Supporting Information

Mitigation of Aβ neurotoxicity in Alzheimer's disease by a non-toxic platinum complex derived from retinamide

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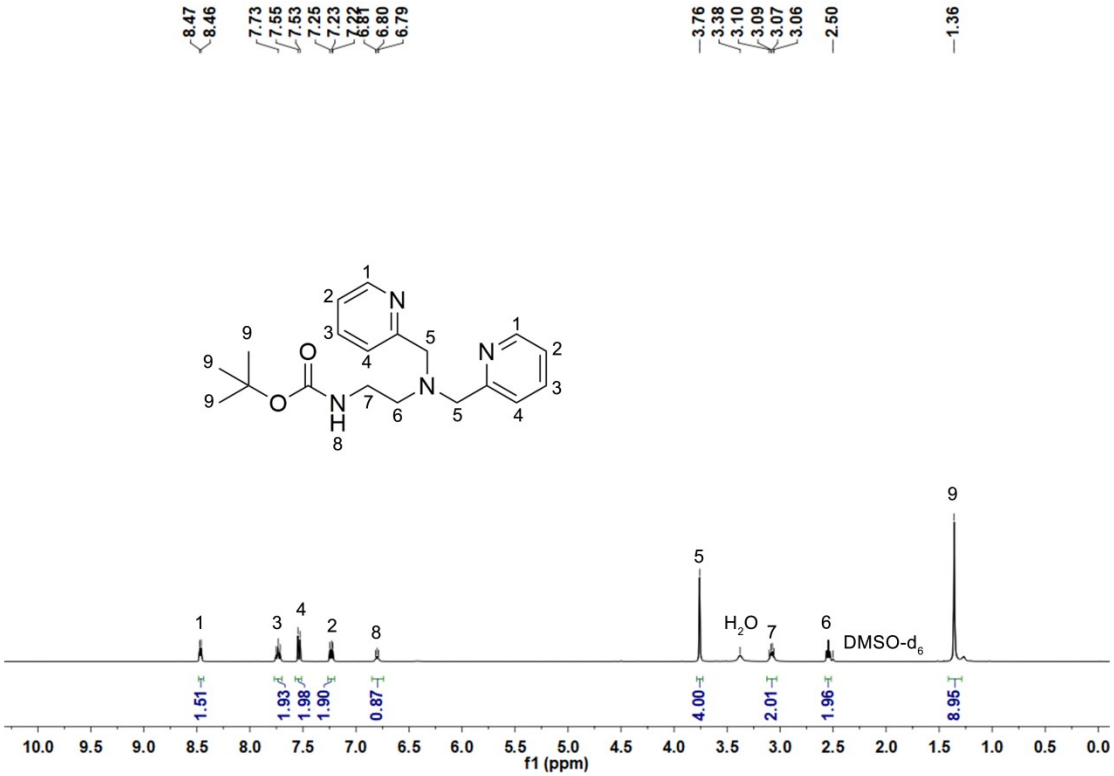
Supplementary Figures



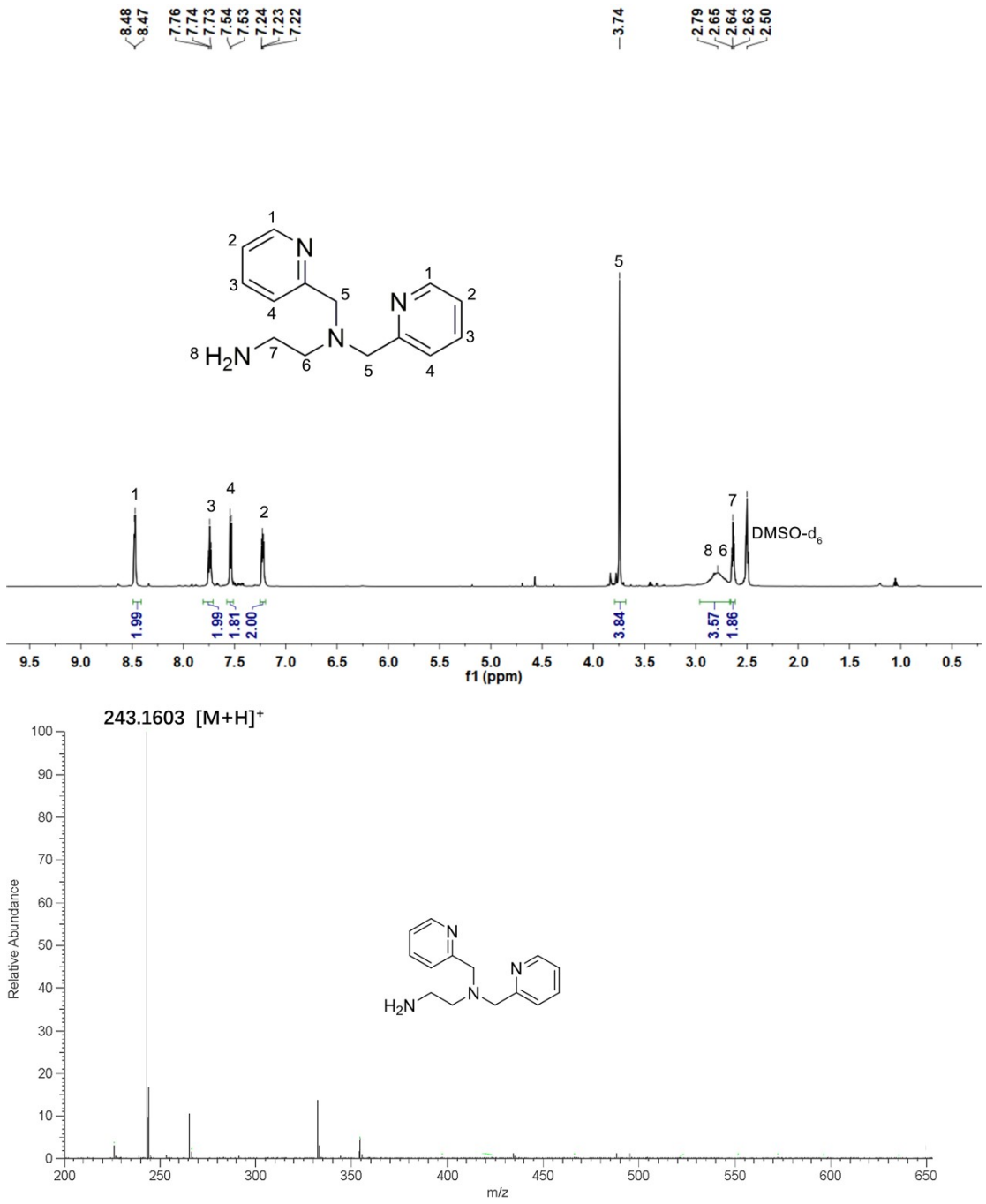


Fig. S1 ^{1}H NMR (DMSO-d_6) for boc-N,N-bis(pyridin-2-ylmethyl)ethane-1,2-diamine; ^{1}H NMR (DMSO-d_6) and ESI-MS spectra for N,N-bis(pyridin-2-ylmethyl)ethane-1,2-diamine.

