Discovery of Dehydroabietylamine Derivatives as Multifunctional Agents for the Treatment of Alzheimer's Disease

Yingying Cao[a,†], Ling Wang[a,†], Zhongxiang Lin[b], Fengyin Liang[c], Zhong Pei[c], Jun Xu[a], Qiong Gu [a, *]

[a]Research Center for Drug Discovery, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou 510006, China.

[b]College of Chemical Engineering Nanjing Forestry University, Nanjing 210037, China

[c]Neurology Department of the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510080, China

[†] Equal contribution

*Correspondence to: guqiong@mail.sysu.edu.cn, Tel: +86-20-3994-3077 Fax: +86-20-3994-3077

Experimental section

1. Anti-oxidant activity in SH-SY5Y cells

The anti-oxidant activities of the dehydroabietylamine derivatives were measured with a fluorescent probe (2',7'-dichlorofluorescein diacetate; DCFH-DA) with minor variations of the previously reported method[1]. Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, GIBCO), 1 mM glutamine, 50 mg/μL penicillin, and 50 mg/μL streptomycin at 37 °C in a 5% CO_2 humidified incubator. The SH-SY5Y cells were incubated in 96-well plates with a density of 1×10^5 cells/well for 24 h with DMEM, FCS, glutamine, penicillin, and streptomycin, as described previously. The cells were treated with the tested compounds at 10 μM. After 24 h of treatment, the cells were washed 3 times with PBS, and then 5 μM DCFH-DA in PBS was added directly to the cells at 37 ℃ in 5% CO_2 for 30 min. The DCFH-DA was then removed, 5 μM t-BuOOH in PBS was added and the cells were maintained for 30 min. After incubation, the fluorescence absorbance of the cells was tested by a monochromator-based multimode microplate reader (INFINITE M1000) at an excitation of 488 nm and an emission of 525 nm. The analysis of the results was performed by calculating the percentage of the average sample and that of the control

group as follows: (OD sample-OD blank) / (OD control-OD blank) × 100%.

2. Thioflavin T (ThT) fluorescence assay

The aggregation and the fibril formation of Aβ_{42} were measured by the Thioflavin T (ThT) fluorometric assay. Aβ_{42} was dissolved in 1% NH_3–H_2O (440 μM), and the tested compounds (10 mM) were dissolved in DMSO. To monitor the inhibition of Aβ_{42} aggregation and fibril formation by these compounds, 100 μM Aβ_{42} was left untreated or mixed with equal amounts of these compounds and incubated at 37 ℃ for 48 h. To assess the disaggregation of pre-formed fibrils, 100 μM Aβ_{42} was incubated for 48 h in a 37 ℃ incubator. Then, the preformed fibrils were mixed with an equal amount of each compound for an additional 2 days at 37 ℃. After incubation, the samples were added to 96-well microtiter plates to a final volume of 200 μL with 50 mM glycine-NaOH buffer (pH 8.5) containing 5 μM ThT. The fluorescence of the samples was monitored by a Therme-type multifunctional microplate reader (Ruishi Di Ken) at an excitation of 450 nm and an emission of 485 nm.

3. CD assay

Aβ_{42} (150 μL of 100 μM) was left untreated or mixed with an equal amount of **3b** (200 μM) in 20 mM sodium phosphate buffer (pH 7.4) at 37 ℃ for 0 h and 48 h. Compound **3b** was dissolved and diluted with methanol to 10 mM. The samples were diluted with 20 mM PBS to a final volume of 750 μL, and the final concentrations of Aβ_{42} and **3b** were 20 and 40 μM, respectively. The CD spectra were determined using a Jasco-810-150S spectropolarimeter (JASCO, Tokyo Japan) with a wavelength range of 190-260 nm, a bandwidth of 0.5 nm, and a 3 s response time. A quartz cell with a 10 mm optical path was used.

