Journal of Inorganic Biochemistry 181 (2018) 56–64

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

RTHLVFFARK-NH_2: A potent and selective modulator on Cu^{2+}-mediated amyloid-β protein aggregation and cytotoxicity

Jie Meng[a,1], Huan Zhang[a,1], Xiaoyan Dong[a,*], Fufeng Liu[b], Yan Sun[a,*]

[a] Department of Biochemical Engineering and Key Laboratory of Systems Bioengineering of the Ministry of Education, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300354, China

[b] Key Laboratory of Industrial Fermentation Microbiology of Ministry of Education, Tianjin Key Laboratory of Industrial Microbiology, National Engineering Laboratory for Industrial Enzymes, National and Local United Engineering Lab of Metabolic Control Fermentation Technology, College of Biotechnology, Tianjin University of Science & Technology, Tianjin 300457, China

ARTICLE INFO

Keywords:
Amyloid β-protein
Cu^{2+} chelator
Aggregation
Alzheimer's disease
Inhibitor
Peptide

ABSTRACT

Dysfunctional accumulation of amyloid-β (Aβ) protein stimulated by Cu^{2+} is considered as a key process in the pathogenesis of Alzheimer's disease (AD). Thus, bifunctional substances capable of chelating Cu^{2+} and inhibiting Aβ aggregation are promising therapeutic agents against AD. Herein, a novel bifunctional decapeptide RTHLVFFARK-NH_2 (RK10) was developed by integrating a metal chelating tripeptide (RTH) and an Aβ aggregation inhibitor Ac-LVFFARK-NH_2 (LK7). The high selectivity of RK10 for Cu^{2+} over other biologically relevant metal ions was demonstrated by isothermal titration calorimetry. RK10 bound Cu^{2+} with a dissociation constant of 0.02 μM. This enabled RK10 to sequester Cu^{2+} from $Aβ_{40}$-Cu^{2+} species and to arrest the production of reactive oxygen species (ROS) catalyzed by Cu^{2+} or $Aβ_{40}$-Cu^{2+} species. Extensive physical, biophysical and biological studies indicate that RK10 targeted free and Cu^{2+}-bound $Aβ_{40}$ species, suppressed $Aβ_{40}$ aggregation, and diminished the cytotoxicity induced by $Aβ_{40}$ and Cu^{2+}-mediated $Aβ_{40}$ in cultured SH-SY5Y cells. Taken together, the results proved the excellent selective roles of RK10 in inhibiting Cu^{2+}-mediated $Aβ_{40}$ aggregation and eliminating ROS generation catalyzed by Cu^{2+}/$Aβ_{40}$-Cu^{2+} species. Thus, this work provided new insight into the design and development of potent bifunctional inhibitors against Aβ aggregation and cytotoxicity.

1. Introduction

Alzheimer's disease (AD), the most widespread form of dementia in the elderly, affects > 30 million people worldwide [1–3]. The clinical characteristics of AD are loss of memory, cognitive decline, behavioral deficits, and other age-related problems, finally leading to death [4]. Up to now, no curable treatment is available for AD. One of the major neuropathological features of AD is the presence of senile plaques, which are predominantly composed of various aggregates of amyloid-β (Aβ) protein [5,6]. It is generally considered that self-assembly of Aβ into neurotoxic oligomers and fibrils is the central process of AD pathophysiology [7]. Hence, inhibition of Aβ aggregation is hypothesized as an effective therapeutic strategy for the disease [8].

Recently, more attention has been paid to the connection between metal ions and AD pathology because some transition metal ions, mainly Cu^{2+}, Zn^{2+} and Fe^{3+}, are enriched in the amyloid plaques of AD patients [9–12]. However, it was also found that Cu^{2+} levels were significantly depressed in several affected regions of AD brain [13]. It was considered that dyshomeostasis of metal ions (decreased copper levels in AD brain tissues and enriched Cu^{2+} in/around amyloid plaques) might contribute to AD pathology by depleting Cu-availability for normal cellular functions [13]. Furthermore, it has been proven that the metal ions modulate the original Aβ aggregation pathways, leading to the formation of more toxic Aβ species [14–17]. In particular, redox-active Cu^{2+} has attracted the most attention since it not only amplifies cytotoxicity of Aβ aggregates, but also leads to the production of neurotoxic reactive oxygen species (ROS) via Fenton's reaction [18–20]. ROS could cause severe oxidative stress and then trigger a series of cell damages including lipid peroxidation and membrane disruption, which is a key feature of AD pathogenesis [21,22].