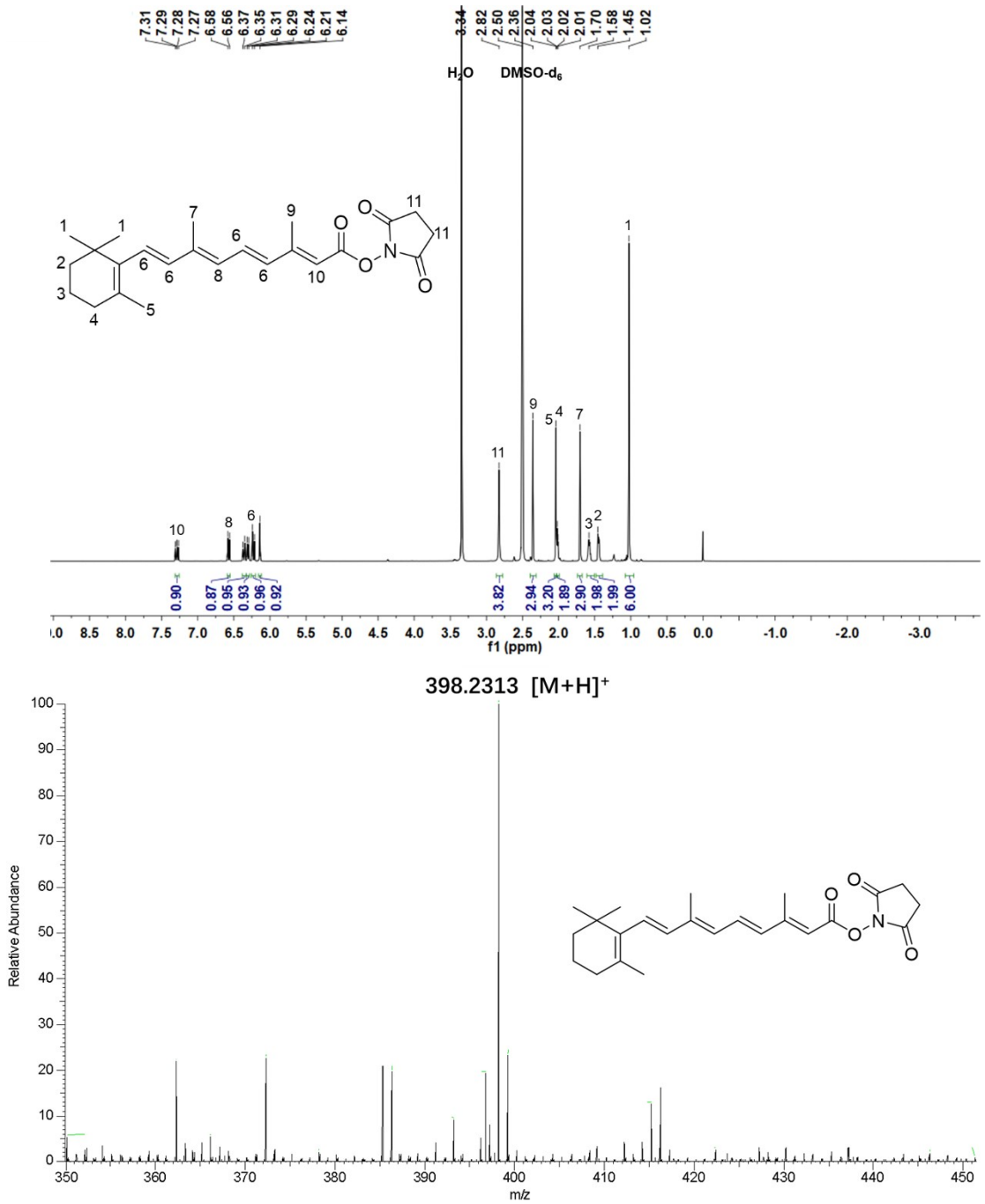
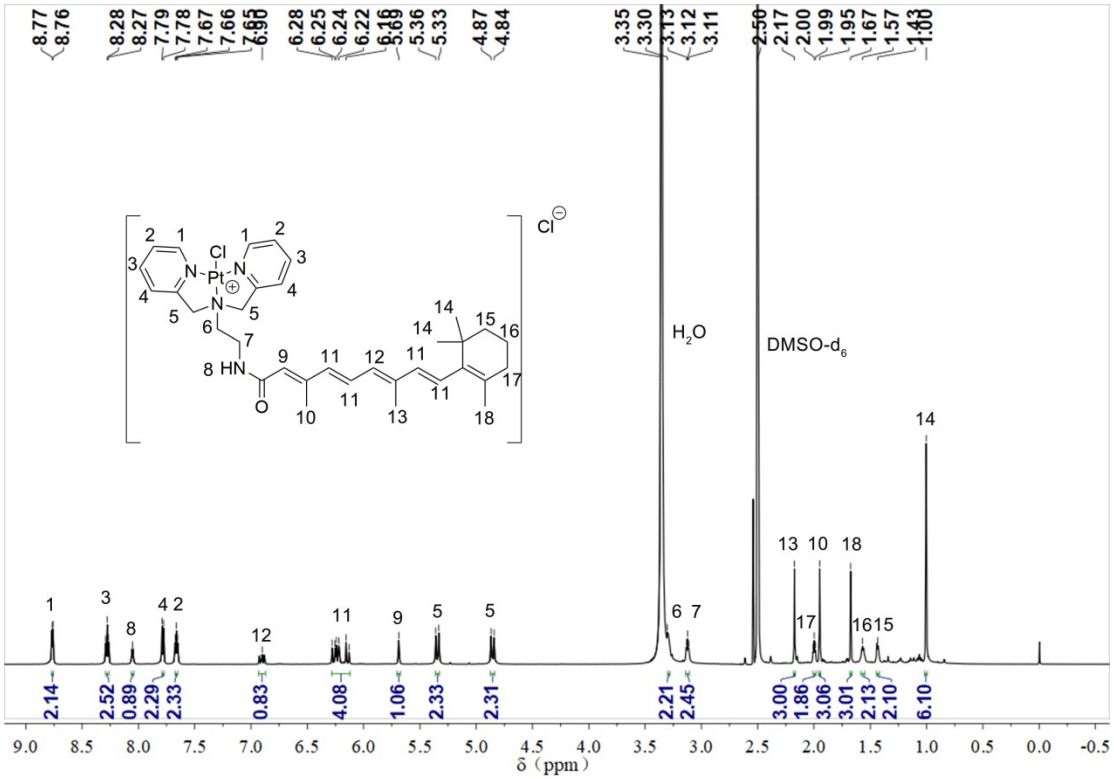
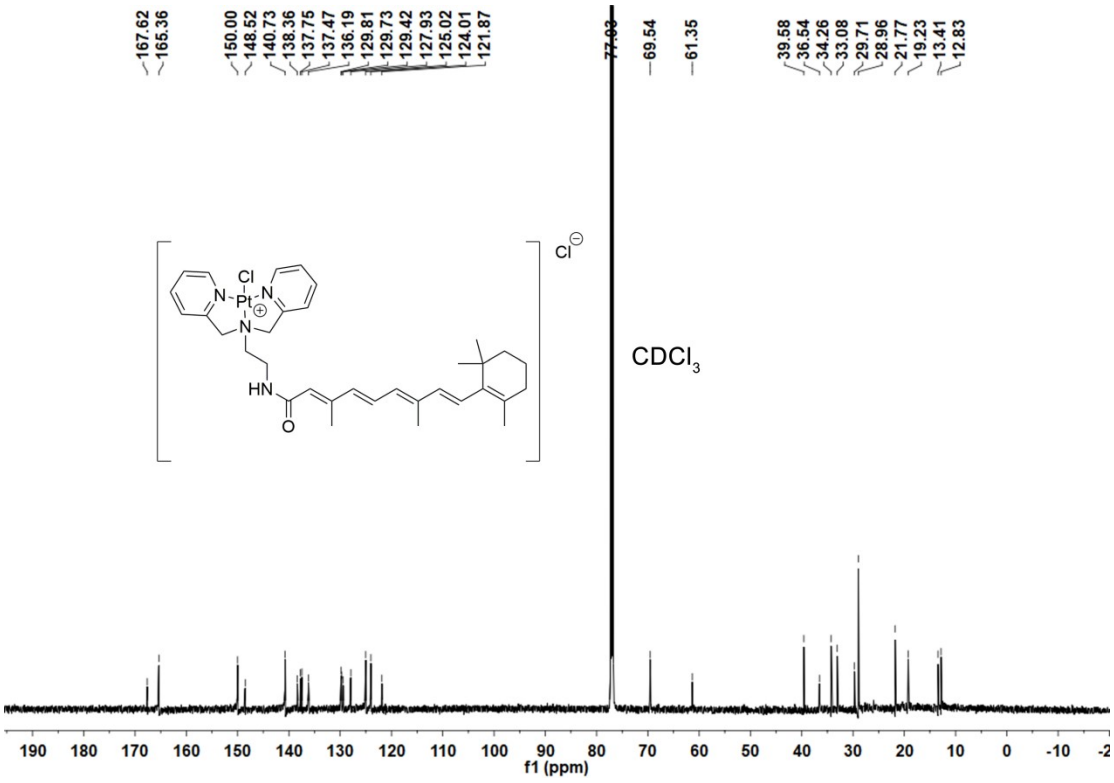


Fig. S2 ^{1}H NMR (DMSO-d_6), and ESI-MS spectra for NHS-RA.


