Protection of primary neurons and mouse brain from Alzheimer’s pathology by molecular tweezers

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Alzheimer’s disease is a devastating cureless neurodegenerative disorder affecting > 35 million people worldwide. The disease is caused by toxic oligomers and aggregates of amyloid β protein and the microtubule-associated protein tau. Recently, the Lys-specific molecular tweezer CLR01 has been shown to inhibit aggregation and toxicity of multiple amyloidogenic proteins, including amyloid β protein and tau, by disrupting key interactions involved in the assembly process. Following up on these encouraging findings, here, we asked whether CLR01 could protect primary neurons from Alzheimer’s disease-associated synaptotoxicity and reduce Alzheimer’s disease-like pathology in vivo. Using cell culture and brain slices, we found that CLR01 effectively inhibited synaptotoxicity induced by the 42-residue isoform of amyloid β protein, including ~80% inhibition of changes in dendritic spines density and long-term potentiation and complete inhibition of changes in basal synaptic activity. Using a radiolabelled version of the compound, we found that CLR01 crossed the mouse blood–brain barrier at ~2% of blood levels. Treatment of 15-month-old triple-transgenic mice for 1 month with CLR01 resulted in a decrease in brain amyloid β protein aggregates, hyperphosphorylated tau and microglia load as observed by immunohistochemistry. Importantly, no signs of toxicity were observed in the treated mice, and CLR01 treatment did not affect the amyloidogenic processing of amyloid β protein precursor. Examining induction or inhibition of the cytochrome P450 metabolism system by CLR01 revealed minimal interaction. Together, these data suggest that CLR01 is safe for use at concentrations well above those showing efficacy in mice. The efficacy and toxicity results support a process-specific mechanism of action of molecular tweezers and suggest that these are promising compounds for developing disease-modifying therapy for Alzheimer’s disease and related disorders.
Introduction

Alzheimer’s disease is the leading cause of dementia, affecting >35 million people worldwide (Alzheimer’s Association, 2011). Neuropathologically, Alzheimer’s disease is characterized by accumulation of neuritic plaques, comprising mainly fibrillar amyloid β protein and neurofibrillary tangles made of filamentous hyperphosphorylated tau (p-tau). The inertive assault on susceptible neurons is believed to be mediated by amyloid β and possibly tau oligomers that disrupt synaptic communication (Kirkadzde et al., 2002; Kayed, 2010) before cognitive symptoms can be detected. Thus, if formation of these toxic assemblies can be prevented before overt neurodegeneration occurs, the brain may be able to mount a defence and possibly recover.

We sought compounds that would modulate the assembly of amyloid β and tau and inhibit their toxicity at the earliest possible step. We identified Lys residues as attractive targets where interference would disrupt assembly because these residues have a unique ability to participate in both hydrophobic and electrostatic interactions involved in the assembly process of amyloidogenic proteins, including amyloid β and tau (Petkova et al., 2002; Lazo et al., 2005; Huang and Stultz, 2008; Li et al., 2009; Usui et al., 2009; Cohen et al., 2011; Vana et al., 2011; Sinha et al., 2012b). Therefore, we conjectured that compounds that bind specifically to Lys residues might inhibit formation of toxic amyloid β and tau assemblies.

Lys-specific ‘molecular tweezers’, originally reported by Fokkens et al. (2005), bind to Lys residues with a dissociation constant of ~20 μM. Their specificity for Lys results from the Lys butylene moiety threading through the molecular tweezer cavity and facilitating hydrophobic interactions with the molecular tweezer sidewalls, and the ε-ammonium group’s electrostatic attraction to the negatively charged bridgehead groups of the molecular tweezers. Thus, molecular tweezers use the same types of interactions involved in early amyloid β assembly (Lazo et al., 2005), allowing them to compete with these interactions and disrupt amyloid β assembly and toxicity. A similar mechanism is expected to inhibit tau toxicity.

