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Original Contribution

# Decreasing oxidative stress and neuroinflammation with a multifunctional peptide rescues memory deficits in mice with Alzheimer disease

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ABSTRACT

Alzheimer disease (AD) is characterized by extracellular senile plaques, intracellular neurofibrillary tangles, and memory loss. Aggregated amyloid-β (Aβ), oxidative stress, and inflammation have pivotal roles in the pathogenesis of AD. Therefore, the inhibition of Aβ-induced neurotoxicity, oxidative stress, and inflammation is a potential therapeutic strategy for the treatment of AD. In this study, a heptapeptide, isolated from a Ph.D.-C7C library by phage display, attenuated Aβ42-induced cytotoxicity in SH-SY5Y neuroblastoma cells and reduced Aβ42-induced oxidative stress by decreasing the production of reactive oxygen species and glutathione disulfide. As a result, glutathione level increased and superoxide dismutase and glutathione peroxidase activities were enhanced in vitro and in vivo. This peptide also suppressed the inflammatory response by decreasing the release of proinflammatory cytokines, such as tumor necrosis factor α and interleukin 1β, in microglia and by reducing microgliosis and astrogliosis in AD transgenic mice. This peptide was intracerebroventricularly administered to APPswe/PS1dE9 transgenic mice. We found that this peptide significantly improved spatial memory and reduced the amyloid plaque burden and soluble and insoluble Aβ levels. Our findings suggest that this multifunctional peptide has therapeutic potential for an Aβ-targeted treatment of AD.



Alzheimer disease (AD), one of the most common forms of dementia among the elderly, is a neurodegenerative disease clinically characterized by global cognitive dysfunction, especially memory loss [1–3]. The neuropathological hallmarks of AD include extracellular senile plaques composed of β-amyloid (Aβ) deposits, intracellular neurofibrillary tangles, and cerebral atrophy [4,5]. Aβ, derived from proteolytic processing of amyloid precursor protein, has been suggested to have a pivotal and probably causal role in the cascade process of AD [6,7]. Aggregated Aβ can elicit neurotoxicity and induce oxidative stress and inflammation in the brain of patients with AD.

Increasing evidence has indicated that oxidative stress and inflammation are important in the mechanisms associated with Aβ-induced neurotoxicity [8–10]. Aggregated Aβ may induce oxidative stress by causing mitochondrial dysfunction and lipid peroxidation. Mitochondrial dysfunction induces the release of reactive oxygen species (ROS) and decreases the levels of antioxidants, such as glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) [11–14]. Lipid peroxidation can directly destroy the structural integrity of cell membranes and lead to apoptosis. Moreover, malondialdehyde (MDA), the product of lipid peroxidation, is a neuronal toxin and may impair protein function. A circular relationship is found between Aβ and Aβ-mediated oxidative stress [15–17]. Aβ aggregates may induce the release of ROS; in turn, the overproduced ROS accelerate Aβ generation and accumulation, facilitating the progression of AD [18]. Moreover, Aβ triggers the accumulation of astroglia and microglia as well as neuroinflammation, inducing the production of proinflammatory factors, such as cytokines and chemokines [19]. Cytokines, particularly tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), can induce chronic inflammation that may promote the loss of

*Abbreviations:* AD, Alzheimer disease; Aβ, β-amyloid; ROS, reactive oxygen species; GSH, glutathione; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; GR, glutathione reductase; MDA, malondialdehyde; MTT, 3-[4,5–dimethylthiazol–2–yl]-2,5-diphenyltetrazolium bromide; ThS, thioflavin S; MWM, Morris water maze

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synapses and cognitive dysfunction and, eventually, neuronal death [19,20]. Therefore, inhibiting Aβ-induced neurotoxicity and suppressing oxidative stress and inflammation are attractive therapeutic and preventive strategies for the treatment of AD.

Many agents, such as small organic molecules, peptides, proteins, and antibodies, may inhibit Aβ aggregation and neurotoxicity; these agents may also reduce the production of ROS and proinflammatory cytokines [21–25]. Among these agents, peptides are considered to be a promising therapeutic tool to promote low immunogenicity, amenable modification, high stability, and high permeability of the blood–brain barrier [26]. Many peptides reported as Aβ inhibitors are derived from the central hydrophobic fragment of Aβ17–21 (KLVFF) or the C-terminus of Aβ42 [27–30]. These peptides possibly interfere with Aβ42 aggregation and neurotoxicity in vitro; however, few peptides can improve cognitive function and reduce amyloid plaques in AD mouse models [31]. This study demonstrates that a multifunctional heptapeptide isolated from a phage display does not inhibit Aβ42 aggregation; instead, this heptapeptide prevents Aβ-induced cytotoxicity, attenuates oxidative stress, decreases the production of proinflammatory cytokines in vitro and in vivo, improves cognitive function, and reduces Aβ deposits in AD transgenic mice.