H2O
DMSO-d6
δ (ppm)


CDCl3
f1 (ppm)

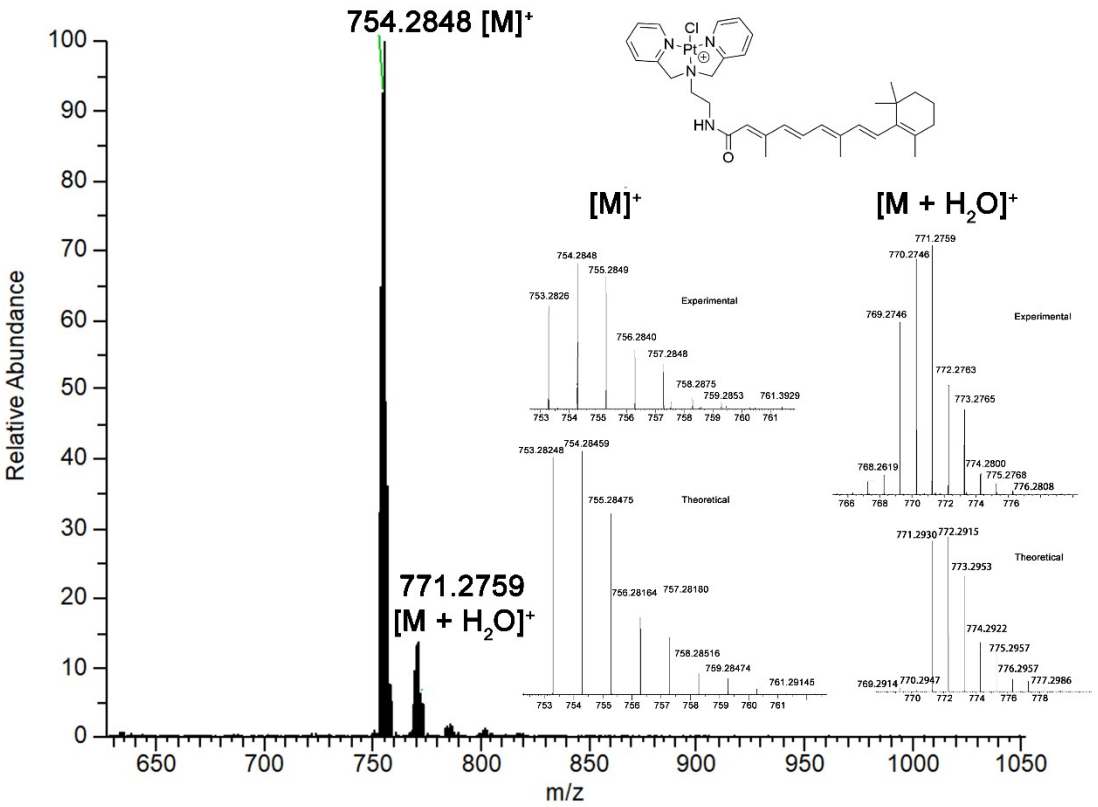


Fig. S3 ^{1}H-, ^{13}C NMR ($CDCl_3$), and ESI-MS spectra for RP.

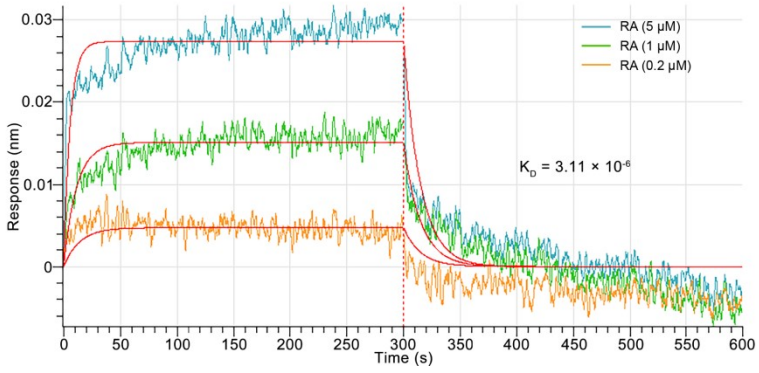
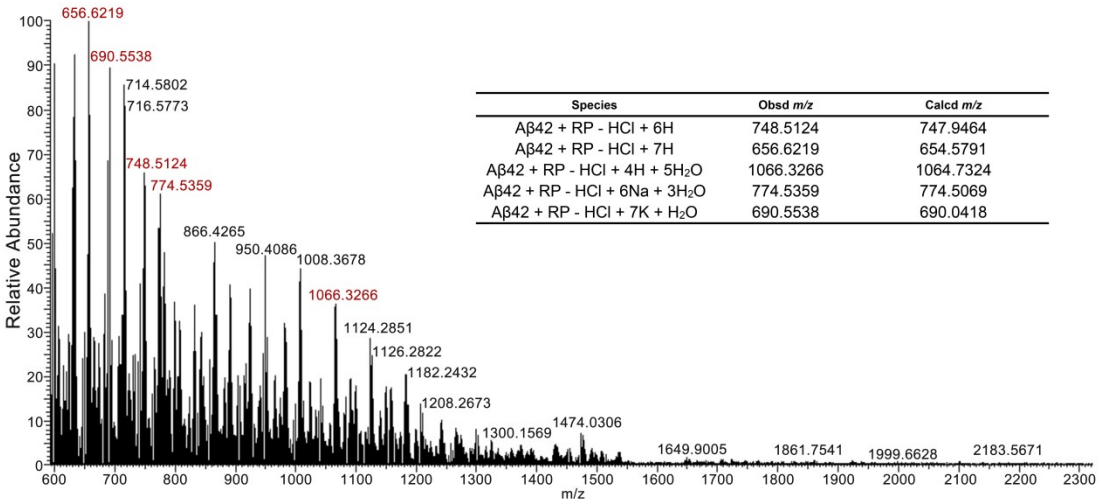


Fig. S4 Binding affinity of RA to Aβ42 assessed by the biolayer interferometry (BLI) assay.



Species	Obsd *m/z*	Calcd *m/z*
Aβ42 + RP - HCl + 6H	748.5124	747.9464
Aβ42 + RP - HCl + 7H	656.6219	654.5791
Aβ42 + RP - HCl + 4H + 5H_2O	1066.3266	1064.7324
Aβ42 + RP - HCl + 6Na + 3H_2O	774.5359	774.5069
Aβ42 + RP - HCl + 7K + H_2O	690.5538	690.0418

Fig. S5 High resolution mass spectra of RP-Aβ42 conjugates in the PBS buffer ([Aβ42] : [RP] = 1 : 1, [Aβ42] = 20 μM).

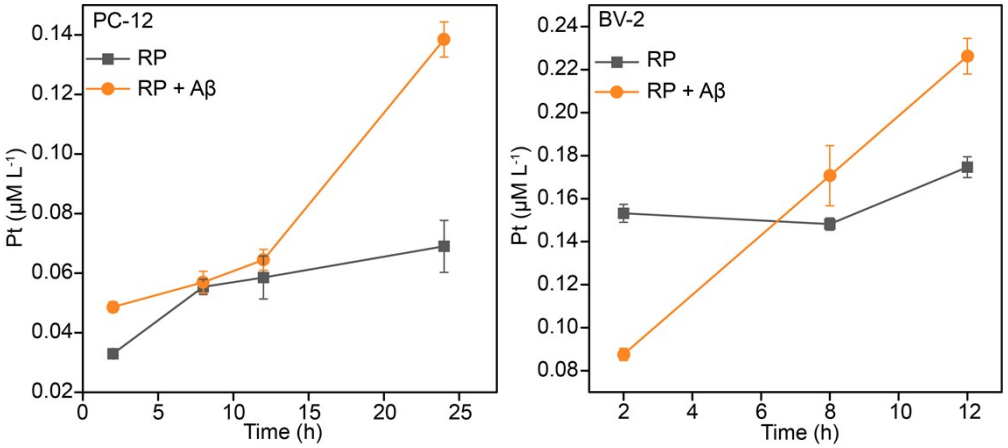


Fig. S6 RP uptake (Pt content) by PC12 and BV-2 cells in the absence and presence of Aβ (20 μM) at different incubation times determined by ICP-MS.

