Inhibitor of the Tyrosine Phosphatase STEP Reverses Cognitive Deficits in a Mouse Model of Alzheimer’s Disease

Jian Xu1, Manavi Chatterjee1, Tyler D. Baguley2, Jonathan Brouillette1, Pradeep Kurup1, Debolina Ghosh1, Jean Kanyo3, Yang Zhang1,4, Kathleen Seyb4, Chimezie Ononenyi1, Ethan Foscue1, George M. Anderson1,5, Jodi Gresack6, Gregory D. Cuny4, Marcie A. Glicksman4, Paul Greengard6, TuKiet T. Lam3, Lutz Tautz7, Angus C. Nairn8, Jonathan A. Ellman2, Paul J. Lombroso1,8,9*

1 Child Study Center, Yale University, New Haven, Connecticut, United States of America, 2Department of Chemistry, Yale University, New Haven, Connecticut, United States of America, 3Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut, United States of America, 4Laboratory for Drug Discovery and Department of Neurology, Brigham and Women’s Hospital, Cambridge, Massachusetts, United States of America, 5Department of Laboratory Medicine, Yale University, New Haven, Connecticut, United States of America, 6Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, New York, United States of America, 7Infectious and Inflammatory Disease Center, Sanford-Burnham Medical Research Institute, La Jolla, California, United States of America, 8Department of Psychiatry, Yale University, New Haven, Connecticut, United States of America, 9Department of Neurobiology, Yale University, New Haven, Connecticut, United States of America

Abstract

STEP (STriatal-Enriched protein tyrosine Phosphatase) is a neuron-specific phosphatase that regulates N-methyl-D-aspartate receptor (NMDAR) and z-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAAR) trafficking, as well as ERK1/2, p38, Fyn, and Pyk2 activity. STEP is overactive in several neuropsychiatric and neurodegenerative disorders, including Alzheimer’s disease (AD). The increase in STEP activity likely disrupts synaptic function and contributes to the cognitive deficits in AD. AD mice lacking STEP have restored levels of glutamate receptors on synaptosomal membranes and improved cognitive function, results that suggest STEP as a novel therapeutic target for AD. Here we describe the first large-scale effort to identify and characterize small-molecule STEP inhibitors. We identified the benzopentathiepin 8-(trifluoromethyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride (known as TC-2153) as an inhibitor of STEP with an IC50 of 24.6 nM. TC-2153 represents a novel class of PTP inhibitors based upon a cyclic polysulfide pharmacophore that forms a reversible covalent bond with the catalytic cysteine in STEP. In cell-based secondary assays, TC-2153 increased tyrosine phosphorylation of STEP substrates ERK1/2, Pyk2, and GluN2B, and exhibited no toxicity in cortical cultures. Validation and specificity experiments performed in wild-type (WT) and STEP knockout (KO) cortical cells and in vivo in WT and STEP KO mice suggest specificity of inhibitors towards STEP compared to highly homologous tyrosine phosphatases. Furthermore, TC-2153 improved cognitive function in several cognitive tasks in 6- and 12-mo-old triple transgenic AD (3xTg-AD) mice, with no change in beta amyloid and phospho-tau levels.

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Abbreviations: 3xTg-AD, triple transgenic Alzheimer mice; Aβ, beta-amyloid; AD, Alzheimer’s disease; AMPAR, z-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; DCF, dichlorofluorescein diacetate; DFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; HPLC, high-performance liquid chromatography; IC50, median inhibition concentration; i.p., intraperitoneal; GSH, reduced glutathione; KO, knock out; MAPK, mitogen-activated protein kinase; MWM, Morris water maze; NMDAR, N-methyl-D-aspartate receptor; NOR, novel object recognition; PAG, polyacrylamide electrophoresis; pNPP, para-nitrophenyl phosphate; ppm, parts per million; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; STEP, STriatal-Enriched protein tyrosine Phosphatase; TC-2153, benzopentathiepin 8-(trifluoromethyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride; Ts-2576, transgenic mouse line that contains mutations in the amyloid precursor protein; WT, wild type.

* Email: paul.lombroso@yale.edu

Introduction

STEP (STriatal-Enriched protein tyrosine Phosphatase (PTPN9)) is a brain-enriched protein tyrosine phosphatase (PTP) targeted in part to postsynaptic terminals of excitatory glutamatergic synapses [1–4]. Recent studies indicate that STEP is overactive in Alzheimer’s disease (AD), schizophrenia, and fragile X syndrome (FXS) [5–9]. The emergent model based on these findings suggests that the increase in STEP activity interferes with synaptic strengthening and contributes to the characteristic cognitive and behavioral deficits present in these disorders.

Elevated levels of STEP activity disrupt synaptic function by dephosphorylation of STEP substrates [10]. These include...
STEP Inhibitor Reverses Cognitive Deficits in AD Mice

Author Summary
A series of recent studies have found that the levels of the enzyme striatal-enriched protein tyrosine phosphatase (STEP) are raised in several different neuropsychiatric and neurodegenerative disorders, including Alzheimer’s disease, fragile X syndrome, and schizophrenia. STEP normally opposes the development of synaptic strengthening, and these abnormally high levels of active STEP disrupt synaptic function by removing phosphate groups from a number of proteins, including several glutamate receptors and kinases. Dephosphorylation results in internalization of the glutamate receptors and inactivation of the kinases—events that disrupt the consolidation of memories. Here we identify the benzopentathiepin 8-(trifluoromethyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride (known as TC-2153) as a novel inhibitor of STEP. We show that the mechanism of action involves the formation of a reversible covalent bond between the inhibitor and the catalytic cysteine residue of STEP, and we demonstrate the activity of TC-2153 both in vitro and in vivo. TC-2153 shows specificity towards STEP compared to several other tyrosine phosphatases and shows no toxicity to cultured neurons. Importantly, the compound reversed cognitive deficits in a mouse model of Alzheimer’s disease in a way that did not involve changes in the usual pathological signs (p-tau and beta-amyloid).

