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Inhibition of amyloid fibril formation and cytotoxicity by a chemical analog of Curcumin as a stable inhibitor

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ABSTRACT

Clinical application of curcumin for Alzheimer's disease treatment is severely limited with regard to its poor bioavailability, high rate of metabolism, and instability under neutral condition. In the current study, we designed three compounds in which the diketone moiety of curcumin was replaced by cyclohexanone. In these compounds, the linker length of the molecules was optimal; and substitution of dioxolane for hydroxyl groups on compound 3 should prevent metabolic inactivation. The inhibitory effect of the compounds was investigated against hen egg white lysozyme (HEWL) fibrillation using AFM (atomic force microscope), ThT (thioflavin T) and MTT assay. We found that all three compounds were able to inhibit HEWL aggregation in a dose-dependent manner and inhibit the cytotoxic activity of aggregated HEWL. Docking results demonstrated that the compounds could bind into lysozyme and occupy the whole active site groove. In conclusion, we present chemical analogs of curcumin with various modifications in the spacer and the phenolic rings as improved inhibitors of amyloid aggregation.

1. Introduction

Progressive neurodegenerative disorders (e.g. Alzheimer's and Parkinson's disease) are responsible for significant, increasing incidence of morbidity and mortality in the world [1]. Deposition of amyloid-like plaques is an early observed histological modification in the brain of the patients [2]. These plaques are related to the symptoms of cognitive impairment and suggested to have their role in the events resulting into the clinical presentation of dementia [3,4]. Aβ oligomers have been demonstrated to induce cognitive dysfunction and synaptic impairment during AD progression [5]. Insoluble and large fibrillar aggregates of amyloid polypeptides were previously supposed to be cytotoxic [6,7] but more recent studies suggest the soluble oligomeric forms of amyloid proteins to have higher cytotoxicity [8–10]. With regard to the role played by the progressive accumulation of Aβ aggregates in the development of neurodegenerative pathology, proposed therapeutic approaches toward AD have focused on decreasing the concentration of cerebral Aβ. On the other hand, amyloid formation is not an exclusive property of the disease-related proteins. In fact formation of *in vitro* fibrillar aggregates has been demonstrated for several other proteins that have no link to pathologies [11]. It has recently been found that a protein has no direct link to AD or other human diseases can induce memory impairment, resulting in an neurological effect similar to that caused by Aβ oligomers [12]. These findings suggest the existence of a common molecular mechanism in amyloid formation, including amyloid diseases [13]. As a consequence, studies on the inhibition or reversal of amyloid formation in disease – unrelated proteins could provide general information about the aggregation processes and finally result in finding potential preventive means related to amyloid diseases [14,15]. Several *in vitro* studies have reported amyloid aggregation of hen egg white lysozyme (HEWL) and wild- type human one under acidic pH and high temperature [16,17]. Lysozyme is considered a useful model in such studies, with regard to the fact that its structure and folding mechanisms are well characterized. Classical suggested approaches to treat amyloidogenic diseases target the amyloidogenic formation of proteins (reduction), or the clearance rate of

Abbreviations: AD, Alzheimer's disease; HEWL, hen egg white lysozyme; ThT, thioflavin T; CR, Congo red; AFM, atomic force microscope; MTT, 3-(4, 5-dimethylthiazol)-2, 5-diphenyltetraazolium bromide; ANS, 8-anilinonaphthalene-sulfonate; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle's Medium.

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misfolded/aggregated proteins (increase); accordingly, the stability of proteins native state could be increased, or direct inhibition of the self-assembly process may be aimed [18]. Multiple studies have reported that small organic molecules containing single or multiple (hetero) aromatic residues, possess *in vitro* anti aggregation activity [19,20]. This inhibitory effect may be exerted through several mechanism: stopping formation of mature fibrils from monomers [19], preventing both oligomers and fibril formation [21], affecting only oligomers without effect on mature fibrils formation, or block mature fibrils while allowing oligomer formation [22]. However, the practical use of these inhibitors is limited due to potential toxicity or possible inability to cross the blood–brain barrier. Clinical studies have demonstrated the chemotherapeutic activity of curcumin in AD and its lack of toxicity even at very high doses (500–8000 mg/day for 3 months) [23]. However, curcumin has not yet been approved as a therapeutic agent due to poor bioavailability and high rate of metabolism as well as instability under neutral-basic condition. Considering these facts, modifications in the core structure of curcumin may result in the generation of improved AD therapeutics. It has been suggested that the chemical scaffold of curcumin, with two aromatic groups separated by a planar backbone, is needed to fulfill its inhibitory role [2,24]. On the other hand, the hydroxyl groups of curcumin are modified in the kidneys, liver, and mucosal intestine, leading into production of curcumin glucuronide and curcumin sulfate, resulting into low bioactivity [25–27]. Moreover, both *in vitro* and *in vivo* experiments have shown that the enolic moiety in curcumin is responsible for its instability [28,29].

