N,N’-1,10-bis (naringin) triethylenetetraamine, synthesis and as a Cu(II) chelator for Alzheimer’s disease therapy

Li-Xia GUO, Bin SUN*

1 Key Laboratory of Natural Medicine Research of Chongqing Education Commission, Chongqing Technology and Business University, 2 College of Environment and Resources, Chongqing Technology and Business University, Chongqing, 400067, PR China

Corresponding author:
Bin Sun
1 College of Environment and Resources, 2Chongqing Key Laboratory of Catalysis and functional Organic Molecules, Chongqing Technology and Business University, Chongqing, 400067, P. R. China

Email: sunbin@ctbu.edu.cn, binsunsh@yahoo.com
Abstract: The bis-Schiff base of N,N-1,10-bis (naringin) triethylenetetraamine (1) was prepared, as a copper (II) ion chelator, compound 1 was used for Alzheimer’s disease therapy in vitro. The MTT assay of compound 1 showed that this Schiff base could promote PC12 cells proliferation, and also, compound 1 could inhibit Cu²⁺-Aβ₁₋₄₂ mediated cytotoxicity on PC12 cells. The ThT assay showed that 1 can effectively attenuate Cu²⁺-induced Aβ₁₋₄₂ aggregation. In addition, compound 1 is determined to be potent antioxidants on the basis of in vitro antioxidant assay, it can effectively decrease the level of ROS in Cu²⁺-Aβ₁₋₄₂-treated PC12 cells and elevate the SOD activity in Cu²⁺-Aβ₁₋₄₂-treated PC12 cells. The results show that N,N-1,10-bis (naringin) triethylenetetraamine is a potential agent for therapy of Alzheimer’s disease.

Key words: naringin; Schiff base; Cu²⁺ ion; amyloid-beta; antioxidant; Alzheimer’s disease
1 Introduction

Alzheimer’s disease (AD) is an age-related neurodegenerative progressive and fatal disorder [1]. Although the development of cure for AD has been hindered by a lack of understanding of both the causes and mechanisms of this disease onset and progression, a series of hypothesis came forth to explain the cause of AD, among which amyloid cascade hypothesis, glycogen synthase kinase-3 (GSK-3) hypothesis, oxidative stress hypothesis, metal ion hypothesis and cholinergic hypothesis are of the central importance [2-6]. Among them, the aggregation of Amyloid-β (Aβ) plays a major rule in neuronal loss and cognitive impairment by forming senile plaques. This Aβ species form unbranched fibrils consisting of parallel and ordering β-sheet structures. Aβ, especially Aβ1-42, is proven to form toxic aggregates, among them, soluble Aβ oligomers are the main toxic aggregates [7-11]. Therefore, preventing Aβ aggregation is a major strategy against AD.

Metals (such as Cu, Zn, Fe) play an important role in the pathogenesis of Alzheimer's disease [12-14], particularly Cu and Zn, bind to Aβ peptides facilitating their aggregation [15-20], moreover, dysregulated redox active metal ions, Cu (I/II) and Fe (II/III), both unbound and bound to Aβ peptides, are observed to promoted overproduction of reactive oxygen species (ROS) that damage biological molecules, [21] such as proteins, DNA and lipid [22-24]. Evidences show that metal chelators can prevent metal induced Aβ aggregation and reactive oxygen species (ROS) production [25-29], and also can solubilize Cu (II) induced Aβ plaques, therefore, metal chelators are potential therapeutic agents of AD [30-34].