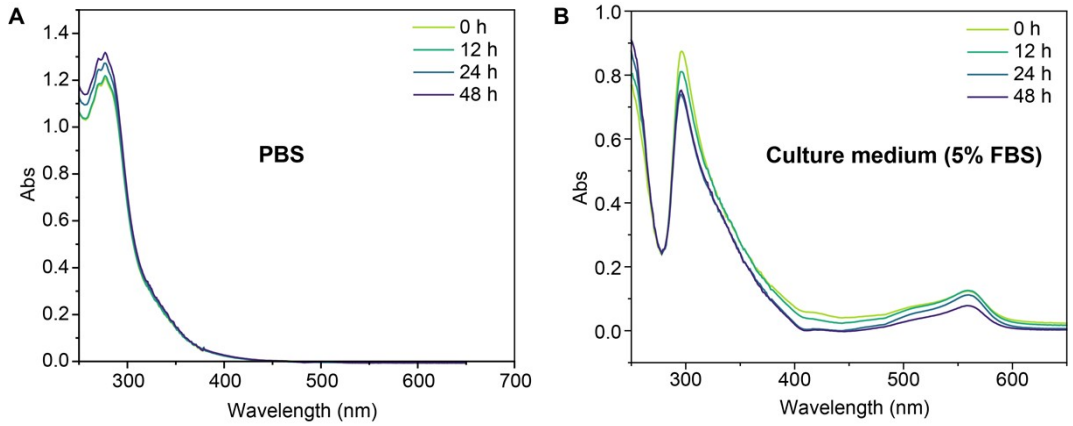


Fig. S7 UV-Vis absorption spectra of RP (100 μM) in PBS buffer (A) and 1640 culture medium (B) after incubation at 37 ℃ for 0, 12, 24 and 48 h.

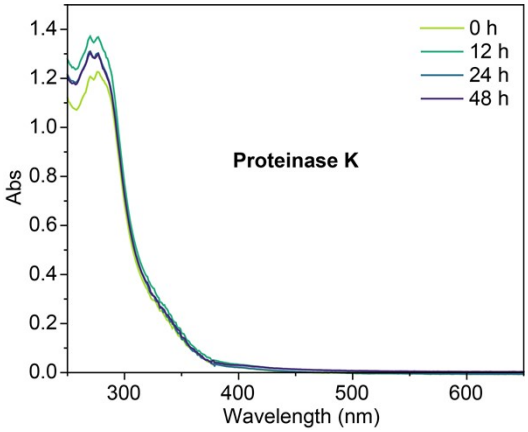


Fig. S8 UV-Vis absorption spectra of RP (100 μM) and proteinase K (1 mg/L) in PBS buffer after incubation at 37 ℃ for 0, 12, 24 and 48 h.

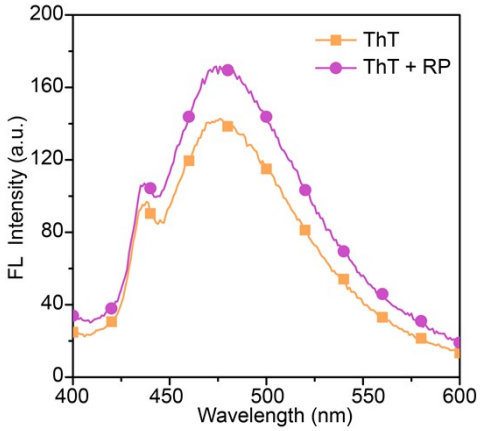


Fig. S9 ThT fluorescence intensity (λ_{ex} = 440 nm, λ_{em}= 484 nm) after incubation with or without RP at 37 °C and pH 7.4 for 24 h ([ThT] : [RP] = 1 : 1, [ThT] = 20 μM).

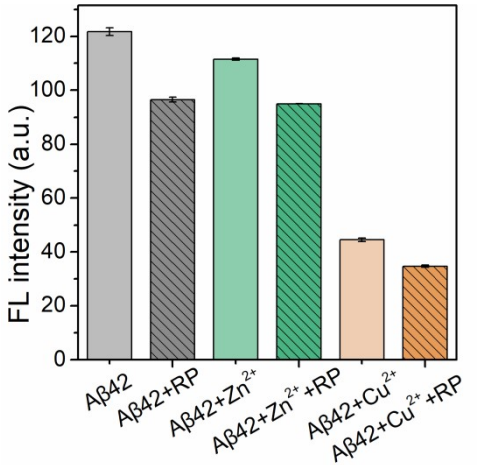


Fig. S10 Fluorescence intensity of ThT pre-incubated with Aβ42 in the absence and presence of Cu^{2+} or Zn^{2+} followed by incubation with or without RP at 37 ℃ and pH 7.4 for 24 h ([Aβ] : [metal ions] : [RP] = 1 : 1 : 1, [Aβ] = 20 mM; λ_{ex} = 440 nm).

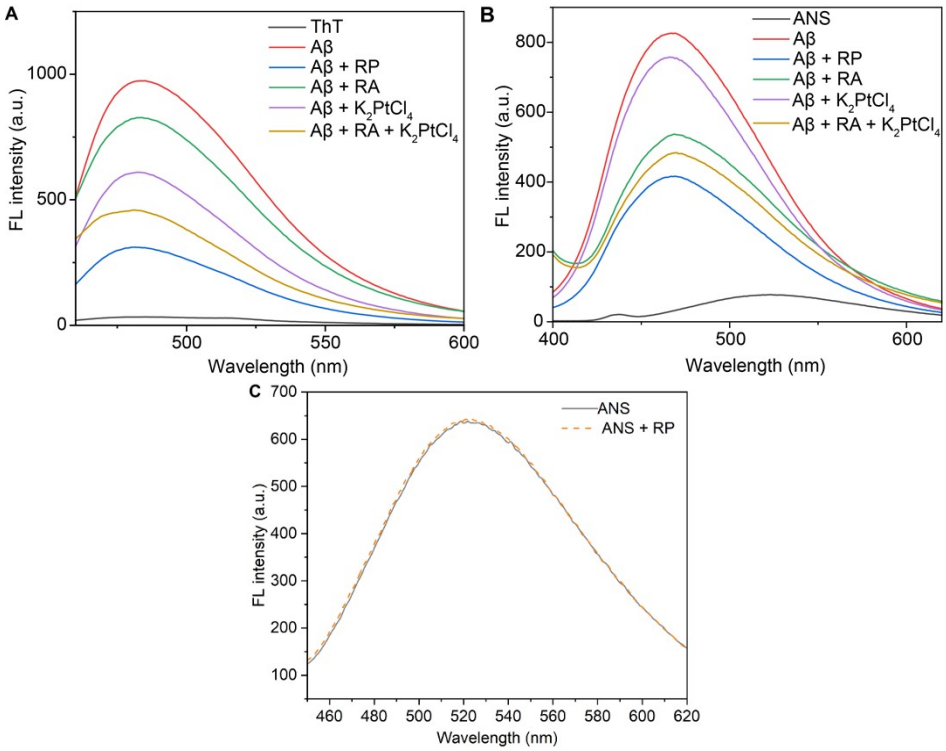


Fig. S11 Fluorescence intensity of ThT (20 μM, λ_{ex} = 440 nm, λ_{em} = 484 nm) (A) and ANS (B) without or with Aβ42, RP, RA, or K_2PtCl_4 after incubation at 37 ℃ and pH 7.4 for 24 h; fluorescence intensity of ANS (20 μM, λ_{ex} = 380 nm) in the presence and absence of RP (20 μM) in PBS buffer after incubation at 37 ℃ and pH 7.4 for 24 h (C). Aβ42 = RP = RA = K_2PtCl_4 = 20 μM.

