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Supplementary Information

for

Tunable Regulatory Activities of 1,10-Phenanthroline Derivatives towards Acid Sphingomyelinase and Zn(II)–Amyloid-β

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Experimental Section

Materials and Methods. All reagents were purchased from commercial suppliers and used as received unless otherwise stated. Two compounds, **phen** (1,10-phenanthroline) (purity 99%) and **5** (phenanthrene) (purity 98%), were purchased from Thermo Fisher Scientific (Waltham, MA, USA). **1** (5-amino-1,10-phenanthroline) (purity 97%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). **2** (5-methyl-1,10-phenanthroline) (purity 99%), **3** (5-chloro-1,10-phenanthroline) (purity 98%), and **4** (5-nitro-1,10-phenanthroline) (purity 98%) were acquired from TCI Chemicals (Tokyo, Japan). ^{1}H and ^{13}C NMR spectra of compounds were recorded using a Bruker AV400 NMR spectrometer [Department of Chemistry, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Republic of Korea]. Absorbance values for the assays of the enzyme activity, turbidity, and cell viability were measured on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Trace metal contamination was removed from all solutions used for Aβ experiments by treating with Chelex (Sigma-Aldrich) overnight. $A\beta_{42}$ (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) was obtained from Anaspec (Fremont, CA, USA) or Peptide Institute, Inc. (Osaka, Japan). Double-distilled water (ddH_2O) used for all experiments was obtained from a Milli-Q Direct 16 system (Merck KGaA, Darmstadt, Germany). Optical spectra were recorded on an Agilent 8453 UV−visible spectrophotometer (Santa Clara, CA, USA). Transmission electron microscopic images were collected on a Tecnai F20 transmission electron microscope [KAIST Analysis Center for Research Advancement (KARA), Daejeon, Republic of Korea].

Cell Culture for Acid Sphingomyelinase (ASM) Activity Assay. The human fibroblast

cell lines (GM05399 for normal and AG08711 for PS1) acquired from the Coriell Institute (Camden, NJ, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells with a passage number 10–15 were used in this study. To measure the ASM activity, each compound (final concentration, 10 μM; 1% v/v DMSO) was treated in presenilin-1 (PS1) fibroblasts. DMSO (1% v/v) was used as a vehicle. After 30 min, cells were harvested and lysed in homogenization buffer [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (GIBCO, Grand Island, NY, USA), 150 mM NaCl (Sigma-Aldrich), 0.2% Igepal (Sigma-Aldrich), protease inhibitor (Calbiochem, Darmstadt, German)] as previously described.[1]

ASM Activity Assay. ASM activity was measured as previously described[2] by an ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA). The samples (3 μL) were mixed with the ASM assay buffer [3 μL; 200 μM Bodipy-C12-sphingomyelin (Invitrogen, D7711), 0.2 M of sodium acetate buffer, pH 5.0, 0.2 mM $ZnCl_2$, 0.2% Igepal CA-630] and incubated at 37 °C for 20 min. The hydrolytic reaction was stopped by adding ethanol (EtOH, 114 μL) and centrifuged at 13,000 rpm for 5 min. The supernatant (30 μL) was then transferred to a sampling glass vial and 5 μL was applied onto a UPLC system for analysis. Quantification was achieved by comparison to Bodipy-C12-ceramide standards using the Waters Millennium software.

Statistical Analysis for ASM Activity Assay. Sample sizes were determined by G-Power software. In cases where more than two groups were compared to each other, a

one way analysis of variance (ANOVA) was used, followed by Tukey's Honestly Significant Difference (HSD) test. All statistical analyses were performed using SPSS statistical software. $^{*}P < 0.05$, $^{***}P < 0.001$ were considered to be significant.

Carbonic Anhydrase (CA) Activity Assay. The CA activity assay was performed using a colorimetric CA inhibitor screening kit (BioVision, San Francisco, CA, USA).[3,4] Following the manufacturer's procedure with minor modifications, **phen** derivatives (final concentration, 10 μM; 1% v/v DMSO, vehicle), acetazolamide[5] (a known CA inhibitor as a positive control) (final concentration, 10 μM; 1% v/v DMSO, vehicle), and an equivalent amount of DMSO (1% v/v; 1 μL) were treated to an assay buffer solution containing CA (5 μL) in a 96-well microplate. The plates were incubated at room temperature for 10 min, followed by addition of a substrate (5 μL). The absorbance was measured at 405 nm by the SpectraMax M5 microplate reader (Molecular Devices). The activity (%) was obtained relative to the reaction velocity (absorbance/min) of the control containing the equivalent amount of DMSO.

Matrix Metalloproteinase-9 (MMP-9) Activity Assay. The activity of MMP-9 was measured using an MMP-9 colorimetric drug discovery kit (Enzo Life Sciences, Farmingdale, NY, USA).[6,7] According to the manufacturer's instruction with minor modifications, **phen** derivatives (final concentration, 10 μM; 1% v/v DMSO, vehicle), *N*-isobutyl-*N*-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid (NNGH,[8] a known MMP-9 inhibitor, as a positive control) (final concentration, 10 μM; 1% v/v DMSO, vehicle), and

an equivalent amount of DMSO (1% v/v; 1 μL) were treated to an assay buffer solution containing MMP-9 (45 mU/μL; 20 μL) in a 96-well microplate. The plates were incubated at 37 °C for 1 h, followed by treatment with chromogenic thiopeptide substrate (200 μM; 20 μL). After incubation at 37 °C for 20 min, the absorbance at 412 nm was obtained by the SpectraMax M5 microplate reader (Molecular Devices). The activity (%) was calculated relative to the reaction velocity (absorbance/min) of the control containing the equivalent amount of DMSO.