## Materials and methods

### *Materials*

The Ph.D.-C7C Disulfide Constrained Peptide Library Kit encoding $1.2 \times 10^9$ random seven-amino-acid insertions was obtained from New England Biolabs. Aβ42 was purchased from American Peptide Co. (Sunnyvale, CA, USA). For aggregation experiments, Aβ42 was dissolved in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to obtain a concentration of 1 mg/ml, sonicated in a water bath for 10 min, aliquotted into microcentrifuge tubes, dried under vacuum, and stored at −20 °C. Immediately before use, HFIP-treated Aβ42 was dissolved in dimethyl sulfoxide to obtain a concentration of 1 mg/ml, diluted to 10 μM in phosphate-buffered saline (PBS) at pH 7.4, and incubated at 37 °C without shaking in a 0.5-ml Eppendorf tube. The peptides isolated from the Ph.D.-C7C library, with or without His tag, Aβ1–17 (DAEFRHDSGYEVHHQKL), Aβ13–25 (HHQKLVFFAEDVG), Aβ22–35 (EDVGSNKGAIIGLM), Aβ33–42 (GLMVGGVVIA), Aβ13–35 (HHQKLVFFAEDVGSNKGAIIGLM), and four different mutated peptides of Aβ13–35 (F20A, F19A+F20A, I31A, I31A+F20A) (Fig. 1A), were synthesized by GL Biochem Co., Ltd. (Shanghai, China). Peptide ZW1 was dissolved in PBS at pH 7.4 to obtain a concentration of 2 mg/ml, aliquotted, and stored at −20 °C. The following antibodies were used: 6E10 (monoclonal antibody reactive to Aβ1–16, Signet Laboratories, Dedham, MA, USA), glial fibrillary acid protein (GFAP; polyclonal antibody, Gold Bridge Co., Beijing, China); Iba-1 (polyclonal antibody, Wako), HRP-9E10 (horseradish peroxidase (HRP)-conjugated anti-M13 antibody, Santa Cruz Biotechnology, USA), and HRP-conjugated mouse anti-His-tag monoclonal antibody (Gold Bridge Co.). The microscale MDA kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The assay kits for ROS, GSH, glutathione disulfide (GSSG), SOD, GSH-Px, and GR were purchased from Beyotime Co. (Jiangsu, China). TNF-α and IL-1β ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA). Aβ40 and Aβ42 kits for Aβ measurement were purchased from IBL Co., Ltd. (Gunma, Japan).

### *Biopanning using the phage display library*

A peptide was selected using four rounds of panning according to a previously described method with slight modifications [22]. In brief, high-binding 96-well plates (Corning, USA) were coated with 1 μg/well of Aβ42 overnight at 4 °C. The plates were blocked with 5% bovine serum albumin (BSA) for at least 2 h at room temperature. An aliquot of $2 \times 10^{11}$ phage units from the Ph.D.-C7C library was incubated with Aβ. The plates were thoroughly washed with TBST solution (0.1% Tween 20 in 50 mM Tris, 150 mM NaCl, pH 7.5) to remove any unbound phage. Bound phage was eluted with 0.2 M glycine–HCl (pH 2.2). Phage titers were determined by infecting *Escherichia coli* cells and using serial dilution on agar plates containing isopropyl-β-D-1-thiogalactopyranoside (IPTG)/X-gal. Eluted phages were subsequently amplified by infecting *E. coli* ER2738. The phages were further purified using polyethylene glycol (MW 8,000)/NaCl precipitation, resuspended in PBS, and then used for further rounds of selection.

### *Screening of positive clones*

At the end point of the fourth round of panning, the eluted phage was diluted, used to infect *E. coli* ER2738, and plated as single colonies on LB/IPTG/X-gal plates. Clones were individually picked, amplified, and purified. High-binding 96-well ELISA plates were coated with 1 μg per well of Aβ42 in PBS (pH 7.4) overnight at 4 °C. The prepared phages were added to the wells and incubated for 2 h at room temperature. HRP-9E10 was also added, followed by 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma). The reaction was stopped after 15 min by the addition of 2 N $H_2SO_4$. Subsequently, data were obtained by reading the absorbance at 450 nm on a Tecan Safire2 microplate reader (Tecan, Männedorf, Switzerland). The clones with higher absorbance than the negative controls were selected for further studies. Twenty-five Aβ42-binding clones were DNA sequenced. One peptide corresponding to the encoded sequence in 20 clones was synthesized by standard solid-phase peptide synthesis with N-terminal Fmoc protection. The peptide was purified to homogeneity by reverse-phase high-pressure liquid chromatography and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

### *Affinity measurement*

ELISA was performed to determine the binding capacity of the synthesized peptide (ZW1) to the full length of Aβ42 peptide. In brief, various concentrations of Aβ42 were coated onto the high-binding polystyrene microtiter plates at 4 °C overnight. Non-specific binding was blocked through incubation with 3% (w/v) BSA at 37 °C for 2 h. The synthesized ZW1 peptide with a His tag was added to each well. The plates were then incubated for 2 h at room temperature. HRP-conjugated mouse anti-His-tag monoclonal antibody was used as the secondary antibody. To determine the affinity of ZW1 with various segments of Aβ and various mutated segments of Aβ13–35, we coated the high-binding polystyrene microtiter plates with 1 μg of Aβ1–17, Aβ13–25, Aβ22–35, Aβ33–42, or Aβ13–35 or its various mutated segments per well in PBS. After blocking, we incubated the plates with the synthesized His-tag-conjugated peptide of ZW1. ELISA was then conducted as previously described.

### *Molecular dynamics simulation*

To determine which residues contribute to the binding of Aβ42 to ZW1, we performed molecular dynamics analysis. Initially, an unfolded and fully extended structure was generated by the PROTEIN program in Tinker software (backbone torsion $\varphi, \psi = -135, 135$) using AMBER99 force-field parameters to assign the atom types. The structure was then optimized by the truncated Newton conjugate gradient method using the GB/SA continuum