) of L\textsubscript{2-b1}, DPA\textsubscript{1}, DPA\textsubscript{2}, PMA\textsubscript{1}, and PMA\textsubscript{2} (30 \SI{}{\mu M}) was titrated with small aliquots of HCl to acquire at least 30 spectra in the range from pH 2.0–9.0. The pK\textsubscript{a} values were calculated using the program HypSpec (Protonic Software, Leeds, UK).\textsuperscript{44} Speciation diagrams of ligands were modeled using the HySS2009 program (Protonic Software, Leeds, UK).\textsuperscript{45}

3.4.9. Metal binding studies

The interaction of L\textsubscript{2-b1}, L\textsubscript{2-b2}, DPA\textsubscript{1}, DPA\textsubscript{2}, PMA\textsubscript{1}, and PMA\textsubscript{2} with Cu(II) and Zn(II) was monitored by UV–Vis and \textsuperscript{1}H NMR, along with previously reported procedures.\textsuperscript{23,24,26,30} A solution of L\textsubscript{2-b1} and L\textsubscript{2-b2} [50 \SI{}{\mu M} and 25 \SI{}{\mu M}, respectively, in acetonitrile (CH\textsubscript{3}CN)], DPA\textsubscript{1}, DPA\textsubscript{2}, PMA\textsubscript{1}, and PMA\textsubscript{2} (50 \SI{}{\mu M} in EtOH) was treated with 1 to 5 (for L\textsubscript{2-b1}, DPA\textsubscript{1}, DPA\textsubscript{2}, PMA\textsubscript{1}, and PMA\textsubscript{2}) or 1 to 2 (for L\textsubscript{2-b2}) equiv of CuCl\textsubscript{2} or ZnCl\textsubscript{2}, and incubated at room temperature for 30 min (for L\textsubscript{2-b1} and L\textsubscript{2-b2}) or 10 min (for DPA\textsubscript{1}, DPA\textsubscript{2}, PMA\textsubscript{1}, and PMA\textsubscript{2}). The optical spectra of the resulting solutions were measured by UV–Vis. In addition, the interaction of L\textsubscript{2-b1}, PMA\textsubscript{1}, or PMA\textsubscript{2} with ZnCl\textsubscript{2} was observed by \textsuperscript{1}H NMR. ZnCl\textsubscript{2} (1 equiv) was added to a solution of L\textsubscript{2-b1}, PMA\textsubscript{1}, or PMA\textsubscript{2} (4 mM) in acetonitrile-d\textsubscript{3} (CD\textsubscript{3}CN).

3.4.10. Spectrophotometric studies

All samples were prepared in Chelex-treated buffered solution [HEPES 20 \SI{}{\mu M}, pH 6.6 for Cu(II) samples or pH 7.4 for metal-free and Zn(II) samples, 150 \SI{}{\mu M} NaCl]. For Aβ-free samples, L\textsubscript{2-b}, or L\textsubscript{2-b2} (50 \SI{}{\mu M}) was treated with CuCl\textsubscript{2} or ZnCl\textsubscript{2} (25 \SI{}{\mu M}) for 2 min. For Aβ-containing samples, Aβ\textsubscript{40} (25 \SI{}{\mu M}) was treated with CuCl\textsubscript{2} or ZnCl\textsubscript{2} (25 \SI{}{\mu M}) for 2
min, and then L2-b, or L2-b2 was added. The absorption spectra of the resulting solution was obtained at 1, 10/30 min, 4, 12, and 24 h incubation time points at room temperature without agitation. Metal-free samples with and without Aβ40 were also monitored by UV–Vis in an anaerobic environment. All solvents required for the preparation of the anaerobic samples were degassed by freeze-pump-thaw cycling three times and stored in a N2-filled glove box. Anaerobic samples were prepared in a N2-filled glove box. UV–Vis spectra were recorded at 0, 4, 12, and 24 h incubation time points at room temperature without agitation.

3.4.11. Docking studies of compounds with metal-free Aβ40 and Zn(II)-bound Aβ16 monomers.

Flexible ligand docking studies for DMPD, L2-b, L2-b1, L2-b2, DPA1, DPA2 PMA1, or PMA2 against metal-free Aβ40 and Zn(II)-bound Aβ16 monomer were conducted using AutoDock Vina.46 The starting conformations of the monomer and fiber were obtained from NMR structures of Aβ40 monomer (PDB 2LFM),33 and Zn(II)–Aβ16 monomer (PDB 1ZE9).34 A model for compounds was first constructed and energy minimized using the MMFF94 energy minimization function in ChemBio3D Ultra 11.0 to optimize their structure for the docking studies. Individual structures of monomeric metal-free Aβ40 or Zn(II)–Aβ40 conformations and compounds were prepared in AutoDock Tools and imported into PyRx. The search space was constrained to the dimensions of the peptide. The exhaustiveness for the docking runs was set at 1024. Docked models of the compounds were visualized with Aβ species using Pymol (The PyMOL Molecular Graphics System, Schrödinger, LLC).

3.4.12. 2D NMR spectroscopy

The interaction of Aβ40 with L2-b, L2-b1, and L2-b2 was monitored by 2D 1H-15N SOFAST-HMQC NMR at 10 °C.47 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 PO4, pH
7.4, 50 mM NaCl; 7% v/v D$_2$O). Each spectrum was obtained using 64 complex $t_1$ points and a 0.1 sec recycle delay on a Bruker Avance 600 MHz spectrometer. The 2D data was processed using TOPSPIN 2.1 (from Bruker). Resonance assignment was performed with SPARKY 3.1134 using published assignments for Aβ$_{40}$ as a guide.$^{31,48}$

### 3.4.13. Saturation transfer difference (STD) NMR spectroscopy

For the STD NMR experiments, a 150 μM solution of fibrillar Aβ$_{42}$ was prepared through incubation for 24 h at 37 °C with constant agitation in 10 mM deuterated Tris-DCl, 95% D$_2$O with or without ZnCl$_2$ (80 μM) at pD 7.4 (corrected for the isotope effect). The samples for STD experiments were prepared by diluting fibrils to 1 μM (effective monomer concentration) into 10 mM deuterated Tris-DCl to which was added 100 μM of ligand (0.5% DMSO-d$_6$). 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$^{47,49,50}$) or 40 ppm (off resonance) with a total saturation time of 2 sec on a Bruker 600 MHz (metal-free samples) or 900 MHz spectrometer (Zn(II) samples).$^{51}$ 1024 scans were recorded for the reference spectrum at 25. An inter-scan delay of 1 sec was used for both the STD and the reference experiments.

### 3.4.14. Mass spectrometric studies

Samples containing L2-b or L2-b2 (50 μM) and Aβ$_{40}$ (25 μM) for experiments were prepared in 100 μL of 1 mM NH$_4$OAc (pH 7.4). The resulting solutions were incubated for 0, 4, 12, and 24 h at 37 °C with constant agitation. The samples were directly injected into the mass spectrometer at a flow rate of 240 μL/h. ESI interface was operated in positive-ion mode; spray voltage was set at 4.5 kV with capillary temperature at 300 °C and capillary exit voltage at 101 V. Mass spectra were taken in the range of m/z 50–500.

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

PAMPA-BBB experiments of compounds were carried out using the PAMPA Explorer kit (pION, Inc. Billerica, MA, USA) with modifications to previously reported protocols.$^{23,26,28,39-41}$ Each stock solution was diluted with Prisma HT buffer (pH 7.4, pION)
to a final concentration of 25 μM (1% v/v final DMSO concentration). The resulting solution was added to the wells of the donor plate (200 μL, number of replicates = 12). BBB-1 lipid formulation (5 μL, pION) was used to coat the polyvinylidene fluoride (PVDF, 0.45 μM) filter membrane on the acceptor plate. This acceptor plate was placed on top of the donor plate forming a sandwich. Brain sink buffer (BSB, 200 μL, pION) was added to each well of the acceptor plate. The sandwich was incubated for 4 h at room temperature without stirring. UV–Vis spectra of the solutions in the reference, acceptor, and donor plates were measured using a microplate reader. The PAMPA Explorer software, v. 3.8 (pION), was used to calculate the value of –logPₑ for each compound. CNS± designations were assigned by comparison to compounds that were identified in previous reports. 