4. TEM assay

To monitor the inhibition of Aβ_{42} fibril formation by morphology, 50 μM *Aβ_{42}* was left untreated or mixed with an equal volume of 100 μM **3b** at 37 ℃ for 48 h. To assess the morphology of the disaggregation of pre-formed fibrils, 50 μM aggregated Aβ_{42} was mixed with 100 μM **3b** and incubated for an additional 48 h. All samples were measured by a transmission electron microscope (JEOL JEM-1400) after incubation. A copper grid was used keep the samples in place, and the samples were

negatively stained for one minute with a 2% uranyl acetate solution. Finally, the staining solution was drained off using filter paper.

5. Molecular dynamics stimulations

For the Aβ_{42} docking study, the initial structure of Aβ_{42} was taken from the NMR structure (PDB ID: 1IYT)[2]. Autodock 4.0 was employed to identify the binding poses of **3b** for Aβ_{42} with a Lamarckian genetic algorithm[3]. To evaluate the binding energies between the ligand and the receptor, a grid map, 80×80×80 points spaced equally at 0.375 Å, was generated using the AutoGrid program. All docked poses of compound **3b** were clustered using a tolerance of 2 Å for the root mean square deviation (RMSD) and were ranked on the basis of the docking binding energies. For each compound, the lowest energy conformation in the most populated cluster was chosen for further study. The initial coordinates for the Aβ_{42}-**3b** complex were taken from the docking results. The complex was first put into a suitable box, where the minimal distance from the peptide to the box wall was 1.2 nm. Then, the box was solvated with the TIP3P water model. The MD simulations were carried out using the GROMACS 4.5.3 package[4] with constant number, pressure, and temperature (NPT) and using periodic boundary conditions. The AMBER ff03 force field[5] was applied for the peptides. The parameters for **3b** were obtained from the ANTECHAMBER module using the Generalized Amber force field (GAFF)[6]. The partial atomic charges for the ligand atoms were assigned using the RESP charge-fitting procedure with input from the Hartree–Fock calculations at the 6-31G* level (through the use of the Gaussian03 program[7]). During the simulations, the pressure and the temperature were coupled to 1 bar with an anisotropic coupling time of 1.0 ps and kept at 300 K with a coupling time of 0.1 ps. The coordinates of the entire 90 ns MD run were saved every 2 ps. The secondary structures were characterized via the DSSP method[8]. All structural diagrams were constructed using the PyMOL molecular graphics package.

6. Cell culture and the MTT assay for cell viability

The SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% fetal bovine serum (GIBCO), 50 mg/μL penicillin, and 50 mg/μL streptomycin. The aforementioned MTT assay was performed to assess cell

viability. The cells were cultured in 96-well plates with a density of 1 x 10^5 cells/well at 37 ℃ in a 5% CO_2 humidified atmosphere. After 24 h, different concentrations of **3b** (0-50 μM) and 10 μM of the dehydroabietylamine derivatives were added to the 96-well plates to a final volume of 100 μL. The blank well was filled with conditioned media alone and the control well was filled with cells and conditioned media. Then, the 96-well plates were incubated at 37 ℃ in a 5% CO_2 incubator for an additional 48 h. MTT (10 μL of 5 mg/mL) was added to the 96-well plates and incubated for 4 h. After incubation, the MTT medium in each well was removed and 100 μL of DMSO was added. The absorbance of each well was then measured using a PowerWave XS2 microplate reader (Bio-Tek) set to monitor 570 nm.

7. Neuroprotective activity in SH-SY5Y cells

The protective effect of **3b** on Aβ_{42}-induced neurotoxicity was measured by the MTT assay in SH-SY5Y cells[9]. Aβ_{42} (2 mM) was dissolved in DMSO and diluted with culture solution to different concentrations. Then, **3b** was diluted into different concentrations (50, 25, 10, and 5 μM) with conditioned media and mixed with 40 μM Aβ_{42}. These 100 μL mixtures were incubated at 37 ℃ for 48 h before they were added into the 96-well plates. The SH-SY5Y cells were added into the 96-well plates and incubated for 24 h. The control was filled with cells and conditioned media and the blank was filled with conditioned media only. Then, the tested compounds were added into the 96-well plates for another 48 h at 37 ℃ in a 5% CO_2 incubator. After incubation, DMSO was added into the 96-well plates after MTT (10 μL of 5 mg/mL) was incubated with the cells for 4 h. Then, the absorbance tests and analysis of the results were conducted according to the MTT method.