Results
Initial High Throughput Screening for STEP Inhibitors
We initially screened ~150,000 compounds from the Laboratory for Drug Discovery in Neurodegeneration library using para-nitrophenyl phosphate (pNPP) as the target substrate (see Text S1 for more information on assay development and secondary screens). Eight compounds were selected for further characterization based on chemical structure and IC_{50} values, which ranged between 1 μM and 9.7 μM (Table S1), and studies of these molecules indicated potent inhibition of STEP activity in neuronal cultures and cortical tissue after intraperitoneal (i.p.) injections in WT mice. However, following resynthesis of several of the lead compounds, we found that they all exhibited essentially no inhibitory activity towards STEP (Figure S1). We therefore tested the possibility that a “contaminant” in the commercial preparations of the lead compounds was inhibiting STEP activity. To address this issue, we performed preparative HPLC on Compound 3 and tested eluted fractions for activity against STEP in the pNPP assay (Figure 1A). Compound 3 appeared as a major peak (fraction 7) on the reverse-phase HPLC preparation and had no inhibitory activity against STEP compared to a second peak that appeared as a late minor peak (fraction 32) that was a potent inhibitor of STEP. Given the high apparent lipophilicity of the unknown, the supplied material was extracted with hexane and recrystallized from methanol. Small pale yellow needle-shaped crystals (0.5–1 cm in length) were obtained in approximately 1% yield. The isolated crystalline material displayed the same HPLC retention, UV absorbance, and STEP inhibitory properties as the initially collected late-eluting peak. The crystalline compound was characterized by X-ray crystallography and found to be sulfur (S8) (Figure 1B).

S8 is poorly soluble in aqueous solution and cannot easily be modified to improve physicochemical properties, redox activity, binding affinity, and selectivity. We therefore sought to identify more conventional inhibitor structures that would improve solubility and enable further refinement through analog preparation and evaluation. We identified the benzopentathiepin core structure present in a number of natural products as the most promising for further investigation (Figure 1B). Natural products incorporating the benzopentathiepin core motif have been reported to have antifungal and antibacterial activity in cell culture as well as cytotoxicity against human cancer cell lines [19,20]. Moreover, amino-substituted derivatives such as varacin and TC-2153 have reasonable solubility in aqueous solution [21,22]. TC-2153 reportedly has a low level of acute toxicity (LD_{50}>1,000 mg/kg) and was proposed to cross the blood brain barrier as evidenced by anxiolytic and anticonvulsant effects in mice [23]. We therefore chose to evaluate the STEP inhibitory activity of TC-2153. We first compared the inhibitory activities of S8 and TC-2153 against recombinant STEP using pNPP assays at several concentrations of the inhibitors. Both S8 and TC-2153 inhibited STEP potently, with IC_{50}s of 17.2±0.4 nM and 24.6±0.8 nM, respectively (Figure 1C–D).

STEP Inhibition Increases the Tyr Phosphorylation of STEP Substrates in Cortical Neurons and in Vivo
We treated cortical neurons for 1 hr with S8 or TC-2153 and determined the Tyr phosphorylation of residues that STEP dephosphorylates on GluN2B (Y1472), Pyk2 (Y402), and ERK1/2 (Y507/175). For S8, there was a significant increase in the Tyr phosphorylation of all three STEP substrates at doses above 0.05 μM, with 1 μM showing maximum inhibition (Figure 2A and Figure S2A for representative blots) (1 μM dose, pGluN2B, 1.33±0.08, p<0.05; pPyk2, 1.49±0.12, p<0.05; pERK1/2, 1.67±0.14, p<0.01). For TC-2153, there was also a significant increase in the Tyr phosphorylation at these sites (Figure 2B and Figure S2B for representative blots) (1 μM dose, pGluN2B, 2.07±0.15, p<0.001; pPyk2, 1.81±0.21, p<0.001; pERK1/2, 2.39±0.18, p<0.001). The decrease in Tyr phosphorylation in the presence of the highest dose of TC-2153 (10 μM) may be due to off-target effects on positive regulatory PTPs. We found similar inverted-U dose–response curves on Tyr phosphorylation of direct PTP targets in previous work with PTP inhibitors [24,25].

We next tested whether S8 and TC-2153 inhibited STEP activity in WT mice in vivo. Six-month-old male mice (C57BL/6)
Figure 1. Compound 3 fractionation and initial characterization. (A) Commercially purchased Compound 3 was dissolved in methanol at 10 mg/mL, and 300 µL portions were injected onto a Zorbax (Agilent) 5 µm 300SB-C18 column (0.94 cm x 25 cm, 3 mL/min 75% methanol/25% pH 4.0 M ammonium acetate). Thirty-five fractions (3 mL each) were collected, evaporated, and reconstituted in 100 µL of DMSO. Fractions were tested with pNPP assays to determine inhibition of STEP activity by using 0.1 µL of each fraction and 100 nM of STEP protein in 96-well plates. DMSO alone...
were injected with vehicle or S8 (0.5, 1, 3 mg/kg, i.p.) and cortices were removed and processed 3 h postinjection. S8 led to a significant increase in the Tyr phosphorylation of GlnN2B, Pyk2, and ERK1/2 [at 1 mg/kg, pGluN2B, 1.31 ± 0.11, p<0.05; pPyk2, 1.46 ± 0.14, p<0.05; pERK1/2, 1.57 ± 0.13, p<0.05] (Figure 2C and Figure S2C for representative blots). Similar results were determined to be 17.2 ± 0.4 nM (mean ± s.e.m., n = 4); (D) The IC50 for TC-2153 was determined to be 24.6 ± 0.8 nM (mean ± s.e.m., n = 4).

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**Mechanism of STEP Inhibition by TC-2153**

We next examined the mechanism by which TC-2153 inhibited STEP. Because the catalytic cysteine in PTPs is prone to sulfhydration, nitrosylation, and oxidative modifications that cause inhibition of phosphatase activity [27–32], we first examined the effect of a reducing agent on STEP inhibition by TC-2153. The addition of reduced glutathione (GSH, 1 mM) decreased the inhibitory activity of TC-2153 by two orders of magnitude in *in vitro* assays [IC50 = 8.79 ± 0.43 μM compared to 24.6 ± 0.8 nM] (Figure 5A). These results suggested an oxidative mechanism for the inhibition of STEP. We established that TC-2153 was stable and did not degenerate in the assay conditions by sensitive 1H NMR monitoring (Figure S4) and was not acting through generation of reactive oxygen species (ROS), which was tested by the addition of catalase or superoxide dismutase to the *in vitro* assay (Table S2). To confirm that ROS are also not released in cortical cultures with TC-2153 treatment, we performed H2O2 colorimetric assay and fluorescence assay with 2,7-dichlorofluorescein diacetate (DCF) and did not observe any significant differences in H2O2 or ROS levels between the TC-2153 treated compared to nontreated control groups (Figure S5).

