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Small Molecule Modulators of Copper-Induced Aβ Aggregation

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The World Health Organization estimates that *ca.* 11 million people worldwide have Alzheimer's disease (AD) and this population is expected to nearly double by 2030.[1] This disease, which manifests in progressive neurodegeneration, is characterized by the presence of amyloid-β (Aβ) peptide aggregates.[2−4] The mechanism for the formation of Aβ aggregates is not entirely understood, though metal ions such as Cu^{II} and Zn^{II} have been shown to facilitate Aβ aggregation.[2−4] In particular, redox-active Cu^{II} is implicated in the generation of reactive oxygen species (ROS), leading to an increase in oxidative stress, which is one proposed neuropathology of AD.[2−8] To elucidate Cu-mediated events in AD pathogenesis, Cu coordination to Aβ has been explored as well as effects on the removal of Cu from Cu-Aβ species using chelating agents.[2−13] These studies have suggested that the extent of metal-induced Aβ aggregation and ROS production can be modulated by metal chelators, which highlights metal-ion chelation therapy as a promising AD treatment. It still is unclear how metal ions are implicated in AD pathology, however. Thus, the development of small molecules that are able to interact with both metal ions and Aβ species is necessary to unravel metal-Aβ-related pathological pathways in AD and contribute to metal-ion chelation therapy.

Many orthodox metal chelators show inhibition of metal-induced Aβ aggregation and ROS formation,[2−4,9,13] but they may not be suitable as chemical probes and potential therapeutics for AD. Most of these chelators cannot cross the blood brain barrier (BBB) and are not able to specifically target metal ions in various Aβ forms without removing vital metals from other biological systems due to lack of an Aβ recognition/interaction ability. The metal chelator clioquinol (CQ) reveals decreased Aβ aggregate deposits and improved cognition in early clinical trials.[14] Long-term use is, however, limited by an adverse side effect, subacute myelo-optic neuropathy.[15,16] Our recent studies suggest that CQ assists, in part, in the disaggregation of Aβ aggregates but could not completely prevent Aβ aggregation.[17] Therefore, rational design of chelating agents capable of targeting metal ions in Aβ species in the brain is essential to understand metal-Aβ-associated neuropathology and develop therapeutic agents for metal-ion chelation therapy for AD. Only limited efforts have been made toward this goal.[3,10−12] Herein we present the preparation of bifunctional metal chelators (**1** and **2**) and their ability to control Cu-induced Aβ aggregation and ROS production. Both compounds exhibit modulation of Cu-associated Aβ aggregation, which is more effective than that by the well-known metal chelating agents CQ, EDTA, and phen in this study.[18]

Our strategy for developing metal chelators as chemical probes and potential therapeutic agents for AD is to create bifunctional small molecules that contain structural moieties for metal chelation and Aβ recognition/interaction (Figure 1). For the latter purpose, the basic frameworks of **1** and **2** are based on the Aβ aggregate-imaging probes ^{125}IMPY and *p*-^{125}I-stilbene,[18] respectively, which show a strong binding affinity to Aβ aggregates.[19] These compounds are small, neutral, lipophilic, and thus able to penetrate the BBB. Furthermore, they are easily removed from normal brain tissue and accumulate in the blood at relatively low levels, which reduces their toxicity for in vivo applications.[19] For metal chelation, we incorporate a nitrogen and/or oxygen donor atom into the Aβ aggregate-imaging agents (Figure 1). Similar approaches have been described by other groups which have used the structure of a probe for detecting Aβ aggregates, thioflavin-T (ThT),[20] as an Aβ recognition/interaction moiety.[11,12] The compounds, however, were composed of truncated structures for Aβ identification and/or nonspecific metal binding sites. Our design principle for bifunctional metal chelators involves the direct introduction of a metal coordination site into an Aβ recognition/interaction molecule without major structural modifications (Figure 1).

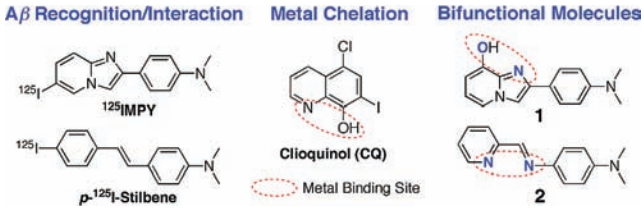

Figure 1. Strategy of designing bifunctional small molecules. Chemical structures of ^{125}IMPY, *p*-^{125}I-stilbene, CQ, **1**, and **2** are depicted.

