CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.201103546

Inhibition of Cu-Amyloid-β by using Bifunctional Peptides with β-Sheet Breaker and Chelator Moieties

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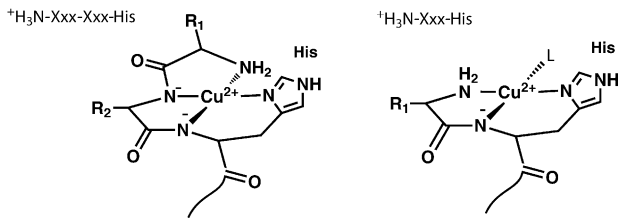
Although the exact origin of the sporadic form of Alzheimer's disease (AD) is not known, it is clear that this pathology is multifactorial. One of the most prominent factors is the peptide amyloid-β (Aβ). β- and γ-secretase cleave this peptide from the amyloid precursor protein. In healthy brain tissue, Aβ exists in a nontoxic, monomeric form; however, AD patients present brain tissue with amyloid plaques, which are insoluble aggregates of peptides with a high concentration of Aβ40 and Aβ42 (two forms of Aβ containing 40 and 42 amino acids, respectively). Plaque formation occurs through a commonly accepted progression termed the amyloid cascade hypothesis in which an accumulation of Aβ monomers leads to the formation of Aβ oligomers, protofibrils, and finally fibrils. Although Aβ fibrils are the main components of plaques, recent studies indicate Aβ oligomers as the most neurotoxic species.[1,2]

Linked to Aβ is another factor thought to be important to AD progression: oxidative stress. Redox-active metal ions such as copper and iron play a key role in formation of reactive-oxygen species (ROS), and evidence has been provided that copper ions bound to Aβ might be involved in the formation of ROS in AD.[3–5] Cu-Aβ catalyzes ROS production under aerobic conditions in vitro in the presence of physiological reductants, such as ascorbate.[3,6,7]

In this respect, inhibition of the formation of toxic Aβ aggregates (oligomers and others) and disruption of Cu-Aβ with subsequent redox-silencing of Cu have been considered promising strategies against AD progression. Several copper-binding ligands have been tested in vitro and the clioquinol derivative PBT2 showed promising results in a clinical phase IIA study.[8,9] In addition, compounds able to inhibit Aβ aggregation have been widely studied,[10] including peptides,[11] such as KLVFF (Aβ16–20).[12,13] The hydrophobic Aβ16–20 region of full-length Aβ is implicated in both Aβ-dimer formation, and in subsequent oligomerization and fibrillization. Thus addition of the short peptide Aβ16–20 is expected to disturb the interaction between two molecules of Aβ to form a dimer and hence inhibit aggregation.

Considering the multifactorial nature of AD etiology, a multifaceted approach for AD treatment is appealing.[14–17] Herein, we present a dual approach to inhibit Aβ aggregation and ROS production by combining two functions on a single peptide, that is, the copper chelator and β-sheet breaker moieties (Scheme 1). The idea was to use a sequence

peptide	*Metal-binding function*	β-sheet breaker function
KLVFF		$^{+}H_3N$-Lys-Leu-Val-Phe-Phe-COO^{-}
Aβ12-20	*$^{+}H_3N$-Val-His-His-*	Gln-Lys-Leu-Val-Phe-Phe-COO^{-}
Aβ13-20	*$^{+}H_3N$-His-His-*	Gln-Lys-Leu-Val-Phe-Phe-COO^{-}



Scheme 1.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201103546.

of the native Aβ, that is, Aβ12–20 and Aβ13–20 (Scheme 1), in which the β-sheet breaker sequence KLVFF is elongated by three or four amino acids to include the well-known Cu^{II}-binding sites of the H_2N-Xxx-His and H_2N-Xxx-Xxx-His, respectively.[18] Peptides with the N-terminal sequence H_2N-Xxx-His and H_2N-Xxx-Xxx-His bind Cu^{II} differently and with a stronger affinity for Cu^{II} than Aβ and their Cu^{II} complexes do not produce ROS efficiently (Scheme 1).[19–22] Note that although the peptides Aβ12–20 and Aβ13–20 contain His13 and 14, which are involved in Cu binding of full length Aβ, the coordination of Cu^{II} is different due to the presence of an N-terminal amine at position 12 or 13, (which is not the case for Aβ). The present conceptual work reports on the efficiency of the bifunctional Aβ12–20 and Aβ13–20 peptides as a β-sheet breaker and a chelator/redox silencer.

