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Submitted date: 03/05/2019 • Posted date: 06/05/2019

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Citation information: Han, Jiyeon; Lee, Hyuck Jin; Kim, Kyu Yeon; Chae, Junghyun; Lim, Mi Hee (2019): Mechanistic Approaches for Modifying the Coordination Sphere of Metal–Amyloid- β Complexes: Covalent Adduct Formation and Oxidation. ChemRxiv. Preprint.

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Mechanistic Approaches for Modifying the Coordination Sphere of Metal–Amyloid- β Complexes: Covalent Adduct Formation and Oxidation

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ABSTRACT

The impaired homeostasis of redox-active metal ions could contribute to neurodegeneration found in neurodegenerative disorders. In the brain of Alzheimer's disease (AD), redox-active metal ions [particularly, Cu(II)] are coordinated to amyloid- β (A β) peptides forming metal-A β complexes able to generate toxic A β oligomers and overproduce reactive oxygen species. To modulate such reactivities of Cu(II)-A β complexes, various chemical reagents have been developed and used to sequester Cu(II) from Cu(II)-A β complexes. No chemical reagent or strategy, however, is currently available to control both Cu(II)-A β complexation and the oligomerization of the resultant metal-free A β upon removal of Cu(II) from Cu(II)-A β complexes. Herein, we report, *for the first time*, a novel mechanistic tactic to directly modify the coordination sphere of Cu(II)-A β complexes, which can prevent Cu(II) binding to A β , modulate the aggregation of Cu(II)-A β , and generate chemically transformed A β with less cytotoxicity, to the best of our knowledge. The chemical modifications towards Cu(II)-A β complexes include covalent adduct formation, oxidation, or both in a metal binding residue (His14)-specific manner and can be achieved using a rationally designed small molecule-based peptide modifier (**L1**). Our new rationale and molecular-level investigations of reactivities and mechanisms demonstrate the feasibility of developing effective strategies to regulate metal-interacting aggregation-prone peptides observed in neurodegenerative disorders.

INTRODUCTION

Transition metals are essential for diverse functions in the brain, playing structural or catalytic roles in metalloproteins and neurotransmission.¹⁻³ Among transition metals, copper is the third most abundant metal in the brain and an indispensable element for energy metabolism, antioxidant defense, and synthesis of neurotransmitters.⁴⁻⁸ Thus, the uptake and efflux of intracellular copper are tightly regulated in the healthy brain.⁴ When the homeostasis of copper is impaired, copper-mediated functions are disrupted, which could be further linked to the development of neurodegenerative disorders, such as Alzheimer's disease (AD).⁴⁻⁹

In the AD-afflicted brain, the level of copper is elevated as high as 400 μM in senile plaques mainly composed of amyloid- β ($\text{A}\beta$) aggregates.^{10,11} The complexation between Cu(II) and $\text{A}\beta$ peptides, along with neurotoxic aspects of $\text{Cu(II)}\text{--}\text{A}\beta$ complexes, has been previously reported.^{4,9-14} The binding affinity (K_d) of Cu(II) for $\text{A}\beta$ peptides is in the picomolar to nanomolar range (ca. 10^{-11} to 10^{-7} M) with distinct coordination modes depending on pH.^{4,14} The first coordination sphere of Cu(II) bound to $\text{A}\beta$ typically consists of three nitrogen (N) donor atoms and one oxygen (O) donor atom.^{14,15} The N donor atoms are a combination of three histidine residues (i.e., His6, His13, and His14), an amide backbone, and the primary amine at the N-terminus, while a carbonyl backbone between Asp1 and Ala2 is the O donor atom under physiological conditions.¹⁴⁻¹⁷ Cu(II) binding to $\text{A}\beta$ can accelerate $\text{A}\beta$ aggregation and stabilize toxic structured $\text{A}\beta$ oligomers.^{4,18} In addition, redox-active $\text{Cu(I/II)}\text{--}\text{A}\beta$ complexes can

overproduce reactive oxygen species (ROS) via Fenton-like reactions, contributing towards oxidative stress.^{4,11,19}

To regulate the reactivities of Cu(II)–A β complexes (i.e., aggregation and ROS generation), several metal chelators have been employed to remove Cu(II) from Cu(II)–A β ;²⁰⁻²² however, since the resultant metal-free A β species upon dissociation of Cu(II) from Cu(II)–A β complexes continued to aggregate generating toxic oligomers, the toxicity triggered by A β species could not be fully controlled using metal chelators. Alternatively, a new class of small molecules was engineered as chemical reagents capable of forming a ternary complex with Cu(II)–A β and consequently varying the reactivities of Cu(II)–A β .²³⁻²⁵ These molecules, however, are not able to chelate Cu(II) out from Cu(II)–A β complexes. To date, no chemical reagent or approach has been invented for sequestering Cu(II) from Cu(II)–A β and subsequently altering the on-pathway oligomerization of the metal-free A β produced upon removal of Cu(II) from Cu(II)–A β . To achieve these two aspects, modifying the coordination sphere of Cu(II)–A β would be an effective tactic to disrupt Cu(II) binding to A β and obtain chemically modified metal-free A β , which could eventually modulate the aggregation and cytotoxicity of both Cu(II)–A β and the resultant metal-free A β .

Herein, *for the first time*, we report a novel mechanistic strategy for directly modifying the Cu(II) coordination site in Cu(II)–A β complexes in a metal binding residue-specific manner (Figure 1a), to the best of our knowledge. The chemical modifications of Cu(II)–

A β complexes, achieved by our newly designed small molecule-based peptide modifier (**L1**; Figure 1b), include (i) the covalent bond formation between His14, a Cu(II)-binding amino acid residue in A β ,¹⁴⁻¹⁷ and a structural fragment of **L1** and (ii) the oxidation of His14 into 2-oxohistidine. Such modifications are demonstrated to effectively block Cu(II)–A β complexation and control the aggregation and toxicity of both Cu(II)–A β and the resultant metal-free A β . Collectively, our overall approaches and findings would contribute to the development of new effective strategies to regulate metal-interacting amyloidogenic peptides associated with neurodegeneration through directly modifying their metal coordination sites.

RESULTS AND DISCUSSION

Rational Design and Characterization of a Small Molecule as a Peptide Modifier for the Cu(II) Coordination Site in Cu(II)–A β Complexes. A small molecule (**L1**) was rationally devised as a peptide modifier capable of transforming the Cu(II)-binding amino acid residues in A β (Figure 1a; covalent adduct formation and oxidation). For the design of **L1** (Figure 1b), two structural moieties, i.e., thiophen-2-ylmethanamine and *N,N*-dimethylaniline (**DMA**), were incorporated into a structural entity for interactions with Cu(II) and A β , respectively. The C–N bond attached to **DMA** at the *para* position in **L1** could be hydrolytically cleaved in the presence of Cu(II)–A β ,²⁵⁻²⁷ generating thiophen-2-ylmethanol (**TM**) and *N,N*-dimethyl-*p*-phenylenediamine (**DMPD**), as depicted in Scheme 1 (i) and (ii). Both fragments (**TM** and **DMPD**²⁸) could be conjugated with A β that contains multiple nucleophilic amino acid residues (e.g., His, Lys, and Tyr). In addition to **L1**'s fragmentation, the oxidation of **L1** to **L1**^{••} can occur while Cu(II)–A β

is reduced to Cu(I)–A β as shown in Scheme (iii) (vide infra, oxidation potential).^{25,29} Cu(I)–A β can undergo the reaction with dioxygen (O₂) affording A β oxidation under aerobic conditions.^{12,30-32} In addition, a cationic radical form of **L1** could abstract a hydrogen atom from A β producing a carbon-centered peptide radical which is able to react with O₂ leading to peptide oxidation.^{30,33,34} Together, the small molecule, **L1**, is rationally engineered to carry out (i) the covalent conjugation of its structural fragment with A β as well as (ii) the oxidation of A β at the Cu(II)-binding site (i.e., His14; vide infra). These peptide modifications towards Cu(II)–A β are able to impact the aggregation and toxicity of Cu(II)–A β and the resultant metal-free A β through disrupting Cu(II)–A β binding and generating chemically transformed metal-free A β .