Naringin is a dihydroflavone glycoside. It exhibits a variety of biological activities and pharmacological effects such as anti-inflammatory, anti-virus, anti-cancer, antioxidant and antihypertensive activities [35]. Also, it can lower blood cholesterol, reduce the formation of blood clots, improve local microcirculation and nutrient supply reduce the formation of blood clots [36-38]. Naringin can be used to produce drug for the prevention and treatment of cardiovascular and cerebrovascular diseases [39]. In addition, naringin can attenuate neuronal apoptosis which induced by
lipopolysaccharide (LPS), and improve the impairment of learning and memory in AD transgenic mouse model APPswe/PSDeltaE9 [37, 40]. Our group found that N,N’-bis (naringin)-1,3-propyldiamine (Figure 1) expressed higher biological activities in inhibition of Cu²⁺ induced Aβ aggregation, attenuation of cytotoxicity of Aβ and anti-oxidation [41]. As a part of our continual interest in searching new medicines for curing AD, here we report the synthesis of N,N’-1,10-bis (naringin) triethylenetetraamine and in vitro studies of N,N-1,10-bis (naringin) triethylenetetraamine as potential agent for Alzheimer’s disease.

Figure 1 The structure of N,N’-bis (naringin)-1,3-propyldiamine

2 Experiment
2.1 Materials and Methods
Chemicals used were reagent grade as supplied except where noted. Analytical thin-layer chromatography was performed using silica gel 60 F254 glass plates; Compound spots were visualized by UV light (254 nm) and by staining with a yellow solution containing Ce(NH₄)₂(NO₃)₆ (0.5 g) and (NH₄)₆Mo₇O₂₄·4H₂O (24.0 g) in 6% H₂SO₄ (500 mL). Flash column chromatography was performed on silica gel 60 (200–400 Mesh). NMR spectra were referenced using Me₄Si (0 ppm). High-resolution mass spectra were recorded on a Q-TOF Ultima API LC-MS instrument with Waters 2795 Separation Module (Waters Corporation, Milford, MA).

Dimethyl sulfoxide (DMSO), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), thioflavine T (ThT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DCFH-DA kit and SOD kit were purchased from Sigma-Aldrich (St. Louis, MO).

BCA protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA,
USA). Aβ1-42 (> 98% purity) was purchased from ChinaPeptides (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). Rat pheochromocytoma PC12 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 Synthesis

*N,N'-1,10-bis (naringin) triethylenetetramine*  Naringin (5.81 g, 10 mmol) was dissolved in a mixture solvent of methanol and ethanol (V/V = 1 : 1)(100 mL). The reaction mixture was stirred and heated to 50°C, a solution of triethylenetetramine (0.74 mL, 5 mmol) in ethanol (10 mL) was added into the reaction mixture. After 30 min, a lot of yellow precipitate was formed. The reaction mixture was continued stirring for 1 h, and then the mixture was cooled down to room temperature. Filtered, the residue was washed with anhydrous ethanol (3 x 20 mL) 3 times and dried over a vacuum to obtain crude product (5.75 g). The crude product was purified by recrystallization with ethanol and water (3 : 1) to give product (5.50 g, 87%). 

$^1$H NMR (600 MHz, DMSO-d$_6$, ppm) $\delta$ 12.02 (s, br, 2 H), 9.58 (s, br, 2 H), 7.30 (dd, J = 12.0, 4.8 Hz, 4H), 6.77, d, J = 12.0 Hz, 4 H), 6.09 (t, J = 3.0 Hz, 2 H), 6.06 (t, J = 4.2 Hz, 2 H), 5.52-5.42 (m, 2H), 5.27 (d, J = 6.3 Hz, 2 H), 5.14-5.05 (m, 6 H), 4.69 (d, J = 6.3 Hz, 2 H), 4.63 (d, J = 6.0 Hz, 2 H), 4.54(t, J = 6.0 Hz, 2 H), 4.44 (d, J = 2.4 Hz, 2 H), 3.71-3.59 (m, 6 H), 3.47-3.36 (m, 9 H), 3.32-3.24 (m, 16 H), 3.19-3.10 (m, 5 H), 2.75-2.65 (m, 2 H), 1.13 (d, J = 9.0 Hz, 6 H). $^{13}$C NMR (150 MHz, D$_2$O, ppm) $\delta$ 169.0, 164.1, 164.0, 163.5, 163.2, 158.0, 128.3, 127.9, 127.8, 127.7, 116.1, 116.0, 115.9, 103.8, 100.9, 97.4, 78.7, 77.5, 76.3, 75.6, 71.8, 70.0, 68.7, 60.3, 60.1, 57.3, 51.7, 48.9, 46.7, 66.5, 38.8, 38.4, 38.1, 16.7. HR-ESI-MS: m/z: calcd for C$_{60}$H$_{78}$N$_4$O$_{26}$Na: [M + Na]$^+$, 1293.4802, Found 1293.4864.

