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Inhibition of amyloid fibril formation and cytotoxicity by caffeic acid-conjugated amyloid-β C-terminal peptides

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ABSTRACT

Amyloid-β (Aβ) deposition and oxidative stress observed in the brains of patients with Alzheimer's disease (AD) are important targets for therapeutic intervention. In this study, we conjugated the antioxidants caffeic acid (CA) and dihydrocaffeic acid (DHCA) to $Aβ_{1-42}$ C-terminal motifs ($Aβ_{x-42}$: $x = 38, 40$) to synthesize CA-$Aβ_{x-42}$ and DHCA-$Aβ_{x-42}$, respectively. Among the compounds, CA-$Aβ_{38-42}$ exhibited potent inhibitory activity against $Aβ_{1-42}$ aggregation and scavenged $Aβ_{1-42}$-induced intracellular oxidative stress. Moreover, CA-$Aβ_{38-42}$ significantly protected human neuroblastoma SH-SY5Y cells against $Aβ_{1-42}$-induced cytotoxicity, with an IC_{50} of 4 μM. These results suggest that CA-$Aβ_{38-42}$ might be a potential lead for the treatment of AD.

Alzheimer's disease (AD) is a neurodegenerative disorder of the brain predominantly affecting the older population. The pathogenesis of AD is characterized by the aggregation and fibril formation of the 40-residue $Aβ_{1-40}$ and 42-residue $Aβ_{1-42}$ peptides, and their subsequent deposition as amyloid plaques, causing neurological dysfunction in the cerebral cortex and hippocampus. The aggregation process is initiated by oligomerization of soluble Aβ monomers, followed by their association into protofibrils and then fibrils, which finally deposit as amyloid plaques.

Oxidative stress and generation of reactive oxygen species (ROS) also play crucial roles in accelerating peptide fibrillization, which in turn can generate more ROS causing a deleterious vicious cycle of neurodegeneration.[1–3] With regard to the role played by the progressive accumulation of Aβ aggregates in the development of neurodegenerative pathology, it has been reported that various compounds inhibit the formation and elongation of Aβ fibrils.[4,5] Among them, several phenolic antioxidants, such as epigallocatechin gallate (EGCG), myricetin, curcumin and resveratrol, can competitively interact with aromatic residues or hydrophobic side chains of Aβ and other amyloidogenic proteins, leading to inhibition of the self-assembly process (i.e., amyloid fibril formation).[6,7] The antioxidative property of polyphenols also plays a role in inhibiting the cytotoxicity of amyloidogenic proteins. In addition to the data from human epidemiological studies[8,9] that suggest drinking polyphenol-containing tea or coffee decreases the incidence of dementia and AD, the inhibitory effect on Aβ fibril formation and antioxidative activity of polyphenols suggests the possibility of using polyphenols as potential lead compounds for treatment and prevention of AD. To the best of our knowledge, however, no phenolic compounds have been designed to selectivity target soluble Aβ oligomers, especially oligomers of $Aβ_{1-42}$.

The relative abundance of $Aβ_{1-42}$ compared to $Aβ_{1-40}$ reflects the fact that even a small elongation (Ile-Ala) of the stretch of hydrophobic residues in the C-terminal region dramatically

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; CA, caffeic acid; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DHCA, dihydrocaffeic acid; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; Gly, glycine; HBTU, *O*-(benzotriazol-1-yl)- *N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HOBt, 1-hydroxybenzotriazole; HRMS, high resolution mass spectrometry; PI, propidium iodide; ROS, reactive oxygen species; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; Th-T, thioflavin T.

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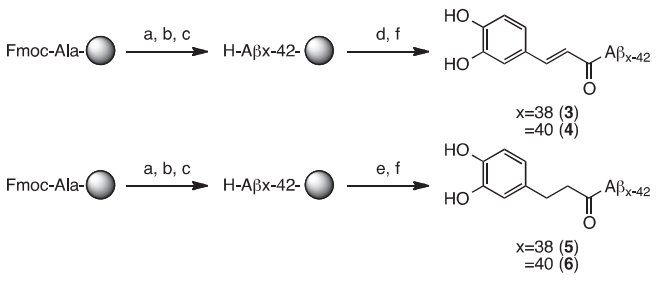
increases the tendency of this peptide to aggregate.[10] The hydrophobicity of the C-terminal region of $A\beta_{1-42}$ can be attributed to the acceleration of the self-assembly process into dimers, trimers and higher-order oligomers via the formation of β-sheet structures based on the underlying secondary structures of amyloid fibrils.[11] Moreover, this implies that peptide fragments encompassing the C-terminal motif of $A\beta_{1-42}$ exhibit the ability to selectively bind $A\beta_{1-42}$ and, thereby, are easily applicable to targeting the $A\beta_{1-42}$ molecule.

