Supporting Information

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SI Text

Chemicals. The btsc compounds Cu^{II}(gtsm) and Cu^{II}(atsm) were synthesized as described previously (1, 2). All other chemicals were purchased from Sigma–Aldrich unless specified elsewhere.

Cell Culture Experiments. SH-SY5Y cells were grown in DMEM:F12 media supplemented with 10% (vol/vol) FBS, 50 mM Hepes, nonessential amino acids (Invitrogen), and penicillin/streptomycin sulfate (Invitrogen). Cultures were maintained at 37 °C in a humidified incubator with 5% (vol/vol) CO_2 and passaged every 5–6 days at a dilution of 1/10. For experiments with Cu^{II}(gtsm) and Cu^{II}(atsm) cells were seeded into 6-well plates. Once the cells had reached ≈80% confluency, the media were removed by aspiration and replaced with fresh, FBS-free media supplemented with 25 μM Cu^{II}(gtsm) or 25 μM Cu^{II}(atsm). Both btsc compounds were prepared freshly as 25 mM stock solutions in DMSO, and control treatments therefore received an equivalent volume of DMSO. Cells were grown for a further 5 h at 37 °C/5% CO_2 and then harvested by scraping the cells into the treatment media (2 wells per replicate) and pelleting by centrifugation (1,000 × *g*, 3 min). Media were removed and pelleted cells resuspended in 50 μL of Cytobuster Protein Extraction reagent (Novagen) supplemented with EDTA-free protease inhibitor mixture (Roche), phenylmethanesulfonyl fluoride, sodium fluoride, sodium vanadate, β-glycerophosphate, sodium pyrophosphate, and deoxyribonucleate 5′-oligonucleotido-hydrolase. Cell extracts were collected by centrifuging samples (15,000 × *g*, 3 min), and storing the supernatant at −20 °C. Protein content of the cell extracts was determined by using a protein content determination kit (Pierce).

Inductively Coupled Plasma Mass-Spectrometry Determination of Metals Ions. SH-SY5Y cells were treated with DMSO, Cu^{II}(gtsm) or Cu^{II}(atsm) as described above except that the cultures treated were growing in T175 cm^2 flasks (1 flask per replicate). After treating for 5 h and collecting cells as described above, pelleted cells were rinsed 3 times with PBS. A small aliquot from each sample was used to determine total cellular protein, and the remaining sample analyzed for cellular Cu, Fe, Zn, and Mn using inductively coupled plasma mass-spectrometry (ICP-MS) as described previously (3). Cellular metal levels were normalized for total cellular protein and are expressed as the fold change in metal content relative to basal metal concentrations in DMSO-treated controls. The basal Cu concentration for DMSO-treated cells was 34 nmol per milligram of cellular protein.

AD Mice and Treatments. The AD mice used in this study expressed mutant human APP (K670N, M671L) and mutant human PS1 (ΔE9) (4). After weaning, animals were genotyped and group housed until 3 weeks before treating, at which time they were individually housed. All animals had free access to water and rodent chow and were maintained on a 12-h light–dark cycle. Two separate studies were performed. In both studies, treatments were administered daily by oral gavage and treatment began when the mice were 5–6 months old. Acid stability studies have shown dissociation of Cu–btsc complexes is unlikely to occur in the gut (5). Approximately equal numbers of males and females were included in each group. For the first study, 29 mice were used. Fifteen were treated with Cu^{II}(gtsm) in standard suspension vehicle (SSV) at 10 mg/kg of body weight and 14 were treated with SSV (sham group). These mice were tested for cognitive performance by using the Y-maze test, and the brains collected after culling were used for all biochemical analyses. In the second study, 37 mice were used. Thirteen were treated with Cu^{II}(gtsm) at 10 mg/kg of body weight, 9 were treated with Cu^{II}(atsm) at 100 mg/kg of body weight, and 16 were treated with SSV. Mice in the second study were again tested for cognitive performance and then the brains collected for immunohistochemistry.