Aβ Aggregation Experiments. $A\beta_{42}$ were dissolved in ammonium hydroxide (NH_4OH, 1% v/v, aq). The resulting solution of $A\beta_{42}$ was aliquoted, lyophilized overnight, and stored at –80 °C. A stock solution of $A\beta_{42}$ was then prepared by dissolving the lyophilized peptide using NH_4OH (1% v/v, aq; 10 μL) and diluting with ddH_2O. $A\beta_{42}$ samples were prepared following the previously reported procedures.[9–13] The concentration of the peptide solution was determined by measuring the absorbance of the solution at 280 nm (ε = 1,490 $M^{-1}cm^{-1}$). The peptide stock solution was diluted to a final concentration of 25 μM in the Chelex-treated buffer (pH 7.4). For Aβ aggregation studies, compounds (final concentration, 25 μM; 1% v/v DMSO) were added to the samples of $A\beta_{42}$ (25 μM) in the absence and presence of a zinc chloride salt ($ZnCl_2$; 25 μM) followed by 24 h incubation at 37 °C with constant agitation.

Gel Electrophoresis with Western Blotting (Gel/Western Blot). The resultant Aβ species from *in vitro* experiments were analyzed through gel/Western blot using an anti-

Aβ antibody (6E10) (Covance, Princeton, NJ, USA).[11–14] Each sample (10 μL) was separated on a 10–20% Tris-tricine gel (Thermo Fisher Scientific). Following separation, the proteins were transferred onto nitrocellulose membranes and blocked with bovine serum albumin (BSA, 3% w/v; Sigma-Aldrich) in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) overnight at 4 °C. The membranes were incubated with the 6E10 (1:2,000) in a solution of BSA (2% w/v in TBS-T) for 4 h at room temperature. After washing with TBS-T (3x, 10 min), a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000 in 2% w/v BSA in TBS-T; Cayman Chemical Company, Ann Arbor, MI, USA) was added for 2 h at room temperature. A homemade ECL kit[11–13,15] was used to visualize gel/Western blot data on a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). The peptide amount shown in each lane of the gel/Western blot was quantified by the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Transmission Electron Microscopy (TEM). Samples for TEM were prepared according to the previously reported methods.[9,11–14,16,17] Glow-discharged grids (Formvar/Carbon 300-mesh; Electron Microscopy Sciences, Hatfield, PA, USA) were treated with Aβ samples (25 μM, 5 μL) for 2 min at room temperature. Excess sample was removed using filter paper followed by washing twice with ddH_2O. Each grid was incubated with uranyl acetate (1% w/v ddH_2O; 5 μL) for 1 min. Upon removal of excess uranyl acetate with filter paper, the grids were dried for at least 20 min at room temperature before measurement. Images for each sample were taken on a Tecnai F20 TEM (200 kV; 29,000 × magnification, KARA).

Dot Blot Assay. The samples containing Zn(II)–Aβ aggregates produced by incubation for 1, 2, 6, 12, and 24 h with and without treatment of **1** and **5** were prepared according to Aβ aggregation experiments (*vide supra*). The solutions (2 μL) were spotted on a nitrocellulose membrane and blocked with the solution of BSA (3% w/v) in TBS containing 0.01% Tween 20 (0.01% TBS-T) at room temperature for 2 h. The membrane was incubated with a primary antibody, 6E10 (1:2,000) or OC[18] (1:1,000; Merck Millipore, Billerica, MA, USA), in the solution of BSA (2% w/v in TBS-T) for 2 h at room temperature. After washing with 0.01% TBS-T (3x, 7 min), the horseradish peroxidase-conjugated goat anti-mouse (1:2,000; for 6E10) or goat anti-rabbit (1:2,000; for OC; Promega, Madison, WI, USA) secondary antibody in the solution of BSA (2% w/v in TBS-T) was added to the membrane and incubated for 2 h at room temperature. The homemade ECL kit[11–13,15] was used to visualize the results on a ChemiDoc MP Imaging System (Bio-Rad). The signals from OC-detectable fibrillary Aβ species were quantified by the ImageJ software.

Turbidity Assay. The samples containing Zn(II)–Aβ aggregates ([$A\beta_{42}$] = 100 μM; [$ZnCl_2$] = 100 μM) generated by incubation for 1, 2, 6, 12, and 24 h with and without treatment of **1** and **5** (100 μM; DMSO 1% v/v) were prepared according to Aβ aggregation experiments (*vide supra*). The samples (80 μL) were delivered to a flat-bottomed 96-well microtiter plate (Corning Costar Corp., Corning, NY, USA). The absorbance was recorded at 395 nm on the SpectraMax M5 microplate reader (Molecular Devices). $|\Delta A_{395nm}|$ was calculated by subtraction of the absorbance of compound-added Zn(II)–$A\beta_{42}$ samples after 1, 2, 6, 12, and 24 h incubation from that of the compound-untreated Zn(II)–$A\beta_{42}$

sample. Error bars were calculated as standard errors.

