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Research article

Supratherapeutic concentrations of cilostazol inhibits β-amyloid oligomerization *in vitro*

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ABSTRACT

Alzheimer disease (AD) is the most common type of dementia, and is currently incurable. The efficacy of existing treatments for AD such as acetylcholinesterase inhibitors is limited to symptom improvement. Research on disease-modifying therapies (DMTs) has conventionally focused on amelioration of CNS pathogenesis. Two neuropathological changes correlate strongly with AD, the appearance of neurofibrillary tangles containing the microtubule-associated protein tau and extracellular amyloid deposits containing amyloid β-protein (Aβ). The aggregation of Aβ is believed to be the key pathogenic event in AD, with oligomeric assemblies thought to be the most neurotoxic form. Inhibitors of oligomer formation, therefore, could be valuable therapeutics for AD patients. The clinical phosphodiesterase type-3 inhibitor cilostazol (CSZ) was recently found to suppress the progression of cognitive decline in patients with stable AD receiving acetylcholinesterase inhibitors. Here we examined the effects of CSZ on *in vitro* aggregations of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ including oligomerization, using the thioflavin T assay, photo-induced cross-linking of unmodified proteins, and electron microscopy. CSZ (25–100 μM) inhibited Aβ aggregation, especially oligomer formation. Considering that CSZ might be a key molecule for DMTs of AD, it cannot be ruled out that the low concentration of CSZ achievable in patient dosing may display some ant-oligomeric activity in synergy with its known therapeutic effects.

1. Introduction

Alzheimer's disease (AD) is characterized by accumulation of intraneuronal neurofibrillary tangles composed of the microtubule-associated protein tau and extracellular deposition of the amyloid β-protein (Aβ) as amyloid plaques and vascular amyloid [39]. Despite recent progress in symptomatic therapy using cholinergic drugs and N-methyl-D-aspartate receptor antagonist, there are currently no effective therapeutic approaches that directly modify the neurodegenerative processes of AD (disease-modifying therapies, DMTs). Research in this area has focused mainly on agents that disrupt the accumulation of Aβ and tau in the CNS [5].

Aβ is widely believed to be a major factor in AD pathogenesis (the amyloid hypothesis) based on human genetic analyses, *in vitro* biochemical and cell viability studies, and a myriad of neurophysiological and behavior studies in animal models, particularly Aβ transgenic mice [3,39,40]. Two major forms of Aβ are produced in AD, the 40-amino acid $A\beta_{1\text{-}40}$ and 42-amino acid $A\beta_{1\text{-}42}$ residues are produced, but the relative amount of $A\beta_{1\text{-}42}$ is particularly critical for AD progression because this longer form is more prone to aggregate than the shorter peptide [16,39]. Aβ molecules tend to aggregate and form oligomers and mature fibrils [25,32,49]. These Aβ aggregates may cause neuronal injury directly by acting on synapses or indirectly by activating microglia and astrocytes; therefore, several pharmacological approaches for DMT have been developed that target the sequential events in Aβ production [25,27,32,40,42,48,49].

Cilostazol (CSZ) (Fig. 1), a selective inhibitor of phosphodiesterase type-3 (PDE3), acts as an antiplatelet agent and is used for the prevention of cerebral ischemia in Japan and other Asian countries [8,10]. It was also reported that CSZ slows cognitive decline in patients with

Abbreviation: Aβ, amyloid β-protein; AD, Alzheimer's disease; APP, amyloid precursor protein; APS, ammonium persulfate; αS, α-synuclein; CREB, cyclic adenosine monophosphate response element-binding protein; CSZ, cilostazol; DMTs, disease-modifying therapies; EM, electron microscopy; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; LMW, low molecular weight; low-n, low molecular weight order; MAPK, mitogen-activated protein kinase; MCI, mild cognitive impairment; Nox, nicotinamide adenine dinucleotide phosphate oxidase; PDE3, phosphodiesterase type-3; PICUP, photo-induced cross-linking of unmodified proteins; ROS, reactive oxygen species; Ru(bpy), tris(2,2′-bipyridyl) dichlororuthenium(II); SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; ThT, thioflavin T