To evaluate the mode of inhibition, we incubated STEP with TC-2153, subjected the sample to dialysis to remove excess inhibitor, and monitored enzyme activity (Figure 5B). After 24 h of dialysis, STEP remained inhibited, suggesting that TC-2153 acts as an irreversible inhibitor under the conditions used. Using the progress curve method [29], inhibition was also found to be irreversible and the second order rate of inactivation was determined (Figure 5C). A k_diss was determined for pNPP in the presence of varying initial inhibitor concentrations (n=4). Values were then analyzed with nonlinear regression to obtain the kinetic constants: k_diss = 0.0176 ± 0.0007 s⁻¹; K_i = 115 ± 10 mM; k_diss/K_i = 153,000 ± 15,000 M⁻¹s⁻¹. However, STEP activity could be recovered following incubation with GSH or DTT (Figure 5D). Aliquots of STEP were incubated with DMSO control or TC-2153 and were then added to assay buffer containing 1 mM GSH, 1 mM DTT, or water control and allowed to incubate for up to 1 h prior to testing for enzymatic activity. STEP activity was rapidly recovered by both reductants, with DTT showing a greater recovery of activity (75% recovery...
after 1 h, where DMSO control represents 100% activity) compared to GSH (29% recovery after 1 h).

We then performed LCMS analysis to determine the intact protein mass of STEP and STEP+TC-2153. Our intact protein analyses suggest a covalent adduct to STEP. Although we were able to obtain the accurate mass for STEP, we were unable to mass spectrally resolve the heterogeneous mixture of intact STEP+TC-2153 and its covalent adducts with sufficient accuracy to fully interpret the results. Therefore, we next used high-resolution tandem mass spectrometry to focus upon whether TC-2153 might

Figure 2. S8 and TC-2153 increases the Tyr phosphorylation of STEP substrates in neuronal cultures and in vivo. Cortical neuronal cultures were treated with (A) S8 and vehicle (Veh) or (B) TC-2153 and vehicle (0.05, 0.1, 1, and 10 μM) for 1 h. Phosphorylation of GluN2B (Y1472), Pyk2 (Y402), and ERK1/2 (Y204/187) were significantly higher after treatment of cultures with S8 (A) or TC-2153 (B) (*p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with post hoc Bonferroni test). Data represent the phospho-signal normalized to total protein and then to GAPDH (mean ± s.e.m., n = 4). C57BL/6 mice (3–6 mo) were injected with (C) S8 (0.5, 1, 3 mg/kg, i.p.) or (D) TC-2153 (i.p., 1, 3, 6, 10 mg/kg, i.p.) and were sacrificed 3 h later. Cortices were microdissected and lysates spun down to P2 fraction and prepared for Western blotting. Tyrosine phosphorylation status was probed with phospho-specific antibodies to pGluN2B: Tyr1472, pPyk2: Tyr402, and pERK1/2: Tyr204/187 (p<0.05; **p<0.01; one-way ANOVA with post hoc Bonferroni test). Data represent the phospho-signal normalized to the total protein signal and then to GAPDH (mean ± s.e.m., n = 3).

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modify the active site cysteine of STEP. For these experiments, we used WT STEP as well as a STEP mutant in which the catalytic cysteine was changed to serine. Greater than 90% of the primary amino acid sequences were identified by LC-MS/MS for WT STEP or for the STEP mutant, following in-gel tryptic digestion of STEP from nondenaturing (native) preparations. We initially analyzed the catalytic cysteine at position 472 of STEP in the absence of TC-2153 and found a disulfide bridge between Cys\(^{465}\) and Cys\(^{472}\) that presumably forms following tryptic digestion given the positions of Cys\(^{465}\) and Cys\(^{472}\) in the three-dimensional X-ray crystal structure of STEP [30]. This modification was not observed when the catalytic site cysteine (Cys\(^{472}\)) was mutated to serine. Incubation of WT STEP with TC-2153 resulted in the presence of a \(\text{de novo}\) trisulfide within the Cys\(^{465}\)/Cys\(^{472}\) bridge, which was not observed for WT STEP alone or when the catalytic site cysteine (Cys\(^{472}\)) was mutated to serine. Greater than 90% of the primary entries were calculated. There was no significant change in arm entries in the novel object recognition (NOR) task. WT or 3xTg-AD mice had no significant differences in baseline locomotor activity as measured during the habituation phase. Mice received an acute injection of vehicle or TC-2153 (10 mg/kg) 3 h prior to the training phase. Twenty-four h later, mice were subjected to the test phase. Discrimination indexes (DIs) were compared for group differences in object memory. The main effect of genotype \([F(1, 23) = 4.342, p < 0.05]\), treatment \([F(1, 23) = 5.895, p < 0.01]\), and Genotype × Treatment interaction was significant \([F(1, 23) = 4.362, p < 0.05]\). Post hoc analysis indicated that the DI in the AD-TC group was significantly higher than those of the AD-Veh group (TC-treated, 0.354 ± 0.094 versus vehicle-treated, \(-0.259 ± 0.104, p < 0.001\)). In the WT groups, the DI in the TC-2153-treated mice did not differ from the Veh-treated mice (vehicle-treated, 0.166 ± 0.057; TC-treated, 0.504 ± 0.095, \(p > 0.05\)) (Figure 6C).

We then tested the effects of TC-2153 in the reference memory version of the Morris water maze (MMW). A three-way ANOVA analysis revealed a significant Genotype × Treatment × Training Day interaction \((p < 0.05)\). Daily injection of TC-2153 3 h prior to training reversed memory deficits in 3xTg-AD mice on days 5 and 6 of the acquisition phase \((p < 0.01)\) (Figure 6D). The longer escape latency of 3xTg-AD mice injected with vehicle was not attributed to slower swimming speed, as no significant differences were found between groups \((p > 0.05\); two-way ANOVA) (Figure 6E). To confirm memory status, the number of entries in a circular zone located around the previous platform location (target zone) and in the opposite quadrants was evaluated during the probe trial 24 h after the last acquisition day. A three-way ANOVA analysis revealed a significant Genotype × Treatment × Quadrant interaction \((p < 0.004)\). The 3xTg-AD mice treated with TC-2153 spent as much time as WT mice in the target zone, whereas AD mice injected with vehicle showed no preference for the target zone (Figure 6F). All groups had similar escape latencies during the cued trial when the platform was visible, indicating the absence of sensorimotor or motivational deficits to escape from water (WT-Veh, 15.1 ± 1.7 s; WT-TC, 15.6 ± 1.7 s; AD-Veh, 15.3 ± 3.0 s; AD-TC, 16.0 ± 2.3 s; mean ± s.e.m.; \(p > 0.05\); two-way ANOVA). There were no differences in thigmotaxic swimming patterns between any of the tested groups (Figure S7). Taken together, these results demonstrate that TC-2153 significantly improved cognitive functioning in 6-mo-old 3xTg-AD mice.