Cu^{2+}-binding to Aβ can be followed by the fluorescence of tyrosine (Tyr) at position 10, because tyrosine fluorescence is quenched when Aβ binds Cu^{2+}, and the fluorescence returns after withdrawal of Cu^{2+} from Aβ. Peptides with the motifs H_2N-Xxx-His and H_2N-Xxx-Xxx-His have been shown to abstract copper from Aβ.[20–23] We observed that addition of up to one equivalent of Cu^{2+} reduced the Tyr fluorescence intensity of Aβ42 (Figure 1), and subsequent addition of peptide (Aβ12–20, Aβ13–20) solutions almost entirely reversed the fluorescence quenching at one equivalent of peptide. This suggests that the peptides have a much stronger affinity for copper than Aβ42. Similar results were obtained for Cu^{II}-Aβ40 and Cu^{II}-Aβ16 (not shown).

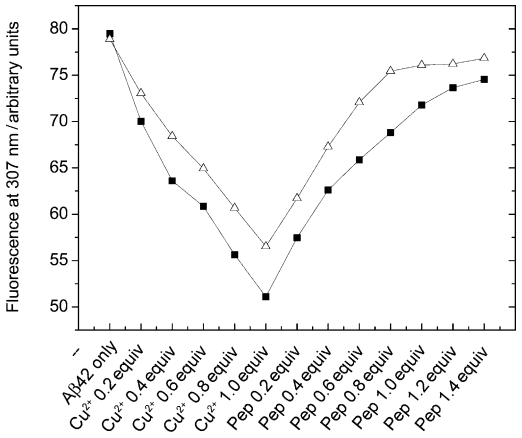

Figure 1. Tyrosine fluorescence from Aβ42 (20 μM) in HEPES buffer (50 mM, pH 7.4) upon the addition of 0–20 μM Cu^{2+} and 0–28 μM peptides (Pep) (Aβ12–20: ■; Aβ13–20: △). The fluorescence intensities were calibrated to account for the change in volume. (Excitation at 274 nm, emission at 307 nm).

The effectiveness of bifunctional peptides in diminishing ROS production by Cu-Aβ42 was assessed by using the $HO^{\bullet}$ trap coumarin-3-carboxylic acid (CCA). The reaction product 7-OH-CCA was measured by fluorescence, which correlates directly with the concentration of $HO^{\bullet}$. The kinetic traces of 7-OH-CCA fluorescence are shown in Figure 2. In the presence of ascorbate, Cu^{II}-Aβ42 produced $HO^{\bullet}$ under aerobic conditions in a time-dependent manner as observed previously.[22] The two bifunctional peptides Aβ12–20 and Aβ13–20 were able to suppress the $HO^{\bullet}$ production of Cu-Aβ42 almost completely (<5%, Figure 2). It seems that Aβ12–20 suppresses $HO^{\bullet}$ more effectively than Aβ13–20, which is in line with the redox inertness of Cu-DAHK compared to Cu-GHK regarding the reduction process.[19] This is in contrast to KLVFF, which does not contain a copper binding sequence and thus only partially suppressed $HO^{\bullet}$ production. This is likely due to trapping of $HO^{\bullet}$ by KLVFF (instead of undergoing a reaction with CCA) and not due to the Cu binding of KVLFF, because addition of a second equivalent of Aβ42 also reduced the fluorescence of 7-OH-CCA.