To confirm whether the structural transformations of **L1** (i.e., hydrolytic cleavage and oxidation; Scheme 1) can be responsible for modifications of the coordination sphere of Cu(II)–A β , two additional compounds, **L2** and **L3**, were constructed through the replacement of the C–N bond of **L1** with the C–S bond, as presented in Figure 1b. Similar to **L1**, **L2** and **L3** contain **DMA** for A β interaction²⁵⁻²⁷ and a thiophen-2-ylmethanethioether group (for **L2**) or a pyridin-2-ylmethanethioether group (for **L3**) for metal interaction; thus, they could interact with Cu(II)–A β . On the other hand, Cu(II)- or Cu(II)–A β -mediated S_N2-type cleavage of **L2** and **L3** is relatively harder than that of **L1** since the *p*-aminothiophenol moiety in their overall structures shows its higher oxidation potential than **DMPD**²⁹ thus serving as a poor leaving group. In addition, the redox property of the *p*-aminothiophenol moiety would make **L2** and **L3** harder to undergo

one-electron oxidation compared to **L1**. Overall, **L1**'s transformations could be validated to be critical for its reactivity against Cu(II)–A β complexes via the studies of **L2** and **L3**.

As summarized in Scheme S1, **L1–L3** were prepared through reductive amination (for **L1**) and substitution reactions (for **L2** and **L3**). More detailed information for the synthesis and characterization of **L1–L3** is provided in the Supporting Information (Scheme S1 and Figures S1–S3). **L1–L3** are expected to cross the blood-brain barrier (BBB) [$\log_{BB}$ = 0.345 (for **L1**), 0.702 (for **L2**), and 0.338 (for **L3**)] adhering to the values of Lipinski's rule (Table S1).^{35,36} Furthermore, the in vitro parallel artificial membrane permeability assay adapted for BBB (PAMPA–BBB) was employed to obtain the experimental permeability values ($-\log P_e$) of the compounds. The values of $-\log P_e$ of compounds were 4.31 (for **L1**), 4.61 (for **L2**), and 4.35 (for **L3**), indicating the possible availability of these small molecules in the central nervous system (Table S1).^{35,36}

Modifications of the Coordination Sphere of Cu(II)–A β Complexes by L1. Chemical variations at the Cu(II) coordination site in Cu(II)–A β complexes by **L1** were identified by electrospray ionization–mass spectrometry (ESI–MS) and tandem MS (ESI–MS²). When A β_{40} was incubated with Cu(II), Cu(II)-bound A β_{40} was detected in the ESI–MS spectrum (Figure 2a; left). Upon treatment of Cu(II)-treated A β_{40} with **L1**, distinctive peptide modifications, i.e., covalent conjugation and oxidation, were indicated, along with the disappearance of the peak corresponding to Cu(II)–A β_{40} (Figure 2a; right), while **L2** and **L3** did not noticeably affect Cu(II) binding to A β_{40} (Figure S4). In the absence of Cu(II), modifications of metal-free A β_{40} were not observed by **L1–L3** (Figure S5).

(i) *Covalent Adduct Formation between A β and the Fragmented L1*. Under Cu(II)-present conditions, a covalent adduct between A β_{40} and the structural portion of the fragmented **L1** was observed at 1476 m/z with a mass shift of 94 Da, relative to metal-free A β_{40} (1443 m/z) (Figure 2a). This peak is assigned to be a conjugation of A β_{40} with 2-methylthiophene (98 Da) followed by the loss of four protons [Scheme 1 (i)]. Moreover, the peak of Cu(II)-bound A β_{40} disappeared as **L1** was introduced into Cu(II)-added A β_{40} (Figure 2a), implying that Cu(II)-A β complexation could be disrupted through the covalent bonding between A β_{40} and 2-methylthiophene.

As shown in Scheme 1 (i) and (ii), **L1** can be hydrolytically cleaved to **TM** and **DMPD** in the presence of Cu(II)-A β based on previously reported studies.²⁵ When **TM** interacts with Cu(II) surrounded by A β , it possibly forms a transient ternary **TM**-Cu(II)-A β complex. In addition, **TM** bound to the Cu(II) center in the transient ternary complex could be first attacked by a nucleophilic amino acid residue in A β (e.g., His; vide infra) producing a covalent bond between A β and the 2-methylthiophene moiety with the dissociation of Cu(II) from Cu(II)-A β . A second nucleophilic attack on thiophene, after possible epoxidation of thiophene in the A β covalently bound to 2-methylthiophene,^{37,38} could afford a covalent adduct with A β as the final product with the addition of 94 Da to native A β [Scheme 1 (i)].³⁹

To support whether the fragmentation of **L1** to **TM** is necessary for its covalent linkage to Cu(II)-treated A β , the **TM**-added Cu(II)-A β sample was further analyzed by ESI-MS

(Figure 2b). Similar to **L1**, the peak corresponding to $[A\beta_{40} + 94]^{3+}$ was detected at 1476 m/z in the sample incubated with **TM**, along with the suppression of the peak assigned as Cu(II)-bound $A\beta_{40}$, indicative of generating a covalent adduct of $A\beta_{40}$ with 2-methylthiophene. Thus, the covalent bond formation with $A\beta_{40}$ observed upon introduction of **L1** requires the molecule's fragmentation affording **TM**.

(ii) *Oxidation of Cu(II)-Treated $A\beta$ by **L1**.* In addition to a covalent adduct of $A\beta_{40}$ with **L1**'s structural fragment, a mass shift of monomeric $A\beta_{40}$ was observed by 16 Da at 1449 m/z corresponding to the singly oxidized $A\beta_{40}$ monomer upon incubation of Cu(II)-added $A\beta_{40}$ with **L1** (Figure 2a; red peak). Cu(II)- $A\beta$ could be reduced to Cu(I)- $A\beta$ (ca. 0.08 V *versus* Ag/AgCl in H_2O) upon **L1**'s oxidation to the cationic radical, **L1**^{•+} (Scheme 1), considering the E_{pa} value of the *p*-diaminobenzene moiety in **L1** (ca. 0.1 V *versus* Ag/AgCl in H_2O).^{29,40,41} Cu(I)- $A\beta$ then reacts with O_2 forming Cu(II)($A\beta_{40}$)($O_2^{\bullet-}$) and Cu(II)($A\beta_{40}$)(O_2^{2-}) intermediates under aerobic conditions [Scheme 1 (iii), top], which could lead to $A\beta$ oxidation.^{12,30-32} In parallel, **L1**^{•+} could abstract a hydrogen atom from $A\beta$ to generate a carbon-centered peptide radical, which could further interact with O_2 to produce a peroxy radical form of $A\beta$ [Scheme 1 (iii), bottom].³³ **L1**-induced $A\beta$ oxidation site in the presence of Cu(II) was assigned to be His14 (*vide infra*). Such His oxidation could affect Cu(II) binding to $A\beta$, supported by the decrease in the peak intensity of Cu(II)- $A\beta$ (Figure 2a). To determine the fragmentation of **L1** is a prerequisite process for $A\beta$ oxidation, the sample containing **TM**, Cu(II), and $A\beta_{40}$ was analyzed. $A\beta_{40}$ oxidation, however, was not observed in this sample (Figure 2b), suggesting that

such peptide oxidation occurs through **L1**'s oxidation in the presence of Cu(II)–A β rather than the compound's fragmentation into **TM** and **DMPD** (Scheme 1).

