Supporting Information

Identification of a small molecule Cyclophilin D inhibitor for rescuing Aβ-mediated mitochondrial dysfunction

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Materials and methods

Synthesis of CypD inhibitors

Compound C-**9** that exhibited the best biological activity based on the measured binding affinities and observed effects on mitochondrial function induced by Ca^{2+} or $A\beta_{1-42}$ was used in all pharmacological studies.

A solution of 2-(3-cyano-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylic acid and thionyl chloride was stirred in dry toluene for 30 min in the presence of a catalytic amount of dimethylformamide to produce an acid chloride intermediate for C-**9**. Slowly increasing the temperature to 90−95 °C with constant stirring for 3 h resulted in a clear solution. The reaction mixture was then cooled slowly to room temperature and concentrated to give a white residue. This residue was dissolved in acetonitrile and an equimolar amount of 4-aminobenzenesulfonamide was added. This solution was stirred for 4 h at 75 °C to give impure solid **9** [1]. This solid that was separated by filtration, washed with acetonitrile, ice-cold water, and then recrystallized from ethanol to give pure C-**9**.

Compound C-**9** was present as an off-white powder with a melting point of 297-299 °C, a molecular weight of 471 and a molecular formula of $C_{22}H_{22}N_4O_4S_2$ (**Figure 1**). High pressure liquid chromatography (HPLC) purity of compound C-**9** was 100%. HPLC: 100% at 21.37 min (retention time) on an ACE C18 column (Mac Mod Analytical, 3µm, Ultra-Inert HPLC Column, 50×2.1mm) protected by a matched ACE guard cartridge and a detection wavelength of 254 nm. Solvents A: H_2O (99%), methanol (1%) and formic acid (0.1%) and B: H_2O (1%), methanol (99%) and formic acid (0.1%) delivered at a flow rate of 400 µl/min. Using a gradient pump program beginning at 15% methanol containing 0.1% formic acid (FA), H_2O (1%), and 85% aqueous containing 0.1% FA and methanol (1%) with a flow rate of 0.4 mL/min for 10 minutes.

The methanol with FA increased linearly from 15 to 80% from 0.5 to 5 minutes and then was maintained at 80% from 5 to 45 minutes.

2-(3-Cyano-4-isobutoxyphenyl)-4-methyl-N-(4-sulfamoylphenyl) thiazole-5-carboxamide (C-9): R_f 0.62; (methylene dichloride: methanol, 9:1 v/v). ^{1}H NMR (DMSO-d_6) δ: 1.03 (d, 6H, J = 13.6 Hz), 2.12-2.08 (m, 1H), 2.65 (s, 3H), 4.02 (d, 2H, J = 12.4 Hz), 7.31 (s, 2H), 7.44-7.40 (m, 1H), 8.87-8.81 (m, 4H), 8.25-8.21(m, 1H), 8.31 (s, 1H), 10.59 (s, 1H); ^{13}C NMR (DMSO-d_6) 164.4, 161.9, 160.0, 156.2, 141.4, 139.5, 132.9, 131.3, 126.5, 125.8, 125.2, 119.8, 115.3, 113.9, 101.5, 75.0, 27.5, 18.6, 17.1; HRMS cald for $C_{22}H_{22}N_4O_4S_2$ (M+H)+ 471.1161; found 471.1164 (TOF MS ES+).

Binding experiment of CypD inhibitors with CypD protein

Surface Plasmon Resonance (SPR) using a BIACORE 3000 at 25°C was used to study the binding interaction between compound C-**9** and cyclophilin D (CypD). The SPR binding experiments used a dual flow cell as previously described[2, 3]. They were conducted in phosphate-buffered saline solution (PBS, pH 7.4, 0.005% surfactant P20) that served as both running and sample buffer. The sensor chip surface was initially activated with mixtures of N-hydroxysuccinimide (NHS, 115 mg/ml) and N-(3-dimethyl-aminopropyl)-N'-ethyl-carbodiimide-hydrochloride (EDC, 750 mg/ml) for 7 min each. CypD protein was first dissolved in PBS (pH 5.0) at a concentration of 10 μg/ml, and then immobilized directly and covalently attached to a hydrophilic carboxy-methylated dextran matrix of the Biacore CM5 sensor chip using the standard primary amine coupling reaction and standard procedures. After the CypD protein was immobilized, the activated carboxylic acid groups were quenched using ethanolamine (1 M, pH 8.5).

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Considerable care was taken to prevent contamination. Samples were carefully injected to avoid carryover effects and the system was carefully washed before injection of each new sample. The sample flow rate was set at 40 μl/min to determine the kinetic and equilibrium constant. The equipment surfaces were washed extensively with buffer solution to restore the surfaces before each binding experiment.