Cell Viability Studies. Murine Neuro-2a (N2a) neuroblastoma cells were maintained in media containing 45% DMEM (GIBCO), 45% Opti-MEM (GIBCO), 10% FBS (GIBCO), 1% L-glutamine (GIBCO), 100 U/mL penicillin (GIBCO), and 100 mg/mL streptomycin (GIBCO). The cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C and seeded in a 96-well plate (3,000 cells/100 μL). Cell viability was determined by the MTT assay [MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, Sigma-Aldrich]. Cell viability upon treatment of compounds was determined in the absence ([compound] = 5, 10, 25, 50, 100, and 200 μM; 1% v/v DMSO) and presence of Zn(II) [[compound] = 5 μM (1% v/v DMSO); [$ZnCl_2$] = 2.5 and 5 μM]. The ability of compounds to recover the toxicity induced by metal-free $A\beta_{42}$ or Zn(II)–$A\beta_{42}$ was evaluated with the species of metal-free Aβ and Zn(II)–Aβ (5 μM) containing compounds (5 μM; 1% v/v DMSO) prepared through Aβ aggregation experiments (*vide supra*). After 24 h incubation, MTT [25 μL of 5 mg/mL in PBS (pH 7.4, GIBCO)] was added to each well and the plate was incubated for 4 h at 37 °C. Formazan produced by cells was solubilized using an acidic solution of DMF (pH 4.5, 50% v/v, aq) and sodium dodecyl sulfate (SDS; 20% w/v) overnight at room temperature in the dark. The absorbance was measured at 600 nm by the SpectraMax M5 microplate reader (Molecular Devices). Cell viability was calculated relative to the cells containing an equivalent amount of DMSO.

Solution Speciation Studies. The pK_a values for **phen** and **1**–**4** were determined

through UV–vis variable-pH titrations based on the previously reported procedures.[11,12,14] To obtain the pK_a values of compounds, the solution of a compound (25 μM; 10 mM NaOH, pH 12, 100 mM NaCl) was titrated with small aliquots of HCl to obtain at least 30 spectra in a range from pH 2 to 9. In addition, to determine Zn(II) binding affinities of a compound, small aliquots of HCl were titrated into a solution containing the compound and $ZnCl_2$ [[Zn(II)]/[L] = 1:2; [Zn(II)] = 12.5 μM]. At least 30 spectra were acquired in the pH range from 2 to 9. The acidity and stability constants were calculated by the HypSpec program (Protonic Software, Leeds, UK).[19,20]

Zn(II) Binding Experiments. Zn(II) binding of compounds was investigated by UV–visible spectroscopy (UV–vis). UV–vis experiments were performed in the Chelex-treated buffered solution [20 μM HEPES, pH 7.4, 150 μM NaCl]. Various concentrations of $ZnCl_2$ (0, 12.5, 25, 50, and 125 μM for **phen** and **1**–**4**; 0, 5, 10, 20, and 50 μM for **5**) were titrated to the solution of a ligand (25 μM for **phen** and **1**–**4**; 10 μM for **5**; 1% v/v DMSO). UV–vis spectra were recorded after incubation for 10 min at room temperature.

Docking Studies of Compounds with ASM and Zn(II)–Aβ_{16}. Flexible ligand docking studies were conducted using AutoDock Vina[21] against ASM and Zn(II)–Aβ_{16}, whose structures have been previously determined by X-ray crystallography (PDB 5I81)[22] and NMR in aqueous solution (PDB 1ZE9),[23] respectively. The representative conformation of Zn(II)–Aβ_{16} was selected for docking within the PDB files (7 from 1ZE9). The MMFF94 energy minimization function in ChemBio3D 15.0 was used to optimize the structures of

compounds prior to the docking studies. Individual structures of ASM, Zn(II)–Aβ_{16}, and compounds were prepared using AutoDock Tools, imported into PyRx,[24] and used to run AutoDock Vina. For ASM, the search space was constrained to possess the dinuclear Zn(II) binding site with or without substrate cavity depending on compounds. The search space for Zn(II)–Aβ_{16} was constrained to contain the whole structure of Zn(II)–Aβ_{16}. The exhaustiveness for the docking runs was set at 1,024. Docked models of compounds with ASM or Zn(II)–Aβ_{16} were visualized using Pymol 2.0.7.