Defined by the restrictive terms of Lipinski's rules (MW $\leq$ 450, clogP $\leq$ 5, HBD $\leq$ 5, and HBA $\leq$ 10), PSA ($\leq$ 90 Å^2), and calculated logBB for potential applications in brains,[21] **1** and **2** fulfill drug-like criteria and possible brain penetration (Table S1, Supporting Information). The bifunctional small molecules **1** and **2**[22] were prepared *via* cyclocondensation and Schiff base condensation, respectively (Scheme S1). The binding stoichiometry of **1** and **2** with $CuCl_2$ was determined by Job's method of continuous variation using UV−visible spectroscopy.[23] The Job plot for **1** revealed a break between 0.33 and 0.5, indicating the formation of a mixture of 1:2 and 1:1 Cu/ligand complexes (Figure S1). For **2**, the break occurred at 0.5, suggesting the generation of a 1:1 Cu/ligand complex.

In addition to metal binding properties of **1** and **2**, their direct interactions with Aβ were investigated via two-dimensional TROSY ^{1}H−^{15}N HSQC-based NMR structural determinations (TROSY = transverse relaxation optimized spectroscopy; HSQC = heteronuclear single quantum correlation).[24] The TROSY spectrum of Aβ itself is consistent with the previously reported one.[24b] Interestingly, upon treatment with **1** or **2**, chemical shifts of the Aβ residues E11 and H13 are significantly shifted, as depicted in Figures 2 and S2. Both **1** and **2** show more influence on the less

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ordered, *N*-terminal portion of Aβ than the *C*-terminus, which is clearly presented in plots displaying the difference of $^{1}H-^{15}N$ shifts ($\Delta\delta$ Hz) as a function of the amino acid sequence (Figures 2b and S2b). These observations reveal that **1** and **2** are capable of interacting with Aβ and, more importantly, they could have close contact with metal coordination sites in Aβ, where H6, H13, and H14 residues are involved.[2−8,25] This suggests that **1** and **2** may easily target metal ions in Aβ species. Along with NMR studies, the competitive binding of **1** to Aβ aggregates with ThT, a fluorescent indicator for Aβ species upon binding, was observed (Figure S3),[26] which suggests that **1** is able to bind to Aβ. Taken together, these studies by NMR and fluorescence demonstrate interactions of **1** or **2** with Aβ, and thus, these compounds would be classified as bifunctional small molecules for metal chelation as well as Aβ interaction.

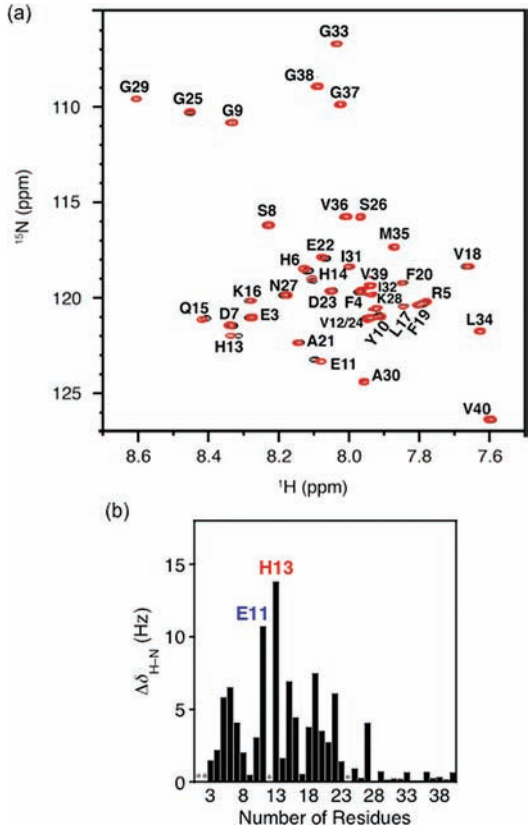
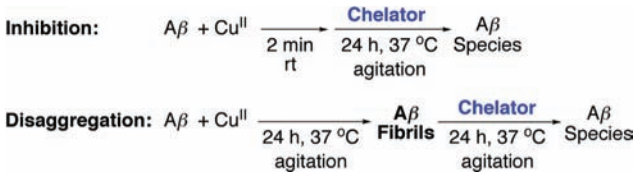


Figure 2. NMR studies of Aβ with **2**. (a) Overaly of 2D TROSY $^{1}H-^{15}N$ HSQC spectra of Aβ upon addition of **2** (900 MHz, 200 mM SDS, 20 mM sodium phosphate, pH 7.3, 25 °C). Black and red chemical shifts were obtained from the Aβ sample (^{15}N-labeled Aβ_{1-40} was used, *ca.* 192 μM) containing *ca.* 2 μL of DMSO-d_6 or *ca.* 1.2 equiv of **2** (*ca.* 1.4 μL DMSO-d_6), respectively. (b) Change in the combined ^{1}H and ^{15}N chemical shifts as a function of the amino acid sequence to identify the major interaction sites of Aβ with **2**. * Denotes absent or overlapped signals.