Recently, we have shown that a molecular tweezer derivative called CLR01 is a potent inhibitor of assembly and toxicity of multiple disease-related amyloidogenic proteins, including amyloid β and tau (Sinha et al., 2011). CLR01 was found to inhibit amyloid β oligomerization, dissociate preformed amyloid β fibrils and stabilize non-toxic amorphous assemblies. Mass spectrometry and nuclear magnetic resonance experiments confirmed binding of CLR01 to Lys in amyloid β at the earliest stages of assembly (Sinha et al., 2011). CLR01 was found to inhibit amyloid β–induced toxicity in differentiated rat pheochromocytoma cells and in primary rat hippocampal cultures or mixed neuronal/gial cultures (Sinha et al., 2012a) at micromolar concentrations. In addition, CLR01 was found to rescue zebrafish expressing human wild-type α-synuclein from severe deformation and early death by keeping the intracellular α-synuclein soluble and allowing its proteasomal clearance (Prabhudesai et al., 2012).

In light of these encouraging results, we evaluated the effect of CLR01 on synaptic dysfunction in vitro and on Alzheimer’s disease–related brain pathology in transgenic mice. We also studied several aspects of CLR01’s drug-like characteristics and possible toxicity to evaluate the potential of molecular tweezers as mechanism-based drugs for Alzheimer’s disease and related diseases. The initial assessment described here suggests that CLR01 is an efficacious and safe drug lead.

Materials and methods

Additional details on methods are available in the online Supplementary material.

Molecular tweezers

CLR01 and CLR03 were prepared and purified as described previously (Talbiersky et al., 2008).

Protein and sample preparation

The 42-residue isoform of amyloid β (amyloid β42) was obtained from the University of California–Los Angeles Biopolymers Laboratory or from AnaSpec. Sample preparation was performed as described previously (Maiti et al., 2010). Briefly, amyloid β42 was disaggregated by treatment with 1,1,1,3,3,3-hexafluoroisopropanol (Sigma) as described (Rahimi et al., 2009). Dried peptide films were stored at −20°C until use. For dendritic spine experiments, 27-μg films were dissolved in 20 μl of 60 mM sodium hydroxide, sonicated for 1 min, and then diluted to 30 μM with 180 μl of Neurobasal® media. For electrophysiological experiments, 50-μg films were dissolved in 11 μl of dimethyl sulphoxide to reach a concentration of 1 mM and sonicated for 10 min. For basal synaptic transmission experiments, 2 μl of the solution was then diluted to 5 ml with culture media to reach a final concentration of 400 nM. For long-term potentiation experiments, 40 μl of amyloid β42 from 100 μM stock solution was incubated for 12 h at 4°C to promote protein oligomerization. This preparation was further diluted with artificial CSF in the absence or presence of CLR01 to reach the final amyloid β42 concentration of 200 nM immediately before experiments.

Primary hippocampal neuronal culture

Hippocampal neurons were prepared as described previously (Maiti et al., 2010) and plated on poly-L-lysine-coated (0.1 mg/ml) 13-mm round glass cover slips in 24-well culture plates at a density of 300 000 cells/well.

Dendritic spine morphology

Experiments were performed as described previously (Maiti et al., 2010). Briefly, rat primary hippocampal neurons were grown for
3 weeks. Half of the growth medium (1 ml) was removed and new media (600 µl) containing 30 µM freshly prepared amyloid β42 (200 µl) and media containing 300 µM CLR01 or CLR03 (200 µl) were immediately added, resulting in a final concentration of 3 µM amyloid β42 and 30 µM CLR01 or CLR03. After 72 h of incubation, neurons were fixed with 4% paraformaldehyde and stained with 1,1'-diocadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (Dil, Invitrogen). The neurons were visualized using a confocal laser-scanning microscope (Leica) at ×2000 magnification. The total number of spines per 100 µm were counted using ImageJ (Abramoff et al., 2004). At least 100 dendritic branches from 10 to 15 individual neurons were selected per experimental condition. All data are shown as means ± standard error measure, and the level of significance was set at P < 0.05. Statistical analysis was performed in Prism 5.0c (GraphPad) using one-way ANOVA with Tukey’s post hoc multiple-comparison test.