We next determined whether inhibition of STEP in 12-mo-old 3xTg-AD mice affected beta amyloid or phospho-tau levels. We first needed to confirm that TC-2153 was effective in attenuating cognitive deficits at 12 mo, as these mice have more robust increases in phospho-tau and Aβ levels. We tested the mice with the NOR task and once again found a significant improvement of memory in AD mice treated with TC-2153 during the choice phase (10 mg/kg, i.p.; TC-2153-AD, familiar versus novel, \(p < 0.05\)). TC-2153 did not affect cognitive function in WT mice (Figure S8A). There were no significant changes in Aβ or phospho-tau levels after administration of TC-2153 (Figure S8B–C).

**Table 1. Selectivity of TC-2153 in Vitro.**

| PTP   | Accession* | IC\(_50\) (nM)* | Reference |
|-------|------------|-----------------|-----------|
| STEP(5) | NP_001265167 | 93.3 ± 1.1 | 6          |
| STEP(6) | 62         | 57.3 ± 1.1 | 6          |
| HePTP | AAH01746   | 3635 ± 1.2 | 6          |
| PTP-SL | NP_002840  | 2206 ± 1.3 | 6          |
| PTP1B | NP_002818  | 7239 ± 1.2 | 6          |
| SHP-2 | AAA6610    | 6896 ± 1.2 | 6          |

*from NCBI database; *calculated using GraphPad Prism 5. mean ± s.e.m. (n = 3).

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**Discussion**

STEP function is disrupted in several neurological disorders in addition to AD, including FXS [9], Parkinson’s disease [31], and schizophrenia [8]. The increase in STEP expression in these illnesses is due to either an increase in its translation (in FXS) or a decrease in its degradation (in AD, Parkinson’s disease, and schizophrenia). In contrast, STEP levels or activity are lower in several other disorders, including stress-related conditions [32,33], excessive EtOH consumption [34], and cerebral ischemia [35]. Thus, the current model is that STEP activity must be within an optimal range and that either high or low levels of STEP disrupt...
Synaptic plasticity. Disruption in STEP function has also been implicated in seizures [36], ethanol abuse [37], amphetamine-induced stereotypies [38], and Huntington’s disease [39,40], although the basis for these changes remain to be determined.

In terms of STEP dysfunction, most is known about its role in AD. 

**Figure 3.** TC-2153 selectively inhibits STEP. (A) TC-2153 failed to increase tyrosine phosphorylation of STEP substrates in STEP KO cortical neurons. WT and STEP KO cultures were treated with TC-2153 (0.1 and 1 µM), vehicle (0.1% DMSO), or sodium orthovanadate (Na$_2$VO$_3$, 1 mM) for 1 h. Phosphorylation of GluN2B Y$^{412}$, Pyk2 Y$^{102}$, and ERK1/2 Y$^{204/187}$ was normalized to total protein level and then to GAPDH as loading control (*p<0.05, **p<0.01 one-way ANOVA with post hoc Bonferroni test, compared with veh-treated controls, n=4). (B–G) TC-2153 increased the phosphorylation of ERK1/2 Y$^{204/187}$ and Pyk2 Y$^{102}$ in frontal cortex and hippocampus, but not in cerebellum, spleen, kidney, or pancreas, all tissues that do not have STEP. Mice were injected i.p. with TC-2153 (10 mg/kg; n=4) or vehicle (n=4) and were sacrificed 3 h later. Changes are expressed as the mean ± s.e.m. of pERK1/2 and pPyk2 normalized to total protein level and then to GAPDH (*p<0.05, **p<0.01, two-way ANOVA follow by Tukey’s H.S.D. test).

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6- and 12-mo-old 3xTg-AD mice, a reflection of the suggested role of STEP in the initial synaptopathology of AD [6,16,56]. The administration of TC-2351 did not affect the Aβ and tau brain pathology of the mice, although the question of the efficacy of TC-2153 at advanced stages of pathology remains open. Longitudinal studies with TC-2153 as well as administration of TC-2153 to other AD models will help address this question. Longitudinal studies will also address the long-term preventive effects of STEP inhibition on cognitive decline. Finally, it is important to determine whether TC-2153 is effective in other animal models of neuropsychiatric diseases in which STEP activity is elevated and these studies have begun.

Figure 4. TC-2153 does not induce neuronal cell death. Cortical cells were incubated with TC-2153 (1, 10, and 100 μM) for 1 h along with positive controls: glutamate (100 μM), SDS (0.02%), and Triton-X-100 (0.15%) (A), and at multiple time points (1 h, 3 h, 24 h, and 48 h) (B). Bovine LDH was used as a LDH-positive control. The media was collected and analyzed for LDH. The assay quantitatively measures LDH, a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a 30-min coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells. doi:10.1371/journal.pbio.1001923.g004
Figure 5. **TC-2153 targets the active site cysteine of STEP.** (A) STEP activity was measured with pNPP and IC\(_{50}\)s were 24.6±0.8 nM and 8.79±0.43 μM in the absence and presence of 1 mM GSH (mean ± s.e.m., n=2). (B) STEP (200 nM) and TC-2153 (1 μM) or DMSO control) were incubated for 60 min to inhibit enzymatic activity prior to dialysis. Aliquots were tested against pNPP (mean ± s.e.m., n=4). (C) The progress curve method was used to determine the second-order rate constant: \(k_{\text{obs}}/K_i = 153,000 \pm 15,000 \text{ M}^{-1}\text{s}^{-1}\) (mean ± s.e.m., n=4). (D) STEP (200 nM) and TC-2153 (5 μM) were incubated for 10 min and then incubated with GSH or DTT (1 mM each) or water (no reductant) for 0, 15, 30, or 60 min, and the enzymatic activity of STEP was measured using the pNPP assay (mean ± s.e.m., n=4). (E) Detection of trisulfide bridge formation between C465 and C472. The peptide sequence in (1) illustrates the trisulfide bridge along with the b and y-ion assignments detected in the MS/MS fragmentations spectrum (see Figure S6). (2) compares the 3D elution profile of the trisulfide peptide (mass = 2,746.242 Da). The trisulfide bridge (modified) peptide is only detected in the WT STEP in the presence of TC-2153. The corresponding disulfide (non-modified) peptide (mass = 2,714.254 Da) was detected in WT STEP (see Figure S6).