2.3 Biological assays

2.3.1. Aβ1-42 peptide solution preparation

In brief, Aβ1-42 powder was firstly dissolved in HFIP at 1.0 mg/mL, sonicated for 10 min in ice bath to disrupt the pre-existing aggregates, and aliquoted into
microcentrifuge tubes to obtain 0.1 mg stocks. The stocks were stored at room temperature and protected from light for 5-24 h. Thereafter, HFIP was removed by evaporation under N$_2$. The thin transparent film of peptides at the internal surface of the tube was stored at −80 °C. In aggregation and inhibition experiments, Aβ$_{1-42}$ was dissolved in DMEM, and then diluted in PBS (pH 7.4) to a final concentration of 10 μM.

2.3.2. **ThT fluorescent assay**
Aβ$_{1-42}$ samples (10 μM) with CuCl$_2$ (5 μM) and different concentrations of N,N’-bis (naringin) triethylenetetraamine (dissolved in DMSO) were incubated at 37 °C. For ThT assays, 200 μL of ThT (ThT was dissolved in 0.1 M glycine buffer at pH 8.9) incubated samples were drawn carefully at 72 h. ThT fluorescence intensities (excitation wavelength 450 nm; emission wavelength 482 nm) were measured by a fluorescence spectrometer. The fluorescence intensity of the samples without Aβ$_{1-42}$ was subtracted as background from each read with Aβ$_{1-42}$. Each value reported is the average of three readings for every sample after subtraction of ThT fluorescence background. All ThT fluorescence experiments were performed in triplicate.

2.3.3. **Cell viability assay**
MTT assays were performed to assess the cytotoxicity of Aβ$_{1-42}$ aggregates using PC12 cells. The PC12 cells were cultured in DMEM supplemented with 10 % FBS at 37 °C under 5 % CO2. Prior to the MTT assays, PC12 cells were seeded onto 12-well plates at a density of 5×104 cells/well and cultured for 24 h. Then, the cells were treated with samples [mixtures of Aβ$_{1-42}$ (10 μM) with Cu$^{2+}$ (5 μM), different concentrations of N,N’-Bis (naringin) triethylenetetraamine], and incubated for additional 48 h. After that, 0.5 mg/mL MTT solutions (dissolved in water, sterile filtration) were added into each well. After 2-4 h incubation, the medium was replaced with DMSO to dissolve the formazan salt. Then, absorbance at 570 nm was measured by a multifunctional microplate reader.

2.3.4 **Intracellular Determination of ROS**
Intracellular ROS accumulation was monitored on a fluorescence spectrofluorometer (Beckman Coulter, Brea CA) with excitation and emission wavelengths of 485 and 530 nm, respectively using the fluorescent probe DCHF-DA. To do the test, cells were incubated with 20 μmol/L of DCHF-DA (DCFH-DA was dissolved in DMSO and prepared into 10 mm storage solution) for 1 h at 37°C in the dark after treatment with various concentrations of test agents. After incubation, the cells were washed twice with PBS and, finally, analyzed by a fluorescence spectrofluorometer. Protein concentration of samples was determined by BCA Protein assay kit with BSA used as a standard. Intracellular ROS accumulations of test compounds were assayed in triplicate.

2.3.5 SOD Activity Determination
Superoxide dismutase (SOD) activity of the complexes was based on the inhibitory effect of SOD on the reduction of nitroblue tetrazolium (NBT) by the superoxide anion generated by the system xanthine/xanthine oxidase, measuring the absorption at 560 nm. PC12 cells were seeded onto 12-well plates at a density of 5×10^4 cells/well and cultured for 24 h. Then, the cells were treated with samples [mixtures of Aβ_{1-42} (10 μM) with Cu^{2+} (5 μM), different concentrations of N,N’-1,10-Bis (naringin) triethylenetetraamine, and incubated for additional 24 h. Then, the cells were washed two times with 0.1 M phosphate buffer saline (PBS, pH = 7.4).