8. Cell culture and toxicity analysis in swAPP HEK293 cells

HEK293 cells were stably transfected with APP695 and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 50 mg/μL penicillin, 50 mg/μL streptomycin, and 200 mg/mL G418 in a 5% CO_2 incubator at 37 ℃. To measure the toxicity of **3b** on the swAPP HEK293 cells, the MTT method was used[10]. HEK293 cells were added into a 24-well plate at a density

of 1×10^4 cells per well for 24 h. After incubation, the conditioned media was removed and the cells were washed with PBS three times. Then, **3b** (25, 10, 5, and 1 μM) was added into each well and incubated in a humidified atmosphere at 37 ℃. Cur (10 mM) was used as the control. After 48 h of incubation, MTT (10 μL of 5 mg/mL) was added into the 24-well plate and incubated for another 4 h. Finally, the measurement of the absorption values and the analysis of the results were conducted according to the MTT method.

9. Sandwich ELISA

HEK293 cells were added into 6-well plates with a density of 4×10^4 cells per well for 24 h in a 5% CO_2 incubator at 37 ℃[11,12]. Then, the conditioned media was replaced with **3b** (25, 10, 5, and 1 μM) or Cur (10 μM) for another 48 h. The cultured media was obtained and the extracellular Aβ_{42} levels were measured using the human Aβ_{42} assay kit (Cusabiao Biotech Co., Ltd., U.S.) according to manufacturer instructions.

10. Caenorhabditis elegans paralysis assay

The *C. elegans* paralysis assay was measured using transgenic strain CL4176[13,14]. The Aβ proteins in CL4176 have high expression levels and accumulate when they were transferred from 15 ℃ to 26 ℃. When the Aβ proteins accumulate to a certain degree, the CL4176 strain undergoes paralysis. After incubation at 15 ℃ for 3 days, the CL4176 were age-synchronized, and they were then transferred onto new 35×10 mm NGM plates with 200 μL OP50 (the *Escherichia coli* strain). Compound **3b** (500 mM) was dissolved in DMSO and diluted to different concentrations (10, 50, 200, and 500 μM) with OP50, then added into the fresh culture plates. The concentration of DMSO in each plate is less than 0.1%. The blank plate was filled with OP50 containing 0.1% DMSO. The plate with 500 μM Huperzine A was regarded as the positive control. Then, the tested compounds (200 μL) were added into the fresh NGM plates. After synchronization for 24 h, the CL4176 worms were washed by 1 mL of ddH_2O and collected in a 1.5 mL centrifuge tube. Approximately 25 worms were added into the plates with **3b** or Huperzine A and incubated at 15 ℃ for another 12 h. Then, all NGM plates were transferred to a 26 ℃ incubator. After 36 h of

incubation, the amount of paralysis in each plate was recorded using a microscope at 2 h intervals and the paralyzed worms were picked out with needle until blank group was almost entirely paralyzed.

11. Western blotting method

The Aβ species from the CL4176 worms were extracted at the time when the paralyzed worms were 50% of the total worms in the blank group. The worms were washed with PBS, centrifuged at 12000 r/s for 15 minutes, and sonicated in RIPA Lysis Buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 1% sodium deoxycholate, and 0.1% SDS) (Bio-Rad Laboratories, Richmond, CA, USA) with protease inhibitor cocktail (PMSF) for 30 minutes at 4 ℃. In the western blot assay, the protein was boiled with loading buffer at 100 ℃ for 5 minutes before being loaded into the gel. An equal amount of protein (80 μg) was added into the lane of the Tricine-SDS-PAGE[15] gel, and β-actin (1:1000) and 6E10 (1:1000) were used as the primary antibodies. Anti-mouse IgG (1:5000) was used as the second antibody, and the mean optical density was analyzed by Image J.

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