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**Materials and Methods**

**Ethics Statement**  
The Yale University Institutional Animal Care and Use Committee approved all proposed use of animals. All animal work was carried out in strict accordance with National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

**Reagents**  
pNPP, 2-(N-morpholino) ethanesulfonic acid (MES), sodium orthovanadate, ATP, and all buffer components were purchased from Sigma-Aldrich (St. Louis, MO). Malachite Green reagent kit was purchased from Bioassay system (Hayward, CA); 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) and EnzChek phosphatase assay kit were purchased from Invitrogen (Carlsbad, CA). The 96- and 384-well clear polystyrene plates were purchased from VWR (Radnor, PA), and 384-well white plates were purchased from Nalge Nunc International (Rochester, NY). Full-length STEP\textsubscript{46} was used in the initial library screen. STEP\textsubscript{46} cDNA was cloned into pGEX2T and transformed into BL21 (DE3) E. coli cells. STEP (20 mg) was purified on a glutathione sepharose column to immobilize the GST-tagged protein [14]. The column was loaded, washed, and bound protein eluted using Fast Protein Liquid Chromatography. For some of the biochemical experiments, we purified WT TAT-STEP\textsubscript{46} and TAT-STEP\textsubscript{44} (C to S) proteins, the latter containing a mutation at its catalytic cysteine within the active site that renders the enzyme inactive [15]. The assay development for the HTS is described in detail in Figure S9, biochemical characterization of STEP is in Figure S10, and the synthesis of TC-2153 (benzopentathiepin 8-(trifluoromethyl)-(C to S)) proteins, the latter containing a mutation at its catalytic cysteine within the active site that renders the enzyme inactive [15]. The assay development for the HTS is described in detail in Figure S9, biochemical characterization of STEP is in Figure S10, and the synthesis of TC-2153 (benzopentathiepin 8-(trifluoro-methyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride) is described in detail in Figure S11.

**Cell-Based Assay**  
Primary cortical neurons were isolated from Sprague Dawley rat embryos (E18) (Charles River Laboratories, Wilmington, MA) as previously described [15]. In some experiments, cortical neurons were made from WT and STEP KO mouse embryos (E18). Neurons were allowed to grow for 18–21 d in CO\textsubscript{2} incubator before addition of compounds at indicated doses for 1 h. Immediately following treatments, neurons were lysed in Radio-Immunno Precipitation Assay (RIPA) buffer supplied with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitors (NaF and Na\textsubscript{3}VO\textsubscript{4}). All experiments were replicated four times with four independent batches of cultures.

**In Vivo Work**  
Wild-type, male C57BL/6 mice (3–6 mo) were used for all studies. An initial dose–response curve was carried out using S8 (0.5, 1, and 3 mg/kg, i.p.) or TC-2153 (1, 3, 6, and 10 mg/kg, i.p.). Pilot studies were conducted to optimize the time after i.p. injection when STEP substrates showed maximum Tyr phosphorylation (1–3 h). Cortical tissues were dissected out 3 h postinjection and processed for subcellular fractionation. We homogenized brain tissue in buffer containing (in mM): 10 Tris-HCl, pH 7.6, 320 sucrose, 150 NaCl, 5 EDTA, 5 EGTA, 20 NaF, 1 Na\textsubscript{3}VO\textsubscript{4} and protease inhibitors (TEVP). Homogenates were centrifuged at 800 \times g to remove nuclei and large debris (P1). Synaptosomal fractions (P2) were prepared from S1 by centrifugation at 9,200 \times g for 15 min. The P2 pellet was washed twice and was resuspended in TEVP buffer. In some experiments, mice were injected with S8 (1 mg/kg, i.p.) or TC-2153 (3 mg/kg, i.p.), and cortex, cerebellum, and spleen were removed to test for the in vivo inhibition of the highly related PTPs, He-PTP, and PTP-βL [57–60].

**Western Blotting**  
Samples were prepared and resolved by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with phosphospecific antibodies (anti-pY\textsuperscript{Y204/187} ERK1/2, anti-pY\textsuperscript{Y492} Pyk2, anti-pY\textsuperscript{Y412} GluN2B) or total protein antibodies (anti-ERK2, anti-Pyk2, and anti-NR2B) overnight at 4°C. All antibodies used are listed in Table S3. Immunoreactivity was visualized using a Chemiluminescent substrate kit (Pierce Biotechnology, Rockford, IL) and detected using a G:BOX with the image program GeneSnap (Syngene, Cambridge, UK). All densitometric quantifications were performed using the Genetools program.

**Isolation and Identification of S8 as Active Constituent of Compound 3**  
Compound 3 was extracted with hexane and the residue obtained after rotary evaporation, then recrystallized from methanol. Small pale yellow needle-shaped crystals (0.5–1 cm) were obtained in approximately 1% yield. The isolated crystalline material displayed the same HPLC retention, UV absorbance, and STEP inhibitory properties as the initially collected late-eluting peak. The crystalline compound was characterized by the X-Ray Crystallographic Facility of the Yale University Department of Chemistry and found to be sulfur (S8).

**General Procedures for Determination of Inhibitor IC\textsubscript{50}**  
Reaction volumes of 100 \muL were used in 96-well plates. We added 75 \muL of water to each well, followed by 5 \muL of 20 \times buffer (stock, 1 M imidazole HCl, pH 7.0, 1 M NaCl, 0.02% Triton-X 100). We added 5 \muL of the appropriate inhibitor dilution in DMSO, followed by 5 \muL of phosphatase (stock, 0.2 \muL, 10 nM in assay). The assay plate was then incubated at 27°C for 10 min with shaking. The reaction was started by addition of 10 \muL of 10 \times pNPP substrate (stock, 5 mM, 500 \muM in assay), and reaction progress was immediately monitored at 405 nm at a temperature of 27°C. The initial rate data collected were used for determination of IC\textsubscript{50} values. For IC\textsubscript{50} determination, kinetic values were obtained directly from nonlinear regression of substrate–velocity curves in the presence of various concentrations of inhibitor using one site competition in GraphPad Prism v5.01 scientific graphing software. The K\textsubscript{m} value of pNPP in this system was determined to be 745 \muM and was used in the kinetic analysis.

For experiments with catalase or superoxide dismutase (SOD), 10 \muL of the appropriate enzyme stocks (catalase, 800 U/mL stock, 80 U/mL in assay; SOD, 1,000 U/mL stock, 100 U/mL in assay) were added prior to addition of the inhibitor and STEP.

For the experiments with glutathione reducing agent, 10 \muL of glutathione (stock, 10 mM, 1 mM in assay) or water control was added before the inhibitor stocks, and only 65 \muL of water was added initially to maintain the 100 \muL assay volume. Once the inhibitor stocks were added, the assay plate was allowed to incubate 10 min at 27°C with shaking. This was followed by addition of phosphatase (stock, 0.4 \muL, 20 nM in assay) and another 10-min incubation at 27°C prior to addition of pNPP substrate.