(iii) Concurrent Modifications of His14 in Cu(II)–A β by L1: Covalent Adduct Formation and Oxidation. As depicted in Figure 2a, the singly oxidized covalent adduct was detected at 1481 m/z assigned as $[A\beta_{40} + 94 + O]^{3+}$, along with a covalent adduct of **L1**'s structural fragment with A β_{40} and the singly oxidized A β_{40} . This observation indicates that both the covalent adduct formation with A β and A β oxidation can concurrently occur when Cu(II)–A β reacts with **L1**. In addition, the reduced peak intensity of the Cu(II)–A β_{40} complex shown in Figure 2a supports the disruption of Cu(II)–A β complexation by these modifications of A β .

To identify which metal-binding residue is chemically modified by **L1**, ESI–MS² was carried out for the singly oxidized covalent adduct monitored at 1481 m/z (Figure 2c). The collision-induced dissociation (CID) on the selected ion produces b fragments useful for analyzing residue-specific modifications of peptides.²⁹ As indicated in Figure 2c, the 94 Da-added b fragments were observed from b_{14} , suggesting that the His14 residue in A β_{40} could be the first nucleophile capable of reacting with **TM** to generate a covalent adduct [Scheme 1 (i)]. In addition, the oxidized b fragments were detected from b_{14} , indicating His14 as a plausible oxidation site in A β . Lastly, the singly oxidized covalent adducts on b fragments were also monitored from b_{14} . This observation confirms that the modifications of Cu(II)–A β_{40} by **L1** occur at His14. Collectively, **L1** is able to chemically modify the metal-binding residue, His14, disrupting the complexation

between Cu(II) and A β and affording structural variations of the resultant metal-free A β , i.e., (i) covalent bond formation and (ii) oxidation. These modifications of Cu(II)–A β at His14 could significantly affect the aggregation and cytotoxicity of Cu(II)–A β and the resultant metal-free A β (vide infra).

Cu(II)–A β -Mediated Transformations of L1. To determine the molecular mechanisms for L1-mediated modifications at the Cu(II) coordination site in Cu(II)–A β complexes, the transformations of L1–L3 were investigated in detail in the presence of Cu(II) and/or A β (Figures 3 and 4).

(i) *Cleavage of L1.* The covalent adduct formation with A β_{40} is directed by L1's transformation, as shown in Figure 2b. The fragmentation of L1 into **TM** and **DMPD** in the presence of Cu(II) with or without A β_{40} was monitored by UV–vis and ESI–MS. When L1 was incubated with CuCl₂ and A β_{40} (Figure 3a; left), an increase in absorbance at ca. 375 nm was observed followed by the disappearance of the same peak after 12 h. This charge transfer band suggests the possible interaction between L1 and Cu(II).⁴² Additionally, the peaks at ca. 260 and 300 nm were slightly enhanced, which may result from the presence of a mixture of **TM** and **DMPD**. As depicted in Figure 4, **DMPD**, one of L1's cleaved products, was also detected in the ESI–MS spectrum when L1 was treated to Cu(II)–A β_{40} .

Under A β_{40} -free conditions (Figure 3a, right), the optical bands of Cu(II)-treated L1 at 260 and 300 nm were noticeably enhanced, suggesting the production of both **TM** and

DMPD. The Cu(II)-induced fragmentation of **L1** without A β was also confirmed by ESI-MS (Figure S6a). Different from **L1**, **L2** and **L3** did not show their noticeable transformation in the presence of Cu(II) and Cu(II)-A β , except for the charge transfer band in the range of ca. 300 to 500 nm (Figures 3b and S6b). This observation supports no modification at the Cu(II) coordination site in Cu(II)-A β by these two compounds (Figure S4).

(ii) *Oxidation of **L1**.* A β oxidation shown in Figure 2b could occur through **L1**'s oxidation into **L1^{•+}** in the presence of Cu(II)-A β_{40} . When **L1** was treated with Cu(II)-A β_{40} , the relatively unstable cationic radical intermediate, **L1^{•+}**, was not detected by either ESI-MS or UV-vis (Figures 3a and 4). Instead, the two-electron oxidized form (**L1²⁺**) was monitored in the low molecular weight region of the ESI-MS spectrum from the **L1**-containing Cu(II)-A β_{40} sample (Figure 4; **M_v⁺**). The observed transformation of **L1** suggests that A β oxidation is linked to the redox communication between of **L1** and Cu(II)-A β [Scheme 1 (iii)].¹²

In the presence of Cu(II) without A β_{40} , the peak at 232 *m/z* corresponding to **L1^{•+}** was detected by ESI-MS (Figure S6a). Moreover, the peaks at ca. 500 and 550 nm were exhibited when **L1** and Cu(II) were co-incubated, indicative of the one-electron oxidation of the compound (Figure 3a, right).^{28,29} Note that these double peaks may be resulted from **DMPD^{•+}** produced one-electron oxidation of **DMPD**^{28,43} (one of **L1**'s fragmented products shown in Scheme 1),^{28,43} which may be also involved in A β oxidation. In the case of **L2** and **L3**, their spectral changes at ca. 500 and 550 nm were not presented

even in the presence of Cu(II) with or without A β , suggesting no Cu(II)-mediated oxidation of these two compounds (Figures 3b and S6b).

In addition to the spectroscopic and spectrometric studies, the oxidation potentials (E_{pa}) of **L1–L3** were measured at various scan rates in DMSO and H₂O (Figures 3c, S7, and S8). The E_{pa} value of **L1** was determined as 0.267 V (*versus* Ag/AgCl in DMSO; Figure S7), which is much lower than those of **L2** (0.770 V) and **L3** (0.787 V). On the basis of their E_{pa} values, the *p*-diaminobenzene group is responsible for stabilizing the oxidized form of **L1**, consistent with the previously reported molecules.^{25,29} The E_{pa} values of **L1** and **L3** were also obtained in H₂O [0.131 V (for **L1**) and 0.538 (for **L3**) *versus* Ag/AgCl; Figure S8]. Note that the E_{pa} value of **L2** could not be measured in H₂O because of its limited solubility. Based on the reduction potential of Cu(II)–A β ₄₂ to Cu(I)–A β ₄₂ (ca. 0.08 V *versus* Ag/AgCl in H₂O),⁴¹ the oxidation potential of **L1** is low enough to reduce Cu(II)–A β to Cu(I)–A β .