3.4.16. Trolox equivalent antioxidant capacity (TEAC) assay

The free radical scavenging capacity of DMPD, L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 were determined by the TEAC assay in EtOH or employing lysates of human neuroblastoma SK-N-BE(2)-M17 (M17) cells purchased from ATCC (Manassa, VA, USA). (a) The assay in solution was performed according to a previously reported method with slight modifications. To generate blue ABTS cation radicals [ABTS⁺⁺; ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Sigma], ABTS (7.0 mM, Sigma, MO) with potassium persulfate (2.5 mM) was dissolved in 5 mL of water and incubated for 16 h in the dark at room temperature. The resulting solution of ABTS⁺⁺ was diluted with EtOH to absorbance of ca. 0.7 at 734 nm. The solution of ABTS⁺⁺ (200 μL) was added to the wells of a clear 96 well plate and incubated at room temperature for 5 min in the plate reader. Various final concentrations (0, 1, 2.5, 5, 7.5, 10, 15, and 20 μM) of DMPD, L2-b1, L2-b2, DPA1, DPA2, PMA1, PMA2, or Trolox (Trolox = 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; dissolved in EtOH) were treated and incubated with the ABTS⁺⁺ solution at room temperature for 10 min. The percent inhibition was calculated according to the measured absorbance at 734 nm [% inhibition = 100 × (A₀ - A)/A₀] and was plotted as a function of ligand concentration. The TEAC value of compounds for each time point was calculated as a ratio of the slope of the compound to that of Trolox. The measurements were carried out in triplicate. (b) The assay employing cell lysates was conducted following the protocol of the antioxidant
assay kit purchased from Cayman Chemical Company (Ann Arbor, MI, USA), with minor modifications. M17 cells were cultured in media containing 1:1 Minimum Essential Media (MEM, GIBCO, Grand Island, NY), Ham’s F12K Kaighn’s Modification Media (F12K, GIBCO), 10% (v/v) fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, USA GIBCO), 100 U/mL penicillin (GIBCO), and 100 mg/mL streptomycin (GIBCO). The cells were grown and maintained at 37 °C in a humidified atmosphere with 5% CO2. For the antioxidant assay using cell lysates, cells were seeded in a 6 well plate and grown to approximately 80-90% confluence. Cell lysates were prepared following a previously reported method with modifications.  M17 cells were washed once with cold PBS (pH 7.4, GIBCO) and harvested by gently pipetting off adherent cells with cold PBS. The cell pellet was generated by centrifugation (2,000 x g for 10 min at 4 °C). This cell pellet was sonicated on ice (5 sec pulses, 3 times with 20 sec intervals between each pulse) in 2 mL of cold Assay Buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% NaCl and 0.1% glucose). The cell lysates were centrifuged at 5,000 x g for 10 min at 4 °C. The supernatant was removed and stored on ice until use. To standard and sample 96 wells, 10 μL of the supernatant of cell lysates was delivered followed by addition of compound, metmyoglobin, ABTS, and H2O2 in order. After 5 min incubation at room temperature on a shaker, absorbance values at 750 nm were recorded. The final concentrations (0.045, 0.090, 0.135, 0.180, 0.225, and 0.330 mM) of compounds and Trolox were used. The antioxidant concentration was calculated according to the measured absorbance (% Inhibition = (A0 – A)/A0, where A0 is absorbance of the supernatant of cell lysates). The measurements were conducted in triplicate.

3.4.17. Cell viability measurement

M17 cells were seeded in a 96-well plate (15,000 cells in 100 μL per well), according to previously reported methods. These cells were treated with various concentrations of L2-b1, L2-b2, DPA1, DPA2, PMA1, and PMA2 (0-10 μM, 1% v/v DMSO) in the absence and presence of CuCl2 or ZnCl2 (1:1 or 1:2 metal/ligand ratio) with and without Aβ40 (Aβ : metal : ligand = 10 : 10 : 20 μM). After 24 h incubation at 37 °C, 25 μL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5 mg/mL in phosphate buffered saline (PBS), pH7.4, GIBCO] was added to each well and the plates
were incubated for 4 h at 37 °C. Formazan produced by the cells was dissolved in a solution containing $N,N$-dimethylformamide (DMF, 50% v/v aq, pH 4.5) and sodium dodecyl sulfate (SDS, 20% w/v) overnight at room temperature. Subsequently, absorbance at 600 nm was measured on a microplate reader.

3.5. Acknowledgment

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3.6. References


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Chapter 4: Interaction and Reactivity of Human Serum Albumin with Metal-free and Metal-associated Amyloid-β Peptides

This chapter is based on work that will be submitted for publication. We thank Professor Hugh I. Kim and Taesu Choi at Pohang University of Science and Technology (POSTECH) for ESI-MS, IM-MS, SAXS, and CID analysis for HSA–Aβ interaction (results and discussion are not shown in the chapter due to incomplete studies); Dr. Yeon Ju Kwak for cell viability studies. I was involved in the experiments of the ThT assay, gel electrophoresis with Western blot, and TEM investigations for identifying Aβ aggregation, as well as cell imaging studies for identifying cellular membrane permeability of Aβ with HSA.

4.1. Introduction

The cause and mechanism of the onset and progression of Alzheimer’s disease (AD) have not been clearly understood.1-8 Among potential pathological features, the aggregation of misfolded proteins, such as amyloid-β (Aβ), have been suggested to be involved in AD etiology.1-8 Especially, soluble and structured oligomeric forms are considered as toxic species.1-8 In addition, previous studies demonstrate that highly concentrated metal ions [i.e., Cu(I/II), Zn(II)] are co-localized in senile plaques (ca. 0.4 and 1.0 mM, respectively) in the AD affected brain.1-9 These metal ions can bind to Aβ peptides and facilitate the peptide aggregation.1-10 Moreover, Aβ peptides have been presented to bind to or interact with various other proteins. In particular, more than ca. 90% of Aβ peptides are bound to human serum albumin (HSA) in human blood plasma.11-19

HSA is the most abundant protein of blood serum and is composed of three hydrophobic domains which can bind to hormones, metal ions, other proteins, or drugs endogenously or exogenously via electrostatic or hydrophobic interactions as well as is observed to transport them.11-16 In the pharmaceutical industry, HSA has been of interest due to their delivery ability.11-16 Since HSA is able to transport proteins, such as Aβ, it is possibly able to decrease the risk of AD by regulating the equilibrium of Aβ between the brain and blood plasma across the blood-brain barrier (BBB).11,15,18 HSA could bind to Aβ
by hydrophobic interactions (with the hydrophobic region of Aβ); upon binding of HSA with Aβ, Aβ aggregation is suggested to be inhibited or interfered.\textsuperscript{11,15,20} Additionally, HSA is presented to serve as a physiological transporter for the essential metal ions, such as Cu(II) and Zn(II), in the blood serum.\textsuperscript{14,19,22} Although Aβ peptides with metal ions, HSA with Aβ, and HSA with metal ions have been studied individually, the interrelationships among all three of Aβ, metal ions, and HSA have been rarely interrogated.

Herein, we report the direct interactions of HSA with metal-free Aβ or metal-associated Aβ (metal–Aβ) as well as its influences on the peptide aggregation. Furthermore, we present the ability of HSA to attenuate cytotoxicity induced by metal-free Aβ or metal–Aβ and penetrate through the cellular membrane with Aβ peptides in both the absence and presence of metal ions. From our studies, HSA is observed to affect metal-free Aβ aggregation as similar to previous studies,\textsuperscript{11,13} as well as metal–Aβ aggregation. HSA is not shown to inhibit Aβ aggregation but delay or redirect the aggregation pathways to produce possibly less toxic species. Taken together, our studies with HSA, Aβ, and metal ions found in the brain of AD, suggest the potential communication between these three elements via direct interactions, indicating the possible link of protein–protein-small molecule (metals) networks to AD onset and progression.