Autaptic neuron culture preparation and synaptic transmission studies

Basal synaptic transmission was studied in autaptic microcultures of hippocampal neurons using the patch-clamp technique in the whole-cell configuration, as described previously (Ripoli et al., 2012). Briefly, neurons were voltage clamped at a membrane potential (V_m) of −70 mV, and stimuli mimicking action potentials (2 ms at 0 mV) were delivered every 6 s to evoke excitatory postsynaptic currents. The amplitudes and frequency of miniature excitatory postsynaptic currents were evaluated in 60-s recordings (V_m = −70 mV). The detection threshold of miniature excitatory postsynaptic currents was set to 3.5 times the baseline standard deviation. These parameters were measured in cells exposed for 24 h to freshly prepared 200 nM amyloid β42, 200 nM amyloid β42 + 2 µM CLR01, or vehicle. Amyloid β42 (2 µl from 1 mM stock solution in dimethyl sulphoxide) was mixed with 20 µl of CLR01 (from 1 mM stock solution in water) and diluted up to 5 ml to the final concentrations of 400 nM and 4 µM of amyloid β42 and CLR01, respectively. Medium (5 ml) was removed and replaced with 5 ml of fresh media containing dimethyl sulphoxide vehicle, amyloid β42 alone or amyloid β42 and CLR01. Additional details are provided in the Supplementary material.

Long-term potentiation recordings

Electrophysiological recordings were performed using standard protocols in coronal hippocampal slices (400-µm thick) obtained from 8-week-old male C57BL/6 mice as described previously (Fusco et al., 2012). Briefly, field excitatory postsynaptic potential (field EPSP) evoked by Schaffer collateral stimulation was recorded from the CA1 subfield of the hippocampus. The stimulation intensity that produced one-third of the maximal response was used for the test pulses, and the long-term potentiation-induction protocol consisted of four trains of 50 stimuli at 100 Hz repeated every 20 s, hereto called the ‘high-frequency stimulation’ paradigm. The magnitude of long-term potentiation was measured for 60 min after tetanus and expressed as a percentage of baseline field EPSP peak amplitude. The mean values observed during the last 10 min of pre-tetanus recordings were considered to represent the baseline at 100%. For experiments, 40 µl of amyloid β42 from 100 µM stock solution and 40 µl of CLR01 from 1 mM stock solution were mixed and either immediately diluted to a final volume of 20 ml, corresponding to final amyloid β42 concentration 200 nM and final CLR01 concentration 2 µM, or incubated for 1 h and then diluted. The diluted mixture was then immediately added to slices and incubated for 20 min followed by long-term potentiation induction with a standard high-frequency stimulation paradigm. Additional details are provided in the Supplementary material. Statistical analysis in all electrophysiological experiments (Student’s unpaired t-test) was performed with SYSTAT 10.2 (Statcom).

Western blot analysis of amyloid β42 species under electrophysiological experimental conditions

The assembly size of the amyloid β42 species was analysed in both denaturing and native conditions using Western blots probed with anti-amyloid β monoclonal antibody 6E10. Amyloid-β42 preparations for these analyses were identical to those in the electrophysiological experiments described earlier. See Supplementary material for additional details.

Blood–brain barrier experiments

Three wild-type and three transgenic mice were anaesthetized by intraperitoneal injection of ketamine and xylazine. Then, 2 µCi/g of 3H-CLR01 as a 11.8 µg/g of 3H-CLR01 + CLR01 mixture, where the 3H-CLR01 made up 10% of the total CLR01, was injected into the jugular vein. Mice remained anaesthetized for 1 h after injection, at which point blood was collected via cardiac puncture, the mouse perfused thoroughly through the heart with PBS and the brain collected. One hemisphere of the brain or 100–350 µl of blood were digested with 1 ml Solvable™ (Perkin Elmer), added to Ultima Gold™ liquid scintillation cocktail (Perkin Elmer) and read in a Triathler liquid scintillation counter (model 425-034). Brain penetration percentage was calculated as activity per gram of brain relative to activity per millilitre of blood. Statistical analysis (Student’s unpaired t-test) was performed using Prism 5.0d (GraphPad).

Treatment of triple-transgenic mice with CLR01

Triple-transgenic mice (14–15 months old, n = 6–7) and wild-type control mice (n = 11–12) were anaesthetized with 3% isoflurane gas (oxygen 2 l/min) and mini-osmotic pumps (model 1004, Alzet) were subcutaneously implanted on the dorsal back. The pumps contained CLR01 in sterile saline (40 µg/kg/d) or saline as vehicle. The 40 µg/kg/d dose was chosen based on limited knowledge, at the time this experiment was initiated, of the solubility and safety of CLR01. On Day 28, mice were anaesthetized with pentobarbital (100 mg/kg), and blood was collected by cardiac puncture. Mice were perfused with cold non-fixative saline buffer containing protease and phosphatase inhibitors (Roche) as described previously (Lim et al., 2000).