The cell lysates were used to determine SOD activity using commercial kits according to the kit’s instructions. Protein concentration of samples was determined by BCA Protein assay kit with BSA used as a standard. The SOD activities of test compounds were assayed in triplicate.

2.3.6 Statistical analysis
All data are presented as means ± SD. Statistical analyses were conducted using Origin version 8.0 software (OriginLab Corporation, MA). Data were analyzed using one-way ANOVA, followed by Tukey’s post hoc test, for differences among treatments. P < 0.05 was considered statistically significant.
3 Results and Discussion

3.1 chemistry

Synthesis of ligand In order to avoid naringin in product, the reactant should be completely dissolved in solvent. Therefore, a solvent having a moderate solubility for naringin is required, which can completely dissolve naringin, and also, the product is easy to precipitate from the solvent after the reaction finished. The solubility of naringin in methanol is good, however, there is no product precipitate from solvent when the solution of naringin in methanol react with triethylenetetraamine. The solubility of naringin in ethanol is much worse than that in methanol, the proper amount of ethanol cannot completely dissolve the naringin. After repeated attempts, the mixture solvent of methanol and ethanol with 1 : 1 ratio can obtain product precipitates with 96% yield.

3.2 Biological activities

Inhibition of Cu$^{2+}$-induced Aβ$_{1-42}$ aggregation by N,N'-$1,10$-bis (naringin) triethylenetetraamine

Cu$^{2+}$ and Zn$^{2+}$ are known to promote the rapid aggregation of Aβ peptides in solution and this has been implicated in several neurodegenerative diseases such as Alzheimer and Parkinson disease [18, 20]. It has been shown previously that metal chelators can attenuate these effects [24-28]. However, treatment of Tg2576 mice with traditional Cu chelator TETA (triethylenetetraamine), didn’t inhibit amyloid deposition [33]. In the ThT assay, we found that TETA also didn’t effectively prevent the Cu$^{2+}$-induced Aβ$_{1-42}$ accumulation. TETA (100 μM) was incubated with Aβ$_{1-42}$ and Cu$^{2+}$ for 72 h, the inhibition rate was only 3% (Figure S3). The activity of naringin for preventing of
Cu\textsuperscript{2+}-induced A\textsubscript{β}\textsubscript{1-42} aggregation is better than that of TETA, naringin (100 μM) was incubated with A\textsubscript{β}1-42 and Cu\textsuperscript{2+} for 72 h, the inhibition rate was 13% (Figure 2, B). Comparing TETA and naringin, N,N'-1,10-bis(naringin) triethylenetetraamine was shown high activity to reduce Cu\textsuperscript{2+}-induced A\textsubscript{β}1-42 aggregation (Figure 2, A), this bis-Schiff base (10 μM) was incubated with A\textsubscript{β}1-42 and Cu\textsuperscript{2+} for 72 h, the inhibition rate was 33%, when its concentration increased to 100 μM, the inhibition rate increased to 55%, these results were obviously better than that of bis-(naringin)-1,3-propyldiamine (when its concentration is separately 10 μM, 100 μM, the inhibition rate is separately 3%, 26%, Figure S4), and the attenuation of this Schiff base was increased with the prolongation of action time (24-72 h). These results indicated that compound 1 has much better ability to inhibit Cu\textsuperscript{2+}-induced Aβ aggregation than TETA, naringin and N,N'-bis (naringin)-1,3-propylenediamine.

![Figure 2](image_url)

**Figure 2.** ThT assay on the Cu\textsuperscript{2+}-induced aggregation of Aβ\textsubscript{1-42} peptide in the absence or presence of N,N'-bis(naringin)-triethylenetetraamine (A) and naringin (B) at indicated concentrations.