4.2. Results and discussion

4.2.1. Investigations of the influence of HSA on Aβ\textsubscript{40} aggregation pathways by thioflavin-T (ThT) assay

The thioflavin-T (ThT) assay is generally used to observe the degree of Aβ fibrillization by measuring the fluorescence intensity induced by binding of ThT to Aβ fibrils.\textsuperscript{12,20,23,24} We applied the ThT assay to monitor the influence of HSA on the formation of metal-free Aβ and Zn(II)–Aβ fibrils. Samples were prepared for the inhibition and disaggregation experiments. For the inhibition experiments (Figure 4.1a), freshly dissolved Aβ with or without Zn(II) was incubated with HSA. For disaggregation experiments (Figure 4.2a), Aβ aggregates were preformed by incubating without HSA for
24 h, and then treated with HSA for additional 24 h incubation. The Aβ fibrilization growth curve was obtained from the ThT assay (Figure 4.1b). Once Aβ peptides were incubated with HSA, the kinetics of the Aβ fibril production were modulated to be delayed in both the absence and presence of Zn(II). As depicted in Figure 4.1b (left), under metal-free conditions, HSA could slow down the generation of Aβ fibrils at early stages and alter the aggregation pathway. Up to 12 h incubation, Aβ samples with HSA showed the less

![Diagram](image)

**Figure 4.1.** Influence of HSA toward both metal-free Aβ40 and metal–Aβ40 aggregation pathways. (a) Scheme of the inhibition experiment. (b) Analysis of Aβ40 fibril formation from the samples incubated with metal-free Aβ40 (left) or Zn(II)–Aβ40 (right) in the absence and presence of HSA, monitored by the ThT assay. (c) TEM images of the 24 h incubated samples. Conditions: [Aβ40] = 20 μM; [ZnCl2 or CuCl2] = 20 μM; HSA = 20 μM; pH 6.6 (for Cu(II) experiment) or pH 7.4 (for metal-free and Zn(II) experiments); 37 °C; constant agitation.
fluorescence intensity than the HSA-free samples, indicating that less Aβ fibrils were formed. The ability of HSA to postpone the generation of metal-free Aβ fibrils is consistent with previous studies.\textsuperscript{12,20,23} Since HSA and Aβ are indicated to bind directly,\textsuperscript{12,15,17,23,25} the HSA–Aβ complex formation may be able to affect and alter Aβ aggregation pathways. Furthermore, when Zn(II) was introduced to Aβ, the fiber generation was slower than that observed from metal-free conditions, as previously reported;\textsuperscript{1-4,10,20} HSA could make the Zn(II)–Aβ fibrillization process much slower. A recent report presents that high negative charges on the surface of HSA could stabilize the protonated form of H13 of Aβ peptide, possibly resulting in weakened Zn(II) binding to Aβ and redirection of the peptide aggregation pathways.\textsuperscript{20} The fibrillization pathway of Zn(II)–Aβ without HSA reached the maximum of fluorescence after 36 h incubation, while Zn(II)–Aβ with HSA presented only ca. 20% of the maximum fluorescence intensity after 36 h incubation; upon 72 h incubation of Zn(II)–Aβ with HSA, the samples exhibited ca. 91% of the maximum
fluorescence intensity. Compared to the metal-free case, HSA is more noticeably observed to postpone the formation of Zn(II)–Aβ fibrils. In contrast, no difference in fluorescence intensity from the samples of Aβ with or without HSA was indicated in the disaggregation experiment in both the absence and presence of Zn(II) (Figure 4.2b). These results suggest that HSA may not interact with higher order of Aβ aggregates (e.g., higher order oligomers, protofibrils, fibrils), and disassemble or alter further aggregation from the peptide aggregates. Taken together, the results from both inhibition and disaggregation experiments, observed by the ThT assay, imply that HSA may prefer to interact with monomeric or smaller-sized Aβ species over high molecular weight (MW) species, and could affect the aggregation pathways of both metal-free Aβ and Zn(II)–Aβ.

4.2.2. Morphologies of Aβ40 upon treatment with HSA, monitored by transmission electron microscopy (TEM)

The morphologies of metal-free Aβ and metal–Aβ aggregates incubated with or without HSA were monitored by TEM. From the inhibition experiments, after 24 h incubation, metal-free Aβ and metal–Aβ aggregates with HSA presented more amorphous and thinner fibers than the Aβ samples without HSA (Figure 4.1c). In the disaggregation experiments, there was no significant difference between HSA-treated and -untreated preformed Aβ aggregates (Figure 4.2c). As anticipated from the results of the ThT assay, gel electrophoresis (vide infra), and TEM, HSA may interact with Aβ monomers or smaller-sized species over higher order aggregates, causing morphological differences depicted only in the inhibition studies.

4.2.3. Visualization of HSA–Aβ40 complexes by gel electrophoresis

As the ThT assay displayed the delay of metal-free Aβ and Zn(II)–Aβ aggregation by HSA, the MW distribution of the resultant Aβ species with or without the treatment of HSA was visualized by gel electrophoresis followed by staining with coomassie blue (Figure 4.3, left) and Western blotting with an anti-Aβ antibody, 6E10 (gel/Western blot; Figure 4.3, right).26-32 Clear bands around 70 and 140 kDa were detected from both coomassie blue stained and Western blotted gels. These bands are possibly corresponding to the complexes of HSA and metal-free Aβ or metal–Aβ [HSA monomers
Figure 4.3. Gel analysis of the formation of HSA–Aβ40 complexes in both the absence and presence of metal ions. Resultant Aβ40 species after (a) 4, (b) 8, (c) 12, and (d) 24 h incubation with or without HSA were visualized by gel electrophoresis with coomassie blue staining (left) and Western blotting using an anti-Aβ antibody (6E10; right). Lanes: (1) Aβ40; (2) Aβ40 + HSA; (3) Aβ40 + ZnCl₂; (4) Aβ40 + ZnCl₂ + HSA; (5) Aβ40 + CuCl₂; (6) Aβ40 + CuCl₂ + HSA. Conditions: [Aβ40] = 20 μM; [ZnCl₂ or CuCl₂] = 20 μM; HSA = 20 μM; pH 6.6 (for Cu(II) experiment) or pH 7.4 (for metal-free and Zn(II) experiments); 4, 8, 12, or 24 h incubation; 37 °C; constant agitation.
or dimers with monomeric or small-sized Aβ or metal–Aβ based on the MWs of HSA and Aβ (around 66.5 and 4.3 kDa, respectively). Such formation of these HSA–Aβ complexes may delay and redirect Aβ aggregation pathways indicated in Figure 4.1b. In addition, since HSA–Aβ complexes are presented from 4 to 24 h incubated samples, HSA may interact with monomeric or small-sized metal-free Aβ or metal–Aβ species and be stabilized as complex forms.

4.2.4. Regulation of metal-free Aβ- and metal–Aβ-induced toxicity by HSA in living cells

The neuroprotective properties of HSA against metal-free Aβ- and metal–Aβ-induced toxicity were examined in SH-SY5Y (5Y) cells. Upon incubation of 5Y cells with metal-free Aβ, ca. 80-85% of viability was shown after 4, 8, 12, and 24 h incubation. With the treatment of HSA to 5Y cells incubated with Aβ, cell viability was recovered by more than ca. 15% (Figure 4.4a). In addition, HSA could attenuate cytotoxicity triggered by metal–Aβ as well [ca. 15% and 11% for Zn(II)–Aβ (Figure 4.4b) and Cu(II)–Aβ (Figure 4.4c)]. These results may be caused from the formation of HSA–Aβ complexes which can redirect Aβ aggregation, possibly generating less toxic species than HSA-free Aβ species. Moreover, since HSA has the anti-oxidant activity,14,33,34 HSA might reduce oxidative stress in cells induced by metal–Aβ species attenuating cytotoxicity. Overall, our cell studies suggest that HSA may have the neuroprotective property against metal-free Aβ and metal–Aβ.

4.2.5. Cellular membrane permeability of Aβ by HSA

Aβ peptides are suggested to be able to be penetrated into 5Y cells.35,36 Since intracellular Aβ species could be more critical and toxic than extracellular Aβ species, it would be valuable to evaluate if HSA could transport Aβ species through cellular membranes. In order to investigate the cellular membrane permeability of Aβ peptides, Hylite Fluor 488 conjugated Aβ40 (HF488Aβ40) was introduced to two different cellular media (fetal bovine serum (FBS) containing and FBS-free media) which were treated to 5Y cells. HF488Aβ40 peptides can be traced by monitoring green HF488 fluorescence by a
Figure 4.4. Effect of HSA on toxicity induced by metal-free Aβ and metal–Aβ species in human neuroblastoma SH-SY5Y (5Y) cells. The cell viability (%) of 5Y cells incubated with (a) metal-free Aβ40 as well as (b) Zn(II)- and (c) Cu(II)-associated Aβ40 with or without HSA for 4, 8, 12, and 24 h, monitored by the MTT assay. Cell viability was calculated in comparison to that treated with water only (1%, v/v). Error bars represent the standard error from four independent experiments. Samples: (M) [ZnCl₂ or CuCl₂]; (1) Aβ40 ± [ZnCl₂ or CuCl₂]; (2) HSA ± [ZnCl₂ or CuCl₂]; (3) Aβ40 ± [ZnCl₂ or CuCl₂] + HSA. Conditions: [Aβ40] = 10 μM; [ZnCl₂ or CuCl₂] = 10 μM; HSA = 10 μM.

fluorescence microscope. When cells were grown with FBS, after 1 h incubation without HSA, Aβ40 species were shown to be outside of cell membranes (Figure 4.5a), which displayed similar results with 4 h incubation. In contrast, more Aβ40 species were observed inside the cells in the presence of HSA than HSA-untreated cells even after 1 h incubation. These trends were also observed from the cells cultured without FBS (Figure 4.5b). Furthermore, monomeric Aβ species are favorable to penetrate cellular
membranes.\textsuperscript{35,37} Once the peptides begin aggregation, it would be difficult to pass through the cellular membrane.\textsuperscript{37} Upon treatment with HSA, however, \(\text{A}\beta\) can form complexes with HSA which can be permeable by endocytosis into the cells as HSA itself can be done,\textsuperscript{36} which could direct more \(\text{A}\beta\) species inside of the cells. From our observations, bovine serum albumin (BSA) in the FBS may not significantly facilitate the cellular uptake of \(\text{A}\beta_{40}\) due to its stronger binding with \(\text{A}\beta_{40}\) than HSA.\textsuperscript{98} This tightly bound complex between BSA and \(\text{A}\beta_{40}\) may interfere the cellular uptake of \(\text{A}\beta_{40}\). In addition, although BSA and HSA share the 76\% sequence identity, the endocytosis mechanisms of each protein are different,\textsuperscript{34} interpreting the different internalization of the complex form of BSA– or HSA–\(\text{A}\beta_{40}\).