Taken together, Cu(II)- or Cu(II)–A β -mediated cleavage of the C–N bond in **L1** takes place to generate **TM** and **DMPD** [Scheme 1 (i)]. The fragmentation of **L1** into **TM** is needed for the covalent bond formation with A β in the presence of Cu(II). Furthermore, **L1**, relatively easily oxidized with the redox chemistry of Cu(I/II)–A β , is able to induce the oxidation of His14 to produce 2-oxohistidine [Scheme 1 (iii)]. In both **L2** and **L3** that contain the C–S bond over the C–N bond, as shown in Figure 1, such transformations (i.e., fragmentation and oxidation) observed in **L1** were not indicated. The chemical modifications of Cu(II)–A β complexes through **L1**'s transformations could, as expected,

vary the aggregation pathways and cytotoxicity of Cu(II)–A β and the resultant metal-free A β (*vide infra*).

Modulation of the Aggregation Pathways of Cu(II)–A β by L1. To determine whether L1-induced modifications at the Cu(II) coordination site (i.e., covalent adduct formation and oxidation) affect the aggregation pathways of Cu(II)–A β , the size distribution and morphology of the resultant A β species were verified by gel electrophoresis with Western blotting (gel/Western blot) and transmission electron microscopy (TEM), respectively. Two types of experiments were conducted: (i) in inhibition experiments (Figures 5a and S11), freshly prepared A β monomers were incubated with L1–L3 in the presence and absence of Cu(II) for 24 h; (ii) in disaggregation experiments (Figures S9 and S12), A β peptides are first pre-incubated with or without Cu(II) for 24 h followed by treatment with L1–L3 for an additional 24 h.

In the inhibition experiments, the aggregation pathways of both Cu(II)–A β_{40} and Cu(II)–A β_{42} were altered by L1 (Figures 5 and S11). When L1 was incubated with Cu(II)-treated A β species, noticeable smearing bands were indicated in the gel/Western blots, compared to those obtained with compound-free Cu(II)–A β samples (Figure 5b; left). The resultant A β aggregates produced in the presence of L1 from the inhibition experiments, visualized by TEM, were observed to be shorter and smaller, relative to compound-free Cu(II)–A β samples (Figures 5c and S11). Aggregation of metal-free A β_{40} and A β_{42} was not affected in the presence of L1, confirmed by the gel/Western (no smearing bands; Figure 5b; right) and TEM (no morphological change; Figures 5c and

S11). As expected from no transformation of the compounds under our experimental conditions, **L2** and **L3** were not able to alter the aggregation pathways of both Cu(II)-added A β_{40} /A β_{42} and metal-free A β_{40} /A β_{42} based on the results obtained by gel/Western blots and TEM (Figures 5b, 5c, and S11).

The disassembly or modulation of further aggregation of preformed Cu(II)-treated and metal-free A β aggregates by **L1–L3** was also investigated (Figures S9 and S12). Similar to the inhibition studies, the gel/Western blots showed (i) the alteration of the aggregation pathways of preformed Cu(II)-treated A β_{40} and A β_{42} aggregates in the presence of **L1**; (ii) no influence on metal-free A β_{40} and A β_{42} aggregates by **L1** (Figures S9b). In the TEM images, short fibrillar A β aggregates were monitored upon treatment of preformed Cu(II)–A β aggregates with **L1**, while long and large A β fibrils were still found under metal-free conditions (Figures S9c and S12). Moreover, **L2** and **L3** exhibited no effects towards the aggregation of preformed Cu(II)–A β_{40} /A β_{42} and metal-free A β_{40} /A β_{42} aggregates according to gel/Western blot and TEM studies (Figures S9 and S12). Overall, the aggregation of both Cu(II)–A β_{40} and Cu(II)–A β_{42} over metal-free A β aggregation is noticeably modulated by **L1**, while **L2** and **L3** cannot significantly affect the aggregation of both Cu(II)-added and metal-free A β . These observations demonstrate that the metal binding residue-specific chemical modifications of Cu(II)–A β by treatment of **L1** are able to notably impact the aggregation pathways of Cu(II)–A β and the metal-free A β produced from removal of Cu(II) from Cu(II)–A β .

Regulation of Cu(II)–A β -Induced Toxicity and Free Radicals by L1. To identify if the metal binding residue-specific modifications of Cu(II)–A β complexes affect Cu(II)–A β -induced toxicity in living cells, cell studies employing the human neuroblastoma SH-SY5Y (5Y) cell line were conducted. 5Y cells were incubated with A β ₄₀, CuCl₂, and **L1** for 24 h, and their survival was determined by the MTT assay [MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The cell viability of Cu(II)-treated A β was improved by ca. 25% in the presence of **L1**, relative to that of compound-free Cu(II)–A β (Figure 6a), indicating that **L1**-induced modifications of Cu(II)–A β complexes at His14 could alleviate the toxicity triggered by Cu(II)–A β complexes. Note that our compounds (**L1–L3**) are relatively less toxic under the conditions used for this work (> 80% of cell survival up to 25 μ M) (Figure S13).

Furthermore, the regulation of free organic radicals by **L1–L3** was monitored by the Trolox equivalent antioxidant capacity (TEAC) assay,⁴⁴ since Cu(II)–A β complexes can produce free radicals in the AD-affected brain (Figure 6b; left).^{11,19} The TEAC values of small molecules indicate their ability to quench free radicals [e.g., ABTS^{•+}; ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)], relative to that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a vitamin E analogue. The TEAC value of **L1** was 1.86 ($\pm$ 0.10), presenting the compound as a better scavenger against free organic radicals than Trolox. The TEAC values of **L2** and **L3** were 0.86 ($\pm$ 0.10) and 0.96 ($\pm$ 0.13), similar to those of Trolox. The trend of the compounds' antioxidant properties is highly correlated to their E_{pa} values (Figure 6b; right). **L1** with a relatively low E_{pa} value exhibited a relatively high TEAC value. These observations are consistent

with the previous reports regarding a direct relation between redox properties of compounds and their antioxidant activity.^{29,45}

CONCLUSIONS

An effective and straightforward mechanistic approach was developed for chemically modifying the coordination sphere of Cu(II)–A β complexes in a metal binding residue-specific manner. The modifications (i.e., covalent adduct formation and oxidation at a Cu(II)-binding amino acid residue, His14) were achieved using a rationally engineered small molecule-based peptide modifier (**L1**). The chemical modifications at the metal-binding residue, His14, are able to (i) block the complexation between Cu(II) and A β and (ii) alter the aggregation characteristics of both Cu(II)–A β and the resultant Cu(II)-free A β species, producing less toxic A β species. Our molecular-level mechanistic investigations propose that such modifications in the coordination sphere of Cu(II)–A β complexes occur through the transformation of **L1** in the presence of Cu(II)–A β . Previous studies have introduced peptide modifications to a broad range of A β or other amyloidogenic peptides to regulate their aggregation pathways;^{46–48} however, no example was reported for directly modifying the coordination sphere of metal–peptide complexes. Our overall multidisciplinary studies with emphasis on novel strategic design, reactivities, and mechanisms demonstrate the validity of developing rational mechanistic tactics for modulating metal-interacting aggregation-prone peptides or proteins related to the pathologies of neurodegenerative disorders.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website.
Experimental Section, Scheme S1, Tables S1–S3, and Figures S1–S13 (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government [NRF-2016R1A5A1009405 and NRF-2017R1A2B3002585 (to M.H.L.); NRF-2015R1D1A1A01060188 (to J.C.)]; KAIST (to M.H.L.). We thank Geewoo Nam for assistance in initial manuscript writing.