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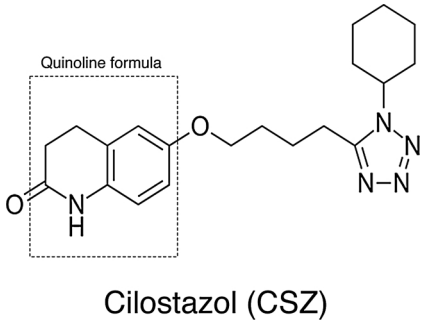

Fig. 1. Structure of Cilostazol (CSZ).

AD and cerebrovascular diseases [14,37,44,45]. In a pilot study including 10 patients with moderate AD receiving the acetylcholinesterase inhibitor donepezil, add-on CSZ treatment for 5–6 months significantly increased Mini Mental State Examination (MMSE) score compared to baseline [2]. In a larger pilot study (30 participants, 12 months), CSZ add-on therapy improved cognitive impairments in patients with stable AD [45]. In addition, CSZ was shown to be effective against cognitive decline in AD patients with cerebrovascular diseases [37] as well as mild cognitive impairment (MCI) [44].

It has also been shown that CSZ decreases $A\beta_{25-35}$ accumulation and attenuates $A\beta_{25-35}$-induced cognitive deficits in animal models of AD [11,35]. Recently, we reported that CSZ suppressed Aβ-induced neurotoxicity in SH-SY5Y cells through inhibition of oxidative stress as evidenced by reduced reactive oxygen species (ROS) accumulation, nicotinamide adenine dinucleotide phosphate oxidase (Nox) activity, and mitogen-activated protein kinase (MAPK) signaling [24].

Although mature fibrils formed from Aβ aggregates have long been thought to be the main neurodegenerative agents in AD, recent evidence suggests that soluble multimers of these pathogenic proteins, referred to here as "oligomers," may initiate the synaptic and neuronal dysfunction associated with AD and the other protein-misfolding diseases (the oligomer hypothesis) [25,27,32,40,42,48,49]. In addition, multiple lines of evidence suggest that a pathogenic change in tau may be mediated by low molecular weight order (low-n) oligomers of Aβ, such as dimers and trimers [15,47]. If so, therapeutic agents must target the assembly or neurotoxic activity of these structures for highest efficacy.

In the present study, we examined the effects of CSZ on aggregation of $A\beta_{1-40}$ and $A\beta_{1-42}$ *in vitro*, including oligomer formation, using the thioflavin binding assay, photo-induced cross-linking of unmodified proteins (PICUP), and electron microscopy (EM).

2. Materials and methods

2.1. Proteins and agents

The $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides were purchased from Peptide Institute Inc. (Osaka, Japan). A stock solution of glutathione S-transferase (GST) (Sigma-Aldrich, St. Louis, MO, U.S.A.) was prepared by dissolving the lyophilizate in 60 mM NaOH to a concentration of 250 μM. Prior to use, peptide aliquots were diluted 5- or 10-fold in 10 mM phosphate buffer, pH 7.4. Cilostazol was purchased from Wako Company (Osaka, Japan). For the thioflavin T (ThT) assay, CSZ was dissolved in assay buffer (50 mM Tris, 150 mM NaCl, pH 7.2) and used at final concentrations of 25, 50, and 100 μM. For the PICUP assay, CSZ was dissolved in dimethyl sulfoxide to a concentration of 2.5 mM and then diluted with 10 mM phosphate (pH 7.4) to final concentrations of 25 and 100 μM as described previously [26]. We confirmed that CSZ did not influence solution pH at 100 μM.