![Figure 4.5](image.png)

Figure 4.5. Cellular membrane permeability of Hylite Fluor 488 conjugated \(\text{A}\beta_{40}\) (HF488\(\text{A}\beta_{40}\)) by HSA with or without Zn(II). Differential interference contrast (DIC) (left), fluorescence (middle), and overlayed (right) images of 5Y cells treated with metal-free HF488\(\text{A}\beta_{40}\) with or without HSA after 1 or 4 h incubation. 5Y cells were cultured under (a) FBS (10\%) containing or (b) FBS-free medium. Conditions: [HF488\(\text{A}\beta_{40}\)] = 250 nM; [ZnCl\(_2\)] = 250 nM; HSA = 250 nM. Scale bar = 20 \(\mu\)m.
4.3. Conclusion

From the experimental results, monomeric or small-sized metal-free Aβ and metal–Aβ species are observed to interact and form a complex with HSA more favorably than higher oligomeric or fibrillar Aβ. The complex formation between HSA and Aβ and potential disruption of metal binding of Aβ20 could delay and modulate the peptide aggregation to generate possibly less toxic Aβ forms than HSA-free Aβ aggregates. In addition, HSA is indicated to be an Aβ transporter passing through the cellular membranes. Overall, our studies provide information on whether HSA and Aβ interact with each other in both the absence and presence of metal ions; how HSA and metal ions affect the peptide aggregation pathways. This could step forward establishing the foundation of understanding a protein–protein–small molecule (metals) interaction toward AD pathogenesis.

4.4. Experimental section

4.4.1. Materials and methods

All reagents were purchased from commercial suppliers and used as received unless otherwise stated. Human serum albumin (HSA) was acquired from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Trace metal contamination was removed from buffers and solutions used for amyloid-β (Aβ) experiments by treating with Chelex (Sigma-Aldrich). Aβ40 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) were purchased from Anaspec (Fremont, CA, USA). All double distilled H2O (ddH2O) used during experiments was obtained from a Milli-Q Direct 16 system (Merck KGaA, Darmstadt, Germany). The concentrations of proteins were determined by an Agilent 8453 UV–Visible (UV–Vis) spectrophotometer (Santa Clara, CA, USA). Transmission electron microscopy (TEM) images were recorded on a JEOL JEM-2100 transmission electron microscope (UNIST Central Research Facilities, UNIST, Ulsan, Korea). Differential interference contrast (DIC) and fluorescence images were taken by a FV1000 confocal microscope (UNIST-Olympus Biomed Imaging Center, UNIST, Ulsan, Korea). A SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used to measure the fluorescence intensity for the ThT assay.
and absorbance for the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

### 4.4.2. Aβ aggregation experiments

Experiments with Aβ were performed according to previously published methods.\textsuperscript{26-32} Aβ peptides were dissolved with ammonium hydroxide (NH\(_4\)OH, 1% v/v, aq), aliquoted, lyophilized, and stored at −80 °C. A stock solution (ca. 200 μM) was prepared by dissolving Aβ with NH\(_4\)OH (1% w/v, aq, 10 μL) followed by dilution with ddH\(_2\)O, as reported previously.\textsuperscript{26-32} The Aβ\(_{40}\) concentration was determined by measuring the absorbance of the solution at 280 nm (ε = 1450 M\(^{-1}\)cm\(^{-1}\)). Buffered solutions containing HEPES [4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (20 mM)] [pH 7.4 for metal-free and Zn(II) samples; pH 6.6 for Cu(II) samples] and 150 mM NaCl were used for both inhibition and disaggregation studies. For the inhibition experiment, freshly dissolved Aβ (20 μM) was treated with HSA (20 μM), and incubated at 37 °C with constant agitation. For the disaggregation experiment, Aβ peptides were initially incubated at 37 °C for 24 h with steady agitation; HSA was added afterward, followed by an additional 24 h of incubation at 37 °C with constant agitation.

### 4.4.3. ThT assay

The kinetics of Aβ fibrillization were monitored as previously published methods.\textsuperscript{12,23,24} Each sample (40 μL; [Aβ] = 20 μM with or without [HSA] = 20 μM) was obtained after 0, 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, and 48 h incubation for metal-free condition; 0, 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 36, 48, 60, 72, 84, and 96 h incubation for Zn(II)-present environment at 37 °C with constant agitation and diluted with buffered solutions (20 mM HEPES, pH 7.4, 150 mM NaCl). The samples were incubated for 20 min with ThT (20 μM) and read fluorescence. Maximum fluorescence of ThT was measured with excitation wavelength of 440 nm and emission wavelength of 490 nm. The fluorescence intensity was measured on a microplate reader and normalized relative to that of metal-free Aβ aggregates generated through 48 h incubation.
4.4.4. Gel electrophoresis

The Aβ peptide experiments described above were analyzed by gel electrophoresis with two different visualization methods; coomassie blue staining and Western blotting (gel/Western blot) using an anti-Aβ antibody (6E10).26-32 Each sample from both inhibition and disaggregation experiments was separated using a 10–20% gradient Tris-tricine gel (Invitrogen, Grand Island, NY, USA). First, the gel was stained with coomassie blue for about 30 min and washed with water to destain the gels for overnight. Gel images were obtained by ChemiDoc MP system (Bio-rad, CA, USA). Then, the dye was completely destained from the gel, and the gel was transferred to a nitrocellulose membrane and blocked 3 h at room temperature with a skimmed milk solution (10% w/v) in Tris-buffered saline (TBS, Fisher, Pittsburgh, PA, USA) containing 0.1% Tween-20 (Sigma-Aldrich; TBS-T). The membrane was treated with the Aβ monoclonal antibody (6E10, Covance, Princeton, NJ, USA; 1:2,000; BSA, 2% w/v, in TBS-T) for overnight at 4 °C and then probed with a horseradish peroxidase conjugated goat anti-mouse secondary antibody (1:5,000; Cayman Chemical, Ann Arbor, MI, USA) in 2% BSA in TBS-T solution for 1 h at room temperature. The protein bands were visualized using Thermo Scientific Supersignal West Pico Chemiluminescent Substrate (Rockford, IL, USA).

4.4.5. Transmission electron microscopy (TEM)

Samples for TEM were prepared following a previously reported method.26-32 Glow discharged grids (Formar/Carbon 300-mesh, Electron Microscopy Sciences, Hatfield, PA, USA) were treated with samples from either inhibition or disaggregation experiments (5 μL) for 2 min at room temperature. Excess sample was removed with filter paper and the grids were washed with ddH2O three times. Each grid was stained with uranyl acetate (1% w/v ddH2O, 5 μL) for 1 min. Uranyl acetate was blotted off and grids were dried for 20 min at room temperature. Images of samples were taken by a JEOL JEM-2100 transmission electron microscope (200 kV, 25,000× magnification).