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Scheme 1. Proposed Mechanisms for Metal Binding Residue-Specific Modifications of Cu(II)–A β by L1.

Figure 1. Peptide modifications and small molecules (**L1–L3**). (a) Chemical modifications (i.e., covalent adduct formation and oxidation) from the reaction of Cu(II)–A β with a newly designed peptide modifier (**L1**). (b) Chemical structures of **L1** (*N*¹,*N*¹-dimethyl-*N*⁴-(thiophen-2-ylmethyl)benzene-1,4-diamine), **L2** (*N,N*-dimethyl-4-((thiophen-2-ylmethyl)thio)aniline), and **L3** (*N,N*-dimethyl-4-((pyridin-2-ylmethyl)thio)aniline).

Figure 2. Modifications of Cu(II)-treated $A\beta_{40}$ by **L1** or **TM**, analyzed by ESI-MS. (a and b) ESI-MS spectra of +3-charged Cu(II)-treated $A\beta_{40}$ monomers incubated with or without **L1** or **TM**. The peaks of a covalent adduct between $A\beta_{40}$ and 2-methylthiophene (1476 m/z) and the singly oxidized $A\beta_{40}$ (1449 m/z) are indicated in orange and red, respectively. The singly oxidized covalent adduct (1481 m/z) is presented as a green peak. One and two Na^+ adducts of $A\beta_{40}$ are shown with black dots. (c) ESI-MS² spectrum of the singly oxidized covalent adduct, $[A\beta_{40} + 94 + O]^{3+}$, detected at 1481 m/z . Chemical bonds of the selected ion are cleaved by CID. The gray, red, orange, and green boxes indicate b_x fragments corresponding to $A\beta$, oxidized $A\beta$, $A\beta$ bound to 2-methylthiophene, and oxidized $A\beta$ bound to 2-methylthiophene, respectively. Conditions: $[A\beta_{40}] = 100 \mu M$; $[CuCl_2] = 100 \mu M$; [compound] = 500 μM ; incubation for 1 h; 20 mM ammonium acetate, pH 7.2; 37 °C; no agitation. The samples were diluted with ddH₂O by 10 fold prior to injection to the mass spectrometer.

Figure 3. Transformations and oxidation potentials of **L1–L3**. UV–vis spectral changes of (a) **L1** upon addition of Cu(II) with and without $A\beta_{40}$ and (b) **L2** and **L3** incubated with Cu(II) and $A\beta_{40}$ under aerobic conditions. The new appearance of the peaks in (a) and (b) is indicated with black arrows. Conditions: $[A\beta_{40}] = 25 \mu\text{M}$; $[CuCl_2] = 25 \mu\text{M}$; [compound] = $50 \mu\text{M}$; room temperature; incubation for 1 (black), 4 (red), and 12 h (blue). (c) Oxidation potentials (E_{pa}) of **L1**, **L2**, and **L3** in DMSO and ddH₂O, measured by cyclic voltammetry. The E_{pa} values of **L1–L3** at various scan rates are summarized in Tables S2 and S3. Conditions: [compound] = 1 mM; 0.1 M *tetra-N*-butylammonium perchlorate (in DMSO) or 1 M NaCl (in ddH₂O); 250 mV/s; room temperature; three electrodes are composed of the glassy carbon working electrode, platinum counter electrode, and Ag/Ag(I) reference electrode. Note that the E_{pa} value of **L2** could not be measured in H₂O because of its limited solubility.

Figure 4. Oxidative transformations of **L1** upon incubation with Cu(II)-treated $A\beta_{40}$. (a) ESI-MS spectrum from the sample containing $A\beta$, Cu(II), and **L1**. (b) Chemical structures of M_i – M_v . Conditions: $[A\beta_{40}] = 100 \mu M$; $[CuCl_2] = 100 \mu M$; $[L1] = 500 \mu M$; incubation for 1 h; 20 mM ammonium acetate, pH 7.2; 37 °C; no agitation. The samples were diluted with ddH₂O by 10 fold prior to injection to the mass spectrometer.

Figure 5. Analysis of Cu(II)-bound and metal-free A β aggregation with or without treatment of **L1–L3**. (a) Scheme of the inhibition experiments. (b) Analysis of the size distribution of the resultant A β ₄₀ (top) and A β ₄₂ (bottom) species by gel/Western blot using an anti-A β antibody (6E10). Lanes: (C) A β $\pm$ CuCl₂; (**L1**) C + **L1**; (**L2**) C + **L2**; (**L3**) C + **L3**. The original gel images are shown in Figure S10a. (c) Morphologies of Cu(II)-bound (left) and metal-free (right) A β ₄₀ aggregates from (b), monitored by TEM. Scale bar = 200 nm. Conditions: [A β] = 25 μ M; [CuCl₂] = 25 μ M; [compound] = 50 μ M; pH 6.6 [for Cu(II)-containing samples] or pH 7.4 (for metal-free samples); 37 $^\circ$ C; constant agitation.

Figure 6. Regulation of Cu(II)–Aβ-induced cytotoxicity and free radicals by compounds. (a) Effect of **L1** on Cu(II)–Aβ-induced toxicity in SHSY-5Y cells, determined by the MTT assay. Cell viability was calculated in comparison to that obtained upon treatment with DMSO only (1% v/v). $P < 0.05$. Conditions: [Aβ₄₀] = 10 μM, [CuCl₂] = 5 μM, [compound] = 10 μM; 37 °C. (b) Scavenging capability of **L1–L3** against free organic radicals (in EtOH), measured by the TEAC assay, and the plot of the TEAC values of the compounds with their E_{pa} values.

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Supporting Information
for
Mechanistic Approaches for Modifying the Coordination Sphere of
Metal–Amyloid- β Complexes: Covalent Adduct Formation and Oxidation

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Experimental Section

Materials and Methods

All reagents were purchased from commercial suppliers and used as received unless otherwise noted. A β ₄₀ and A β ₄₂ (A β ₄₂ = DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAILGLMVGGVVIA) were purchased from Anaspec (Fremont, CA, USA). Trace metal ions were removed from the solutions used for the studies by treatment with Chelex overnight (Sigma-Aldrich, St. Louis, MO, USA). ESI-MS and ESI-MS² analyses were performed by a Waters Synapt G2-Si quadrupole time-of-flight (Q-Tof) ion mobility mass spectrometer (Waters, Manchester, UK) equipped with an ESI source [DGIST Center for Core Research Facilities (CCRF), Daegu, Republic of Korea]. Optical spectra were recorded on an Agilent 8453 UV-vis spectrophotometer. Cyclic voltammograms were obtained under N₂ (g) on a CHI620E potentiostat (Qrins, Seoul, Republic of Korea). TEM images were taken by a JEOL JEM-2100 transmission electron microscope [UNIST Central Research Facilities (UCRF), Ulsan, Republic of Korea]. Absorbance values for the PAMPA-BBB measurements, the MTT assay and the Trolox equivalent antioxidant capacity (TEAC) assay [MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were obtained by a Molecular Devices SpectraMax M5e microplate reader (Sunnyvale, CA, USA). ¹H and ¹³C NMR spectra were recorded on a 400 MHz Agilent NMR spectrometer (UCRF). The high-resolution mass spectra of compounds were taken by a Q exactive plus orbitrap mass spectrometer (Thermo Fisher Scientific, Carlsbad, CA, USA).

