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A Prochelator Activated by β-Secretase Inhibits Aβ Aggregation and Suppresses Copper-Induced Reactive Oxygen Species Formation

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Amyloid plaque formation in the brain plays a central role in the cognitive dysfunction characteristic of Alzheimer's Disease (AD).[1,2] The plaques are composed primarily of 39−43 amino acid amyloid-β peptides (Aβ) that are derived by enzymatic cleavage of the amyloid precursor protein (APP) by β- and γ-secretases.[3] The extracellularly released Aβ is then able to bind metal ions such as copper and zinc, which not only exacerbate plaque formation but, in the case of copper, can also contribute to the formation of neurotoxic reactive oxygen species (ROS).[4]

Currently approved treatments for AD are limited and only temper symptoms without preventing or reversing disease progression. To change this paradigm, a focus on targeting the molecular basis of pathogenesis has led to searches for secretase inhibitors, particularly of β-secretase (BACE).[5] Additionally, with the implication of metals in AD pathogenesis,[6] new drugs such as clioquinol and PBT2 that attenuate metal localization have also made their way into clinical trials.[7] While general metal chelation strategies may address some of the underlying processes associated with AD, they have a shortcoming in their limited ability to differentiate toxic metals associated with Aβ plaques from those associated with normal metal homeostasis.

We previously introduced the concept of a prochelator as an agent that does not interact with metal ions until activated to its chelator form under specific conditions, for example elevated H_2O_2 levels.[8−10] Here we present a peptide prochelator that is enzymatically activated by BACE to yield a high affinity copper chelator (Figure 1). The utilization of a prodrug design allows the metal-binding functionality to be activated at the site of Aβ production and specifically target copper in the Aβ-Cu complex. Furthermore, once copper is bound its reactivity to promote ROS formation via Fenton chemistry is significantly reduced.

To generate a prochelator that would be a good substrate for BACE and that would release a copper chelating agent as the product, we used the known APP Swedish mutant sequence (EVNLDAEF, representing residues 668−675 and abbreviated SW) as a blueprint, since it is a better substrate for BACE than native APP.[11] The cleavage site is located between the leucine and aspartic acid residues. For the prochelator SWH, we replaced the second glutamic acid of SW with a histidine so that BACE cleavage would release the chelator peptide CP, with the N-terminal sequence DAHF. Peptides with an N-terminal free amine and a histidine in the third position are known as ATCUN motifs (amino terminal Cu and Ni binding) and are found natively on human serum albumin (HSA), one of the proteins responsible for binding Cu^{2+} in serum.[12] To facilitate concentration determination and product identification, residues WHDR and WADR were incorporated onto the ends of the SW peptide and the SWH prochelator peptide,[13] respectively, to give the final sequences in Table 1. The H was changed to A in SWH to avoid metal binding. Peptides were prepared by standard Fmoc solid-phase peptide synthesis.

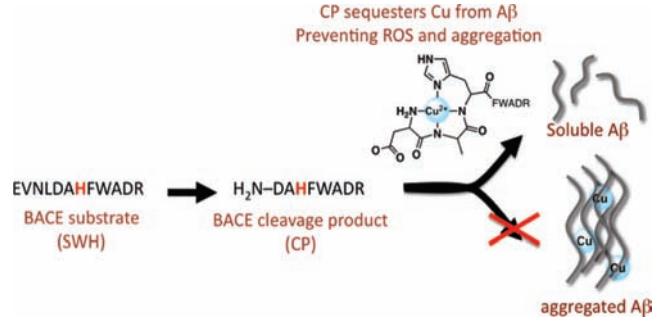

Figure 1. The β-secretase substrate has weak metal affinity, but after selective proteolytic cleavage, the product sequesters copper from Aβ, preventing aggregation. When copper is coordinated to Aβ it catalyzes production of ROS, but sequestration by CP prevents such deleterious reactions.

Conversion of SWH to CP was achieved by incubating SWH with BACE and analyzing aliquots at various time intervals by liquid chromatography−mass spectrometry (LC−MS). The tryptophan tag in both SWH and CP allowed for a direct comparison of peak areas at 280 nm on the LC trace, while the in-line electrospray mass spectrometer provided mass identification of the products to confirm that cleavage occurred at the intended location. This analysis provided initial rates of 0.2% per min for both SWH and SW at 155 μM substrate concentration, indicating that SWH is as good a substrate as SW for BACE (Supporting Information, SI).

Once activated, the cleavage product CP has a high affinity for Cu^{2+}. Competition studies with nitrilotriacetic acid in HEPES buffer at pH 7.4 provide a conditional stability constant (K') corrected for the ternary NTA(Cu)(HEPES) complex of $10^{12.6}$, which is similar to other reports on the ATCUN motif found in HSA.[14] In contrast, SWH at pH 7.4 is unable to compete with even the weak Cu^{2+} ligand glycylglycine (SI). This result indicates that copper binding by prochelator SWH would be inconsequential in a biological system. The summary of relevant conditional stability constants provided in Table 1 predicts that CP should be able to strip Cu^{2+} from Aβ.