(a)

phen

(b)

1

(c)

2

(d)

3

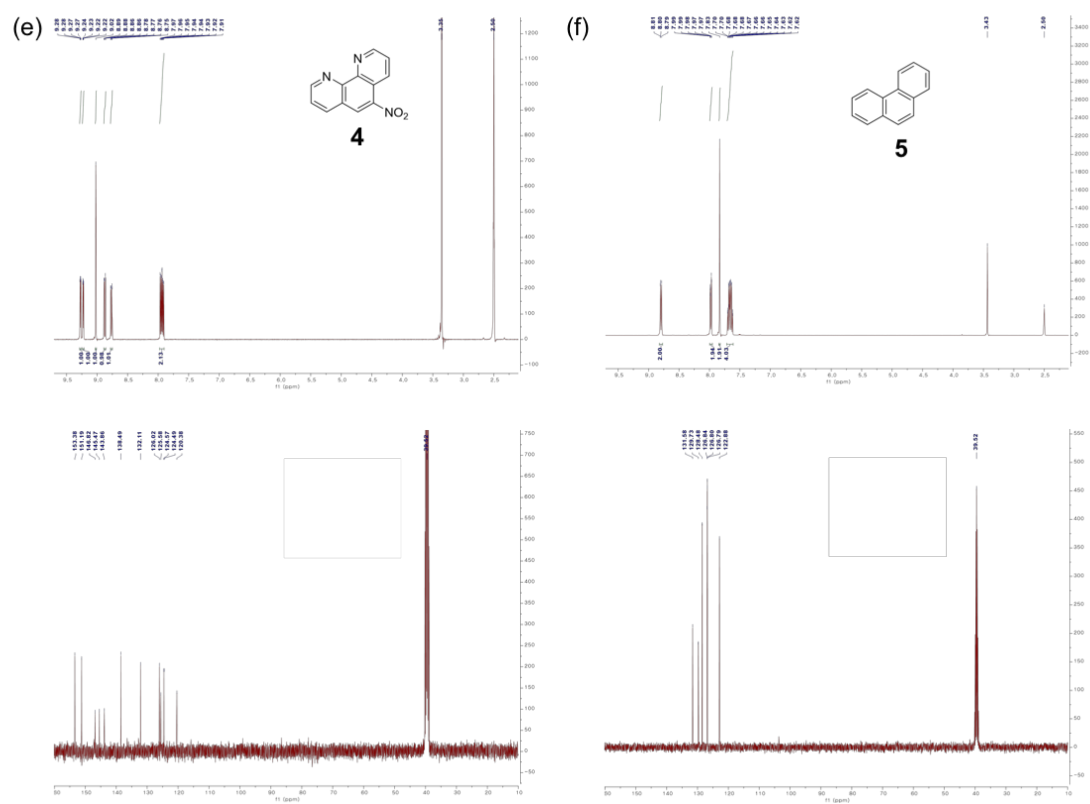

Fig. S1 ^{1}H (400 MHz, top) and ^{13}C (100 MHz, bottom) NMR spectra of (a) **phen**, (b) **1**, (c) **2**, (d) **3**, (e) **4**, and (f) **5** in DMSO-d_6.

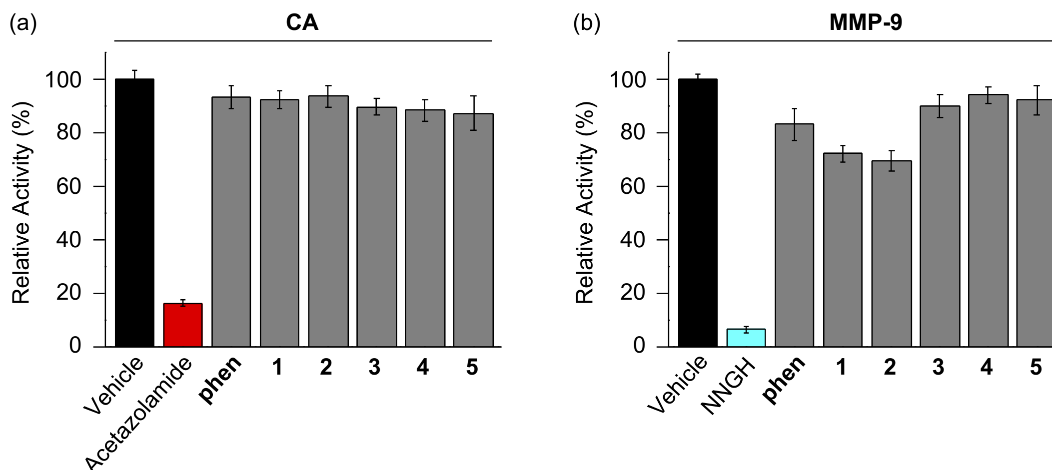

Fig. S2 Effects of **phen** derivatives against CA and MMP-9. The activity of (a) CA or (b) MMP-9 with or without treatment of compounds (final concentration, 10 μM; 1% v/v DMSO) was measured. DMSO (1% v/v) was used as a vehicle. Acetazolamide and NNGH were used as positive controls for CA and MMP-9, respectively.

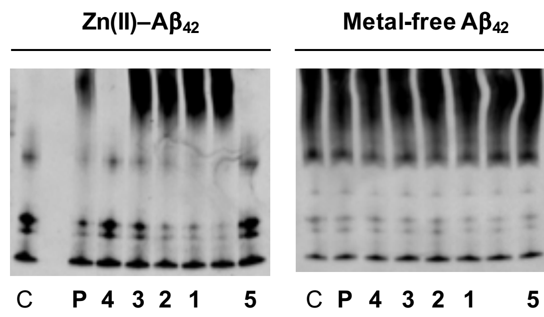

Fig. S3 Original gel images from the Aβ aggregation experiments (Fig. 3b).