4.4.6. Cell viability measurements (MTT assay)

The human neuroblastoma SH-SY5Y (5Y) cell line was purchased from the
American Type Culture Collection (ATCC, Manassas, VA, USA). The cell line was maintained in media containing 1:1 Minimum Essential Media (MEM, GIBCO, Grand Island, NY, USA) and Ham’s F12K Kaighn’s Modification Media (F12K, GIBCO), 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich), and 1% (v/v) penicillin (GIBCO). The cells were grown and maintained at 37 °C in a humidified atmosphere with 5% CO₂. Cell viability upon treatment of Aβ with or without metal ions and/or HSA was determined using the MTT assay. 5Y cells were seeded in a 96 well plate (15,000 cells in 100 µL per well) and treated with Aβ40 (10 µM), ZnCl₂ or CuCl₂ (10 µM), and/or HSA (10 µM). After 4, 8, 12, and 24 h incubation at 37 °C, MTT (25 µL of 5 mg/mL in phosphate buffered saline, PBS, pH 7.4, GIBCO) was added to each well and the plates were incubated for 4 h at 37 °C. Formazan produced by the cells was dissolved overnight at room temperature by addition of a solubilization buffer (100 µL) containing N,N-dimethylformamide (DMF, 50% v/v, aq, pH 4.5) and sodium dodecyl sulfate (SDS, 20% w/v). The absorbance (A₆₀₀) was measured on a microplate reader, cell viability (%) was determined relative to that of cells untreated by Aβ and HSA.

4.4.7. Imaging studies of cellular uptake

Cell uptake experiments were conducted with Hylite Fluor 488 conjugated Aβ40 (HF₄₈₈Aβ₄₀) peptides using a FV1000 confocal microscope (Olympus, Tokyo, Japan).³⁵⁻³⁷ HF₄₈₈Aβ₄₀ (250 nM) was incubated with 5Y cells at 37 °C in a humidified atmosphere with 5% CO₂ for 1 or 4 h with or without HSA (250 nM). Cells were washed with PBS (GIBCO) twice, and images were recorded to avoid further uptake while observing the cells on the microscope. The samples were excited at 488 nm (λₑₘ = 520 nm).

4.5. Acknowledgment

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4.6. References

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Chapter 5: Concluding Remarks and Perspectives

The interrelationships between multiple features [i.e., amyloid-β (Aβ), metal ions, metal-associated Aβ (metal–Aβ), reactive oxygen species (ROS), other proteins] have been suggested to be involved in the onset and progression of Alzheimer's disease (AD).\textsuperscript{1-7} To advance our understanding of such interconnections, natural products or synthetic compounds, as chemical tools, which can target multiple pathological factors and control their reactivities have been screened or designed.\textsuperscript{5-17} These small molecules have been applied to uncover the roles of metal ions, Aβ, metal–Aβ and ROS as well as probe their association with AD pathogenesis \textit{in vitro} and/or \textit{in vivo}.\textsuperscript{8-17}

The studies described in Chapters 2-4 in this thesis present the investigations of a structure-interaction-reactivity relationship between flavonoids (Chapter 2) or synthetic small molecules (Chapter 3) and metal-free Aβ/metal–Aβ as well as of the protein–protein interaction (Chapter 4) related to AD pathogenesis. In Chapter 2, Upon structural variations of the number and position of hydroxyl groups on myricetin which was reported to show its inhibitory abilities toward metal–Aβ aggregation and recovery of cytotoxicity induced by metal-free Aβ and metal–Aβ species,\textsuperscript{13} four flavonoids morin, quercetin, galangin, and luteolin, were selected and presented their ability to interact with Aβ species, redirect metal–Aβ aggregation pathways, and alleviate metal-free Aβ/metal–Aβ associated toxicity to different extents. These results lead us to advance knowledge about the importance of hydroxyl groups on the B ring of flavonoid for their reactivity toward metal–Aβ. In the future, flavonoids with other structural variations (i.e., the position and number of hydroxyl groups on the A ring) will be examined and further optimized for the desired reactivity. Furthermore, to improve the blood-brain barrier (BBB) permeability of flavonoids, some structural adjustments could be required, such as glycosylation.\textsuperscript{18} The glycosylated flavonoids have been reported to show better BBB permeability.\textsuperscript{18} Once structural modified flavonoids can pass through BBB, they can be applied to \textit{in vivo} systems.

From the previous studies, L2-b and DMPD demonstrated their ability to modulate metal-free Aβ and/or metal–Aβ aggregation pathways, \textit{in vitro} and \textit{in vivo}.\textsuperscript{9,15,16} Unfortunately, the critical motif for such reactivity has not been clearly identified; thus, as
discussed in Chapter 3, a chemical library was established to investigate the responsible moiety of L2-b for its redirecting activity toward metal–Aβ aggregation. The L2-b derivatives (L2-b1 and L2-b2) with the dimethylamino functionality located at different positions were examined. L2-b1 could not alter both metal-free Aβ and metal–Aβ aggregation pathways while L2-b2 could modulate the peptide aggregation pathways. Moreover, PMA1 and PMA2, structural moieties from the metal binding regions of L2-b and L2-b2, respectively, as well as DPA1 and DPA2, small molecules designed to increase the denticity and flexibility of L2-b and L2-b2, respectively, were also included in our chemical library in order to complete our investigations for identifying a structural group critical for interacting with metal-free Aβ and metal–Aβ, subsequently altering their aggregation. PMA1, PMA2, DPA1, and DPA2 are not able to redirect metal-free Aβ and metal–Aβ aggregation pathways and noticeably scavenge free radicals. From the comparison of experimental results from our chemical series, the structural component essential for the reactivity of L2-b and L2-b2 is the DMPD motif. Furthermore, the dimethylamino group itself may not be enough to present its inhibitory activity toward Aβ aggregation unless it is located at the proper position of the entire structure of L2-b or DMPD. Therefore, the DMPD derivatives (i.e., the number and position of the dimethylamino group, different substituents) can be the next series of compounds to be investigated their reactivity toward metal-free Aβ and metal–Aβ species for obtaining a greater understanding of structure-interaction-reactivity relationships and further optimizing the reactivity of DMPD.

In addition, in Chapter 4, human serum albumin (HSA), the most abundant protein in blood serum is observed to interact with small-sized metal-free Aβ and metal–Aβ species and form the complexes. Upon binding to Aβ, HSA may delay the fibrillization of both metal-free Aβ and Zn(II)–Aβ and possibly recover toxicity associated with Aβ species in living cells. Although we have studied the influence of HSA on the fibril formation of Aβ, the binding modes and sites of two proteins are not clearly revealed. By employing biophysical methods [e.g., mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy], the complexes of HSA and Aβ are indicated to be formed in the absence and presence of metal ions.
The works presented in this thesis focus on understanding the interaction between pathological features (i.e., metal ions, metal-free Aβ, metal–Aβ, Aβ–interacting proteins) and the potential influence on AD. Moving forward, based on the findings from Chapters 2 and 3, small molecules with the DMPD functionality (i.e., DMPD, L2-b, L2-b2) can be applied to examine whether these are able to affect the actions of different amyloidogenic proteins involved in other neurodegenerative diseases [i.e., α-synuclein (α-Syn) in Parkinson’s disease\textsuperscript{20,21}]. The small molecules may be necessary to be modified for other targets; however, it can offer efficient and economical benefits once these compounds as chemical tools can help identify multiple pathological factors linked to the onset and progression of diverse amyloidogenic diseases.

Overall, the investigations from this thesis can be the first step for understanding of protein–protein–small molecule (metals) interactions in AD. The knowledge obtained from our studies provides insight into fundamental pathogenesis of various diseases related to aggregate-prone proteins, more importantly with new directions for discovery of effective therapeutics for the devastating disease.

References


Appendix A: Influence of the Inflammatory Response Proteins, S100 Protein Families on the Amyloid-β Aggregation

The work presented herein was conducted in collaboration with Professor Elizabeth Nolan and Toshiki Nakashige at MIT. We thank Professor Ayyalusamy Ramamoorthy and Kyle J. Korshavn for NMR experiments. In this present work, I have contributed to the experiments of the ThT assay, gel electrophoresis with Western blot, and TEM analyses for Aβ aggregation with Dr. Masha Savelieff.