Preparation of L1. 2-Thiophenecarboxaldehyde (0.098 mL, 1 mmol) was added into a solution of *N,N*-dimethyl-*p*-phenylenediamine (140 mg, 1 mmol) in EtOH (5 mL). After refluxing the solution for 1 h, the reaction mixture was cooled to room temperature. The solvent was removed under vacuum yielding an imine product (brown solid). Sodium borohydride (NaBH₄; 230 mg, 6 mmol) was added into a solution of the imine product (230 mg, 1 mmol) in CH₃OH (10 mL) at 0 °C. After 10 min, the reaction mixture was stirred at room temperature for 30 min. The reaction was quenched with H₂O and extracted with CH₂Cl₂ (3x). The combined organic phase was washed with brine (1x) and dried over anhydrous magnesium sulfate (MgSO₄). The crude compound was purified by column

chromatography (SiO₂; EtOAc:hexanes = 1:3) yielding a yellow solid product (130 mg, 54%). ¹H NMR [400 MHz, DMSO-*d*₆, δ (ppm)]: 7.33 (1H, dd, J = 1.2 Hz, J = 5.0 Hz), 7.01 (1H, dq, J = 3.6 Hz), 6.95 (1H, dd, J = 3.6 Hz, J = 5.0 Hz), 6.62-6.59 (2H, m), 6.58-6.54 (2H, m), 5.60-5.57 (1H, m), 4.36 (2H, d, J = 5.6 Hz), 2.70 (6H, s). ¹³C NMR [100 MHz, DMSO-*d*₆, δ (ppm)]: 145.1, 143.2, 140.5, 126.6, 124.4, 124.2, 115.1, 113.8, 43.0, 41.7. HRMS (m/z): [M + H]⁺ Calcd. for C₁₃H₁₇N₂S: 233.1112, found: 233.1099.

Preparation of L2. 2-(Bromomethyl)thiophene (62 mg, 0.4 mmol) was added dropwise to a solution of potassium hydroxide (KOH; 33 mg, 0.6 mmol) and 4-(dimethylamino)benzenethiol (30 mg, 0.2 mmol) in DMF/H₂O (2 mL/0.2 mL). The reaction mixture was stirred at room temperature for 24 h and then cooled to 0 °C. The reaction was quenched with H₂O and extracted with EtOAc (4x). The combined organic layer was dried over anhydrous MgSO₄ and concentrated under vacuum. The crude compound was purified by column chromatography (SiO₂; EtOAc:hexanes = 1:6) yielding a yellow liquid product (36 mg, 73%). ¹H NMR [400 MHz, DMSO-*d*₆, δ (ppm)]: 7.36 (1H, dd, J = 5.1 Hz, J = 1.3 Hz), 7.22-7.18 (2H, m), 6.88 (1H, dd, J = 5.1 Hz, J = 3.4 Hz), 6.82-6.81 (1H, m), 6.66-6.62 (2H, m), 4.21 (2H, d, J = 0.7 Hz), 2.80 (6H, s). ¹³C NMR [100 MHz, CD₃OD, δ (ppm)]: 150.5, 141.8, 134.4, 126.0, 125.7, 124.1, 120.1, 112.3, 39.2, 35.5. HRMS (m/z): [M + H]⁺ Calcd. for C₁₃H₁₆NS₂: 250.0724, found: 250.0713.

Preparation of L3. 2-(Bromomethyl)pyridine hydrobromide (100 mg, 0.4 mmol) was added dropwise to a mixture of KOH (57 mg, 1 mmol) and 4-(dimethylamino)benzenethiol (30 mg, 0.2 mmol) in DMF/H₂O (3 mL/0.3 mL). The reaction mixture was stirred for 4 h at room temperature and then cooled to 0 °C. The mixture was diluted with H₂O and extracted with EtOAc (4x). The combined organic layer was dried over anhydrous MgSO₄ and concentrated under vacuum. The crude compound was purified by column chromatography (SiO₂; EtOAc:hexanes = 1:4) yielding a yellow liquid product (33 mg, 65%). ¹H NMR [400 MHz, CD₃OD, δ (ppm)]: 8.40 (1H, ddd, J = 0.8 Hz, J = 4.8 Hz), 7.67 (1H, td, J = 7.6 Hz, J = 1.6 Hz), 7.25 (1H, ddd, J = 1.2 Hz), 7.22-7.14 (3H, m), 6.64-6.60 (2H, m), 4.01 (2H, s), 2.90 (6H, s). ¹³C NMR [100 MHz, CD₃OD, δ (ppm)]: 159.8, 152.0,

149.5, 138.4, 136.0, 125.2, 123.4, 120.6, 113.9, 43.6, 40.6. HRMS (m/z): $[M + H]^+$ Calcd. for $C_{14}H_{17}N_2S$: 245.1112, found: 245.1099.

Parallel Artificial Membrane Permeability Assay Adapted for the Blood-Brain Barrier (PAMPA–BBB). PAMPA–BBB experiments were conducted employing the PAMPA Explorer kit (pION Inc., Billerica, MA, USA) following previously reported protocols.^{1–3} Compounds (25 μ M, 200 μ L) in Prisma HT buffer (pH 7.4, pION) were added to the wells of a donor plate (number of replicates = 12). The polyvinylidene fluoride (PVDF, 0.45 μ M) filter membrane on the acceptor plate was coated with BBB-1 lipid formulation (5 μ L, pION). The acceptor plate was then placed on the top of the donor plate. Brain sink buffer (BSB, 200 μ L, pION) was added to each well of the acceptor plate and was incubated for 4 h at room temperature without agitation. UV–vis spectra of the solutions in the reference, acceptor, and donor plates were measured by a microplate reader. The PAMPA Explorer software v. 3.5 (pION) was used to calculate the $-\log P_e$ values for compounds. CNS $\pm$ designations were assigned by comparison with compounds that were identified in the previous reports.^{4,5}

A β Aggregation Experiments. A β_{40} or A β_{42} was dissolved in ammonium hydroxide [NH_4OH (aq); 1% v/v]. The resulting solution was lyophilized and stored at $-80^\circ C$. A stock solution of A β was then prepared by dissolving the lyophilized peptide with NH_4OH (1% v/v, 10 μ L) and diluting with ddH $_2O$. All A β samples were prepared following the previously reported procedures.^{1–3,6} The concentration of the peptide solution was determined by measuring the absorbance of the solution at 280 nm ($\epsilon = 1,450\text{ M}^{-1}\text{cm}^{-1}$ for A β_{40} ; $\epsilon = 1,490\text{ M}^{-1}\text{cm}^{-1}$ for A β_{42}). The peptide stock solution was diluted to a final concentration of 25 μ M in the Chelex-treated buffer [20 μ M HEPES, pH 6.6 [for Cu(II) samples] or pH 7.4 (for metal-free samples), 150 μ M NaCl]. For the inhibition studies, compounds (final concentration, 50 μ M; 1% v/v DMSO) were added to the samples of A β (25 μ M) in the absence and presence of a copper chloride salt ($CuCl_2$; 25 μ M) followed by incubation at $37^\circ C$ with constant agitation for 24 h. For the disaggregation studies, A β (25 μ M) was incubated with and without $CuCl_2$ (25 μ M) for 24 h at $37^\circ C$ with constant agitation to

generate preformed A β aggregates. The resulting peptide aggregates were then treated with compounds (50 μ M) and incubated with constant agitation for an additional 24 h.