Immunohistochemistry

Analysis was performed according to previously published protocols (Lim et al., 2000; Frautschy et al., 2001). Paraffin-embedded mouse brain regions from −2.97 to −3.08 bregma were deparaffinized, and endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol. The tissue was steamed for 60 min in 2% citrate-buffered antigen-unmasking solution (Vector Labs). Sections were blocked with Tris-buffered saline containing 5% normal horse serum (Vector Labs), 3% bovine serum albumin, and 0.1% Tween-20 and antibodies were diluted in the same solution. Sections
were incubated with monoclonal antibody 6E10 (Covance) diluted 1:1000, anti–p-tau monoclonal antibody AT8 (Thermo Scientific) for phosphorylated paired helical filaments diluted 1:45, anti-tau monoclonal antibody HT7 (Thermo Scientific) for total tau diluted 1:1000, anti-Iba1 polyclonal antibody for microglia (Wako) diluted 1:1000 or anti–glial fibrillary acidic protein monoclonal antibody for astrocytes (Sigma) diluted 1:5000 at 37 °C for 1 h and then overnight at 4 °C. This was followed by incubation with a secondary biotinylated anti-rabbit immunoglobulin-G diluted 1:100 for Iba1 in 1.5% normal horse serum with 3% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 for 1 h at 37 °C. Slides were then incubated with an avidin:biotinylated enzyme complex (ABC Elite Vectastain® kit, Vector Labs) using a peroxidase detection system for 80 min at 37 °C as described previously (Frautschy et al., 2001). Antigen was visualized using metal-enhanced 3,3′-diaminobenzidine tetrahydrochloride (Thermo Scientific). Microscopic quantification used images analysed with macros written in-house for NIH-Image (http://rsb.info.nih.gov/nih-image) or ImageJ (http://rsweb.nih.gov/ij/) to assess deposit size and number. Linear mixed effects models were used to evaluate the treatment effect on the outcome of amyloid β and p-tau load (see Supplementary material for details). For analysis of total tau, microglia and astrocytes, statistical analyses (Student’s unpaired t-test) were performed using Prism 5.0d (GraphPad).

**Mouse behavioural analysis**

General activity and exploratory behaviour were assessed during a single 7-min session and quantified using an automated tracking system (EthoVision 3.0) as described previously (Gale et al., 2009). Multiple locomotor-based endpoints, including velocity (cm/s), mobility (% time) and meander (∫/cm), were quantified. Habituation rates for each endpoint were quantified by calculating the per cent change between the observed mean during the initial 90 s interval and the final 90 s interval. Statistical analysis for all endpoints was performed using two-way (treatment, genotype) ANOVA.

**Brain extraction and western blot for amyloid β protein precursor cleavage products**

Relevant brain regions (hippocampus, entorhinal and piriform cortices) were dissected out of one hemisphere of CLR01- or vehicle-treated triple-transgenic mouse brains at euthanasia. Brain regions were sonicated in a volume of Tris-buffered saline (20 mM Tris–HCl, 150 mM NaCl, pH 7.4) four to seven times the tissue weight, then pelleted at 157 000g for 15 min at 4 °C. The supernatant was saved as the soluble fraction. The pellet was homogenized in Tris-buffered saline with 1% Triton® X-100 and pelleted again. The supernatant was saved as the detergent-soluble fraction. Both fractions were subjected to a biocinchononic acid protein assay (Thermo Fisher Scientific) following the manufacturer’s protocol. The soluble fraction was fractionated on 10% Tris-Tricine SDS-PAGE gels and subjected to western blot using monoclonal antibody 22C11 (Millipore), which recognizes the N-terminal region of amyloid β protein precursor at 1:1000 dilution. The detergent-soluble fraction was fractionated on 10–20% gradient Tris-Tricine gels (Invitrogen) and subjected to western blot analysis probed with polyclonal antibody APP369 (Buxbaum et al., 1990), specific for the C-terminal region of amyloid β protein precursor at 1:1000 dilution. All blots were stripped and re-probed with an anti-β-actin polyclonal antibody (AbFrontier) at 1:2000 dilution as a loading control. Blots were visualized using Enhanced Chemiluminescence (GE Healthcare) and bands quantified densitometrically using ImageJ. Statistical analysis (two-way ANOVA for treatment and genotype) was performed using Prism 5.0d (GraphPad).