**Selectivity of TC-2153 Against STEP in Vitro**  
Purification of GST-tagged STEP\textsubscript{46} and STEP\textsubscript{44} constructs was as previously described [13,14]. The GST-SHP-2 construct was a generous gift from Dr. A. M. Bennett (Yale University). The
STEP Inhibitor Reverses Cognitive Deficits in AD Mice

A

Number of entries

|   | Veh | TC |
|---|-----|----|
| WT |     |    |
| AD |     |    |

B

% alternation

|   | Veh | TC |
|---|-----|----|
| WT |     |    |
| AD |     |    |

C

Discrimination Index

|   | Veh | TC |
|---|-----|----|
| WT |     |    |
| AD |     |    |

D

Escape latency (s)

|   | AD Veh | WT Veh |
|---|--------|--------|
| AD TC |        |        |
| WT TC |        |        |

E

Speed (cm/s)

|   | AD Veh | WT Veh |
|---|--------|--------|
| AD TC |        |        |
| WT TC |        |        |

F

Number of entries

|   | Veh | TC |
|---|-----|----|
| WT |     |    |
| AD |     |    |

Target

|   | Veh | TC |
|---|-----|----|
| WT |     |    |
| AD |     |    |

Opposite

|   | Veh | TC |
|---|-----|----|
| WT |     |    |
| AD |     |    |
Figure 6. TC-2153 improves cognitive deficits in 3xTg-AD mice. WT and 3xTg-AD mice (male, 6 mo old) were treated with vehicle or TC-2153 (10 mg/kg, i.p.) and tested in the Y-maze, NOR, and MWM tasks. (A and B) Y-maze, number of arm entries and percentage spontaneous alternations were calculated (p < 0.05, paired t test, AD-TC versus AD-Veh) (WT, n = 20/group; AD, n = 11/group). (C) NOR, the DI of each group was calculated (**p < 0.001, AD-TC versus AD-Veh) (WT, n = 9/group; AD, n = 16/group). (D) MWM, the 3xTg-AD mice injected with vehicle (n = 6) showed longer escape latency before entering the hidden platform (3 trials/day; 60 ± 30 m intertrial interval) when compared to AD mice treated with TC-2153 (n = 7) or WT mice injected with vehicle (n = 12) or TC-2153 (n = 13) (three-way ANOVA). * and ** represents a statistical significant variation between AD-Veh mouse and AD-TC or WT-Veh, respectively. (E) Swim speed at each training day was not significantly different between groups (three-way ANOVA). (F) Number of entries in a circular zone positioned around the previous platform location and in the opposite quadrants. * indicates a difference for the target and opposite quadrant within each group. Data are mean ± s.e.m. * p < 0.05; ** p < 0.01.

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Determination of TC-2153 Stability in Imidazole Buffer

To monitor the stability of TC-2153 in the imidazole buffer, 20 μL of 20 mM TC-2153 stock in DMSO was added to an Eppendorf tube. The solution was diluted to 400 μL (1 mM TC-2153 final concentration, 5% final DMSO) with either water or the pH 7.0 imidazole buffer. The tube was allowed to incubate at ambient temperature with shaking for 1 h. The mixture was diluted with 150 μL of DMSO-d6, and transferred to an NMR tube containing a capillary of trifluoroacetic acid as an external standard (~76.53 ppm). The stability of the compound in the buffer was confirmed by observing no differences in the sensitive 19F-NMR spectra (Figure S4). As a control for compound modification, the experiment was repeated with the addition of 1 mM GSH in the incubation buffer.

Dialysis

STEP was diluted into 1× assay buffer with either inhibitor or DMSO control (final volume, 2.9 mL; final concentration, 1 μM STEP, 5 μM TC-2153; 50 mM imidazole HCl, pH 7.0, 50 mM NaCl, 0.001% Triton-X 100, 5% v/v DMSO). The samples were shaken at room temperature for 1 h to inhibit STEP. Each sample was then transferred to a separate Thermo Scientific Slide-A-Lyzer dialysis cassette with a 10,000 MW cutoff and 0.5–3.0 mL sample volume and was dialyzed into 1 L of 1× assay buffer over 24 h in a 4 °C cold room. Aliquots of approximately 100 μL were removed from the dialysis cassette at 0, 4, and 24 h time points. Protein concentration was determined by reading absorbance at 280 nm compared to a standard curve for STEP. The samples were diluted to 100 nM in 100 μL of 1× assay buffer. The reaction was started by addition of 10 μL of 10× pNPP substrate (stock, 20 mM, 1.81 mM in assay; total assay volume, 110 μL), and reaction progress was immediately monitored at 405 nm at a temperature of 27 °C. The initial rate data collected were used to determine enzyme activity standardized to the DMSO controls.

Recovery of STEP Activity by Reducing Agents

STEP was diluted to 200 nM in water, and aliquots of this stock were mixed with DMSO (5% by volume) or TC-2153 (5 μM final concentration, 5% DMSO by volume) and incubated at ambient temperature on a shaker for 10 min. Each sample was aliquoted out and 30 μL was transferred to wells of a 96-well microtiter plate containing 40 μL of 2× assay buffer with added reductant (GSH or DTT, 1 mM final concentration) and shaken for 0, 15, 30, or 60 additional minutes at ambient temperature. The reaction was started by addition of 10 μL of 10× pNPP substrate (stock, 20 mM, 2 mM in assay; total assay volume, 100 μL), and reaction progress was immediately monitored at 405 nm at a temperature of 27 °C. The initial rate data collected were used to determine enzyme activity standardized to the DMSO controls.

Determination of Inhibition Constants

The second-order rate constant of inactivation for TC-2153 was determined under pseudo-first-order conditions using the progress curve method [29]. Assay wells contained a mixture of the inhibitor (800, 400, 200, 100, 50, 0 nM) and 745 μM of pNPP (Km = 745 μM) in buffer (50 mM imidazole pH 7.0, 50 mM NaCl, 0.01% Triton-X 100). Aliquots of STEP were added to each well to initiate the assay. The final concentration of STEP was 10 nM. Hydrolysis of pNPP was monitored spectrophotometrically for 30 min at an absorbance wavelength of 405 nm. To determine the inhibition parameters, time points for which the control ([I] = 0) was linear were used. A kobs was calculated for each inhibitor concentration via a nonlinear regression of the data according to the equation \[ P = \frac{(v_i/k_{obs})(1-exp(-k_{obs}t))}{(P_i + K_i + (1 + [S]/K_m))} \] (where P, product formation; vi, initial rate; t, time) using Prism 5 (GraphPad). Because kobs varied hyperbolically with [I], nonlinear regression was performed to determine the second-order rate constant, ksteady/Ki, using the equation kobs = ksteady/[I] + K (1 + [S]/Km). Assays were done in triplicate or quadruplicate on two separate occasions. The average and standard deviation of the assays is reported.