Electrospray Ionization–Mass Spectrometry (ESI–MS) and Tandem MS (ESI–MS²).

The experiments were performed following the previously reported methods.^{7–9} A β ₄₀ (100 μ M) was incubated with compounds (500 μ M; 1% v/v DMSO) and/or CuCl₂ (100 μ M) in 20 mM ammonium acetate (pH 7.2) at 37 °C without agitation. Incubated samples were diluted by 10 fold with ddH₂O and then injected into the mass spectrometer. The capillary voltage, sampling cone voltage, and source temperature were set to 2.8 kV, 70 V, and 40 °C, respectively. The backing pressure was adjusted to 2.7 mbar. Tandem MS (ESI–MS²) analyses were additionally performed on the **TM**–Cu(II)–A β complexes. The ESI parameters and experimental conditions were the same as above. The collision-induced dissociation (CID) was conducted by applying the collision energy in the trap and adjusting the low mass (LM) resolution to 15. More than 200 spectra were obtained for each sample and averaged for the analysis.

Cyclic Voltammetry. Cyclic voltammograms of compounds (dissolved in DMSO; final concentration, 1 mM) were recorded in 0.1 M *tetra*-N-butylammonium perchlorate (in DMSO) and 1 M NaCl (in ddH₂O; 1% v/v DMSO) at various scan rates (25, 50, 100, 150, 200, and 250 mV/s) at room temperature. A three-electrode setup is composed of an Ag/Ag(I) reference electrode [RE-1B Reference electrode Ag/Ag(I); Qrins], a Pt wire auxiliary electrode (SPTE Platinum electrode; Qrins), and a glassy carbon working electrode (Qrins).

Gel Electrophoresis with Western Blot (Gel/Western Blot). The resultant A β species from in vitro experiments were analyzed by gel/Western blot using an anti-A β antibody (6E10).^{1–3,6} The samples (10 μ L) were separated on a 10–20% Tris-tricine gel (Thermo Fisher Scientific). Following separation, the proteins were transferred onto nitrocellulose membranes and blocked with bovine serum albumin (BSA, 3% w/v, Sigma-Aldrich) in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 2 h (at room

temperature) or overnight (at 4 °C). The membranes were incubated with the anti-A β antibody (6E10) (1:2,000, Covance, Princeton, NJ, USA) in a solution of BSA (2% w/v in TBS-T) for 4 h (at room temperature) or overnight (at 4 °C). After washing with TBS-T (3x, 10 min), a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000 in 2% w/v BSA in TBS-T; Cayman Chemical Company, MI, USA) was added for 1.5 h at room temperature. A homemade ECL kit^{7,8,10} was used to visualize the gel/Western blots on a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Transmission Electron Microscopy (TEM). Samples for TEM were prepared following the previously reported methods.^{1-3,6-8} Glow-discharged grids (Formvar/Carbon 300-mesh, Electron Microscopy Sciences, Hatfield, PA, USA) were treated with A β samples (25 μ M, 5 μ L) for 2 min at room temperature. Excess sample was removed using filter paper followed by washing twice with ddH₂O. Each grid, incubated with uranyl acetate (1%, ddH₂O, 5 μ L) for 1 min, was blotted off and dried for 15 min at room temperature. Images for each sample were taken with 200 kV and 25,000x magnification.

Cell Viability Studies. The human neuroblastoma SH-SY5Y cell line was purchased from the American Type Culture Collection (ATCC, VA, USA). The cell line was maintained in media containing 50% minimum essential medium (MEM) and 50% F12 (GIBCO, NY, USA), and supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin, and 100 mg/mL streptomycin (GIBCO). Cells were grown and maintained at 37 °C in a humidified atmosphere with 5% CO₂. The cells used for our studies did not indicate mycoplasma contamination. Cell viability upon treatment with compounds was determined by the MTT assay. Cells were seeded in a 96 well plate (15,000 cells in 100 μ L per well) and treated with compounds (5, 10, and 25 μ M, 1% v/v DMSO). After 24 h incubation, MTT [25 μ L of 5 mg/mL in PBS (pH 7.4, GIBCO)] was added to each well, and the plate was incubated for 4 h at 37 °C. Formazan produced by cells was solubilized using an acidic solution of DMF (pH 4.5, 50% v/v, aq) and sodium dodecyl sulfate (SDS; 20% w/v) overnight at room temperature in the dark. The absorbance was measured at

600 nm by a microplate reader. Cell viability was calculated relative to that of the cells containing an equivalent amount of DMSO.

Trolox equivalent antioxidant capacity (TEAC) Assay. The ability of compounds to scavenge free organic radicals in EtOH was determined by the assay according to a previously reported method with slight modifications.^{1,7} 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) with potassium persulfate [K₂(SO₄)₂; 2.5 mM] was dissolved in 5 mL of ddH₂O 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. Our compounds and Trolox (Trolox = 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were dissolved in EtOH and treated to the solution of ABTS^{•+}. The final concentrations (2.1, 4.3, 6.4, 8.6, 11, and 16 µM) of compounds and Trolox were used. After 10 min incubation at room temperature, the absorbance at 750 nm was recorded. The percent inhibition was calculated according to the measured absorbance [% inhibition = 100 × (A₀ – A)/A₀, where A and A₀ are the absorbance of the samples with and without compound treatment, respectively] and was plotted as a function of compound concentration. The TEAC values of compounds for each time point were calculated as a ratio of the slope of the compound to that of Trolox.

Scheme S1. Synthetic routes to **L1–L3**.

Table S1. Values (MW, clog*P*, HBA, HBD, PSA, logBB, and -log*P*_e) of **L1–L3**.

	L1	L2	L3	Lipinski's rules and others
MW ^a	232	249	244	≤ 450
clog <i>P</i> ^b	15.3	4.08	2.94	≤ 5
HBA ^c	2	1	2	≤ 10
HBD ^d	1	0	0	≤ 5
PSA (Å ²) ^e	15.3	3.24	16.1	≤ 90
logBB ^f	0.345	0.702	0.338	< -1.0 (poorly)
-log <i>P</i> _e ^g	4.31 ± 0.13 (CNS+)	4.61 ± 0.22 (CNS+)	4.35 ± 0.07 (CNS+)	-log <i>P</i> _e < 5.4 (CNS+) -log <i>P</i> _e > 5.7 (CNS-)

^aMW, molecular weight; ^bclog*P*, calculated log of water-octanol partition coefficient; ^cHBA, hydrogen bond acceptor atom; ^dHBD, hydrogen bond donor atom; ^ePSA, polar surface area; ^flogBB = 0.152 × clog*P* - 0.0148 × PSA + 0.139; ^g-log*P*_e, the value obtained using the PAMPA–BBB assay with the PAMPA Explorer software v. 3.5. Compounds assigned to be CNS+ are able to penetrate the BBB and thus be available in the central nervous system (CNS).

Table S2. Quantitative data of cyclic voltammograms of compounds in DMSO.