Table 1. Description of Peptides Discussed in the Text, Along with Conditional Stability Constants for Cu^{2+} at pH 7.4 (log K')[a]

Name	Description	Sequence	Log K'
SW	Swedish APP	Ac-EVNLDAEFWHDR-NH_2	
SWH	Prochelator	Ac-EVNLDA**H**FWADR-NH_2	< 4.7
CP	Chelator	H_2N-DA**H**FWADR-NH_2	12.6
Aβ	Aβ(1-42)	see Supp Info	9.4 [15]
HSA	albumin	N-term = H_2N-DAHK...	12.0 [14]

[a] "Ac" denotes an N-terminal acetyl cap and "−NH_2" a C-terminal amide.

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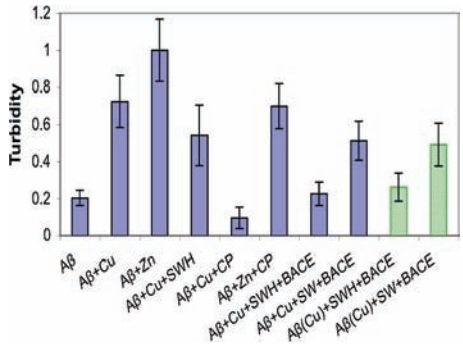

Figure 2. Turbidity of 10 μM Aβ samples in HEPES buffer pH 7.4, as determined by the normalized change in $A_{405\ nm}$. Where indicated, 1 equiv of $Cu(Gly)_2$, $ZnCl_2$, CP, or SWH was added and incubated at 37 °C for 1 h to monitor aggregation prevention (blue bars). Products from SW or SWH plus BACE reactions were also tested for disaggregation of preformed CuAβ aggregates (green dotted bars).

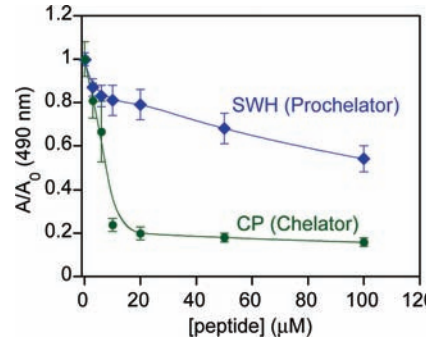

Figure 3. Effect of peptides on deoxyribose degradation by OH$^\bullet$ generated by Cu-promoted Fenton chemistry. A decrease in A/A_0 indicates a protective effect. Conditions: 100 μM H_2O_2, 10 μM $Cu(SO_4)$, 2 mM ascorbic acid, and 15 mM 2-deoxyribose in 50 mM NaH_2PO_4 buffered to pH 7.4.

As predicted and shown by fluorescence titration experiments in the SI, Cu^{2+} readily transfers from Aβ to CP. The binding of paramagnetic metal ions to peptides quenches the fluorescence of aromatic residues tryptophan and tyrosine, and indeed emission from the sole Tyr in Aβ decreases in the presence of Cu^{2+}. When Trp-containing CP is added to this solution, the fluorescence signal remains quenched until more than 1 equiv of CP is added, after which point emission increases linearly with CP concentration. This response is consistent with CP extracting Cu^{2+} from Aβ, which prevents Trp emission until the concentration of CP exceeds that of Cu^{2+}. When the experiment is repeated using SWH as the competitor, fluorescence increases linearly with added SWH, confirming that the prochelator is unable to strip Cu^{2+} from Aβ, as predicted from the thermodynamic data.

By sequestering Cu^{2+} from Aβ, CP also displays an ability to inhibit Cu^{2+}-induced Aβ aggregate formation, as verified by the light-scattering turbidity assay shown in Figure 2. As expected, SWH is unable to inhibit aggregate formation while CP shows a protective effect at 1:1 CP:Cu stoichiometry. This result is consistent with peptides of similar sequence reported by others.[16] Predictably, CP is not as effective at binding Zn^{2+} and preventing Zn^{2+}-induced aggregation. Importantly, the reaction mixture from SWH and BACE incubations (but not SW + BACE) is able to both prevent and disaggregate preformed Aβ-Cu aggregates, as shown by the green bars in Figure 2.

Along with sequestering copper and preventing aggregate formation, CP also shows an ability to prevent ROS formation promoted by copper and Aβ-Cu species. Redox-active $Cu^{2+/+}$ can catalyze OH$^\bullet$ formation from H_2O_2 in Fenton-like reaction cycles.[17] As shown in Figure 3, CP effectively protects against copper-catalyzed OH$^\bullet$ formation, as determined by the deoxyribose assay, a result that is consistent with others in the literature on similar peptide sequences.[16,18] SWH, on the other hand, offers only limited protection, even at concentrations as high as 100 μM. The observed response for SWH may be due to some radical quenching at high concentrations, but the data corroborate the inability of the prochelator to bind copper.

Under reducing conditions, the Aβ-Cu complex reacts with O_2 to generate H_2O_2.[19] An Amplex Red assay was used to show that CP also prevents this ROS formation, whereas SWH does not show an inhibitory effect (see SI).

In summary, we present a prochelator SWH that, once activated by BACE, is able to sequester copper from Aβ, prevent and disassemble aggregate formation, and protect against copper-promoted H_2O_2 and OH$^\bullet$ formation. Because these activities require activation by BACE, an enzyme active in AD brains, this strategy imparts site specificity for chelating copper only when BACE activity is elevated. Because a peptide drug is unlikely to cross the blood brain barrier or withstand the multitude of proteases found in the blood, future work includes improving CP's drug-like properties.

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Supporting Information Available: Full experimental details and additional results. This material is available free of charge via the Internet at http://pubs.acs.org.

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