Y-Maze Test for Spatial Memory and Learning. All mice were subjected to a 2-trial Y-maze test (6), with all testing performed during the light phase of the circadian cycle. Y-Mazes were of uniform gray color and consisted of 3 arms with an angle of 120° between each arm. Each arm was 80 mm × 300 mm ×150 mm (width × length × height). The 3 identical arms were randomly designated start arm, novel arm, and other arm. The maze was placed in a separate room with minimal lighting and the floor of the maze covered with sawdust that was mixed after each individual trial to eliminate olfactory stimuli. Visual cues were placed on the walls of the maze. The Y-maze tests were performed after 6 weeks of treatment and consisted of 2 trials separated by a 1-h intertrial interval to assess spatial recognition memory. The first trial (training) was for 10 min, and the mice were allowed to explore only 2 arms (starting arm and other arm). For the second trial (retention) mice were placed back in the maze in the same starting arm, and allowed to explore for 5 min with free access to all 3 arms. By using a ceiling-mounted CCD camera, all trials were analyzed for the number of entries the mice made into each arm. Data are expressed as the percentage of novel arm entries made during the 5-min second trial.

Preparation of Mouse Brain Samples for Biochemical Analyses. Mice were administered Lethobarb (Virbac) by intraperitonel injection after treating for 15 weeks. They were dissected to expose the chest cavity and then perfused with ≈10 mL of Dulbecco's PBS (Invitrogen) supplemented with 0.5 mg/ml (wt/vol) heparin, 5 μM butylated hydroxytolulene, and Phosphatase Inhibitor Cocktails I and II. Brains were removed and left and right hemispheres stored separately at −80 °C. Right hemispheres were homogenized in 1 mL of PBS supplemented with phosphatase inhibitors as above by sonication. PBS-soluble and -insoluble fractions were collected by centrifuging at 100,000 × *g* for 30 min. The PBS-insoluble material was resuspended in 1 mL of PBS supplemented with phosphatase inhibitors. ERK1/2 could not be detected in PBS extracts. To analyze levels of this protein, left hemispheres were homogenized in Protein Extraction Buffer as per SH-SY5Y samples. Cell lysis buffer soluble and insoluble material was separated by centrifugation (16,000 × *g*, 5 min), and the insoluble material were resuspended in cell lysis buffer. Protein content of soluble and insoluble fractions of PBS and cell lysis buffer homogenized samples was determined by using a protein content determination kit (Pierce).

Mass Spectrometry (MS) Analysis of Aβ in Brain Tissues. Analysis of Aβ from brain tissues was performed with surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) MS using the ProteinChip system (Ciphergen Biosystems). This method enables direct mass-spectrometry identification of individual proteins after immunoaffinity capture purification of the proteins of interest from tissue samples. PBS-soluble and -insoluble extracts of AD mouse brains were subjected to antibody capture using the WO2 antibody for human Aβ (epitope = Aβ residues 5–8) (7), and the mass of each Aβ species was deter-

mined by using the ProteinChip reader. Affinity arrays were constructed by coupling WO2 onto PS10 ProteinChip arrays. Aliquots of antibody (2 μL at 0.25 mg of protein per milliliter) were incubated on the ProteinChip arrays in a humidity chamber at 4 °C overnight to allow covalent binding of the antibody to the array. Unreacted sites were blocked by incubating the arrays with 10 μL of 1 M ethanolamine in PBS (pH 8.0) for 1 h at room temperature. The arrays were then placed in a ProteinChip bioprocessor and washed by shaking for 5 min with 20 μL of PBS containing 0.5% (vol/vol) Triton X-100, then with 20 μL of PBS without Triton X-100. Brain samples were diluted 1:10 with PBS containing 0.5% (vol/vol) Triton X-100 and 1 M urea, and 180 μL of this solution was loaded onto each spot on the array. Arrays were incubated for 3 h at room temperature with constant shaking (300 rpm). The arrays were then washed twice with 200 μL of PBS containing 0.5% (vol/vol) Triton X-100, twice with 200 μL of PBS, then twice with 200 μL of 1 mM Hepes. Arrays were allowed to air-dry, and 1 μL of matrix (1:1 mixture of acetonitrile and 0.5% (vol/vol) sinapinic acid in trifluoroacetic acid) was applied twice to each spot. Mass analysis was performed by averaging a minimum of 60 shots on the ProteinChip reader (version 3.2.1). Data generated by ProteinChip Software was analyzed by using Biomarker Patterns Software for differential peak intensities of each individual peptide. All SELDI-TOF MS brain Aβ data are expressed as log-normalized Aβ peak intensity per milligram of brain protein.