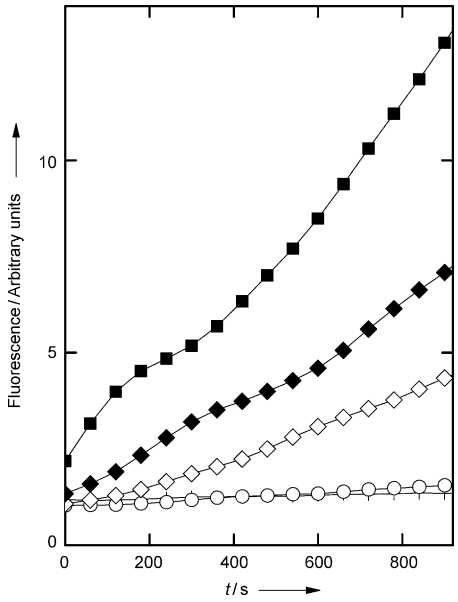

Figure 2. Initial slope of 7-OH-CCA fluorescence intensity for solutions of Cu^{II}-Aβ42 (■) and Cu^{II}-Aβ42 with either one equivalent of Aβ42 (◆), Aβ12–20 (+), Aβ13–20 (○) or KLVFF (◇). $HO^{\bullet}$ production was quantified by measurements of 7-OH-CCA fluorescence for solutions with the conditions: phosphate buffer (6 mM, pH 7.4), desferal (1 μM), CCA (0.5 mM, pH 9), Cu^{2+} (9 μM), and ascorbate (0.5 mM), with or without peptides (10 μM each).

The formation of amyloid fibrils can be followed by monitoring the fluorescence emission of Thioflavin T (ThT), a benzothiazole dye, which exhibits increased fluorescence intensity at ≈486 nm when bound to amyloid fibrils. An increase in ThT fluorescence intensity reports on the formation of amyloid fibrils, and thus increased aggregation (Figure 3). For the aggregation experiments with ThT, the 40-amino acid form Aβ40 was used because Aβ42 aggregated too fast to measure the effect of the bifunctional peptides on the nucleation phase. The aggregation of apo-Aβ40 resulted in a typical sigmoidal trace (Figure 3a), whereas Cu-Aβ40 had a higher aggregation rate, as previously reported[24,25] (Figure 3b). The peptides were added at the beginning of the Aβ aggregation event to assess their capability

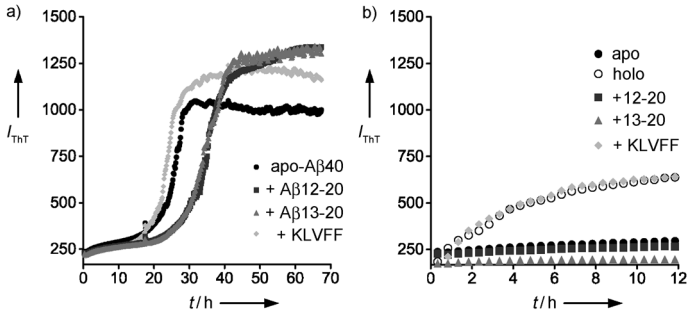

Figure 3. a) Aggregation of apo-Aβ40 (40 μM, black circles) followed by ThT fluorescence with the peptides (40 μM) Aβ12–20 (dark-grey squares), Aβ13–20 (mid-grey triangles), or KLVFF (light-grey diamonds) added at the outset. b) Aggregation of Aβ40 (40 μM, black circles) followed by ThT fluorescence with Cu^{2+} (20 μM, open circles) and peptides (40 μM) Aβ12–20 (dark-grey squares), Aβ13–20 (mid-grey triangles), or KLVFF (light-grey diamonds) added at the outset.

to inhibit the formation of amyloid fibrils. Both bifunctional peptides Aβ12–20 and Aβ13–20 increased the apparent lag-time of Aβ aggregation (Figure 3a; the retardation of Aβ aggregation by Aβ12–20 and Aβ13–20 cannot be due to the binding of residual Cu by Aβ12–20 and Aβ13–20, because the strong Cu^{II}-binding peptide GHK had no retarding effect (data not shown)). Thus, the bifunctional peptides most likely retard the nucleation step in the aggregation process of apo-Aβ40.[26] Surprisingly, the KLVFF peptide, which only has the beta-sheet breaker functionality, did not inhibit aggregation of Aβ40 under these conditions.