Given the recognized interactions of Aβ with transition metal ions, chelation of metal ions using chelating agents has been considered as a promising method for AD therapy. For instance, clioquinol (CQ) and 5,7-dichloro-2-((dimethylamino)methyl) 8-quinolinol (PBT2) have been proven to improve cognitive performance in clinical trials [23]. However, long-term use of CQ is limited by an adverse side effect of

* Corresponding authors.
E-mail addresses: d_xy@tju.edu.cn (X. Dong), ysun@tju.edu.cn (Y. Sun).
[1] The authors contributed equally to this work.

https://doi.org/10.1016/j.jinorgbio.2018.01.012
Received 27 October 2017; Received in revised form 9 January 2018; Accepted 19 January 2018

subacute myelo-optic neuropathy [24]. In addition, most metal-chelators cannot inhibit Aβ aggregation, which would largely restrict their effectiveness in AD treatment [25]. Thus, bifunctional inhibitors, including small chemical molecules [24,26–28] and peptides [25,29], with both capacities of Aβ aggregation modulation and metal ions chelation have been developed recently. However, the poor specificity of bifunctional inhibitors would be limited in applications by some adverse side effects. Most of the current bifunctional chelators show nonspecific chelation or do not have a proper affinity for Cu^{2+} under specific biological conditions [30]. Thus, various metal ions (such as K^+, Ca^{2+}, and Mg^{2+}), which play an important role in maintaining the normal physiological functions of organisms, could be removed by these bifunctional inhibitors. Therefore, it is very urgent to develop such a suitable bifunctional molecule that could selectively chelate Cu^{2+} and prevent Aβ aggregation.

Human protamine HP2 ($HP2_{1–15}$) binds to Cu^{2+} via the tripeptide motif RTH in the N-terminal of $HP2_{1–15}$ to form the Cu^{2+}-$HP2_{1–15}$ complex with high affinity and stability [31]. Previously, a heptapeptide inhibitor Ac-LVFFARK-NH_2 (LK7) was designed to inhibit Aβ fibrillization [32]. Herein, RTH and LK7 were selected as the chelating agent and amyloid inhibitor, respectively, and a novel bifunctional inhibitor RTHLVFFARK-NH_2 (RK10) was designed by conjugating RTH and LK7 (Scheme. 1). The inhibitory effects of RK10 on $Aβ_{40}$ aggregation and its associated cytotoxicity were examined using thioflavin T (ThT) fluorescence assay, atomic force microscopy (AFM), and cell viability assays. The chelating ability and the specificity of RK10 for Cu^{2+} were validated by the tyrosine fluorescence spectroscopy and isothermal titration calorimetry (ITC). Additionally, the inhibitory effects of RK10 on Cu^{2+}-mediated $Aβ_{40}$ aggregation and its associated cytotoxicity were also examined. Finally, coumarin-3-carboxylic acid (3-CCA) fluorescence assay was employed to analyze the inhibitory effect of RK10 on ROS production.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), ThT, 2-[4-(2-hydroxyethyl)-1-piperazine] ethanesulfonic acid (HEPES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 3-CCA were purchased from Sigma-Aldrich (St. Louis, MO). Ascorbate (Asc) was obtained from J&K Scientific (Beijing, China). $Aβ_{40}$ and LK7 (> 95% purity) were purchased from GL Biochem (Shanghai, China). RK10 and RTH (> 95% purity) was purchased from ZiYu Biotech (Shanghai, China). Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F12) and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). Human neuroblastoma SH-SY5Y cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Other chemicals were all of the highest purity available from local sources.