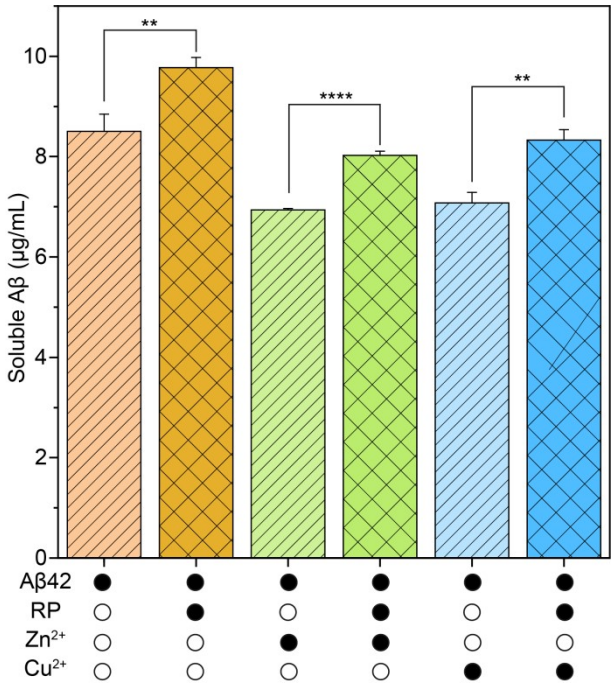


Fig. S12 Soluble Aβ in the absence and presence of Zn^{2+} or Cu^{2+} after incubation with

or without RP in PBS buffer at 37 ℃ and pH 7.4 for 24 h determined by the BCA assay. [Aβ] : [metal ions] : [RP] = 1 : 1 : 1, [Aβ42] = 20 μM; $^{**}p < 0.01$, $^{****}p < 0.0001$.

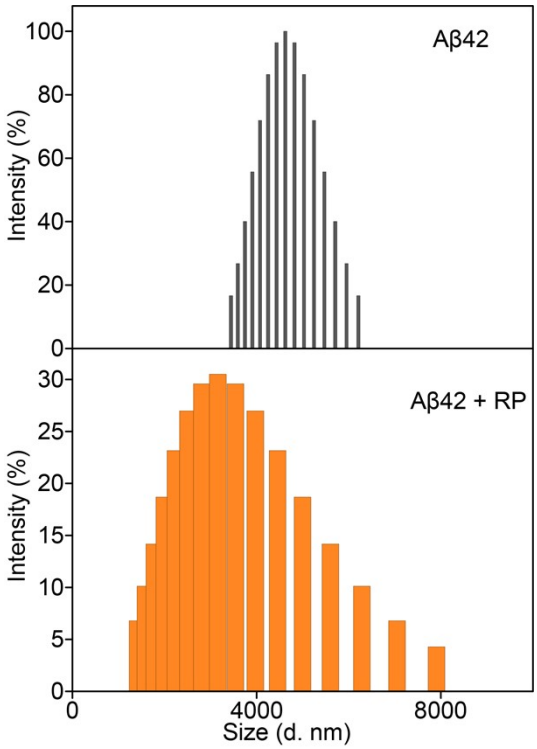


Fig. S13 DLS analysis of Aβ fibrils and RP-induced Aβ aggregates ([Aβ] : [RP] = 1 : 1 and [Aβ] = 20 μM).

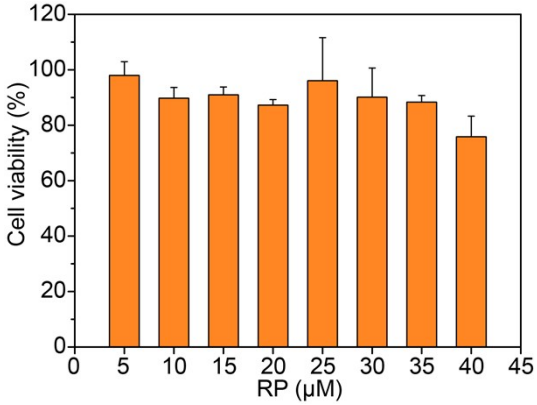


Fig. S14 Viability of PC12 cells after treatment with different concentrations of RP for 24 h.

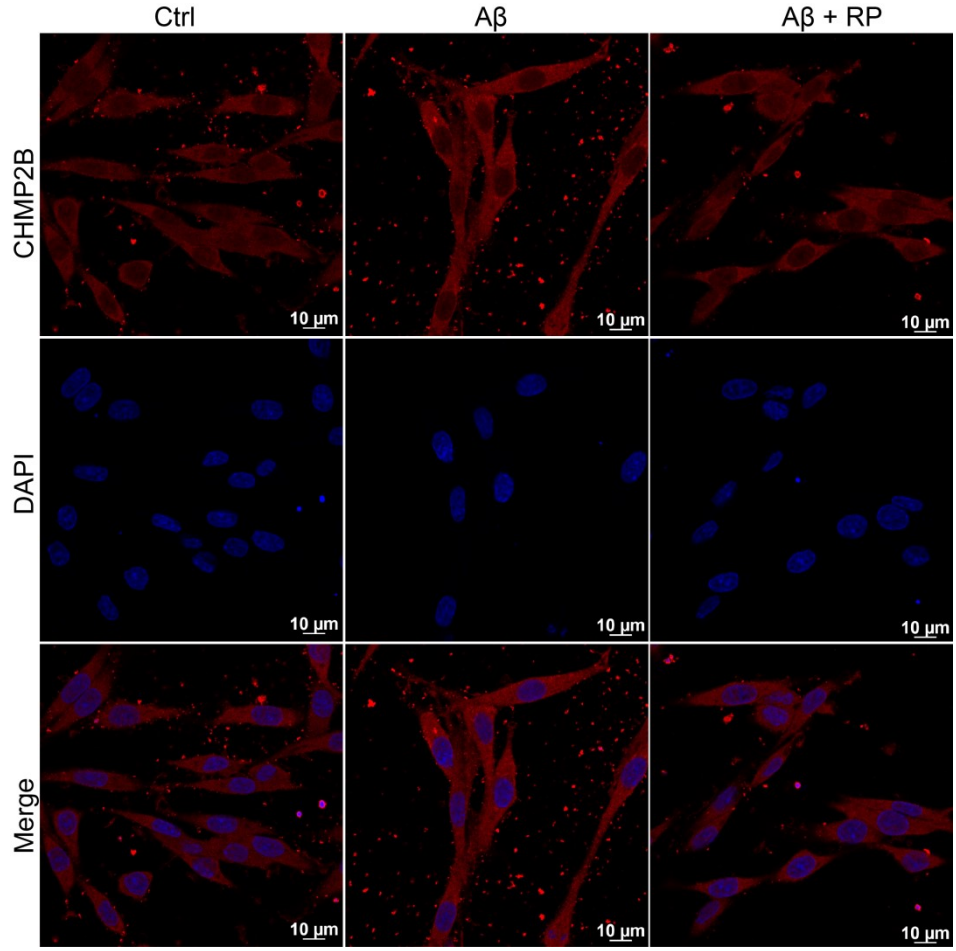


Fig. S15 Immunofluorescence images showing the expression of CHMP2B in Aβ42-exposed PC12 cells in the presence or absence of RP ([Aβ] : [RP] = 1 : 1, [Aβ] = 20 μM).

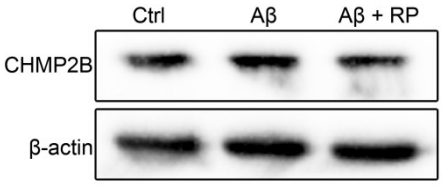


Fig. S16 Western blots of CHMP2B in Aβ42-exposed PC12 cells in the presence or absence of RP ([Aβ] : [RP] = 1 : 1, [Aβ] = 20 μM).

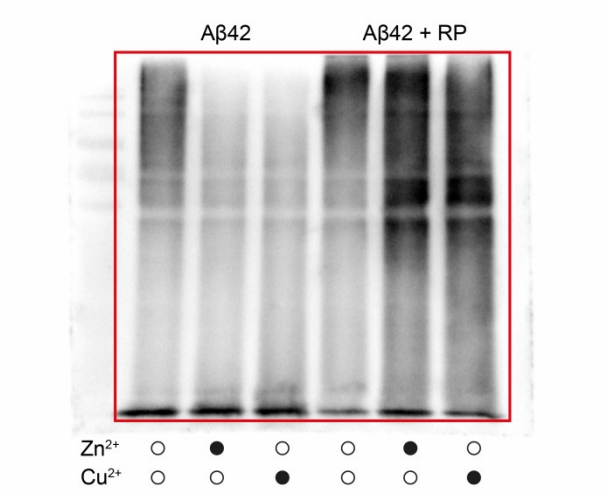


Fig. S17 Uncropped image of Fig 2.