2.2. Preparation of Aβ solutions

Aggregate-free solutions of Aβ were prepared using size exclusion chromatography [26]. The nominal monomer fraction has been termed low molecular weight (LMW) Aβ because at experimental peptide concentrations this fraction comprises a mixture of monomer and low molecular oligomers in rapid equilibrium [26]. Here, we refer here to LMW Aβ simply as "Aβ". To prepare Aβ, 200 μL of a 2 mg/mL peptide solution in dimethyl sulfoxide was sonicated for 1 min in a bath sonicator (Branson Ultrasonics, Danbury, CT, U.S.A.) and then centrifuged for 10 min at 16,000 × *g*. The resulting supernatant was fractionated on a Superdex 75 HR column using 10 mm phosphate buffer (pH 7.4) at a flow rate of 0.5 mL/min. The middle of the LMW peak was collected over 50 s and used immediately for aggregation assays and EM analysis.

2.3. Detection of Aβ fibril formation

Aggregation of $A\beta_{1-40}$ or $A\beta_{1-42}$ was measured using the SensoLyte® ThT $A\beta_{1-42}$ Aggregation Kit (AnaSpec, Fremont, CA, U.S.A.) according to the manufacturer's instructions. To measure $A\beta_{1-40}$ or $A\beta_{1-42}$ fibril formation in 96-well black microplates, 10 μL of 200 μM ThT and 5 μL of each indicated CSZ concentration were added into each well and mixed with 85 μL of 54 μM Aβ solution. The final $A\beta_{1-40}$ or $A\beta_{1-42}$ peptide concentration was ~50 μM, and final CSZ concentrations were 25, 50, and 100 μM. The ThT fluorescence signal was monitored at intervals of 10 min for 6–10 h at 37 °C with an excitation wavelength (λex) of 440 nm and an emission wavelength (λem) of 484 nm using a SpectraMax i3 (Molecular Devices, Sunnyvale, CA, U.S.A.). The mixture was shaken for 5 s between readings to facilitate aggregation. This protocol yielded 6 readings for each well sample. Experiments were performed in triplicate.

2.4. PICUP assay

Immediately after preparation, Aβ samples were cross-linked using PICUP as described [26,30]. Briefly, to 18 μL of 50 μM $A\beta_{1-40}$ or $A\beta_{1-42}$ solution were added 1 μL of 4 mM tris(2,2′-bipyridyl)dichlororuthenium(II) (Ru(bpy)) and 1 μL of 80 mM ammonium persulfate (APS). The final protein:Ru(bpy):APS molar ratios of $A\beta_{1-40}$ or $A\beta_{1-42}$ was 1:4:80. The mixture was irradiated for 1 s with visible light and then the reaction was quenched with 2 μL of 1 M dithiothreitol (Invitrogen, Carlsbad, CA, U.S.A.) in ultrapure water. A control protein, GST, was cross-linked under similar conditions. The frequency distribution of monomers and oligomers was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining as described [26,27,32]. Briefly, 8 μL of each cross-linked sample was electrophoresed on a 10%-20% gradient tricine gel and visualized by silver staining (Invitrogen). Uncross-linked samples were used as controls in each experiment.

2.5. Electron microscopy (EM)

Based on ThT and PICUP results, we performed EM imaging of Aβ assemblies in the presence and absence of CSZ. A 10 μL aliquot of each sample was spotted onto a glow-discharged carbon-coated Formvar grid (Okenshoji Co. Ltd, Tokyo, Japan) and incubated for 20 min. The droplet was displaced with an equal volume of 2.5% (v/v) glutaraldehyde in water and incubated for an additional 5 min. Finally, the peptide was stained with 8 μL of 1% (v/v) filtered (0.2 μm) uranyl acetate in water (Wako Pure Chemical Industries Ltd, Osaka, Japan). This solution was wicked off and then the grid was air-dried. Samples were examined using a H-7600 transmission electron microscope (Hitachi, Tokyo, Japan). The diameters of assemblies were measured using a 10 × magnifier eyepiece containing a graticule (Electron Microscopy Sciences, Hatfield, PA, U.S.A.).

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