A.1. Introduction

Alzheimer’s disease (AD) is the most prevalent form of dementia and accounts for ca. 60% of neurodegenerative diagnoses.1-10 This illness begins with mild cognitive impairment and progresses to severe dementia, and ultimately death.2-10 Senile plaques which are consist primarily of the amyloid-β (Aβ) aggregates are present in the AD-affected brain.2-10 Aβ is produced in the brain as a result of the proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases.2-9 A spectrum of various sized Aβ peptides is produced (from 38 to 43 amino acid residues), but the predominant peptides are Aβ40 (ca. 90%) and Aβ42 (ca. 10%).7-15 Both peptides have a tendency to aggregate into mature fibrils and generated soluble oligomeric species have been indicated as toxic species.6-15 In addition, chronic inflammation, elevated oxidative stress, and metal ion dyshomeostasis are also pervasive in the brain of AD.2,7-16 From previous studies, highly concentrated transition metal ions [i.e., Cu, Zn ions (ca. 0.4 and 1.0 mM, respectively)] are co-localized in senile plaques in the AD-affected brain.3-10 These metal ions can interact with Aβ peptides, facilitate the peptide aggregation and form toxic oligomeric Aβ species.3-10 Particularly, redox active metal ions, [i.e., Cu(I/II), Fe(II/III)] can generate reactive oxygen species (ROS) with or without Aβ peptides which may damage the membranes and disrupt signaling.3-10 Although the efforts have focused principally on Aβ and metal ions, the AD etiology is still uncertain thus, unidentified parameters along with Aβ and metal ions, may be involved in AD pathogenesis.2-10 As a result, much effort has been expended to investigate the interaction of Aβ with various proteins present in the brain that may participate in pathways associated with AD pathology.16-22 The S100
protein family, particularly calprotectin (S100A8/A9; CP) and S100B, an inflammatory response protein whose expression level is upregulated in response to elevated inflammation, has been suggested to be linked to AD etiology. CP and S100B have been detected in senile plaques from the tissue of AD-affected brains and upregulated in AD mouse models.

CP is a calcium responsive protein that adopts a heterodimeric (composed of monomeric subunits; S100A8 and S100A9) or heterotetrameric structures. CP has two calcium binding EF hands (one canonical, one noncanonical); Ca(II) binding induces the formation of CP dimer and tetramer. In addition to Ca(II) binding sites, CP can bind to other transition metal ions, such as Mn(II) or Zn(II) with binding affinities in the range of μM and nM, respectively, at other metal binding sites. Due to its Zn(II) binding property, CP can indirectly affect the elimination of Aβ peptides by deactivating matrix metalloproteases (MMPs) which require Zn(II) to be activated. It, however, has been rarely studied the direct influence of CP on Aβ aggregation. Another S100 protein family, S100B, is also detected to be overexpressed in aged and AD-affected brains suggesting its involvement in AD pathogenesis.

We report herein the interaction of CP and S100B with metal-free Aβ and metal-associated Aβ (metal–Aβ) at the molecular level. Additionally, we demonstrate the ability of CP and S100B to interact with certain conformations of Aβ and modulate Aβ aggregation pathways in both the absence and presence of metal ions [i.e., Cu(II), Zn(II)]. In order to avoid the generation of disulfide bonds during the purification of CP, mutated CPs [C42 of S100A8 and C3 of S100A9 to serine residue (CP-Ser)] were employed in this study. Furthermore, to investigate the alternation of metal binding properties of CP-Ser and S100B on the interactions between S100 proteins and metal-free Aβ/metal–Aβ, and its effects on Aβ aggregation pathways, the proteins with mutation of the histidine and glutamic acid residues at metal binding sites of CP-Ser and S100B to alanine were prepared; CP-SerΔΔ and S100BΔ. From our investigations, CP-Ser and S100B could redirect or inhibit both metal-free Aβ40 and metal–Aβ40 fibrillization under Zn(II)-present conditions (2 and 1 equiv, respectively), respectively, while CP-SerΔΔ and S100BΔ could only modulate metal-free Aβ40 and metal–Aβ40 fibril formation processes with less than 1
equiv of Zn(II). Moreover, all S100 proteins herein could not influence on Aβ42 fibrillization. Not only for Aβ aggregation, but also all the S100 proteins shown in this work present a greater radical scavenging capability than Trolox, a water-soluble vitamin analogue. Overall, our studies employing CP-Ser, CP-SerΔΔ, S100B, and S100BΔ, Aβ, and metal ions can provide insights into some protein–protein-small molecule (metals) interactions which could be linked to AD etiology.

A.2. Results and discussion

A.2.1. Effects of S100 proteins on Aβ aggregation pathways, observed by the thioflavin-T (ThT) assay.

Fibrillization of metal-free Aβ and metal–Aβ in both the absence and presence of proteins, CP-Ser, CP-SerΔΔ, S100B and S100BΔ, was monitored by the ThT assay. Freshly dissolved Aβ with or without metal ions [Cu(II) or Zn(II)] was incubated with the proteins for 24 h at 37 °C as depicted in Figure A.1a. Upon incubation of Aβ peptides with CP-Ser, CP-SerΔΔ, S100B or S100BΔ, less metal-free Aβ40 fibrillation is shown to occur than that from protein-untreated Aβ40 samples. The production of metal–Aβ40 fibrils was also reduced. Moreover, based on the stoichiometry of metal ions to Aβ and S100 proteins, the inhibitory ability of S100 proteins toward metal–Aβ aggregation is observed to different extent.

CP-Ser could inhibit the formation of Zn(II)–Aβ40 fibrils up to 2 equiv of Zn(II) to Aβ40. Interestingly, if excess of Zn(II) (5 or 10 equiv) was introduced, CP-Ser may not be able to affect Aβ40 aggregation pathways (Figure A.1b). On the other hand, CP-SerΔΔ may not have significant effects on Aβ40 aggregation with more than 1 equiv of Zn(II) (Figure A.1b). Similar results were observed in the presence of Cu(II) (Figure A.2a). Up to 1 equiv of Cu(II) to Aβ, CP-Ser could redirect the fibril formation, while CP-SerΔΔ only affects Aβ peptide aggregation in the presence of less than 1.0 equiv of Cu(II) to Aβ. These results indicate that CP-Ser and CP-SerΔΔ may inhibit metal-unbound Aβ40 aggregation. CP-Ser capable of binding to metal ions (up to 2 equiv) could modulate Aβ40
fibrillization by effectively rendering metal-unbound Aβ40, due to their high metal binding affinities and influence on the Aβ fibrillization; however, CP-SerΔΔ which cannot interact with metal ions could not alter the Aβ40 fibrillization in the presence of metal ions. To prove this, it may require further studies about the protein–protein interaction between CP-Ser/CP-SerΔΔ and Aβ40 with or without metal ions.

In contrast, inhibition experiments with Aβ42 showed different results from those with Aβ40. Both CP-Ser and CP-SerΔΔ may not be able to modulate the Aβ42 fibrillization
in both the absence and presence of metal ions (Figures A.1d and A.2b). These observations suggest that differentiated conformations and aggregation pathways of Aβ_{40} and Aβ_{42} can cause CP-Ser/CP-Ser_{ΔΔ} to affect the peptide aggregation pathway to different extents. Moreover, these ThT assay results were consistent to those obtained by TEM (Figures A.2c,d, *vide infra*).

Another S100 family protein, S100B, and its mutant, S100B_{Δ}, have been examined their anti-amyloidogenic ability toward both metal-free Aβ_{40}/Aβ_{42} and Zn(II)–Aβ_{40}/Aβ_{42}. These proteins also showed similar results to the experiments with CP-Ser and CP-Ser_{ΔΔ}. S100B could inhibit metal-free Aβ_{40} and Zn(II)–Aβ_{40} aggregation (Figure A.1c) up to 1 equiv of Zn(II) present environment and S100B_{Δ} could not alter the Aβ_{40} aggregation treated with same amount of Zn(II) (Figure A.1c). Moreover, both S100B and S100B_{Δ} could not affect the both metal-free Aβ_{42} and Zn(II)–Aβ_{42} fibrillization, significantly (Figure A.1e). From these results, S100B which possibly bind to metal ions could influence on metal–Aβ_{40} fibrillization and S100B_{Δ} which cannot interact with metal ions may not be able to prohibit metal–Aβ_{40} fibril formation, as similar to CP-Ser and CP-Ser_{ΔΔ}. Overall, S100 protein family may prefer to interact with Aβ_{40} over Aβ_{42} and modulate the peptide aggregation, potentially, metal-unbound Aβ species.