2.2. $Aβ_{40}$ monomer solution preparation

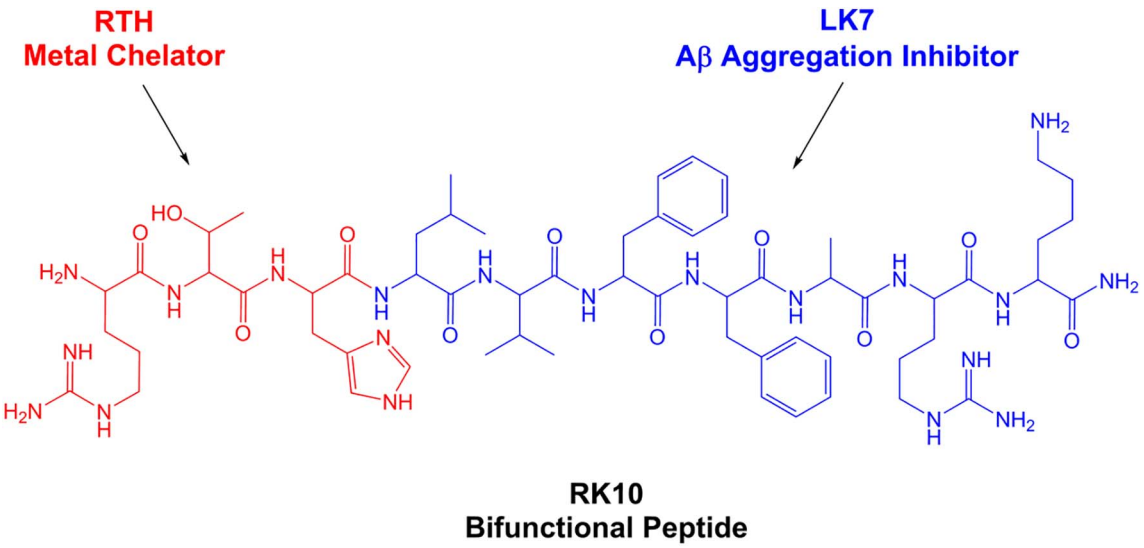
$Aβ_{40}$ was monomerized as described in the literature [32]. In brief, $Aβ_{40}$ powder was firstly dissolved in HFIP at 1.0 mg/mL and left undisturbed for 2 h. It was then sonicated for 30 min in ice bath to disrupt the pre-existing aggregates. Thereafter, HFIP was removed by vacuum freeze-drying. The lyophilized protein was stored at −20 °C. In aggregation and inhibition experiments, Aβ was dissolved in 20 mM NaOH and sonicated for 20 min in ice bath, and then 11-fold diluted in buffer solution (20 mM HEPES, 100 mM NaCl, pH 7.4) to a final concentration of 25 μM.

2.3. ThT fluorescent assay

$Aβ_{40}$ samples (25 μM) without or with $CuCl_2$ (10 μM) and different concentrations of peptides (i.e., RTH, LK7 and RK10) were incubated by continuous shaking at 150 rpm and 37 °C. For ThT assays, 200 μL incubated samples were drawn carefully at 72 h, and mixed uniformly with 2 mL of ThT solution (25 μM ThT in 20 mM HEPES, pH 7.4) in the quartz cell. ThT fluorescence intensities (excitation wavelength 440 nm; emission wavelength 480 nm) were measured by a fluorescence spectrometer (Perking Elmer LS-55, MA, USA) with a slit width of 5 nm at room temperature. The fluorescence intensity of the samples without $Aβ_{40}$ was subtracted as background from each read with $Aβ_{40}$. Each measurement was performed in triplicate and the data were averaged. The standard deviation was reported as an error bar in figures.

2.4. Aggregation kinetics

The samples of 200 μL were mixed in a 96-well plate, containing 25 μM $Aβ_{40}$ monomers, 25 μM ThT and different concentrations of peptides (i.e., RTH, LK7, and RK10) in 20 mM HEPES (pH 7.4). The plates were sealed to avoid evaporation of the sample and incubated at 37 °C. The fluorescence intensities (excitation at 440 nm and emission at 480 nm) of each well were recorded by a multifunctional microplate



Scheme 1. Structure-based design strategy for the bifunctional peptide RK10. Conjugation of a metal chelator (RTH) and a peptide inhibitor (LK7) on Aβ aggregation is the design basis for RK10.