In this study, novel multifunctional antioxidants (CA-$A\beta_{x-42}$ and DHCA-$A\beta_{x-42}$) were designed by conjugating caffeic acid (CA; **1**), a phenolic antioxidant found in numerous dietary plants,[12] and dihydrocaffeic acid (DHCA; **2**), to C-terminal motifs of $A\beta_{1-42}$, namely $A\beta_{38-42}$ and $A\beta_{40-42}$, which are considered essential elements for Aβ fibril formation. Complementary binding of these conjugated molecules to the C-terminus of $A\beta_{1-42}$ was expected to inhibit the self-aggregation of $A\beta_{1-42}$, while the antioxidative properties of CA and DHCA were expected to protect neuronal cells from $A\beta_{1-42}$-induced oxidative stress. The aim of this exercise was to synthesize CA-$A\beta_{x-42}$ and DHCA-$A\beta_{x-42}$ and evaluate the potential of the conjugated molecules for AD therapy by measuring their anti-amyloid aggregation activities and their abilities to neutralize H_2O_2- and Aβ-included oxidative stress in SH-SY5Y cells.

Preparation of CA-$A\beta_{x-42}$ (x = 38, 40; **3**, **4**) was accomplished by Fmoc solid phase peptide synthesis (Scheme 1). Details of the preparation methods are provided in the Supplementary material. Starting from Fmoc-Ala Wang resin, removal of the Fmoc group and elongation with the appropriate Fmoc amino acids (Fmoc-AA-OH) were repeated until peptides with the required length were obtained. The CA moiety was then introduced under the same conditions. After cleavage from the resin, the resultant CA-$A\beta_{x-42}$ peptides were purified by RP-HPLC and characterized using ^{1}H NMR and HRMS. To characterize the structural influence of CA on the anti-aggregation activities of CA-$A\beta_{x-42}$ (**3**, **4**), DHCA-$A\beta_{x-42}$ (x = 38, 40; **5**, **6**), in which the catechol moiety and the $A\beta_{1-42}$ C-terminal motif were connected by saturated aliphatic linkers, were also synthesized.

As the fibrillar aggregates of Aβ are the major components of senile plaques, and may result in neurodegeneration, we first examined the inhibitory effects of the conjugated compounds on $A\beta_{1-42}$ aggregation using a thioflavin T (Th-T) assay, which allows for detection of $A\beta_{1-42}$ fibrils by fluorescence spectroscopy.[13] In this assay, 20 μM $A\beta_{1-42}$ with or without the conjugated test compounds was incubated in 0.2 M phosphate buffer (pH 7.4, 100 mM NaCl). At an arbitrary point in time, a portion of the solution was mixed with 5 μM Th-T in 50 mM Gly-NaOH solution (pH 8.7) and the fluorescence intensity at 485 nm (450 nm excitation) was measured.



Scheme 1. Preparation of **3**–**6**. Reagents and conditions: (a) 20% piperidine in DMF, rt, 20 min; (b) Fmoc-AA-OH, HBTU, HOBt, DIPEA, rt, 30 min; (c) repeat steps (a), (b), (a) until the peptides were elongated to the required length; (d) CA, HBTU, HOBt, DIPEA, rt, 30 min; (e) DHCA, HBTU, HOBt, DIPEA, rt, 30 min; (e) TFA/thioanisole/H_2O (95.0/2.5/2.5), rt, 2 h.