SDS/PAGE and Western Blot Analyses. SH-SY5Y samples and mouse brain samples were diluted with appropriate homogenization buffer (PBS or cell lysis buffer) and 4× gel loading buffer [250 mM Tris, 20% (vol/vol) glycerol, 8% (wt/vol) SDS, 2% (vol/vol) β-mercaptoethanol, 0.01% (wt/vol) bromophenol blue] and then heated at 100 °C for 5 min. Denatured and reduced samples were loaded onto 1.5-mm, 15-well 4–20% glycine gels (Invitrogen) for all tau analyses, or onto 1.0-mm, 15-well 12% glycine gels for all other proteins. The amount of protein loaded per lane varied for each protein analyzed. Proteins were electrophoresed at 125 V for 2–2.5 h and then transferred onto PVDF membranes (Roche) at 25 V for 2 h by using transfer buffer (Invitrogen) supplemented with 20% (vol/vol) methanol. PVDF membranes were incubated in PBST (PBS supplemented with 0.05% (vol/vol) Tween-20) containing 4% (wt/vol) blocking reagent (GE Healthcare). Membranes were then incubated with primary antibody in PBST/blocking reagent solution overnight at 4 °C, washed 3 times for 5 min each with PBST, and then incubated with secondary antibody in PBST/blocking reagent for 2–3 h at room temperature. A full list of antibodies used is provided (Table S1). Secondary antibody was discarded and membranes washed 5 times for 5 min each with PBST before visualizing chemiluminescence of protein bands using ECL Advance (GE Healthcare) and a FujiFilm LAS-3000 imager. Chemiluminescence images were saved as TIFF files and relative abundance of proteins determined using NIH ImageJ 1.38x software. All Western blot densitometry data are expressed relative to levels of the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Hippocampal LTP. Fourteen- to 40-day-old C57Bl6 mice were decapitated under anesthesia by halothane inhalation using procedures approved by institutional ethics committees. Brains were removed and chilled in ice-cold artificial cerebrospinal fluid (ACSF) with reduced $CaCl_2$ (0.75 mM) and supplemented with 8 mM $MgCl_2$ and 2 mM ascorbic acid. The forebrain and cerebellum were removed by transverse cuts, and hemisected brains were sliced horizontally into 350-μm sections taken between 1.75 and 4.6 mm from the dorsal surface of the brain using a Vibratome 3000. Slices were incubated at 34 °C for 30 min in ACSF (24 mM NaCl, 2.5 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM D-glucose, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$) supplemented with 2 mM ascorbate, then chilled to room temperature (≈24 °C). The ACSF was bubbled vigorously with 95% O_2/5% CO_2 (pH 7.38, HCl/$NaHCO_3$) and incubated for 1.5 to 5 h before electrophysiological recordings.

Field potential recordings were made in the hippocampus by stimulating and recording orthodromically in the stratum radiatum of the CA1 region with an interelectrode distance near 350 μm. The stimulating electrode was a bipolar concentric electrode and the recording electrode was 1.5-mm borosilicate glass (Harvard Apparatus) filled with 2 M NaCl and pulled to a resistance of ≈5 MΩ. Recordings were made with an NPI microelectrode amplifier in bridge-mode, connected to a 20× preamplifier with 10-kHz low-pass 8-pole Bessel filtration (model 3381 filter/amplifier; Krohn-Hite). Signals were digitized with a Digidata 1322A A/D converter (Axon Instruments) and stored on the hard drive of a personal computer using pClamp 8.2 software (Axon Instruments). Slices were stimulated at 0.033 Hz with a 0.1-ms square pulse using a Master 8 stimulator (A.M.P.I. Instruments) with a variable current stimulus isolation unit (Getting Instruments). Voltage–time data were collected at 50 kHz. Baseline stimulation intensity was that required to produce 20–30% of the maximum slope of the field excitatory post synaptic potential (fEPSP) and was calibrated at the beginning of each experiment. Tetanic stimulation was a 1-s 100-Hz pulse delivered in substitution for the test stimulus at the test intensity. Data were analyzed offline by using pClamp 8.2 and Microsoft Excel software. The slope of each fEPSP was determined by linear regression of the data points after the presynaptic fiber volley with the fitted data range chosen by visual inspection. Normalized fEPSP slopes were expressed as the percent of the average slope of the baseline data points between 30 and 20 min before tetanus. Long term potentiation (LTP) was quantified by averaging the data for each slice from 55 to 60 min after tetanus. Results are presented as mean values (±SEM).

ELISA for Aβ Detection. PBS-soluble and -insoluble fractions of mouse brain samples were mixed with guanidine HCl to a final concentration of 0.5 M. Aβ content of the samples was determined by using the DELFIA Double Capture ELISA as described previously (3).