To investigate the influence of **1** and **2** on Cu^{II}-induced Aβ aggregation, we performed two individual studies (Scheme 1): inhibition (the prevention of forming metal-induced Aβ aggregates)

Scheme 1. Experiments of Inhibition and Disaggregation



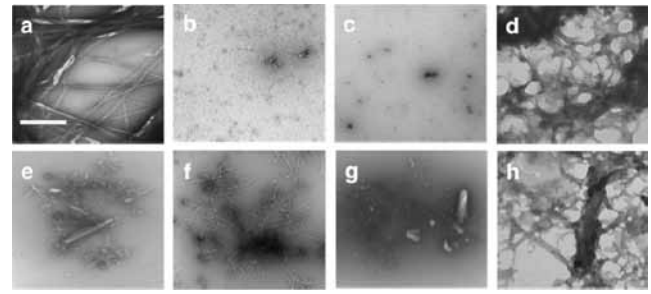


Figure 3. Inhibition experiments. TEM images of samples of Cu^{II}-treated Aβ (a) incubated with the chelator [(b) **1**, (c) **2**, (d) CQ, (e) EDTA, or (f) phen] or with the control molecule [(g) MPY or (h) stilbene] ([Aβ] = 25 μM, [Cu^{II}] = 25 μM, [chelator] = 50 μM, 24 h, 37 °C, constant agitation). The scale bar indicates 500 nm.

and disaggregation (the transformation of metal-Aβ fibrils by chelators). The degree of Aβ aggregation was probed mainly by transmission electron microscopy (TEM).[27]

For the inhibition studies (Scheme 1 and Figure 3), Aβ peptide (25 μM) was treated with 1 equiv of Cu^{II} for 2 min at room temperature followed by incubation with a chelator (50 μM) for 24 h at 37 °C with constant agitation. The Cu^{II}-induced Aβ aggregation is modulated by treatment with **1** or **2** (Figure 3b,c). Less Cu^{II}-triggered Aβ aggregation is indicated in the presence of **1** and **2** over the well-known chelators CQ, EDTA, and phen (Figure 3b−f). Even upon treatment with the chelators CQ, EDTA, and phen, development of Aβ aggregates is still visible, but their morphology is different from that of Cu^{II}-Aβ aggregates (Figure 3a). These observations suggest that metal chelation by these compounds may be one of the driving forces in altering the structural organization of Aβ aggregates. Our previous studies reveal that CQ is capable of chelating metal ions from metal-Aβ aggregates, but it does not completely prevent Aβ aggregation.[17] Furthermore, the control molecules MPY and stilbene that do not contain a metal binding site, but interact with Aβ species, also exhibit Aβ aggregation (Figure 3g,h). Like other chelators, conformational transformation of Aβ species is observed by MPY and stilbene (Figure 3g,h). This may be a result of interactions of Aβ with MPY or stilbene, suggesting the direct Aβ contact as an important parameter for modulation of Aβ aggregation. Overall, based on these TEM results using the control molecules and the known chelators, interaction with either only Aβ or only Cu^{II} is not sufficient to block the Aβ aggregation. Synergistic interactions of **1** and **2** with Aβ and Cu^{II} could result in better modulation of Aβ aggregation, as described in our design strategy.

Along with the TEM analysis, the Aβ species from the inhibition experiments were visualized by native gel electrophoresis followed by Western blotting using an anti-Aβ antibody 6E10 (Figure S5).[28] In the samples of Cu^{II}-Aβ incubated with **1** or **2**, low molecular weight Aβ species are shown, while the samples containing CQ, phen, and EDTA indicate only transformation of Aβ to high molecular weight Aβ species. Thus, these observations also support that modulation of Cu^{II}-induced Aβ aggregation can be better obtained using small molecules having bifunctionality for metal chelation and Aβ interaction.