When 20 μM $A\beta_{1-42}$ was incubated alone, the fluorescence intensity was increased in a time-dependent manner and saturated over 4 h (Fig. 1), indicating aggregation of $A\beta_{1-42}$ into fibrils. Meanwhile, 20 μM $A\beta_{1-42}$ co-incubated with either 80 μM CA (**1**), CA-$A\beta_{40-42}$ (**4**), or CA-$A\beta_{38-42}$ (**3**) showed less of an increase in Th-T fluorescence and, at 4 h incubation, fluorescence intensity was significantly reduced by 78.2 ± 5.8, 86.4 ± 1.2, and 63.8 ± 0.9%, respectively, relative to that of $A\beta_{1-42}$ alone (Fig. 1A). These results suggest that CA (**1**) and CA conjugated with the C-terminal motif of $A\beta_{1-42}$ were able to inhibit $A\beta_{1-42}$ fibril formation, and that the inhibitory effect of CA (**1**) was enhanced by conjugating with the longer of the two $A\beta_{1-42}$ C-terminal motifs investigated, i.e., $A\beta_{38-42}$. Previously, we have reported that no significant inhibitory effects on $A\beta_{1-42}$ aggregation were found for $A\beta_{X-42}$ (x = 36, 38, 40).[14] Even if C-terminal motif that is not conjugated to CA has an ability to bind to $A\beta_{1-42}$, the binding between the C-terminal motif and amyloid $A\beta_{1-42}$ might not affect the fibril formation. Alternatively, the strong inhibition of CA-$A\beta_{38-42}$ (**3**) on $A\beta_{1-42}$ aggregation indicates that the motif exerted synergistic effect with CA. Further studies on the structural insight into interactions between CA-$A\beta_{38-42}$ (**3**) and $A\beta_{1-42}$ are in progress.

To characterize the structural influence of CA on the anti-aggregation activities of CA-$A\beta_{x-42}$ (**3**, **4**), dihydrocaffeic acid (DHCA; **2**) and DHCA-$A\beta_{x-42}$ (x = 38, 40; **5**, **6**), in which the catechol moiety and the $A\beta_{1-42}$ C-terminal motif were connected by saturated aliphatic linkers, were also synthesized and subjected to the anti-aggregation Th-T assay. When 20 μM of $A\beta_{1-42}$ was co-incubated with 80 μM of these saturated compounds, the Th-T fluorescence intensity was significantly reduced compared with that of $A\beta_{1-42}$ alone. However, the inhibitory activities were weaker than those observed for CA (**1**) and CA-$A\beta_{x-42}$ (x = 38, 40; **3**, **4**), and, interestingly, similar inhibitory activities against $A\beta_{1-42}$ aggregation were shown for DHCA (**2**), DHCA-$A\beta_{38-42}$ (**5**) and DHCA-$A\beta_{40-42}$ (**6**) (80.1 ± 3.8, 87.4 ± 2.5 and 83.8 ± 5.4%, respectively, at 4 h) (Fig. 1B).

The catechol moiety in polyphenols is known to play an essential structural role in the anti-amyloid aggregation activity, which makes polyphenols attractive as potential protective agents against amyloid-induced cytotoxicity. Our results suggest that, similar to CA, the catechol structure in DHCA also plays a significant role in its anti-aggregation activity. Despite these similarities, however, DHCA conjugation to the $A\beta_{1-42}$ C-terminal motif had no apparent effect on the anti-aggregation activities of the catechol moiety, compared with the effects observed for CA conjugation in CA-$A\beta_{x-42}$, which resulted in increased anti-aggregation activities of the catechol moiety. It is likely that the double bond in CA-$A\beta_{x-42}$ plays a significant role in increasing the anti-aggregation activity of the catechol moiety by the binding affinity of the C-terminal motif.

Intriguingly, the assay showed further decreases of $A\beta_{1-42}$ aggregation at 48 h in the presence of all of the test compounds, compared to those at 4 h (Supplementary Fig. 1). We hypothesized that this decrease in aggregation was due to auto-oxidation of the catechol rings, resulting in subsequent dissociation of $A\beta_{1-42}$ aggregates, as reported in other catechol compounds.[15–17] Thus, these compounds were found to have long-lasting inhibitory effects on $A\beta_{1-42}$ aggregation and may disaggregate already formed amyloid fibrils. As CA-$A\beta_{38-42}$ (**3**) showed the most potent anti-aggregative activity of $A\beta_{1-42}$, it was the primary focus for assessment in subsequent assays.

During the progression of AD, $A\beta_{1-42}$ plays a critical role in promoting oxidative stress and, consequently, may play a central role in the pathogenesis of the disease. Thus, we assessed the antioxidative activities of CA (**1**) and CA-$A\beta_{38-42}$ (**3**) toward $A\beta_{1-42}$-induced intracellular oxidative stress in human neuroblastoma SH-SY5Y cells using the fluorescent probe DCFH-DA to detect intracellular ROS production,[18,19] When SH-SY5Y cells were treated with 5 μM