**CLR01 stability and cytochrome P450 inhibition**

**In vitro** evaluation of CLR01’s stability in plasma and liver microsomes, and inhibition of cytochrome P450 was performed by Wolfe Laboratories, Inc. The experimental details are proprietary and therefore only a brief description of each experiment is given. For stability measurements, CLR01 was incubated with mouse or human plasma or liver microsomes and an Nicotinamide adenine dinucleotide phosphate (NADPH)-regenerating system. Testing was conducted at 15 min intervals up to 60 min. After protein precipitation by an organic solvent, samples were analysed by high-performance liquid chromatography/mass spectrometry to determine overall stability and half-life of clearance. Testosterone was used as a positive control. For cytochrome P450 inhibition, CLR01 was prepared at eight concentrations ranging from 0 to 25 μM with each of the following individual human recombinant cytochrome P450 isozymes (1A2, 2C9, 2C19, 2D6 and 3A4) and the appropriate cytochrome P450 substrate. Aliquots of the test samples were extracted using an organic solvent and analysed by high-performance liquid chromatography/mass spectrometry to determine the cytochrome P450 half-maximal inhibition concentration values.

**Cytochrome P450 induction by pregnane X receptor reporter gene assay**

African green monkey kidney cells were plated in 96-well plates at a density of 7000 cells per well in Dulbecco’s modified Eagle medium (Invitrogen) with 10% foetal bovine serum (Fisher Scientific) containing penicillin and streptomycin. Twenty-four hours post-plating, cells were transfected with the appropriate plasmids using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. The total DNA per well was 115 ng and contained a mixture of each of the following plasmids: pSV40-β-galactosidase (40 ng), XREM-Luc (20 ng), pSG5-hPXR (5 ng) and pBluescript (50 ng). Luciferase activity was determined using a standard luciferase assay system (Promega). The β-galactosidase activity was determined using standard methods by the o-nitrophenolgalactoside assay and was read at 420 nm. Cells were treated with 10 mM rifampicin (Sigma) or CLR01 for 24 h. Data are normalized to β-galactosidase activity (n = 8).

**Results**

CLR01 rescues neurons from amyloid β protein–induced retraction of dendritic spines

The strongest anatomical correlate for the degree of cognitive impairment in Alzheimer’s disease is synapse loss (DeKosky and Scheff, 1990; Terry et al., 1991). Post-mortem ultrastructural stereological analysis of the hippocampal CA1 regions of brains
of patients with mild cognitive impairment showed 18% synapse loss that progressed to 55% in mild Alzheimer’s disease (Schell et al., 2007). In primary neuronal cultures, oligomers of amyloid β42 have been shown to cause substantial decrease in dendritic spine density (Shankar et al., 2008). Additionally, amyloid β toxicity has been shown to lead to neuritic abnormalities and axonal varicosities in Alzheimer’s disease mouse models (Tsai et al., 2004; Stokin et al., 2005).

To assess the effect of molecular tweezers on amyloid β42–induced synapse loss, we treated primary hippocampal neurons with 3 μM amyloid β42 for 72 h in the presence or absence of 10-fold excess of CLR01 or a negative control derivative, CLR03 (Sinha et al., 2011), and quantified spine density. Amyloid β42 induced abundant varicosities (Fig. 1A) and caused a decline in the number of dendritic spines to 20.9% ± 1.3% of baseline (Fig. 1B). In the presence of CLR01, spine density was rescued to 79.6% ± 2.3% of baseline (P < 0.001 compared with amyloid β42 alone) and varicosities were reduced, whereas CLR03 was inactive (22.5% ± 1.2% of baseline). In these and further experiments addressing synaptotoxicity, we adjusted the amyloid β42 concentration empirically to elicit a sufficiently robust toxic response allowing measurement of inhibition by molecular tweezers (see Supplementary material for additional details).