For the disaggregation studies (Scheme 1 and Figure S6), a chelator (50 μM) was added to Aβ fibrils generated by reacting Aβ (25 μM) with 1 equiv of Cu^{II} (25 μM) for 24 h at 37 °C with constant agitation. Compounds **1** and **2** induce more disaggregation of Aβ fibrils, compared to CQ,[17] phen, and EDTA (Figure S6). These results show that **1** and **2** are capable of disassembling the well-structured Aβ fibrils.

The effects of the chelators on the generation of H_2O_2 by Cu-bound Aβ was examined in cell-free solutions using a horseradish peroxidase (HRP)/Amplex Red assay.[6,7] Samples containing Cu^{II}, Aβ, and either **1** or **2** show 70% lower [H_2O_2] (Figure S7), revealing that **1** and **2** can reduce H_2O_2 production by Cu-Aβ. As expected, the sample containing Aβ, Cu^{II}, and phen in the presence of ascorbate as a reducing agent produces a significant amount of H_2O_2, compared to that of Cu-Aβ, since the $Cu^{I/II}$ redox cycle can be supported by phen.[29] Lastly, the ability of **1** and **2** to modulate the Cu-induced Aβ aggregation was investigated in human neuroblastoma cells (SK-N-BE(2)-M17). The bifunctional molecules **1** and **2** exhibit less toxicity than the clinically available compound CQ in the presence of Cu^{II} (Figure 4). Also, **2** shows no toxicity up to 200 μM (Figure S8). Importantly, toxicity arising from Cu-Aβ is diminished upon incubation with **2**, affording *ca.* 90% cell survival. This is a better survival rate than that for other chelators including CQ (Figure 4). These observations suggest that **2** may be a good candidate to be further studied in vitro and in vivo.

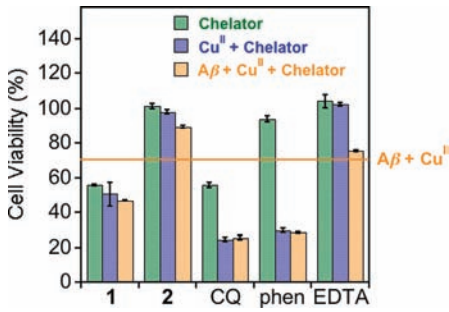


Figure 4. Cytotoxicity of Cu-associated Aβ with the chelators in SK-N-BE(2)-M17 cells using a MTT assay. Cell viability (%) with [chelator] (green), [the chelator and Cu^{II}] (blue), or [Cu^{II}, Aβ, and chelator] (orange) after 24 h incubation ([Aβ] = 20 μM, [Cu^{II}] = 20 μM, [chelator] = 40 μM). Treatment of Aβ in the absence and presence of Cu^{II} for 24 h results in ~90 and ~70% survival of cells, respectively.

In summary, to specifically target divalent metal ions in Aβ aggregates, two bifunctional small molecules, **1** and **2**, were prepared based on a design strategy that integrates metal binding properties into Aβ imaging agents without major structural modifications. The bifunctionality for metal chelation and Aβ interaction of **1** and **2** was verified by spectroscopic investigations. These bifunctional molecules modulate the generation of Cu-triggered Aβ aggregates and promote their disaggregation. Furthermore, studies of our chelators in living cells demonstrate their ability to regulate the cytotoxicity of Cu-induced Aβ species and prompt further investigations in vitro and in vivo. Our approach shown herein may lead to new alternatives for multifunctional chelators as chemical tools to elucidate metal-associated events in AD and as potential therapeutic agents for metal-ion chelation therapy.

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Note Added after ASAP Publication. Due to a production error, some of the changes requested by the author were not included in the version published ASAP October 30, 2009; the corrected version published ASAP November 2, 2009.

Supporting Information Available: Experimental procedures, preparation and characterization of **1** and **2**, Table S1, Scheme S1, and Figures S1−S8. This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) Abbreviatios: **1** = 2-(4-(dimethylamino)phenyl)imidazo[1,2-*a*]pyridine-8-ol; **2** = N^1,N^1-dimethyl-N^4-(pyridin-2-yl-methylene)benzene-1,4-diamine; CQ = clioquinol; EDTA = 2,2′,2″,2‴-(ethane-1,2-diyldinitrilo)tetraacetic acid; phen = 1,10-phenanthroline; ^{125}IMPY = 4-(6-iodoimidazo[1,2-*a*]pyridin-2-yl)-*N*,*N*-dimethylaniline; *p*-^{125}I-stilbene = (*E*)-4-(4-iodostyryl)-*N*,*N*-dimethylaniline.
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