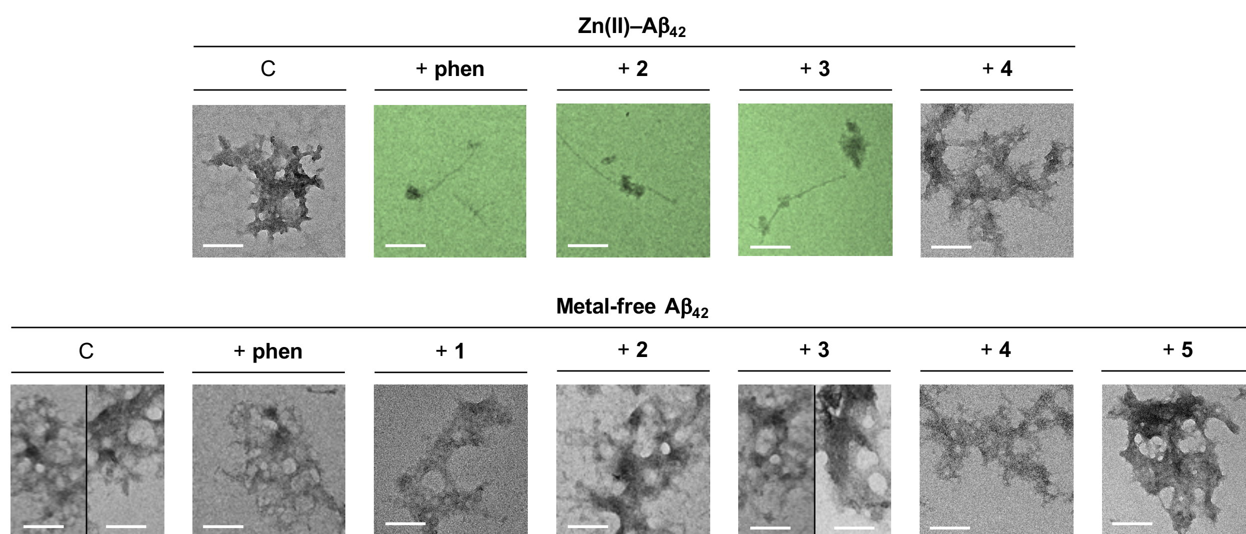

Fig. S4 TEM images of Zn(II)–$A\beta_{42}$ and metal-free $A\beta_{42}$ incubated with or without **phen** derivatives. Conditions: [$A\beta_{42}$] = 25 μM; [$ZnCl_2$] = 25 μM; [compound] = 25 μM; pH 7.4; 37 °C; 24 h incubation; constant agitation. C indicates Aβ ± Zn(II) without treatment of **phen** derivatives. Scale bar = 200 nm.

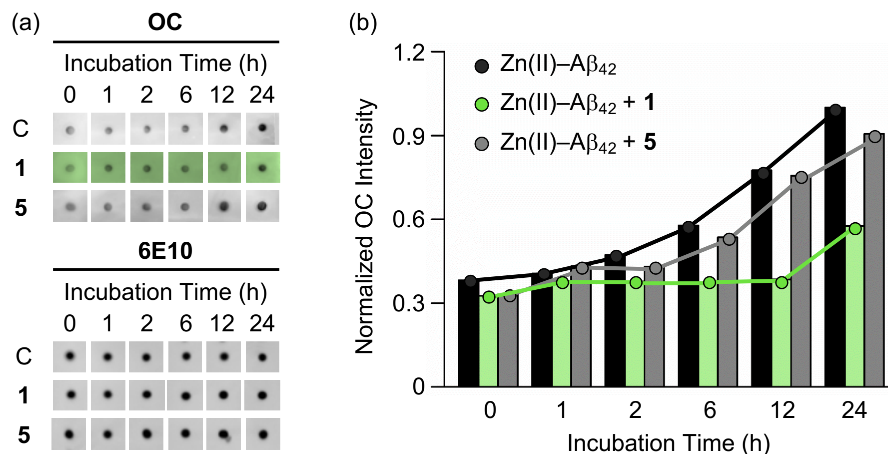

Fig. S5 Formation of fibrillary Aβ species upon treatment of Zn(II)–$A\beta_{42}$ with and without **1** and **5**, monitored by the dot blot assay. (a) Aggregation of Zn(II)–$A\beta_{42}$, detected by (top) an anti-amyloid fibril antibody, OC, and (bottom) an anti-Aβ antibody, 6E10. (b) Quantification of OC-detectable fibrillary Aβ species. Conditions: [$A\beta_{42}$] = 25 μM; [$ZnCl_2$] = 25 μM; [compound] = 25 μM; pH 7.4; 37 °C; 1, 2, 6, 12, and 24 h incubation; constant agitation.