The present study aimed at presenting new curcumin derivatives which would possess the anti-amyloidogenic potential of the natural compound, and concurrently, be improved with regard to stability and bioavailibity. Designed compounds (Table 1) have the following characteristics: first, all three have curcumin scaffold with two aromatic ends and a rigid linker. Second, we found that all three are more or less active in amyloid disaggregation processes. Third, investigators have reported that the optimal length of curcumin derivatives linker lies within a 6–19 Å distance [24] and linkers of the selected compounds fall in that range (8.84 Å).

2. Materials and methods

2.1. Materials

HEWL (EC 3.2.1.17), Curcumin, Congo red, Thioflavin T (ThT), 2, 6 Bis (3, 4-methylenedioxy benzylidene) 1-cyclohexanone, 2, 6-divanillylidene cyclohexanone, 2, 6 Bis (3,4-dimethoxybenzylidene)-1-cyclohexanone were purchased from Sigma (St Louis, MO, USA). 8-anilinonaphthalene-sulfonate (ANS), all salts and organic solvents were obtained from Merck (Darmstadt, Germany). Protein concentration was determined spectrophotometrically at 280 nm, using an extinction coefficient (ε_{280}) of 2.65 L g^{-1} cm^{-1} [30].

2.2. Preparation of compounds solution

All the compounds were dissolved in DMSO to a concentration of 30 mM and then diluted with 50 mM glycine buffer, pH 2.5 to a specific final concentration (containing 2% (v/v) DMSO).

2.3. Defining linker length and flexibility

Linker length was quantified according to Reinke and Gestwick [24] by summing the bond lengths according to the following values: C–C, 1.54 Å; C=C, 1.34 Å; C–O, 1.43 Å. Linker flexibility was quantified based on simple quantification of sp^3 carbons and not based on dynamic simulations. This was described by Reinke and Gestwick [24].

2.4. UV–visible absorption spectra study

Absorption spectra measurements were made in the range of 250–600 nm using a Cecil 7200 UV–visible spectrophotometer (England). A stock solution of 20 mg/mL of all three compounds and curcumin in DMSO were prepared. Each stock underwent dilution with phosphate buffer 50 mM pH 7.4 to achieve a final concentration of 40 μM in the cuvette, then the UV–vis spectra of compounds were separately recorded at 25 °C immediately and after 15 min [31].

2.5. Amyloid preparation

Lysozyme was dissolved at 2 mg/mL in 50 mM glycine buffer (pH 2.5), and then incubated at 57 °C for the specified durations while stirred gently by Teflon magnetic bars [32].

2.6. Fluorescence spectroscopy

Fluorescence experiments were performed on a Cary Eclipse VARIAN fluorescence spectrophotometer. The intrinsic emission spectra were obtained at protein concentration of 0.05 mg/mL. The excitation wavelength was 280 nm and the emission spectra were collected between 290 and 450 nm. The excitation and emission slit widths were both set at 5 nm. ANS fluorescence studies were measured using a final ANS concentration of 250 μM, and the molar ratio of protein to ANS was 1:50. ANS fluorescence emission was scanned between 400 and 700 nm with an excitation wavelength of 380 nm.

2.7. ThT assay

All fluorescence experiments were carried out on a Cary Eclipse VARIAN fluorescence spectrophotometer (Mulgrave, Australia) at room temperature. Stock solution of ThT was prepared in phosphate buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7) at a concentration of 2.5 mM, passed through a 0.45 μm filter paper and stored at 4 °C. At different time intervals, an aliquot (10 μL) of incubated solution was mixed with 490 μL of ThT solution in a quartz cuvette with 1 cm path length. Fluorescence emission spectra were then taken using excitation at 440 nm. The excitation and emission slit widths were set as 5 nm and 10 nm, respectively [33].

2.8. CR assay

Samples were prepared as described above. At different time intervals, 60 μL aliquots of each sample were mixed with 440 μL of a solution containing 20 mM CR, 5 mM sodium phosphate buffer, and 150 mM NaCl at pH 7.4. The Congo red solution was filtered using a center-glass N4 filter. Optical absorption spectra were acquired from 400 to 700 nm, after a 2–3 min equilibration at 25 °C, using a 1 cm path-length cell and a Cecil 7200 UV–visible spectrophotometer (England).

2.9. Atomic force microscopy (AFM)

In order to visualize HEWL amyloid fibrils, atomic force microscopy was used. For this purpose, an aliquot (10 μL) from the incubated solution (with or without the compounds) was placed on freshly cleaved mica at room temperature. After a few minutes mica was slowly washed with 100 μL of deionized water, followed by drying with nitrogen gas. Each image was acquired in a tapping mode at a scan speed of 30 μm/s, loop filter of 3 Hz and force of