**A.2.2. Morphological changes of Aβ_{40} upon treatment of proteins, monitored by transmission electron microscopy (TEM)**

The morphologies of both metal-free Aβ and metal–Aβ aggregates treated by proteins were monitored by transmission electron microscopy (TEM). After 24 h incubation, metal-free Aβ_{40} and metal–Aβ_{40} aggregated generated with CP-Ser were indicated to be more amorphous than the protein-untreated Aβ_{40} samples (Figures A.2c,d), consistent with those monitored by the ThT assay. On the other hand, CP-Ser_{ΔΔ} with 1 equiv metal ions could not present morphological changes of the resultant Aβ species. Furthermore, as expected from the results indicated by the ThT assay, both CP-Ser and CP-Ser_{ΔΔ} could not inhibit Aβ_{42} fibril formation.
A.2.3. Visualization of protein–Aβ40 complexes by gel electrophoresis

Since the ThT assay and TEM displayed the inhibition of metal-free Aβ40 and metal–Aβ40 fibrillation by S100 proteins, the molecular weight (MW) distribution of the resultant Aβ40 species treated or untreated with the proteins was visualized by gel electrophoresis followed by Western blotting with an anti-Aβ antibody (gel/Western blot; upper row) or anti-S100A9 antibody (lower row) for CP-Ser or CP-SerΔΔ treated samples (Figure A.3a,b); gel/Western blot with anti-Aβ antibody (upper row) and coomassie blue staining (lower row) for the samples incubated with S100B or S100BΔ (Figure A.3c,d). Bands of proteins (littler higher than 10 kDa) were detected from the samples of both metal-free Aβ40 and metal–Aβ40 incubated with CP-Ser or CP-SerΔΔ. As the MWs of the
S100A9 subunit in CP-Ser/CP-Ser_{ΔΔ} and Aβ_{40} are ca. 13.2 and 4.3 kDa, respectively, the clear bands on the gels may correspond to the complex formation between the

Figure A.3. Gel analysis of the formation of CP-Ser/CP-Ser_{ΔΔ} and S100B/S100B_{ΔA}–Aβ_{40} complexes in the absence and presence of metal ions. Resultant Aβ_{40} species after 24 h incubation with or without (a) CP-Ser or (b) CP-Ser_{ΔΔ} were visualized by gel/Western blot using an anti-Aβ antibody (upper row) and anti-S100A9 antibody (lower row); Aβ samples treated with or without (c) S100B or (d) S100B_{ΔA} were also visualized by gel/Western blot using an anti-Aβ antibody (upper row) and coomassie blue staining. Lanes: (1) Aβ (2) Aβ ± protein; (3) Aβ ± 0.1 equiv of [ZnCl_2 or CuCl_2]; (4) Aβ ± 0.1 equiv of [ZnCl_2 or CuCl_2] + protein; (5) Aβ ± 1.0 equiv of [ZnCl_2 or CuCl_2]; (6) Aβ ± 1.0 equiv of [ZnCl_2 or CuCl_2] + protein. Conditions: [Aβ_{40}] = 20 μM; [ZnCl_2 or CuCl_2] = 2 or 20 μM; [protein] = 20 μM; pH 6.6 (for Cu(II) experiment) or pH 7.4 (for metal-free and Zn(II) experiments); 24 h incubation; 37 °C; constant agitation.

S100A9 subunit of CP-Ser/CP-Ser_{ΔΔ} and metal-free Aβ_{40} or metal–Aβ_{40}. Moreover, based on the MW of heterodimers of CP (S100A8 and S100A9) is approximately 24 kDa, the bands (little less than 35 kDa) on the gels could correspond to CP-Ser/CP-Ser_{ΔΔ}–Aβ_{40}
complexes (Figure A.3a,b). In addition, CP-Ser or CP-Ser$_{\Delta \Delta}$-treated samples displayed different trends of smearing bands compared to CP-Ser- or CP-Ser$_{\Delta \Delta}$-untreated A$\beta_{40}$ samples. These results suggest that the CP proteins may affect A$\beta_{40}$ aggregation pathways (i.e., less fibril formation) by generating the protein complexes. Furthermore, as anti-S100A9-blotted gels presented, CP-Ser may have the potential to aggregate itself by hydrophobic interactions. Previous studies demonstrate that once Zn(II) is introduced to CP-Ser, this protein can aggregate faster,$^{16}$ which is consistent with our observation.

In addition, S100B/S100B$_{\Delta}$-added A$\beta_{40}$ samples also indicated the protein bands having little higher than 10 kDa which are also possibly the complexes of S100B/S100B$_{\Delta}$ and metal-free A$\beta_{40}$ and metal–A$\beta_{40}$ (Figure A.3c,d), based on the MWs (approximately 10 and 4.3 kDa) of the monomeric subunit of S100B/S100B$_{\Delta}$ and A$\beta_{40}$. Furthermore, S100B/S100B$_{\Delta}$–A$\beta_{40}$ complexes were also visualized considering the MW of S100B dimer (ca. 21 kDa; Figure A.3c,c). Overall, such complex formation between S100 proteins and A$\beta$ could alter metal-free A$\beta_{40}$ and metal–A$\beta_{40}$ aggregation pathways.

**A.2.4. Interaction of CP-Ser with monomeric A$\beta_{40}$**

The direct interaction of CP-Ser with monomeric A$\beta_{40}$ was investigated by 2D band-selective optimized flip-angle short transient heteronuclear multiple quantum correlation (SOFAST-HMQC) NMR. Chemical shifts of $^{15}$N-labeled A$\beta_{40}$ residues upon titration of CP-Ser were detected and monitored to determine the potential binding site and modes of CP-Ser to A$\beta_{40}$ peptide (Figure A.4). Less than 0.02 ppm of chemical shifts were induced upon addition of CP-Ser to the solution containing $^{15}$N-labeled A$\beta_{40}$. Almost entire A$\beta_{40}$ residues were evenly involved in the relatively weak interaction with CP-Ser, along with noticeably perturbed residues; E11, F19, E22, and I32. As expected from gel electrophoresis, CP-Ser and A$\beta$ peptide may interact with each other forming complexes; however, it may not be strongly bound.
A.2.5. Free radical scavenging capability of proteins

The free radical scavenging capability of CP-Ser, CP-SerΔΔ, S100B, and S100BΔ was determined by Trolox (vitamin E analogue) equivalent antioxidant capacity (TEAC) assay which can evaluate the ability to quench ABTS cation radicals \(\text{ABTS}^{+}\); \(\text{ABTS} = 2,2'\text{-azino-bis}(3\text{-ethylbenzothiazoline-6-sulfonic acid})\) in SK-N-BE(2)-M17 (M17) cell lysates.\(^{28-30}\) All the proteins could scavenge free organic radicals better (more than ca. 1.5 times) than Trolox (Figure A.5).\(^{28-30}\)

A.3. Conclusion and future directions

Four different proteins from S100 protein family, CP-Ser, CP-Ser\(_{\Delta\Delta}\), S100B, and S100B\(_{\Delta}\), were investigated for their ability to redirect metal-free A\(\beta\) and metal–A\(\beta\) aggregation pathways (particularly, the formation of fibrils), along with their free radical scavenging capacity. From the experimental results presented herein, all four proteins could affect the A\(\beta\) fibrillization with or without metal ions. Moreover, these proteins are shown to scavenge free radicals. In order to reveal the interaction between S100 protein...
family and Aβ species in detailed, several biophysical methods (e.g., mass spectrometry, NMR) should be applied and analyzed. Overall, our studies provide information on whether the overexpressed protein in AD-affected brains, S100 protein, and Aβ interact with each other with or without metal ions and their effects on Aβ aggregation pathways. This could lead better understandings of a protein–protein-small molecule (metals) interaction in AD etiology.

**Figure A.5.** The free radical scavenging capability of CP-Ser, CP-SerΔΔ, S100B, and S100BΔ, determined by the TEAC assay in SK-N-BE(2)-M17 (M17) cell lysates. The TEAC values are relative to that of a vitamin E analogue, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

### A.4. Experimental section

**A.4.1. Materials and methods**

All reagents were purchased from commercial suppliers and used as received unless otherwise stated. CP and S100B proteins were obtained from Professor Elizabeth Nolan. Trace metal contamination was removed from buffers and solutions used for amyloid-β (Aβ) experiments by treating with Chelex (Sigma-Aldrich). Aβ40 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) and Aβ42 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV) were purchased from Anaspec (Fremont, CA, USA). All double distilled H₂O (ddH₂O) used during experiments was obtained from a Milli-Q Direct 16 system (Merck KGaA, Darmstadt, Germany). The concentrations of proteins were determined by an Agilent 8453 UV–Visible (UV–Vis) spectrophotometer (Santa Clara, CA, USA). Transmission electron microscopy (TEM) images were recorded.
on a Philips CM-100 transmission electron microscope (Microscopy and Image Analysis
Laboratory, University of Michigan, Ann Arbor, MI, USA). A SpectraMax M5 microplate
reader (Molecular Devices, Sunnyvale, CA, USA) was used to measure the fluorescence
intensity for the ThT assay.