Scan rate (mV/s)	L1		L2		L3	
	E _{pa} (V)	i _{pa} (μA)	E _{pa} (V)	i _{pa} (μA)	E _{pa} (V)	i _{pa} (μA)
25	0.209	7.22	0.716	18.6	0.730	22.5
50	0.225	14.0	0.732	25.6	0.748	31.9
100	0.241	18.9	0.749	39.1	0.763	44.6
150	0.251	20.0	0.756	48.4	0.770	49.4
200	0.256	26.4	0.761	48.7	0.775	56.7
250	0.267	41.9	0.770	53.3	0.787	60.5

Table S3. Quantitative data of cyclic voltammograms of compounds in ddH₂O.^a

Scan rate (mV/s)	L1			
	E _{pa} (V)	i _{pa} (μA)	E _{pc} (V)	i _{pc} (μA)
25	0.117	2.58	0.0721	0.106
50	0.116	3.96	0.0764	0.341
100	0.118	5.73	0.0798	0.373
150	0.122	9.33	0.0734	0.993
200	0.129	12.2	0.0793	1.61
250	0.131	15.2	0.0812	1.57

Scan rate (mV/s)	L3			
	E _{pa} (V)	i _{pa} (μA)	E _{pc} (V)	i _{pc} (μA)
25	0.544	4.92	0.481	2.72
50	0.539	7.01	0.481	4.21
100	0.536	11.0	0.483	6.30
150	0.538	13.3	0.483	7.75
200	0.539	16.3	0.483	9.52
250	0.538	21.0	0.48	11.0

^aNote that the E_{pa} of **L2** could not be measured in 1 M NaCl due to its limited solubility in H₂O.

Figure S1. ¹H (a, 400 MHz) and ¹³C (b, 100 MHz) NMR spectra of **L1** in DMSO-*d*₆.

Figure S2. ¹H (a, 400 MHz) and ¹³C (b, 100 MHz) NMR spectra of **L2** in DMSO-*d*₆ and CD₃OD, respectively.

Figure S3. ^1H (a, 400 MHz) and ^{13}C (b, 100 MHz) NMR spectra of **L3** in CD_3OD .

Figure S4. Interactions of **L2** and **L3** with Cu(II)-treated $A\beta_{40}$, analyzed by ESI-MS. Metal-free and Cu(II)-bound $A\beta_{40}$ species are indicated in gray and blue, respectively. Conditions: $[A\beta_{40}] = 100 \mu\text{M}$; $[\text{CuCl}_2] = 100 \mu\text{M}$; [compound] = $500 \mu\text{M}$; incubation for 1 h; 20 mM ammonium acetate, pH 7.2; 37 °C; no agitation. The samples were diluted with ddH₂O by 10 fold prior to injection to the mass spectrometer.

Figure S5. Interactions of **L1–L3** with metal-free $A\beta_{40}$, analyzed by ESI–MS. Na^+ and K^+ adducts of $A\beta_{40}$ were marked with black dots and diamonds, respectively. Conditions: $[A\beta_{40}] = 100 \mu M$; $[compound] = 500 \mu M$; incubation for 3 h; 20 mM ammonium acetate, pH 7.2; 37 °C; no agitation. The samples were diluted with ddH₂O by 10 fold prior to injection to the mass spectrometer.

Figure S6. Transformations of **L1–L3** in the presence of Cu(II). (a) Cu(II)-mediated transformation of **L1**, monitored by ESI–MS. The fragmentation and oxidation of **L1** were observed at 135 and 232 m/z , respectively. Conditions: [**L1**] = 50 μM ; [CuCl_2] = 25 μM ; 20 mM ammonium acetate, pH 7.4; 37 $^\circ\text{C}$; no agitation. The samples were diluted with ddH₂O by 10 fold prior to injection to the mass spectrometer. (b) Optical spectra of **L2** and **L3** upon incubation with Cu(II) for 0, 4, and 12 h. Conditions: [**L2** or **L3**] = 50 μM ; [CuCl_2] = 25 μM ; pH 7.4; room temperature; incubation for 1 (black), 4 (orange), and 12 h (blue).

Figure S7. Oxidation potentials of **L1–L3** at various scan rates in DMSO, measured by cyclic voltammetry. The dashed black lines indicate the oxidation potentials of compounds at 250 mV/s. Conditions: [compound] = 1 mM; 0.1 M *tetra-N*-butylammonium perchlorate (in DMSO); various scan rates [25 (red), 50, 100, 150, 200 and 250 (blue) mV/s]; room temperature; three electrodes composed of the glassy carbon working electrode, platinum counter electrode, and Ag/Ag(I) reference electrode.

Figure S8. Oxidation potentials of **L1** and **L3** in ddH₂O. The dashed black lines indicate the oxidation potentials of compounds at 250 mV/s. Note that the E_{pa} values of **L2** could not be measured due to its limited solubility in H₂O. Conditions: [compound] = 1 mM; 1 M NaCl (in ddH₂O); various scan rates [25 (red), 50, 100, 150, 200 and 250 (blue) mV/s]; room temperature; three electrodes composed of the glassy carbon working electrode, platinum counter electrode, and Ag/Ag(I) reference electrode.

Figure S9. Impact of **L1–L3** on preformed Cu(II)-bound and metal-free A β aggregates. (a) Scheme of the disaggregation experiments. (b) Analysis of the size distribution of the resultant A β_{40} (top) and A β_{42} (bottom) species by gel/Western blot using an anti-A β antibody (6E10). Lanes: (C) A β $\pm$ CuCl₂; (L1) C + L1; (L2) C + L2; (L3) C + L3. The original gel images are presented in Figure S10b. (c) Morphologies of the Cu(II)-treated (left) and metal-free (right) A β_{40} aggregates from (b), monitored by TEM. Scale bar = 200 nm. Conditions: [A β] = 25 μM ; [CuCl₂] = 25 μM ; [compound] = 50 μM ; pH 6.6 [for Cu(II)-containing samples] or pH 7.4 (for metal-free samples); 37 $^\circ\text{C}$; constant agitation.

Figure S10. Original gel images from the (a) inhibition (Figure 5b) and (b) disaggregation (Figure S9b) experiments.

Figure S11. TEM images of the $A\beta_{42}$ aggregates generated upon incubation with **L1–L3** from the inhibition experiments. (a) Scheme of the inhibition experiments. (b) Morphologies of the Cu(II)-treated $A\beta_{42}$ (left) and metal-free $A\beta_{42}$ (right) aggregates, monitored by TEM. Scale bar = 200 nm. Conditions: $[A\beta] = 25 \mu\text{M}$; $[\text{CuCl}_2] = 25 \mu\text{M}$; $[\text{compound}] = 50 \mu\text{M}$; pH 6.6 [for Cu(II)-containing samples] or pH 7.4 (for metal-free samples); 37 °C; constant agitation.

Figure S12. TEM images of the A β_{42} aggregates formed upon incubation with **L1–L3** from the disaggregation experiments. (a) Scheme of the disaggregation experiments. (b) Morphologies of the resultant Cu(II)-treated A β_{42} (left) and metal-free A β_{42} (right) aggregates, monitored by TEM. Scale bar = 200 nm. Conditions: [A β] = 25 μ M; [CuCl₂] = 25 μ M; [compound] = 50 μ M; pH 6.6 [for Cu(II)-containing samples] or pH 7.4 (for metal-free samples); 37 °C; constant agitation.

Figure S13. Toxicity of **L1–L3** in SH-SY5Y cells. Cytotoxicity of compounds (5, 10, and 25 μM) was determined by the MTT assay. Cell viability was calculated in comparison to that obtained upon treatment with DMSO only (1% v/v). Error bars represent the standard deviation from three independent experiments ($P < 0.1$).

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