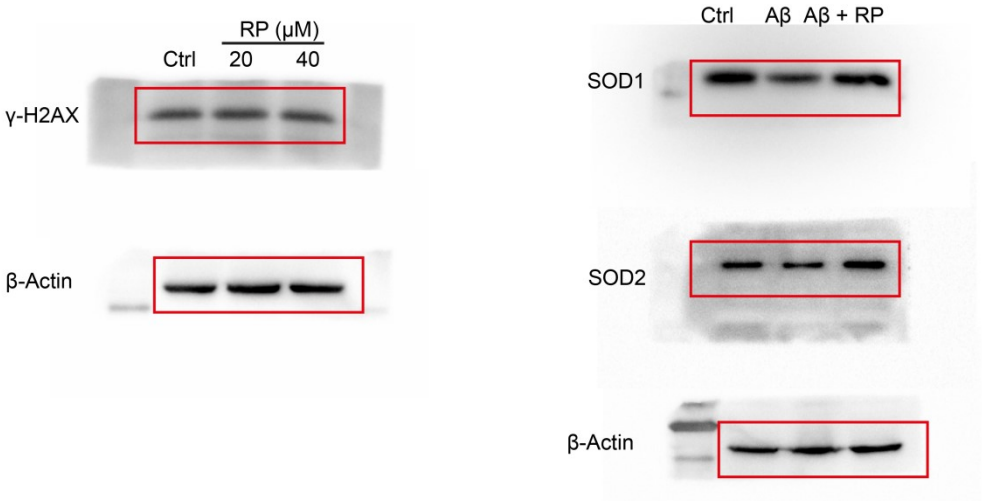


Fig. S18 Uncropped image of Fig 3.

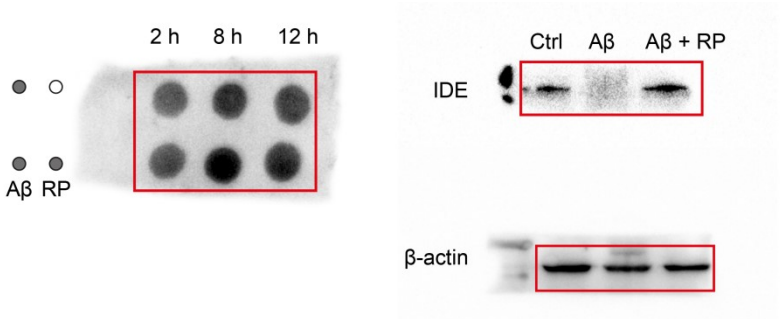


Fig. S19 Uncropped image of Fig 4.

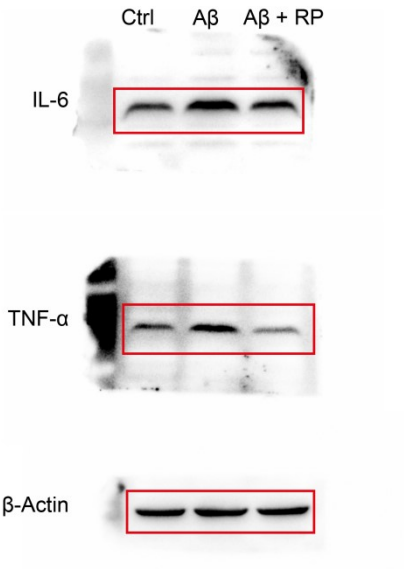


Fig. S20 Uncropped image of Fig 5.

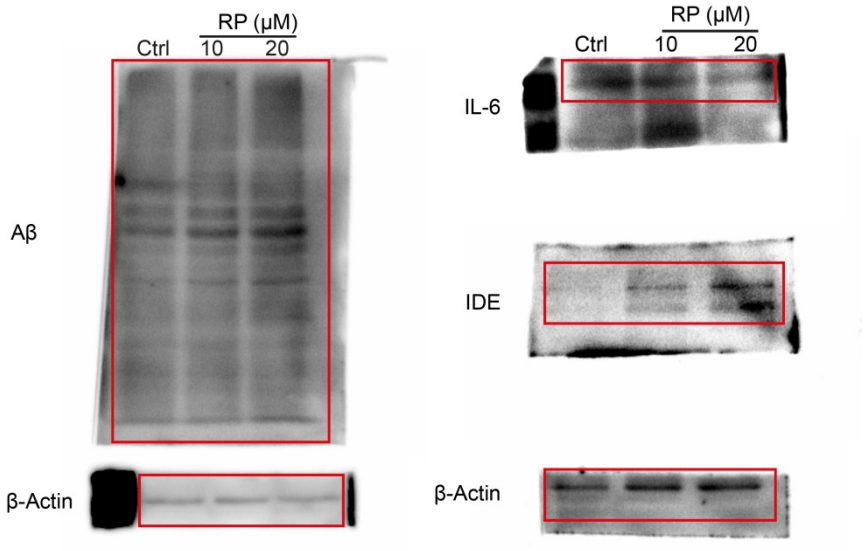


Fig. S21 Uncropped image of Fig 6.

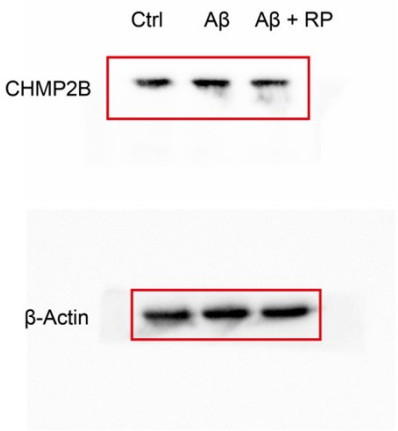


Fig. S22 Uncropped image of Fig S16.

Experimental Section

General methods and materials

All reagents used in this study were purchased commercially and used as received unless otherwise stated. Human Aβ42 was purchased from GL Biochem Ltd. (Shanghai, China). Stock solution of Aβ42 was prepared according to the literature methods. Stocking solution of RP was prepared by dissolving the compound in DMSO and filtering by a 0.22 μM filter (organic system). All solutions and buffers were prepared using Milli-Q water and filtered through a 0.22 μM filter (Millipore) before use. RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) was purchased from Procell and Keygen Biotech, respectively. Fetal bovine serum (FBS) was purchased from Gibco. Mouse PC12 and BV-2 cells were cultured in RPMI-1640 and DMEM, respectively, supplemented with 10% FBS at 37 ℃ and 5 % CO_2.

C. elegans CL4176 strains were obtained from the caenorhabditis genetics center (University of Minnesota, Minneapolis, MN, USA), which express Aβ42 gene in the muscle tissue (dvIs27 [myo-3p::Aβ (1-42)::let-851 3′UTR + rol-6(su1006)]). The strains were cultured on nematode growth media (NGM) plates with *E.coil* strain OP50 as food sources in Luria Broth (LB) medium. $CaCl_2$ (0.6 mL, 1 M), cholesterol (0.6 mL, 5 g L^{-1} in ethanol), $MgSO_4$ (0.6 mL, 1 M), and phosphate buffer (15 mL, 1 M; 17.8 g KH_2PO_4, 500 mL, pH 6.0) were added to the autoclaved NGM (12.0 g agar, 1.5 g peptone, 1.8 g NaCl, 590 mL H_2O). Aliquot (5 mL) of liquid NGM was added into each petri dish to solidify overnight at room temperature. After autoclavation of LB medium (10.0 g NaCl, 5.0 g yeast extract, 10.0 g tryptone, 1.0 L H_2O), *E.coil* strain OP50 were grown in LB medium. Each NGM plate was spotted with OP50 (100 μL) and allowed to dry for 6 h, then transferred to 37 ℃ overnight.

Methods

^{1}H- and ^{13}C NMR spectra were recorded on a Bruker DRX-600 spectrometer. Electrospray ionization mass spectra (ESI-MS) were obtained on an LCQ fleet electrospray mass spectrometer. Fluorescence spectra were recorded on a HORIBA Fluoromax-4P fluorescence spectrometer. Transmission electron microscopic (TEM) images were obtained on a transmission electron microscope (JEOL, JEM-2100).

Elemental analysis was recorded on Unicube (Elementar).