A.4.2. Preparation and purification of proteins

E. coli codon-optimized genes for each subunit of human CP-Ser and (S100A8 and
S100A9) were subcloned into pET41a plasmids (pET41a-S100A8 and pET41a-
S100A9), and then transformed into E. coli BL21(DE3) cells. The fully assembled protein
CP-Ser heterodimer was expressed and purified according to previously established
methods. Briefly, each subunit was overexpressed separately; transformed BL21(DE3)
cells were grown to saturation overnight in LB supplemented with 50 µg/mL kanamycin,
used to inoculate (1:100) larger scale LB media (1 to 4 L), which were shaken at 150 rpm
at 37 °C. Protein expression was induced with 500 µM IPTG at ca. 0.6 OD$_{600}$ over 3-4
hours till ca.1.5 OD$_{600}$. Bacteria were pelleted at 4200 rpm for 30 min at 4 °C, flash frozen
and kept at −80 °C. The CP-Ser$_{AL}$ was prepared in an analogous manner.

S100B and S100B$_{AL}$ were overexpressed in E. coli BL21(DE3) in the pET41a
vector, and purified by 100% ammonium sulfate precipitation, anion exchange
chromatography, and size-exclusion chromatography (SEC). The proteins were
partitioned into 50 µL aliquots in pre-sterilized microcentrifuge tubes.

A.4.3. Aβ aggregation experiments

Experiments with Aβ were performed according to previously published
methods.$^{28,31-36}$ Aβ peptides were dissolved with ammonium hydroxide (NH$_4$OH, 1% v/v,
aq), aliquoted, lyophilized, and stored at −80 °C. A stock solution (ca. 200 µM) was
prepared by dissolving Aβ with NH$_4$OH (1% w/v, aq, 10 µL) followed by dilution with
ddH$_2$O, as reported previously.$^{28,31-36}$ The Aβ$_{40}$ concentration was determined by
measuring the absorbance of the solution at 280 nm ($ε = 1450$ M$^{-1}$cm$^{-1}$ for Aβ$_{40}$; $ε = 1490$
M$^{-1}$cm$^{-1}$ for Aβ$_{42}$). Buffered solutions containing HEPES [4-(2-hydroxyethyl)-1-
piperazineethanesulfonic acid (20 mM)] [pH 7.4 for metal-free and Zn(II) samples; pH 6.6
for Cu(II) samples] and 150 mM NaCl were used for inhibition studies. For the inhibition experiment, freshly dissolved Aβ (20 μM) was treated with S100 proteins (20 μM), and incubated for 24 h at 37 °C with constant agitation.

A.4.4. ThT assay

The kinetics of Aβ fibrillization were monitored as previously published methods.\(^\text{17,37,38}\) Each sample (40 μL; [Aβ] = 20 μM with or without [S100 proteins] = 20 μM) was obtained after 24 h incubation for both metal-free and metal present environment at 37 °C with constant agitation and diluted with buffered solutions (20 mM HEPES, pH 7.4 for metal-free and Zn(II) experiments, pH 6.6 for Cu(II) experiments, 150 mM NaCl). The samples were incubated for 20 min with ThT (20 μM) and read fluorescence. Maximum fluorescence of ThT was measured with excitation wavelength of 440 nm and emission wavelength of 490 nm. The fluorescence intensity was measured on a microplate reader and normalized relative to that of metal-free Aβ aggregates generated through 24 h incubation.

A.4.5 Gel electrophoresis with Western blot

The Aβ peptide experiments described above were analyzed by gel electrophoresis with two different visualization methods; coomassie blue staining and Western blotting (gel/Western blot) using two different antibodies; an anti-Aβ antibody (6E10) or anti-S100A9 antibody (Santa Cruz Biotechnology, Dallas, TX, USA).\(^\text{28,31-36}\) Each sample from inhibition experiments was separated using a 10–20% gradient Tris-tricine gel (Invitrogen, Grand Island, NY, USA). First, the gel run with CP samples was transferred to a nitrocellulose membrane and blocked 3 h at room temperature with a skimmed milk solution (10% w/v) in Tris-buffered saline (TBS, Fisher, Pittsburgh, PA, USA) containing 0.1% Tween-20 (Sigma-Aldrich; TBS-T). The membrane was treated with the Aβ monoclonal antibody (6E10, Covance, Princeton, NJ, USA; 1:2,000; BSA, 2% w/v, in TBS-T) or anti-S100A9 antibody for overnight at 4 °C and then probed with a horseradish peroxidase conjugated goat anti-mouse secondary antibody (1:5,000; Cayman Chemical, Ann Arbor, MI, USA) or donkey anti-goat secondary antibody (1:5,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in 2% BSA in
TBS-T solution for 1 h at room temperature. The protein bands were visualized using Thermo Scientific Supersignal West Pico Chemiluminescent Substrate (Rockford, IL, USA).

The gel run with S100B samples was stained with coomassie blue for about 30 min and washed with water to destain the gels for overnight. Gel images were obtained by ChemiDoc MP system (Bio-rad, CA, USA). Then, the dye was completely destained from the gel, and the gel was transferred to a nitrocellulose membrane for Western blot with 6E10 (1:2000; BSA, 2% w/v, in TBS-T) and a horseradish peroxidase conjugated goat anti-mouse secondary antibody (1:5,000) as same as the gel run with CP samples.

A.4.6. Transmission electron microscopy (TEM)

Samples for TEM were prepared following a previously reported method. Glow discharged grids (Formar/Carbon 300-mesh, Electron Microscopy Sciences, Hatfield, PA, USA) were treated with samples from inhibition experiments (5 µL) for 2 min at room temperature. Excess sample was removed with filter paper and the grids were washed with ddH₂O three times. Each grid was stained with uranyl acetate (1% w/v ddH₂O, 5 µL) for 1 min. Uranyl acetate was blotted off and grids were dried for 20 min at room temperature. Images of samples were taken by a Philips CM-100 transmission electron microscope (80 kV, 25,000× magnification).

A.4.7. 2D NMR spectroscopy

The interaction of Aβ₄₀ with CP-Ser was monitored by 2D ¹H-¹⁵N SOFAST-HMQC NMR at 10 °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 PO₄, pH 7.4, 50 mM NaCl; 7% v/v D₂O). Each spectrum was obtained using 64 complex t₁ points and a 0.1 sec recycle delay on a Bruker Avance 600 MHz spectrometer. The 2D data was processed using TOPSPIN 2.1 (from Bruker). Resonance assignment was performed with SPARKY 3.1134 using published assignments for Aβ₄₀ as a guide.
A.4.8. Trolox equivalent antioxidant capacity (TEAC) assay

The free radical scavenging capacity of CP-Ser, CP-Ser\Delta\Delta, S100B, and S100B\Delta were determined by the TEAC assay employing lysates of human neuroblastoma SK-N-BE(2)-M17 (M17) cells purchased from ATCC (Manassa, VA, USA).\textsuperscript{28-30} The assay employing cell lysates was conducted following the protocol of the antioxidant assay kit purchased from Cayman Chemical Company (Ann Arbor, MI, USA), with minor modifications.\textsuperscript{28} M17 cells were cultured in media containing 1:1 Minimum Essential Media (MEM, Gibco, Grand Island, NY), Ham’s F12K Kaighn’s Modification Media (F12K, Gibco), 10% (v/v) fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, USA Gibco), 100 U/mL penicillin (Gibco), and 100 mg/mL streptomycin (Gibco). The cells were grown and maintained at 37 °C in a humidified atmosphere with 5% CO\textsubscript{2}. For the antioxidant assay using cell lysates, cells were seeded in a 6 well plate and grown to approximately 80-90% confluence. Cell lysates were prepared following a previously reported method with modifications.\textsuperscript{41} M17 cells were washed once with cold PBS (pH 7.4, Gibco) and harvested by gently pipetting off adherent cells with cold PBS. The cell pellet was generated by centrifugation (2,000 x g for 10 min at 4 °C). This cell pellet was sonicated on ice (5 sec pulses, 3 times with 20 sec intervals between each pulse) in 2 mL of cold Assay Buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% NaCl and 0.1% glucose). The cell lysates were centrifuged at 5,000 x g for 10 min at 4 °C. The supernatant was removed and stored on ice until use. To standard and sample 96 wells, 10 μL of the supernatant of cell lysates was delivered followed by addition of proteins, metmyoglobin, ABTS, and H\textsubscript{2}O\textsubscript{2} in order. After 5 min incubation at room temperature on a shaker, absorbance values at 750 nm were recorded. The final concentrations (0.045, 0.090, 0.135, 0.180, 0.225, and 0.330 mM) of proteins (CP-Ser, CP-Ser\Delta\Delta, S100B and S100B\Delta) and Trolox were used. The antioxidant concentration was calculated according to the measured absorbance (% Inhibition = (A\textsubscript{0} – A)/A\textsubscript{0}, where A\textsubscript{0} is absorbance of the supernatant of cell lysates). The measurements were conducted in triplicate.

A.5. References