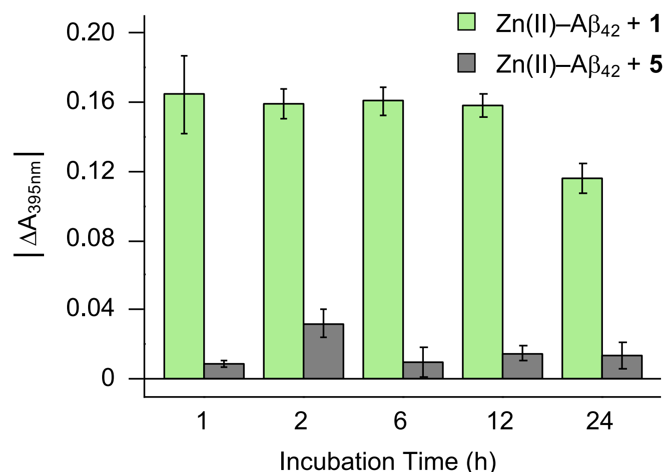

Fig. S6 Turbidity measurements of Zn(II)–Aβ_{42} incubated with **1** (green) and **5** (black). $|\Delta A_{395nm}|$ was calculated by subtraction of the absorbance of compound-added Zn(II)–Aβ_{42} samples after 1, 2, 6, 12, and 24 h incubation from that of the compound-untreated Zn(II)–Aβ_{42} sample. Error bars were calculated as standard errors. Conditions: [Aβ_{42}] = 100 μM; [$ZnCl_2$] = 100 μM; [compound] = 100 μM; pH 7.4; 37 °C; 1, 2, 6, 12, and 24 h incubation; constant agitation.

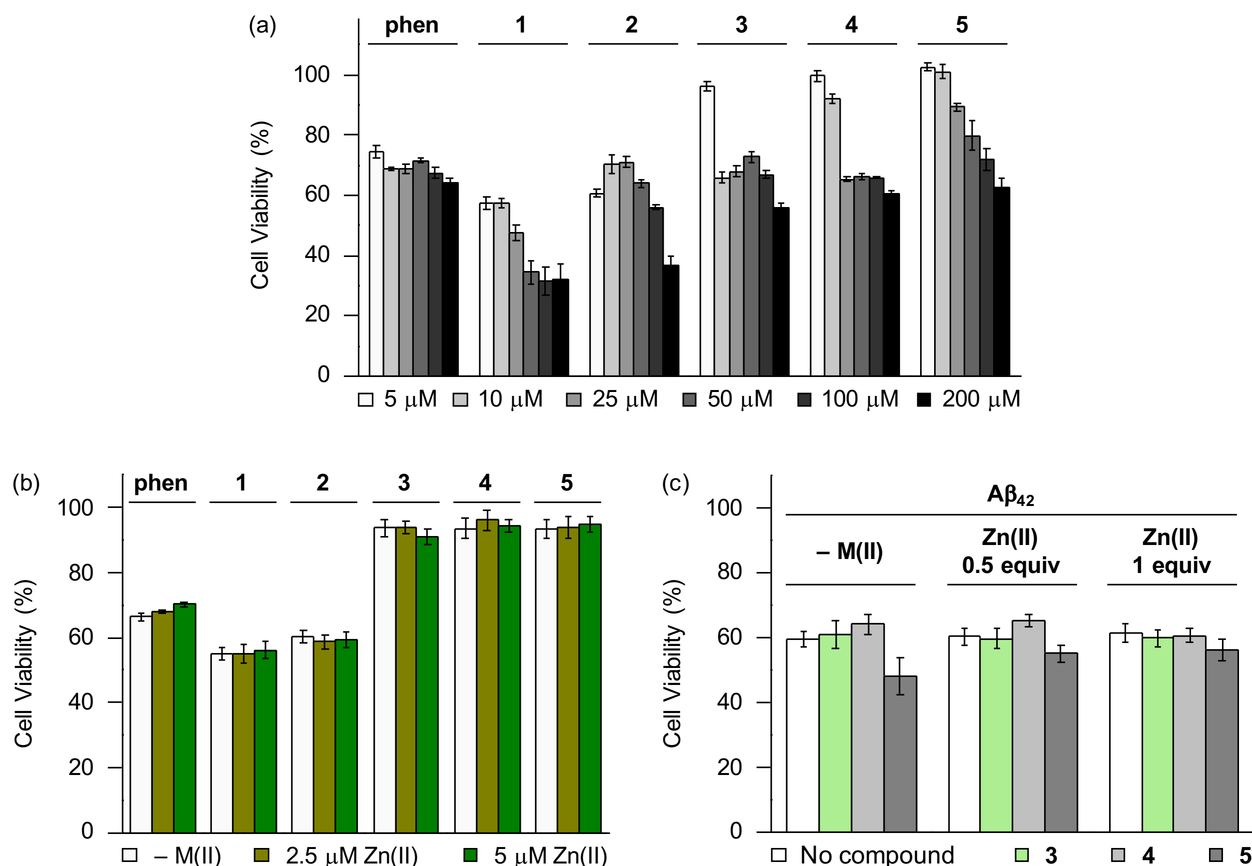

Fig. S7 Toxicity of compounds in murine N2a neuroblastoma cells. Cell survival (%) was measured upon treatment of the compounds in the (a) absence and (b) presence of Zn(II). (c) Effect of **3**, **4**, and **5** on cytotoxicity induced by metal-free $A\beta_{42}$ or Zn(II)–$A\beta_{42}$. Conditions: [$A\beta_{42}$] = 5 μM; [$ZnCl_2$] = 2.5 and 5 μM; [compound] = 5, 10, 25, 50, 100, and 200 μM [for (a)] or 5 μM [for (b) and (c)] (1% v/v DMSO).