Data analysis was performed using BIA evaluation software. Data analysis and sensor grams were automatic corrected for nonspecific bulk refractive index effects. Standard procedures for the 1:1 Langmuir binding fit model were used for the kinetic analysis of ligand binding to the protein.

Real time fluorescence based CypD Prolyl Isomerase Assay[4]

Purified recombinant CypD enzyme (10 nM,) diluted in Working Buffer (WB, 25 mM HEPES, 100 mM NaCl, 0.01% Triton X-100, pH 7.5) was added to the wells of a 384 well plate containing DMSO vehicle controls or compound 9. Compound **9** was synthesized as previously described and transferred using ECHO 555 (Labcyte) to produce solutions with concentrations ranging from 0.19 uM to 100 uM (n=3) while keeping the final DMSO concentration less than 0.4%. The CypD was preincubated with compound **9** at 25°C for 1 hr. Chymotrypsin (25 uM) was added to the wells and the pre-reads were captured for 5 seconds before the addition of the substrate (4 uM, Suc-AAPF-MCA in TFE/LiCl Substrate Dilution Buffer). The increase in fluorescence at Excitation /Emission: 340/510 nm was captured every millisecond for a total time of 2 min using Hamamatsu FDSS 7000.

Isolation of brain mitochondria

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Brain cortex without white matter from mice was used for mitochondrial isolation. Mitochondria were prepared as formerly described[3, 5]. Briefly, brain cortical tissues were homogenized in 9 ml of ice-cold EB buffer (EDTA 1 mmol, bovine serum albumin 1-6 mg/ml) using a Dounce homogenizer until particles were no longer seen in the solution. Homogenates were centrifuged at 1300 x *g* for 5 min. Supernatant from this fraction was carefully deposited on top of 15% percoll solution (10 ml), and then centrifuged at 16000 RPM for 10 min. Next, the pellet was mixed with 9 ml of mitochondrial buffer (D-mannitol 4.098%, Sucrose 2.56%, K_2HPO_4 0.034%, pH 7.3-7.4) and 200 µl of 1% digitonin. After 5 min incubation on ice, the mixture was centrifuged at 8000 RPM for 10 min. Finally, the mitochondrial pellet was resuspended in 100 µl of mitochondrial buffer and used for further experiments.

Brain mitochondrial swelling and Calcium uptake/release inhibition assays[6]

Appropriate amounts of mitochondria were resuspended in 1 ml swelling assay buffer (150 mM KCl, 2 mM KH_2PO_4, 10 mM HEPES, pH 7.4) and energized with the addition of 1 mM Glutamate and 1 mM Malate. Calcium (Ca^{2+}, 200 µM) was added to the assay buffer to trigger mitochondrial swelling. The mitochondrial permeability transition was determined by measuring the rate of change in absorbance at 540 nm with an Amersham Biosciences Ultrospec 3100 pro spectrophotometer.

Initial solubilization of Aβ peptide and preparation of oligomer Aβ

$A\beta_{1-42}$ peptide (lyophilized powder, 1 mg, GenicBio, catalog number A-42-T-1) was stored in sealed glass vials inside desiccated containers at −80 °C. Before resuspension, the lyophilized peptide was allowed to equilibrate to room temperature for 30 min, thereby avoiding condensation. Under a chemical fume hood, the lyophilized $A\beta_{1-42}$ was resuspended in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma-Aldrich, catalog number 105228) in a

polypropylene vial using a glass GasTight® Hamilton syringe with a Teflon plunger. Following vortexing, the solution was divided between 3 polypropylene vials and vortexed again. The HFIP was allowed to evaporate in the fume hood for 2-3 hour. The resultant pellet was stored in desiccated form at -20 °C. Directly before use, the aliquots were resuspended in 5 mM anhydrous dimethyl sulfoxide (DMSO, Sigma-Aldrich, catalog number D-2650) by pipette mixing followed by bath sonication for 10 min.[3, 7]

Preparation of $A\beta_{1-42}$ Oligomer [8]

$A\beta_{1-42}$ oligomers were prepared by diluting 5 mM of $A\beta_{1-42}$ aliquot in PBS. The solution was immediately mixed by vortexing for 30 sec, and incubated at 4 °C for 24 hours. Prior to the experiment, the aliquot was diluted in ice-cold culture media to the required concentration. As demonstrated in our published studies, we have successfully prepared oligomer $A\beta_{1-42}$.[3, 9-12]

Culturing of SK-N-SH cells

The human neuroblastoma-derived cell line SK-N-SH (ATCC HTB-11, American Type Culture Collection, Manassas, VA) was used to assess CypD inhibitor effect on mitochondrial function. The cells were maintained in DMEM media supplemented with 10% fetal bovine serum in a humidified 37 °C, 5% CO_2 incubator.