Mass Spectrometry

To explore the protein modification(s) of STEP upon TC-2153 inhibition, reduced and nonreduced gel-purified STEP (WT or C4728 mutant) proteins were analyzed by high-resolution tandem mass spectrometry. Briefly, purified STEP WT or C-S mutant proteins (10 μg each) were incubated with vehicle (1% DMSO) or TC-2153 (10 μM in 1% DMSO) in assay buffer (50 mM imidazole, pH 7.0) at room temperature (25 °C) for 30 min. Samples were resolved on 8% SDS-PAGE or nondenaturing PAGE, and proteins were visualized by Coomassie Blue staining. Gel bands were excised and kept at -80°C until use. Excised gel bands corresponding to the mutant and WT STEP with and without TC-2153 were in-gel trypsin digested under native conditions (w/o reducing agent) overnight. Peptides were extracted from the digested samples with 80% acetonitrile containing 0.1% trifluoroacetic acid, and then dried under SpeedVac. Samples were then reconstituted in minimum solution containing 0.1% TFA, and loaded onto a RP C18 nanoACQUITY UPLC column (1.7 μm BEH130 C18, 75 μm×250 mm, with a 5 μm
Symmetry C18 2G-V/M Trap [180 μm×20 mm]). Eluted peptides were directly infused into an Orbitrap Elite LC MS/MS system running data-dependent acquisition. Acquired data were processed utilizing Progenesis LCMS software (Nonlinear Dynamics) and Mascot Search engine with user-defined possible modification(s) search criteria.

Behavioral Analysis

A previous study showed that genetic reduction of STEP significantly reversed cognitive deficits in 6-mo-old 3xTg-AD mice [16]. Here we were interested in testing whether pharmacologic inhibition of STEP with TC-2153 had a similar beneficial effect in this AD mouse model. We also wanted to test whether TC-2153 had any effects on cognition in WT mice. Mice completed all tests in the following order: Y-maze alternation, NOR, and MWM. For all behavioral tests, WT or 3xTg-AD mice were randomly allocated to treatment with either vehicle or TC-2153.

Open Field Activity

To assess locomotor activity and exploratory behavior, mice were placed in a square box (60 cm×60 cm) and habituated for 5 min. Mice were treated with vehicle or TC-2153 (10 mg/kg, i.p.) 3 h prior to the exploration phase of the experiment. A video camera mounted directly above the box recorded the trials and ANY-maze software analyzed the distance traveled and time spent in the center of the box.

Y-Maze Alternation Task

A crossover design was used in the Y-maze and NOR tasks, such that mice initially treated with vehicle (or TC-2153) were retested following a 15-d drug-free period and received TC-2153 (or vehicle). The Y-maze apparatus consisted of three dark gray arms (42 cm×8.2 cm). Each mouse was treated with vehicle or TC-2153 (10 mg/kg, i.p.) 3 h prior to the experiment, after which they were placed at the end of one arm (the designated “start arm”) and allowed to freely explore the maze for 5 min. The total number of arm entries was recorded, as was the number of entries representing alternation behavior (i.e., sequential entry into all three arms). All four paws of the mouse had to enter an arm for it to count as an arm entry. Percentage spontaneous alternation = (number of alternations)/(total arm entries – 2). A crossover design was used after a drug-free period of 15 d, with groups previously treated with vehicle then receiving TC-2153 and vice versa. A total number of 20 WT and 11 AD mice were used in the Y-maze task.

NOR Task

Mice were first habituated to the task by allowing them to explore an empty white open field box (60 cm×60 cm) for 5 min. Twenty-four hours later, mice were treated with vehicle or TC-2153 (10 mg/kg, i.p.) 3 h prior to the sample phase. After the elapsed time, the mice completed the sample phase in which they were placed into the open field box with two identical objects located in the right and left corners. Mice were allowed to freely explore until they had accumulated a total of 30 s of object exploration (i.e., contact with the object with the nose and/or front paws), at which point the trial ended. The time spent with each object was recorded. Mice that were unable to complete the 30 s exploration within 20 min during the sample phase were excluded from the study (WT = 1 and AD = 3). Twenty-four hours later, mice completed the choice phase that was conducted in an identical manner to the sample phase except that one of the objects was substituted by a novel object and trial duration was set at 5 min. No drug treatments were given during the choice phase. Fifteen WT mice from the initial cohort were used to optimize the novel object conditions (to identify object pairings of inherent equal interest). Location of the novel object (left or right side) was counterbalanced to minimize possible bias. A crossover design was used, with a different set of objects after a 15-d drug-free period. DI was used to evaluate the effects of the TC-2153 compound on object memory in 6-mo-old 3xTg-AD mice. The DI was calculated for each subject by using the following formula: DI = (time spent exploring novel object – time spent exploring familiar object)/(total time spent exploring both objects). A DI of 0 is indicative of chance performance (i.e., no preference for one object compared to another), whereas a positive index (ranging from 0 to 1) indicates preference for novel object compared to familiar. In order to achieve greater statistical power, a second cohort of AD mice (n = 7) was run in the novel object test using a crossover design. Any value lower or higher than two times standard deviation away from the mean was considered an outlier and was excluded from the study (AD = 1). A total number of 9 WT and 16 AD mice were used in the NOR task.

MWM

The reference memory version of the MWM task was performed as described previously [61]. A crossover design was not used in the MWM task, as the mice were randomly assigned to each treatment condition and can be exposed to the task only once. Briefly, animals were trained to swim in a 1.4 m diameter pool to find a submerged platform (14 cm in diameter) located 1 cm below the surface of water (24°C), rendered opaque by the addition of nontoxic white paint. Animals were pseudo-randomly started from a different position at each trial and used distal visual-spatial cues to find the hidden escape platform that remained in the center of the same quadrant throughout all training days. Training measures included escape latency to reach the platform, swim speed, and thigmotaxis. When animals failed to find the platform, they were guided to it and remained there for 10 s before removal. At 24 h after the acquisition phase, the platform was removed and a probe test of 90 s was given to evaluate the number of entries in a circular zone (three times the platform diameter) positioned around the previous platform location (target zone) and in the opposite quadrants. To assess visual deficits and motivation to escape from water, the probe test was followed by a cued task (60 s; three trials per animal) during which the platform was visible. The visible platform was moved to different locations between each trial. After each trial, animals were immediately placed under a warming lamp to dry to prevent hypothermia. The experimenter was blind to mouse genotype when administering TC-2153 or vehicle to AD mice (AD-TC, n = 7; AD-Veh, n = 6) or WT mice (WT-TC, n = 13; WT-Veh, n = 12). Behavioral data from training, probe, and cued trials were acquired and analyzed using the ANY-maze automated tracking system (Stoelting, IL, USA).