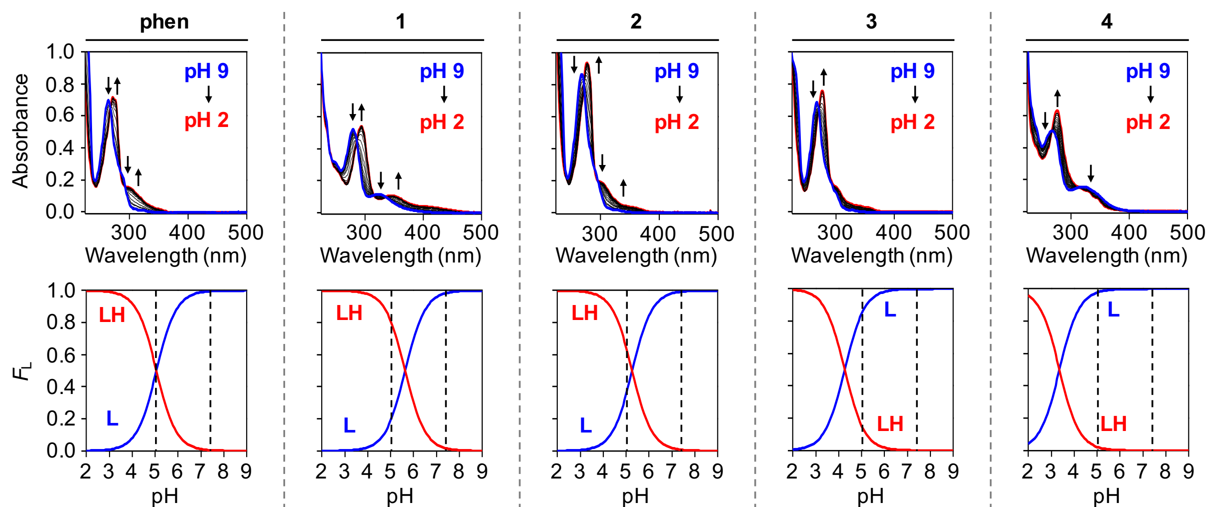

Fig. S8 Solution speciation studies of **phen** and **1**–**4**. Top: UV−vis variable-pH titration spectra. Bottom: Solution speciation diagrams (F_L = fraction of species at given pH). Charges are omitted for clarity. Conditions: [L] = 25 μM; room temperature; I = 0.10 M NaCl. Two pHs, pH 5.0 and 7.4, are indicated with dashed lines.

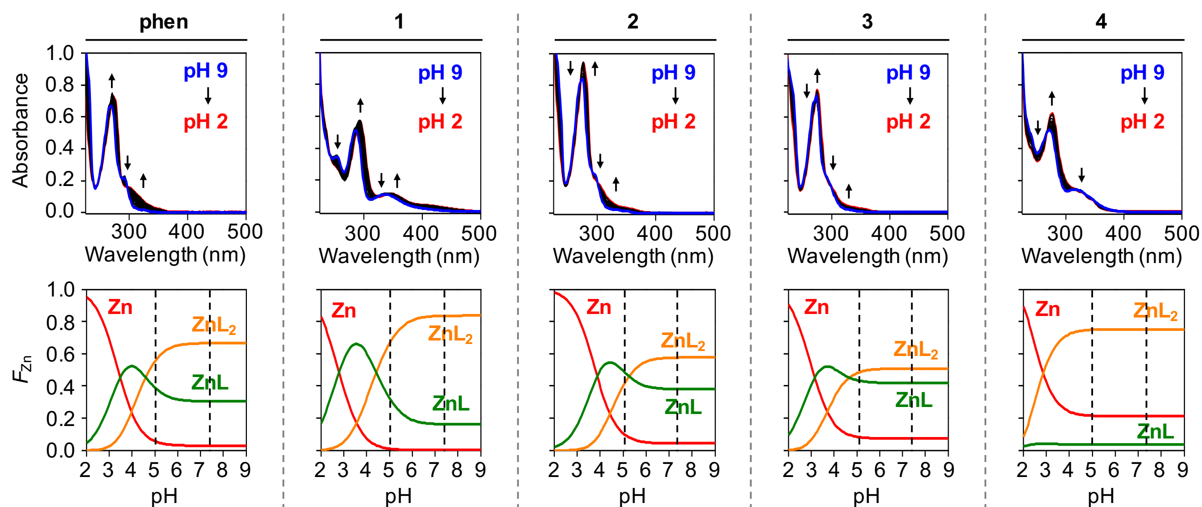

Fig. S9 Solution speciation studies of Zn(II)–L complexes (L = **phen** and **1–4**). Top: UV–vis variable-pH titration spectra. Bottom: Solution speciation diagrams (F_{Zn} = fraction of species at given pH). Charges are omitted for clarity. Conditions: [L] = 25 μM; [$ZnCl_2$] = 12.5 μM; room temperature; I = 0.10 M NaCl. Two pHs, pH 5.0 and 7.4, are indicated with dashed lines.