**Protection effects of N,N'-1,10-bis(naringin) triethylenetetraamine on Aβ\textsubscript{1-42}-Cu\textsuperscript{2+}-induced cytotoxicity of PC12 cells**

To study the influence of N,N'-1,10-bis(naringin) triethylenetetraamine itself on the cell viability, the PC12 cell line was chosen, as it is a neuronal cell line, and the brain is the target organ of the compound. The compound was added to PC12 cells at increasing concentrations (10-200 μM) and incubated for 48 h or 72 h. The cell cytotoxicity was assessed with the MTT assay. (Figure S5) illustrates cell viability in relation to concentrations of compound; as can be seen, the compound had not a significant effect on cytotoxicity, and also, it can promote PC12 cell proliferation,
when the concentration of compound 1 exceeded 25 μM, it incubated with PC12 cells for 72 h, the cell viability was more than 100% (in the same condition, the cell viability of control is 100%).

The ability of N,N'-1,10-bis(naringin)triethylenetetramine to inhibit Cu^{2+}-Aβ₁₋₄₂ assembly suggested that it might be helpful in blocking Aβ-mediated cellular toxicity. As shown in Figure 3 (A), treatment of the PC12 cells with 5 μM Aβ–Cu^{2+} for 48 h the cell viability reduced to 50.2%. When the PC12 cells with 5 μM Aβ–Cu^{2+} and 10 μM naringin Schiff base were incubated for 48 h, the survival of the cells increased to about 60.2%. These results showed that the compound was not only effective as a Cu^{2+}-induced Aβ assembly inhibitor, but also could decrease Aβ-mediated cellular toxicity in PC12 cell lines. The same assays were done for naringin (Figure 3, B) and N,N'-bis (naringin)-1,3-propylenediamine (Figure S6), the results showed that naringin didn’t obviously increase the cell viability of PC12 (P>0.05), and when the concentration of N,N'-bis (naringin)-1,3-propylenediamine is 10μM, the cell viability of PC12 cell isn’t obvious increasing yet. In addition, When the PC12 cells with 5 μM Aβ–Cu^{2+} and 10 μM triethylentetramine (TETA) were incubated for 48 h, the cell viability reduced to 45.2% (Figure S7). These results indicated that N,N'-1,10-bis (naringin) triethylenetetraamine had more ability to attenuate Aβ-mediated cellular toxicity in PC12 cell lines than that of naringin, TETA and N,N'-bis (naringin)-1,3-propylenediamine.
N,N'-1,10-bis(naringin) triethyleneamine attenuates the production of ROS in Aβ\(_{1-42}\)-Cu\(^{2+}\)-treated PC12 cells

The interaction of the Aβ peptides with redox-active metal ions such as Cu\(^{2+}\) has been proposed to lead to formation of ROS and oxidative stress associated with Aβ neurotoxicity [42]. The oxidative stress which associated with high levels of the bio-metals copper (or iron) in the neurodegenerative AD naturally has triggered the potential use of chelating agents to connected with this metal-induced brain damage [42,43]. To evaluate the effect of N,N'-1,10-bis (naringin) triethylenetetraamine on cell viability at the molecular level, we detected the reduction of the amount of ROS by this naringin bis-Schiff bases with the fluorescent probe DCFH in PC12 cells in the presence of Aβ\(_{1-42}\)-Cu\(^{2+}\). The results demonstrated that incubation with 5 μM Aβ\(_{1-42}\)-Cu\(^{2+}\) increased ROS levels compared to control, and by contrast, N,N'-1,10-bis(naringin) triethylenetetraamine dose-dependently decreased the level of ROS in Aβ\(_{1-42}\)-Cu\(^{2+}\) treated PC12 cells, when the concentration of this bis-Schiff base was 25μM, the ROS level was 74 (Figure 4). The same assay was carried out for N,N'-bis (naringin)-1,3-propylenediamine, when its concentration is 25μM, the ROS level was 115 (Figure S8). These results showed that the decreasing of the level of ROS was not simply caused by hydroxyl group on naringin. It maybe that compound 1 can chelate Cu\(^{2+}\) from Aβ-Cu complex, so as to relieve the oxidative stress which caused by Aβ-Cu complex.
N,N’-1,10-bis(naringin)-triethylenetetraamine elevates the SOD activities in Aβ1-42-Cu2+-treated PC12 cells