Immunohistochemical Quantitation of Mouse Brain Aβ Plaques. Left hemispheres (minus cerebellum) freshly removed from AD mice after 8 weeks of treatment were fixed in 10% (vol/vol) Neutral Buffered Formalin. Histochemical analysis of brain amyloid plaques was performed as described previously by using the anti-Aβ antibody 1E8 (8). The total number of amyloid plaques per hemisphere cross-section was determined by using Image Pro Plus image analysis software, and data are expressed as the total number of 1E8 immunoreactive plaques per hemisphere cross-section.

Statistical Analyses. The number of samples used for various analyses in this study was 3–4 for all in vitro experiments and 7–11 for all in vivo experiments. As appropriate for these sample sizes, statistical significance of data when comparing effects of a treatment relative to a control was determined by using the 2-sample t test assuming unequal variance with a hypothesized mean difference of 0 and an α-value of 0.05. Correlation analysis of Y-maze performance and brain Aβ oligomer levels was performed by using straight-line regression analysis and an α-value of 0.05.

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Fig. S1. Western blot (*A*) and densitometry (*B*) analysis showing Cu^{II}(gtsm) decreases phosphorylation of tau at serine-396 in SH-SY5Y cells. Compared with the antibody for pTau ser404 (Figure 2*C*) the antibody for pTau ser396 was immunoreactive for multiple protein bands, possibly different tau isoforms. Phosphorylation at ser396 for some apparent tau isoforms decreased due to treatment with Cu^{II}(gtsm) (46-kDa isoform) and some were unchanged (60-kDa isoform). Decreased phosphorylation of the 46-kDa isoform appears consistent with data shown in Figure 2*C* when using the antibody for pTau ser404. Data in *B* are mean values ± standard error ($n = 4$); *, pTau ser396 levels were significantly lower ($P = 0.0295$).

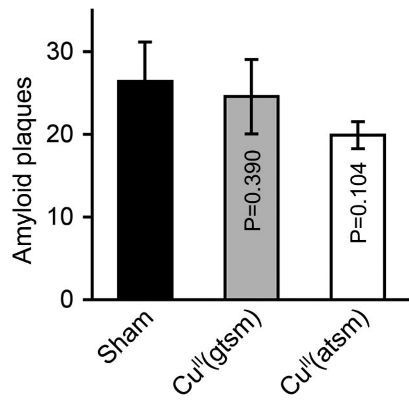

Fig. S2. Aβ plaque density (total number of amyloid plaques per hemisphere cross-section) determined from immunohistochemistry analysis of AD mouse brains. Aβ plaques counts demonstrate that, unlike Aβ trimers, treatment with Cu^{II}(gtsm) did not alter the Aβ plaque load of the AD mouse brain, indicating that Cu^{II}(gtsm)-mediated improvement in the Y-maze test of cognition (Fig. 3) did not correlate with Aβ plaque load. Data shown are mean values ± standard error (n = 9–18 mice), P values show lack of statistical significance, despite an apparent 24% decrease in plaque load for Cu^{II}(atsm)-treated mice.

Table S1. Antibodies used for Western blot analyses and SELDI-TOF MS

Antibody	Company/reference	Source	Catalogue No.
α-Akt	Cell Signaling Technology	Rabbit	9272
α-Phospho Akt	Cell Signaling Technology	Rabbit	9271S
α-ERK1/2	Cell Signaling Technology	Rabbit	9102
α-Phospho ERK1/2	Cell Signaling Technology	Rabbit	9101S
α-GSK3β	Cell Signaling Technology	Rabbit	9315
α-Phospho GSK3α/β	Cell Signaling Technology	Rabbit	9331S
α-GAPDH	Cell Signaling Technology	Rabbit	2118
α-Aβ (WO2)	Ida *et al.* 1996	Mouse	
α-Tau	DAKO Cytomation	Rabbit	A0024
α-Phospho TauSer404	Millipore	Rabbit	AB9660
α-Phospho TauSer396	Zymed	Mouse	35–5300
α-P35	Santa Cruz Biotechnology	Rabbit	SC-820
α-Cdk5	Santa Cruz Biotechnology	Rabbit	SC-173
HRP-linked α-mouse 2°	GE Healthcare	Sheep	NA931V
HRP-linked α-rabbit 2°	Cell Signaling Technology	Goat	7074

Ida N, *et al.* (1996) Analysis of heterogeneous bA4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive Western blot assay. *J Biol Chem* 271: 22908–22914.