Synthesis of N,N-bis(pyridin-2-ylmethyl)ethane-1,2-diamine

N-boc-ethylenediamine (0.32 g, 2 mmol), 2-(chloromethyl) pyridine hydrochloride (0.66 g, 4 mmol) and anhydrous potassium carbonate (1.11 g, 8 mmol) were dissolved in anhydrous ethanol (20 mL). The mixture was stirred for 6 h, heated to 98 ℃ and refluxed overnight under stirring. The mixture was poured into water, extracted with dichloromethane. The combined organic phases with water and brine were dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography with PE/EA eluent. The relevant fractions were collected and concentrated with a rotary evaporator. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.47 (d, J = 6 Hz, 2 H), 7.73 (t, J = 13.8 Hz, 2H), 7.54 (d, J = 12 Hz, 2H), 7.23 (t, J = 9.6 Hz, 2H), 6.80 (t, J = 7.8 Hz, 1H), 3.76 (m, 4H), 3.08 (q, J = 9 Hz, 2H), 2.55 (t, J = 9.6 Hz, 2H), 1.36 (m, 9H). The product was dissolved in the dichloromethane and trifluoroacetic acid (1:1) under ice bath, and stirred at room temperature for 4 h. The mixture was concentrated under reduced pressure and dried under vacuum to give a brown oil. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.47 (d, J = 4.2, 2H), 7.75 (t, J = 7.8, 2H), 7.54 (d, J = 4.8, 2H), 7.23 (t, J = 6, 2H), 3.75 (s, 4H), 2.79 (m, 4H), 2.64 (t, 6.6, 2H). ESI-MS found (calcd) for $C_{14}H_{18}N_4$ (m/z): 243.1610 (243.1603) $[M + H]^+$.

Synthesis of activated ester of RA

Retinoic acid (0.60 g, 2 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.58 g, 3 mmol), and N-hydroxy succinimide (0.23 g, 2 mmol) were dissolved in the N,N-dimethylformamide (5 mL). The mixture was stirred at room temperature for 24 h. Ice water (20 mL) was poured into the reaction mixture and the precipitate was filtered. The product was washed with ice water (10 mL) thrice and dried under vacuum to give a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.29 (dd, J = 11.4, 15, 1H), 6.57 (d, J = 15.6, 1H), 6.36 (d, J = 16.2, 1H), 6.30 (d, J = 11.4, 1H), 6.23 (d, J = 16.2, 1H), 6.14 (s, 1H), 2.83 (s, 4H), 2.36 (s, 3H), 2.04 (s, 3H), 2.02 (t, J = 6, 2H), 1.70 (s, 3H), 1.58 (m, 2H), 1.45 (m, 2H), 1.03 (s, 6H). ESI-MS found

(calcd) for $C_{24}H_{31}NO_4$ (m/z): 398.2313 (398.2331) $[M + H]^+$.

Synthesis of RP

N,N-bis(pyridin-2-ylmethyl)ethane-1,2-diamine (0.24g, 1 mmol) and activated ester of RA (0.40 g, 1 mmol) were dissolved in the dichloromethane, and K_2PtCl_4 (0.42g, 1 mmol) was added dropwise and refluxed at 100 ℃ for 3 h. After stirring overnight at room temperature, the yellow precipitate was collected, washed with methanol, and dried in vacuo. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.76 (d, *J* = 5.4, 2H), 8.28 (t, *J* = 7.2, 3H), 8.05 (t, *J* = 5.4, 1H), 7.78 (d, *J* = 7.8, 2H), 7.66 (t, *J* = 6.6, 2H), 6.90 (m, 1H), 6.22 (m, 4H), 5.69 (s, 1H), 5.35 (d, *J* = 16.2, 1H), 4.86 (d, *J* = 15.6, 2H), 3.30 (m, 1H), 3.12 (t, *J* = 6.6, 2H), 2.17 (s, 3H), 2.00 (t, *J* = 6, 2H), 1.95 (s, 3H), 1.67 (S, 3H), 1.57(m, 2H), 1.43 (m, 2H), 1.00 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$, δ, ppm): 167.62, 165.36, 150.00, 148.52, 140.732, 138.36, 137.75, 137.47, 136.19, 129.81, 129.73, 129.42, 127.93, 125.02, 124.01,121.87, 69.54, 61.35, 39.58, 36.54, 34.26, 33.08, 29.71, 28.97, 21.77, 19.23, 13.41, 12.83. HR-MS (m/z) found (calcd) for $[C_{34}H_{44}N_4OClPt]^+$: 754.2848 (754.2851). Elemental analysis found (calcd) for $C_{34}H_{44}N_4Cl_2Pt$ (%): C, 51.83 (51.64); H, 5.78 (5.61); N, 7.03 (7.09).

ThT fluorescence

Aβ42 (20 μM) was co-incubated with RP (20 μM) in Tris-HCl buffer (20 mM Tris-HCl/150 mM NaCl, pH 7.4) for different times. ThT was added to the solution to reach a final concentration of 20 μM. The ThT fluorescence was recorded at 484 nm with excitation wavelength of 440 nm.

Western blot of Aβ aggregates

Aβ42 (20 μM) and RP at equimolar ratio were co-incubated for 24 h, respectively. Anti-Aβ antibody 6E10 (SIG-39320, Covance) was used to indicate the distribution of Aβ42 species. In the disaggregating assay, Aβ42 aggregates were formed by pre-incubating Aβ42 in PBS buffer in the absence and presence of metal ions for 24 h to reach the plateau region, and then were incubated with RP for 24 h.

Hydrophobicity of Aβ

Aβ42 (20 μM) and Zn^{2+} or Cu^{2+} (20 μM) were co-incubated for 24 h in the

absence or presence of RP (20 μM). 8-Anilino-1-naphthalenesulfonate (ANS, final concentration 20 μM) was added to the solution and fluorescence emission spectra were measured with excitation wavelength of 380 nm.

Solubility of Aβ

Aβ42 (20 μM) were prepared in the PBS buffer. After incubation for 24 h, the solutions were centrifuged at 12000 rpm for 30 min and the peptide concentration in the supernatant was analyzed by the BCA protein assay. λ_{abs} = 562 nm (P. K. Smith, et al. *Anal. Biochem.*, 1985, 150, 76–85).

Morphology of Aβ

Samples were prepared as described in the ThT assay. An aliquot of each solution (10 μL) was spotted on the 300-mesh, 3-mm copper grid carbon support film for 2 min at room temperature and excess sample was removed. Each grid was stained with phosphotungstic acid (4%, w/v) for 1 min, and excess phosphotungstic acid was removed. Imaging was carried out on a JEOL JEM-2100 transmission electron microscope.

Dynamic light scattering

Samples were prepared as described in the TEM experiment. To obtain the actual size of the aggregates, the hydrodynamic radii were directly measured by Zetasizer Nano S90 ZEN1690 (Malvern Instruments, Ltd., United Kingdom).

Octanol/water partition coefficient (Log $P_{o/w}$)

The lipophilicity of RP was measured in an ocatanol/buffer system using the shake-flask method and ICP-MS. Solutions of RP (10, 25, 50 μM) containing DMSO (1%) were prepared in the PBS pre-saturated with octanol. Equal volumes (2 mL) of the solution and octanol pre-saturated with PBS were mixed and shaken at room temperature for 24 h and then separated into two phases by centrifugation. The concentration of Pt was measured by ICP-MS. The aqueous phase concentration of PBS solution was C_w, and the concentration of C_o in n-octanol was calculated according to the law of mass conservation. The lipid-water distribution coefficient of RP was calculated by the formula ($\log P = \log C_o/C_w$).

Cellular uptake

Aβ42 (20 μM) was pre-incubated for 2 h and added to the cell culture medium of PC-12 or BV-2 cells in the absence or presence of RP (20 μM). After incubation for different periods of time, the cells were collected, washed with PBS, digested by concentrated HNO_3 at 95 ℃, 30% H_2O_2 and concentrated HCl at 37 ℃. The platinum amount in the cell lysates was determined by ICP-MS.

Cytotoxicity

The cytotoxicity of RP was tested by the MTT assay on PC12 cells. Briefly, PC12 cells were planted in a 96-well microplate at 6000 cells per well and incubated at 37 ℃ in a humidified atmosphere with 5% CO_2. Aβ42 and RP were added into the medium. After incubation at 37 ℃ for 24 h, MTT (40 μL, 5 mg mL^{-1}) was added to each sample solution. The samples were re-incubated at 37 ℃ for 4 h. The supernatants were removed and the formazan crystals were dissolved in DMSO (200 μL). The absorbance at 490 nm was determined using a Varioskan Flash microplate reader.