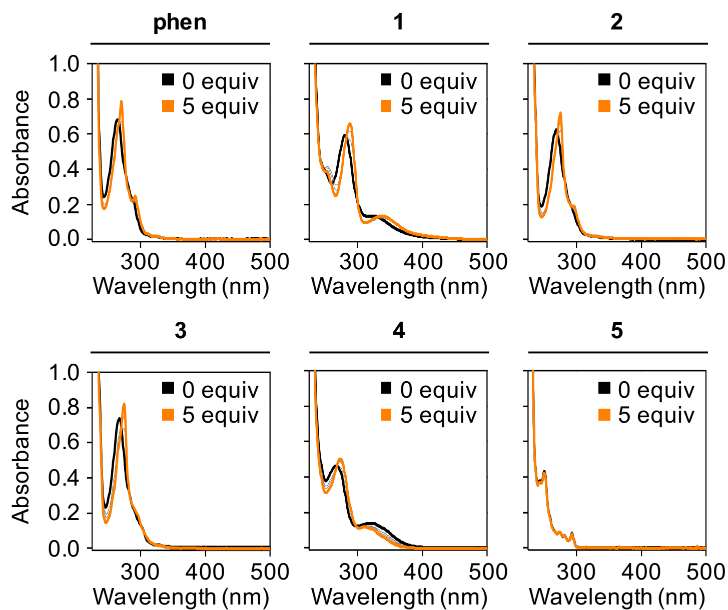

Fig. S10 Zn(II) binding of compounds, monitored by UV–vis. Spectral changes were observed upon addition of Zn(II) into the solution of compounds. Conditions: [compound] = 25 μM (for **phen** and **1**–**4**) and 10 μM (for **5**); [$ZnCl_2$] = 0 (black), 125 μM (for **phen** and **1**–**4**), and 50 μM (for **5**) (orange); pH 7.4 buffered solution; room temperature; incubation for 10 min.

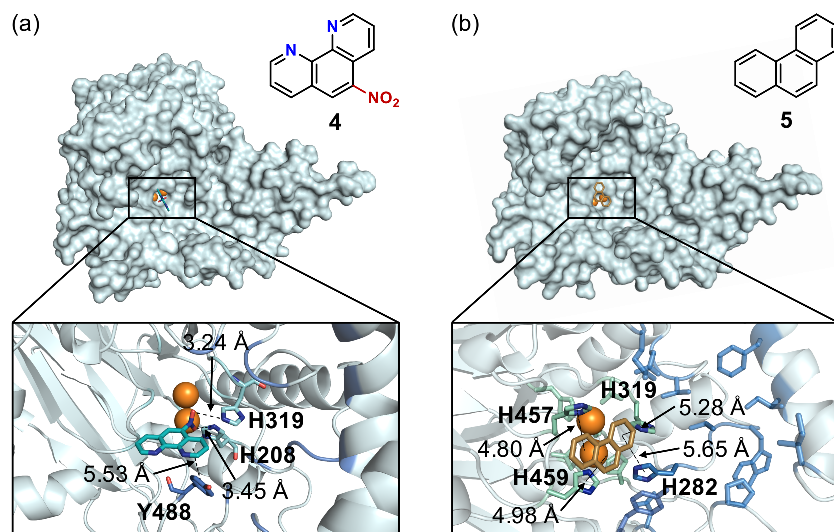

Fig. S11 Docking studies of **4** and **5** with ASM (PDB 5I81[22]). Possible conformations of (a) **4** and (b) **5** docked with ASM were visualized. The amino acid residues of the dinuclear Zn(II) binding site (green-cyan) and the substrate cavity (blue) as well as the ligands (*i.e.*, **4** and **5**) are shown in sticks. Distances are labeled in Å with dashed lines.

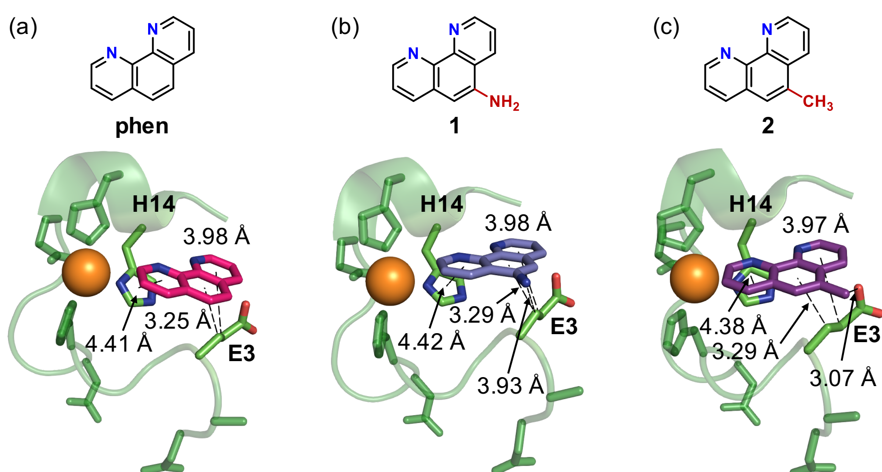

Fig. S12 Docking studies of **phen**, **1**, and **2** with Zn(II)–Aβ_{16} (PDB 1ZE9[23]). Possible conformations of (a) **phen**, (b) **1**, and (c) **2** docked with Zn(II)–Aβ_{16} were visualized. The amino acid residues of Zn(II)–Aβ_{16} and the ligands (*i.e.*, **phen**, **1**, and **2**) are shown in sticks. Distances are labeled in Å with dashed lines.

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