In the case of the Cu-induced aggregation of Aβ40 the situation was different. First, Cu^{II} binding to Aβ40 induced fast aggregation as previously reported.[25] Both bifunctional peptides inhibited the Cu-induced aggregation of Aβ40. This is in line with the capability of the bifunctional peptide to withdraw Cu^{II} from Aβ40 (see above). As expected, KLVFF had no effect, which is in agreement with the absence of a Cu-binding domain.

To test the capability of the bifunctional peptides to reverse aggregation, that is, to resolubilize Aβ40 aggregates, the bifunctional peptides were added after completed aggregation of apo- and Cu^{II}-Aβ40. No significant resolubilization was observed (see the Supporting Information, Figure S1) indicating that these peptides have a retarding effect on aggregation, but are not able to reverse already aggregated amyloids. This suggests that the effect of the bifunctional peptides is primarily kinetic, and that they are interacting on the initially formed oligomers rather than on the end-stage aggregates.

To probe the observed properties of the bifunctional peptides in a more biological environment, we measured the effect of the bifunctional peptides on cell viability in the presence of ascorbate and Cu^{2+} with Aβ42. The conditions were similar to the in vitro ROS measurements (see above) and to our previous work.[23,27] 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used as an indicator of cell toxicity. The survival rate of the treated cells was determined based on a comparison with the absorbance of untreated cells, which were set to 100% viability (Figure 4). The cell viability was significantly decreased (to about 45%) after addition of Cu-Aβ42 in the presence of ascorbate. Addition of Aβ12–20 rescued the cells partially, resulting in 66% cell viability. In contrast, Aβ13–20 did not show any protecting effect.

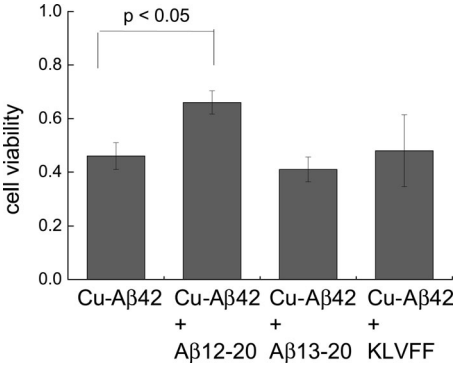

Figure 4. SH-SY5Y cell viability observed based on incubation (24 h) with Aβ42 (50 μM), Cu^{2+} (40 μM), ascorbate (300 μM), and peptides (50 μM): Aβ12–20, Aβ13–20, and KLVFF. Relative cell viability is given compared to the control.

In conclusion, the two bifunctional peptides used here have demonstrated two designed functions, that is, they were able to withdraw Cu^{II} from Aβ, inhibiting $HO^{\bullet}$ formation and they showed β-sheet breaking activity. Although it is clear that the two bifunctional peptides can exert the two individual functions, further studies are needed to determine whether or not an overall enhanced performance (synergism) is present. Since Aβ12–20, and not Aβ13–20, showed protecting activity against Cu-Aβ cell toxicity in the presence of ascorbate in cultured cells, this might support the importance of having a H_2N-Xxx-Xxx-His motif compared with H_2N-Xxx-His. This work shows a proof of concept for bifunctional compounds by using simple and easily synthesized peptides. Peptide Aβ12–20 could also be easily modified into a peptidic bifunctional prodrug, which could be activated by β-secretase as has been shown for the H_2N-Xxx-Xxx-His motif.[28] Further studies are needed to improve and better understand the action of these bifunctional peptides, before attempts for stabilization and vectorization can be envisaged. This is necessary due to the fast degradation of peptides and their low brain penetration, but is expected to be achievable as has been shown for other β-sheet breaker peptides.[11,13,29]

Acknowledgements

This research was sponsored by the National Science Foundation through the US/France IREU program at the University of Florida (NSFaward CHE-0755225 for M.J.), from the ANR NEUROMETALS (Agence Nationale de la Recherche, Grant Neurometals NT09-488591) and from the "Region Midi-Pyrénées" (Research Grant APRTCN09004783). J. T. Ped-

ersen received financial support from the Drug Research Academy at the Faculty of Pharmaceutical Sciences, and Statens Serum Institut.

Keywords: aggregation · amyloid beta peptides · bioinorganic chemistry · copper · redox chemistry

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Received: November 10, 2011
Published online: March 15, 2012