Measurement of intracellular ROS

Aβ42 (20 μM) was pre-incubated for 2 h and added to the cell culture medium of PC-12 cells on glass coverslips for 15 min in the absence or presence of RP (20 μM). The cells were loaded with CM-H_2DCFDA (10 μM) to detect the intracellular ROS. The resulting fluorescence was determined by confocal microscopy system LSM-710 (Zeiss, Germany) and analyzed by ZEN software.

Immunocytochemistry

BV-2 cells were treated as described above, washed with PBS, fixed with 4% paraformaldehyde for 10 min, and permeabilized with 0.5% Triton for 15 min. The cells were washed by PBS for 5 min (×2), blocked for 15 min at room temperature with 0.5% BSA (dissolved in PBS), and incubated with the primary antibody (1:200) at 4 ℃ overnight. After incubation, the cells were washed twice with PBS and incubated with fluorophore-labeled secondary antibody at room temperature for 1 h. The primary antibodies were rabbit anti-CD206 (ab125028, Abcam), mouse anti-

CD86 (ab220188, Abcam), rabbit anti-CHMP2B (ab157208, Abcam); and the fluorophore-labeled secondary antibodies were goat anti-rabbit IgG H&L (Alexa Fluor® 647, ab150079, Abcam), goat anti-mouse IgG H&L (Alexa Fluor® 488, ab150113, Abcam).

Acridine orange (AO) staining

Aβ42 (20 μM) was pre-incubated for 2 h and added to the cell culture medium of BV-2 cells and incubated in the absence or presence of RP (20 μM) for 8 h. The cells were stained with AO (1 μg mL^{-1}) in PBS at 37 ℃ in the dark for 30 min. The cells were washed three times with PBS and observed under Zeiss LSM710 laser scanning microscope (excitation: 488 nm; emission: 500–560 nm or 600–700 nm). The images were analyzed using ZEN software.

Propidium iodide (PI) diffusion

Aβ42 (20 μM) was pre-incubated for 2 h and added to the cell culture medium of PC12 cells in the absence and presence of RP (20 μM) and cultured for 24 h. The cells were stained with PI (120 μg mL^{-1}) in PBS at 37 ℃ in the dark for 10 min, and then washed with PBS and observed under Zeiss LSM710 laser scanning microscope.

Aβ internalization and dot blotting

BV-2 cells were cultured in a 6-well plate for 12 h. The cells were treated with RP and Aβ42 (20 μM) for 2, 8 and 12 h, respectively, at 37 ℃. After incubation, the medium and cells were collected and washed with PBS. The cell lysates were added and incubated on ice for 30 min, and centrifuged. The cellular supernatant was used for dot blotting. Aliquots of each sample (4 μL) were spotted onto PVDF membranes and dried at room temperature. The membranes were blocked for 1 h at room temperature with fat-free milk powder and incubated at 4 ℃ overnight with monoclonal anti-Aβ antibody 6E10. The membranes were washed with PBST (×3) and incubated with HRP-conjugated anti-mouse antibody for 1 h at room temperature. The membrane was washed with PBST (×3) and bands were visualized by SuperSignal (Millipore).

Release of inflammation cytokine

BV-2 cells were cultured in a 6-well plate for 12 h and then treated with RP and Aβ42 (20 μM) at 37 ℃ for 12 h in the FBS-free medium. After incubation, the medium and cells were collected and the cells were washed with PBS. The content of IL6 in the medium was measured by ELISA kit (JingMei, Biotechnology). Protein concentration of the cells was measured to calibrate the values.

Paralysis of *C. elegans*

Synchronized *C. elegans* CL4176 strains that express muscle-specific Aβ42 were cultured onto NGM plates containing *E. coil* OP50 mixed with RP. The worms were allowed to lay eggs at 15 ℃ for 2 h, and the adults were picked off and the eggs were hatched to grow into adults. The temperature was raised to 25 ℃. The number of paralyzed worms was counted every 2 h until all worms were paralyzed.

Analysis of Aβ in *C. elegans*

CL4176 elegans was treated in the same way as in the paralysis assay. The worms were collected and washed with M9 buffer to remove *E. coil.* The RIPA cell lysis buffer was added to lyses the worms for 10 min under ultrasonication. The supernatant was collected by centrifugation, quantified by the BCA protein assay kit, dissolved by loading buffer and heated at 95 ℃ for 10 min. The final samples were detected by western blotting. β-Actin was used as internal reference to ensure protein loading.

Fluorescent staining and ROS measurement in *C. elegans*

Age-synchronized transgenic CL4176 elegans was fed with vehicle and RP (10, 20 μM), respectively, and incubated at 25 ℃ for 32 h. The worms were washed with M9 buffer and collected, and stained with CM-H_2DCFDA (10, 20 μM) and ThS (10, 20 μM) respectively for 1 h. The samples were destained by transferring to fresh NGM plates for 2 h. The fluorescence of CM-H_2DCFDA was measured in the black 96 well microplates (each well > 30 worms), and ThS was observed under Zeiss LSM710 laser scanning microscope (λ_{ex} = 405 nm).

Detection of cellular proteins

PC12 cells or BV-2 cells were planted into a 6-well cell culture plate (medium

with 10% fetal bovine serum). RP (20 μM) and Aβ42 (20 μM) were added in the medium and the cells were incubated at 37 ℃. The cells were collected and lysed on the ice in a cell lysis RIPA buffer and centrifuged at 4 ℃ for 20 min. The supernatant was collected and the protein concentration was determined by the BCA protein assay kit. The loading buffer was added and boiled at 95 ℃ for 10 min. Each sample was separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked at room temperature for 1 h with fat-free milk powder (5%) and incubated with various primary antibody (1:1000 diluted by PBST buffer) at 4 ℃ overnight. The membranes were washed thrice with PBST buffer, incubated with HRP-conjugated goat anti-rabbit antibody (1:1000) at room temperature for 1 h, washed with PBST buffer, and the bands were visualized by SuperSignal (Millipore). The following antibodies were used: rabbit anti-SOD1 (ab308181, Abcam), rabbit anti-SOD2 (ab68155, Abcam), rabbit anti-IDE (ab133561, Abcam), rabbit anti-IL-6 (ab290735, Abcam), rabbit anti-TNF-α (ab183218, Abcam), rabbit anti-γ-H2AX (ab81299, Abcam), and rabbit anti-β-Actin (#4970, Cell Siganling Technology).

BLI assay

Measurement of binding kinetics was performed by the BLI method using the Octet K2 system (Fortebio), according to the manufacturer's protocols. Recombinant human Aβ42 protein (l mg mL^{-1}) was diluted in PBS (pH 7.4)/0.04% Tween 20, and treated by biotinylated kit (Fortebio). The biotinylated Aβ42 was eluted through a desalting column. The protein concentration was measured using the BCA method and diluted to 10 μg mL^{-1} and immobilized on streptavidin (SA) biosensors. The assay buffer is consisted of PBS with 0.04% Tween and 0.1% DMSO. The assay was performed at 30 ℃ with continuous shaking at 1000 rpm. Sensors were pre-wetted with assay buffer for 10 min before each round of association and dissociation and establish a baseline. The binding constants were generated using Data Acquisition software (ForteBio). The resulting data were analyzed on the basis of a 1:1 binding model. K_D values were calculated and fitted using the Octet Data Analysis Software

(Fortebio).

ANS competition assay

The fluorescence of ANS (20 μM, λ_{ex} = 380 nm) in the presence and absence of RP (20 μM) in PBS buffer was detected after incubation at 37 ℃ and pH 7.4 for 24 h.

Stability assay

RP (100 μM) was incubated in PBS buffer and culture medium (1640 + 10% FBS) at 37 ℃ for 0, 12, 24, and 48 h, respectively. The absorption spectra were recorded on a UV-Vis spectrophotometer.

Amide bond stability

RP (100 μM) was incubated in PBS buffer containing proteinase K (1 mg mL^{-1}) at 37 ℃ for 0, 12, 24, and 48 h, respectively. The absorption spectra were recorded on a UV-Vis spectrophotometer.