**CLR01 prevents disruption of basal synaptic transmission**

Changes in gene expression and synaptic vesicle trafficking in the brains of patients with Alzheimer’s disease and transgenic mice suggest that synaptic function is compromised before the physical degeneration of the synapses (Westphalen et al., 2003; Yao et al., 2003). Electrophysiological experiments allow measurement of the earliest neuronal insults by amyloid β. Previous reports have documented changes in basal excitatory synaptic neurotransmission due to amyloid β42 deposition (Malinow et al., 2008 and references therein). In a set of experiments designed to test the capability of CLR01 to rescue amyloid β42–induced inhibition of basal synaptic transmission in autaptic microcultures of hippocampal neurons, we measured the amplitudes of excitatory postsynaptic currents, evoked by stimuli mimicking action potentials, in hippocampal neurons exposed for 24 h to 200 nM amyloid β42 in the absence or presence of 2 μM CLR01. Culture treatment with amyloid β42 alone produced a 51.8% reduction of mean excitatory postsynaptic current amplitude [3.6 ± 0.3 nA (n = 38) versus 7.4 ± 0.5 nA in controls (n = 25), P < 0.001, Fig. 2A]. In contrast, in autaptic hippocampal neurons exposed to amyloid β42 in the presence of CLR01 (or with CLR01 alone), excitatory postsynaptic current amplitudes were not significantly different from controls [6.2 ± 0.6 nA (n = 18); 6.8 ± 0.7 nA (n = 18), respectively].

Application of amyloid β42 to hippocampal CA1 pyramidal neurons has been reported to reduce 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid receptor-mediated spontaneous miniature excitatory postsynaptic current amplitude by 60% and 45%, respectively (Parameshwaran et al., 2007). Miniature excitatory postsynaptic current frequency is thought to be a reflection of presynaptic glutamate release, whereas the amplitude reflects the postsynaptic AMPA [2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid] receptor response to glutamate (Parameshwaran et al., 2007).

Spontaneous neurotransmitter release was studied by recording miniature excitatory postsynaptic current (Fig. 2B) under the same experimental conditions as described earlier. We found that amyloid β42 treatment reduced miniature excitatory postsynaptic current amplitude by 34.5% [17.6 ± 1.0 pA (n = 38) versus 26.9 ± 1.6 pA in controls (n = 25), P < 0.001, Fig. 2C] and miniature excitatory postsynaptic current frequency by 41.6% [4.9 ± 0.5 Hz (n = 38) versus 8.4 ± 0.8 Hz in control (n = 25), P < 0.005; Fig. 2D]. In neurons treated with amyloid β42 in the

**Figure 1** CLR01 protects neurons from amyloid β protein–induced changes in dendritic spine number and morphology. (A) Rat primary hippocampal neurons were incubated for 72 h with media alone or with amyloid β42 in the absence or presence of molecular tweezers. Yellow arrows point to amyloid β42–induced varicosities. Scale bar = 5 μm. (B) The number of dendritic spines per 100 μm was quantified. ***P < 0.001 compared with control; +++ P < 0.001 compared with amyloid β42 + CLR01.
The amyloid β concentration selected, 200 nM, did not influence basal synaptic transmission in brain slices, thus avoiding potentially producing confounding effects on long-term potentiation, as documented by the stability of pre-tetanus field EPSP recordings and the absence of significant differences between the pre- and post-amyloid β application input/output curves. Higher amyloid β concentrations (e.g., 500 nM) did inhibit basal synaptic transmission in brain slices (data not shown). Under control conditions, i.e. when hippocampal slices were perfused with vehicle alone for 20 min before high-frequency stimulation, the field EPSP amplitude recorded 60 min post-high-frequency stimulation displayed increases of +115.0% ± 8.8% over baseline (n = 11). In hippocampal brain slices perfused with 200 nM amyloid β42 for the 20 min preceding high-frequency stimulation, the long-term potentiation was significantly smaller at +53.7% ± 5.1% over baseline (n = 10, P < 0.001, Fig. 3A). Co-application of 200 nM amyloid β42 and 2 μM CLR01 significantly ameliorated long-term potentiation inhibition relative to amyloid β42 alone (field EPSP amplitude increases of +75.4% ± 7.7% (n = 8), P < 0.05, not shown in graph). Though this rescuing effect was statistically significant, its magnitude was small relative to the protective effects of CLR01 in cell culture (Figs 1 and 2). A potential explanation is differences in diffusion to the cellular targets between amyloid β42 oligomers and CLR01, which may diminish the effectiveness of CLR01 in brain slices relative to cultured neurons. To test this hypothesis, we examined whether a 1-h incubation of amyloid β42 with CLR01 before application to hippocampal slices would produce stronger protection. Indeed, 1-h preincubation of amyloid β42 with CLR01 provided a stronger protective effect, raising the field EPSP amplitude potentiation to +94.2% ± 7.4% over baseline (n = 12, P < 0.001 versus amyloid β42 alone, and P < 0.05 versus controls; Fig. 3A and B).