Some research suggests that the possible mechanisms for neurotoxicity in AD are an imbalance in the production and detoxification of free radicals both an increased production of ROS and insufficient antioxidant defense [44, 45]. The immediate evidence is the alterations in enzymatic antioxidant metabolism in postmortem AD brains [46]. SOD multigene family play an important role in the clearance of ROS and imbalance in oxidative stress. Mn-SOD deficiency has been shown to accelerate the onset of a number of behavioral deficits in hAPP mice [47]. And impaired Cu/Zn-SOD activity contributes to increased oxidative damage in APP transgenic mice [48,49]. Instead, overexpression of Mn-SOD reduces hippocampal superoxide and Aβ levels, improves the axonal transport deficits and mitochondrial respiratory function in the Tg2576 Alzheimer model mice [50, 51]. Considering the fact that the active centers of SOD are metal complexes, we speculate that N,N’-1,10-bis (naringin) triethylenetetraamine, as a chelator, could capture Cu from Aβ1-42-Cu2+ complex, and chelator-Cu complex may form a dinuclear Cu complex with naringin Schiff bases (Figure 6, the complex structure was certificated by ESI-HRMS, FTIR, UV and the results of elemental analysis, in supporting information), and this dinuclear Cu complex could mimic superoxide dismutase. The SOD activity was quantified using a modified NBT assay system. The results indicated that Aβ1-42-Cu2+ complex as expected showed little SOD activity compared to control (Figure 5), and by contrast, N,N’-1,10--bis (naringin) triethylenetetraamine dose-dependently increased the activity of SOD in Aβ1-42-Cu2+ treated PC12 cells, when the concentration of compound 1 is separately 0, 10, 20, 50 and 100 μM, the activity of SOD is separately 22, 25, 43, 50, 82. The same assay was done for N,N’-bis (naringin)-1,3-propylenediamine, when its concentration is separately 0, 10, 20, 50 and 100 μM, the activity of SOD is separately 22, 18, 28, 36, 50 (Figure S9). These results indicated that the copper (II) complex with N,N’-1,10-bis (naringin) triethylenetetraamine can more stimulate the action of superoxide dismutase than the
copper (II) complex with N,N’-bis (naringin)-1,3-propylenediamine.

Figure 6 The structure of copper complex with N,N’-1,10-bis (naringin)triethylenetetraamine bis-Schiff bases

In conclusion, in this study, we synthesized N,N’-1,10-bis (naringin)triethylenetetraamine bis-Schiff bases, and studied the bioactivities of this compound in vitro. This compound is designed to attenuate the Aβ aggregation caused by Cu^{2+}, inhibit cellular toxicity mediated by the accumulation of Aβ and prevent the production of reactive oxygen species. The results of biological assays show that this compound can effectively prevent Cu^{2+}-induced Aβ_{1-42} aggregation, attenuation of Aβ_{1-42}-mediated cytotoxicity in PC12 cell lines, decrease the level of ROS in Aβ_{1-42}-Cu^{2+} treated PC12 cells, and increase the activity of SOD in Aβ_{1-42}-Cu^{2+} treated PC12 cells. These studies show that N,N’-1,10-bis (naringin)triethylenetetraamine will be a potential drugs for AD therapy.

ACKNOWLEDGEMENTS
This work was supported by the Natural Science Foundation of China (81603173), the Chongqing Municipal Education Committee (KJ1600607), the Chongqing Science & Technology committee (cstc2018jcyjAX0754; cstc2020jcyj-msxmX0830), the Key Projects of Basic Science and Advanced Technology Research of Chongqing City (cstc2015jcyjBX0125), Research program of Chongqing technology and Business University (1352002) and the open project of the Key Laboratory of Natural Medicine Research of Chongqing Education Commission (CQCM-2017-09, CQCM-2016-09).

Conflict of Interest
The authors declare no conflict of interest.
**Supplementary Materials**

The online version of this article contains supplementary materials.
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