Measurement of mitochondrial cytochrome c oxidase (CcO) activity

Cytochrome c oxidase (CcO) activity was measured as described previously[3, 7] using a cytochrome c oxidase kit (Sigma). Briefly, SK-N-SH cells were incubated with CypD inhibitor **9** and $A\beta_{1-42}$ oligomer. After incubating at 37 °C for 48 hours, cells were washed twice with PBS, followed by harvesting of cell lysates. The protein concentration was determined with the

Bradford method. Next, an appropriate volume of cells and enzyme solution was added to 475 µl of assay buffer provided with the kit. The reaction was triggered by the addition of 25 µl freshly prepared ferrocytochrome c substrate solution. The rate of change in absorbance at 550 nm was recorded immediately with Shimadzu (Kyoto, Japan) UV1200 spectrophotometer programmed for a 5 sec delay and 10 sec intervals for a total of six readings.

Measurement of adenosine-5'-triphosphate (ATP) levels

ATP levels were evaluated using an ATP Bioluminescence Assay Kit (Roche) as was formerly described[3, 13]. In brief, SK-N-SH cells were incubated with CypD inhibitor C-**9** and $A\beta_{1-42}$ oligomer at 37 ºC for 48 hours. Next, cells were harvested using ATP lysis buffer followed by incubation for 15 min on ice. The mixture was centrifuged at 13,000 x *g* for 10 min at 4°C, and the supernatant was collected for the assay. The content of ATP levels were measured using a Luminescence plate reader (Molecular Devices) with an integration time of 10 s [3, 7]. Essentially, light emitted from the luciferase-mediated reaction was captured by a luminescence plate reader (Molecular Devices) at 37 ºC with an integration time of 10 sec.

Cell survival and toxicity assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium_bromide**)** assay is commonly used to examine cell proliferation for the overall assessment of cell viability. SK-N-SH cells were treated with a range of CypD compound C-**9** (at 0, 5, 10, 25, 50, and 100 µM) for 48 hours and then analyzed using the MTT reduction assay following the manufacturer's instructions. Each experiment was run three times.

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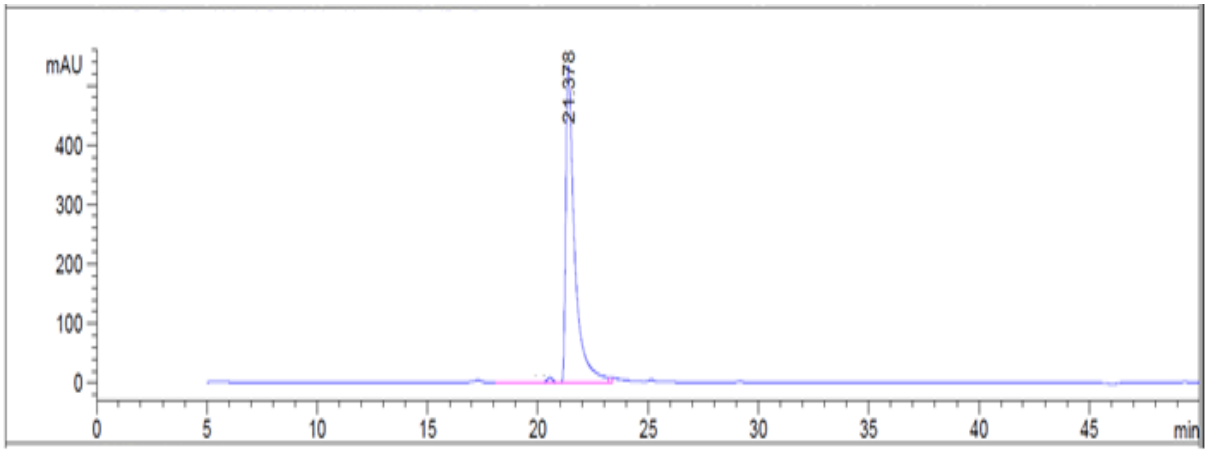

Figure 1: HPLC Chromatogram for compound C-9

Peak #	Ret Time [min]	Width [min]	Area [Mau*s]	Height	Area %
1	21.376	0.5368	7.11374e4	2208.65479	100.00
Total:			7.11374e4	2208.65479	

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