Data Analysis

A two-way analysis of variance (ANOVA) with genotype as the between-subject factor and treatment as the within-subject factor was used for the Y-maze and object recognition tasks. Percent alternation (Y-maze) and DI (object recognition) were the dependent measures. Post hoc analyses were carried out using Bonferroni’s multiple comparison tests as appropriate (GraphPad Prism, La Jolla, CA). In an older (12 mo) cohort of WT and 3xTg-AD mice, the exploration time (NOR task) did not meet the assumption of normality and equal variance, and raw data (seconds) were converted using square-root transformation.
followed by t test. For the MWM training and probe sessions, a three-way repeated measures ANOVA with two-between-subject (Genotype, Treatment) and one within-subject (training day or quadrant) factor was used. Escape latency (training) and number of entries (probe) were the dependent measures (StatView, Cary, NC). Swim speed and escape latency during the probe and cued trials, respectively, were analyzed using a two-way ANOVA with genotype and treatment as the between-subject factors. Post hoc analyses were conducted on significant results. For cell-based assays, one-way ANOVA with post hoc Bonferroni test was used to determine statistical significance. All data were expressed as mean ± s.e.m.

Supporting Information

Figure S1 Compounds were resynthesized and found to be inactive against STEP in the pNPP assay. Dose-response inhibition of STEP activity by commercial or resynthesized compounds was measured in the pNPP assay. Curves were obtained by fitting data to a second-order polynomial model.

Figure S2 Representative Western blots for histograms shown in Figure 2.

Figure S3 Representative Western blots for histograms shown in Figure 3.

Figure S4 TC-2153 stability in imidazole buffer. TC-2153 dissolved in water (A) and pH 7.0 imidazole buffer (B) were incubated for 1 h. For each experiment, the compound purity was determined using sensitive 19F-NMR, which is a sensitive technique for monitoring compound purity. As a control for monitoring modification of TC-2153, the compound was also incubated with 1 mM GSH in pH 7.0 imidazole buffer for 1 h (C), with compound modification clearly observed by 19F-NMR due to the appearance of multiple new peaks at a different chemical shift.

Figure S5 TC-2153 treatment does not generate ROS in cortical neuronal cultures (18 d in vitro). (A) H2O2 levels remain unchanged with 0.1, 1, or 10 μM TC-2153 treatment or with 200 U/ml superoxide dismutase (SOD) and catalase treatment. (B) ROS level, measured with the DCF fluorescence, is not increased with the indicated TC-2153 treatment.

Figure S6 MS/MS verification for the presence of a trisulfide peptide between C4105 and C4172 in TAT-STEP. The upper MS/MS spectrum shows the peaks observed for the fragmentation of the trisulfide peptide and assignments of the b- and y-ions. The inverted lower MS/MS spectrum shows the corresponding fragmentation of a peptide with a disulfide (from WT STEP in the absence of TC-2153), which has a mass difference of 32 Da (corresponding to a sulfur mass) from the trisulfide peptide. Peaks labeled in the lower spectrum with **“*”** are 32 Da less (corresponding to a sulfur mass difference in the fragment ions) than their counterpart y-ions in the upper mass spectrum. The inset details the 32 Da mass differences for the y24**”** fragment between the disulfide and trisulfide. Peaks labeled as “**“*”** in the lower MS/MS spectrum does not have a mass shift between the modified and nonmodified peptide fragments because they do not contain the two cysteines that form the di- and tri-sulfide bridge.

Figure S7 No excessive and persistent thigmotaxic problem in 3xTg-AD mice in the MWM. There was no significant difference in percent time spent in zone A at the periphery of the tank as well as in zone B and C between 3xTg-AD and WT mice following treatment with vehicle or TC-2153 (three-way ANOVA).

Figure S8 TC-2153 has no effect on Aβ or phospho-tau levels in 12-mo-old 3xTg-AD mice. (A) Three hours prior to training, WT and 3xTg-AD mice were given vehicle or TC-2153 (10 mg/kg, i.p.). Time spent with either a novel or familiar object was recorded using ANY-maze software. Square-root transformation was used to meet the assumptions of normality and equal variance of the raw data. All histograms are presented as means ± s.e.m. Student’s t test was applied to determine significance differences (*p* < 0.05; WT-Veh, *n* = 10; WT TC, *n* = 11; AD-Veh, *n* = 22; AD-TC, *n* = 19). (B) Cortical homogenates from vehicle or TC-2153–treated WT or 3xTg-AD mice were immunoprecipitated using 6E10 antibody and blotted with 6E10 antibody, CTFs and Aβ are indicated by arrowheads. Representative 7PA2-CM (Aβ-enriched conditioned medium) immunoprecipitation is shown on right panel. Data are presented as means ± s.e.m. Quantification of Aβ levels showed no significant difference in vehicle or TC-2153–treated 3xTg-AD brain samples (Student’s t test, *p* > 0.05; *n* = 3). (C) Cortical membrane fractions of vehicle or TC-2153–treated WT or 3xTg-AD mice were probed with p-tau (AT810) and total tau (HT7) antibody. Data are presented as means ± s.e.m. Quantification of p-tau levels showed no significant difference in vehicle or TC-2153–treated 3xTg-AD brain samples (Student’s t test, *p* > 0.05; *n* = 6).

Figure S9 Assay development. (A) Determination of Km for pNPP with STEP. We reacted 200 nM STEP with different concentrations of pNPP. The OD405 was read at 5 min after the reaction was initiated. The Km was determined to be 170 μM (n = 5). (B) Determination of Km for DiFMUP with STEP. We reacted 200 nM STEP with different concentrations of DiFMUP. The fluorescence was read at 5 min after reactions started. Km was determined to be 18.3 μM. The DiFMUP final concentration used in confirmatory screening was 20 μM. (C) Z’ factor from representative plates from the primary screen for STEP inhibitors. The majority of the plates were between 0.7 and 0.9, indicating a robust assay.

Figure S10 Characterization of STEP. (A) pH dependency of STEP. Enzyme activity was assayed in buffers with varying pHs. (B) Salt dependency of STEP. Activity was assayed in the presence of increasing concentration of NaCl. (C) DMSO tolerance of STEP. STEP activity was determined in the presence of increasing concentrations of DMSO. (D) Stability of STEP at room temperature. STEP was left at room temperature for indicated time periods prior to initiation of the reaction by addition of pNPP substrate.

Figure S11 Synthesis of TC-2153. Scheme of large-scale synthesis of TC-2153.

Table S1 Eight compounds were selected for further characterization based on chemical structure and IC50 values.

Table S2 In vitro inhibition of STEP by TC-2153. No change was observed in vitro in the activity of STEP or inhibition by
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