Control long-term potentiation experiments were performed in brain slices exposed to 2 μM CLR01 alone or 200 nM amyloid β42 in the presence of 2μM CLR03. Application of CLR01 alone did not significantly affect long-term potentiation (field EPSP amplitude potentiation +113.2% ± 11.7% over baseline; n = 6), whereas amyloid β42 + CLR03 caused long-term potentiation inhibits not significantly different from those produced by amyloid β42 alone (+53.6% ± 5.9%; n = 8; P < 0.001 versus controls, Fig. 3B).

Analysis of the amyloid β42 assembly size distribution in the neurobasal media or artificial CSF preparations used in the experiments described previously by native- or SDS-PAGE western blots showed a mixture of species ranging from monomer to large oligomers. Little difference was observed between distributions of species in the absence or presence of molecular tweezers (Supplementary Fig. 1).

**CLR01 protects synaptic plasticity against amyloid β42-induced insults**

Signalling through AMPA and N-methyl-D-aspartate receptors is critical for long-term potentiation, a cellular correlate for learning and memory that is expressed as an increase in efficiency of synaptic transmission (Bliss and Collingridge, 1993). Inhibition of long-term potentiation, manifested as a decrease in field EPSP amplitudes and slopes, has been observed on application of different amyloid β oligomers to rat hippocampal slices (Lambert et al., 1998; Puzzo et al., 2008).

To further evaluate the protective effect of CLR01 on amyloid β42-induced synaptotoxicity, we studied long-term potentiation at the Schaffer collateral–CA1 synapses in hippocampal brain slices.

**CLR01 is pharmacologically stable and penetrates the brain at similar levels in wild-type and transgenic mice**

Towards evaluating the potential of molecular tweezers for drug development, we studied the stability of CLR01 in plasma and liver microsomes. Biotransformation of CLR01 was measured during
60-min incubation in human and mouse plasma or liver microsome preparations, an abundant source of drug metabolizing enzymes (Obach et al., 2006; Paine et al., 2006). In comparison with testosterone, which was used as a positive control and was degraded down to 29% in human and 1% in mouse liver microsomes, no degradation of CLR01 was observed in these preparations.

Similarly, CLR01 was found to be 100% stable in human and mouse plasma for 60 min at 37°C. The blood–brain barrier is suggested to be compromised in humans with Alzheimer’s disease (Matsumoto et al., 2007; Zipser et al., 2007) and in transgenic animal models of Alzheimer’s disease (Ujiie et al., 2003). Thus, we analysed brain permeability of CLR01 both in triple-transgenic (Oddo et al., 2003) and in healthy wild-type mice to assess how much of the penetration is due to the disruption of the blood–brain barrier seen with disease and how much may be due to other mechanisms, such as transporters. Tritium-labelled CLR01 (3H-CLR01) was administered intravenously to 12-month-old wild-type or triple-transgenic mice. One hour post-injection, blood was collected, mice were perfused to remove blood from the brain vasculature, euthanized and the brains were collected. Brain penetration percentage was calculated as activity per gram of brain relative to activity per millilitre of blood. There was a small non-significant difference in brain penetration of CLR01 between wild-type (1.74% ± 0.35%) and triple-transgenic mice (1.98% ± 0.11%). There was no difference in per cent of injected CLR01 found in the blood 1 h post-injection between wild-type and transgenic mice, 15.5% ± 1.3% and 15.7% ± 3.5%, respectively.

CLR01 reduces brain amyloid β protein and tau burden and ameliorates microgliosis in transgenic mice without apparent toxicity

In light of the promising in vitro data, we next conducted an initial in vivo study to assess the efficacy of peripherally administered CLR01 in transgenic mice using immunohistochemical changes of amyloid β and p-tau burden, and brain inflammation as endpoints. Similar to the blood–brain barrier experiments described earlier, in these experiments we used the triple-transgenic mouse model of Alzheimer’s disease, which overexpresses mutant forms of the human genes encoding presenilin 1 (mutation M146V) and amyloid β protein precursor (mutation KM670/671NL), each of which causes early-onset familial Alzheimer’s disease, and tau (mutation P301L), which causes frontotemporal dementia. This mouse model is particularly relevant to pathological features of Alzheimer’s disease by encompassing both amyloid plaques and neurofibrillary tangles (Oddo et al., 2003).

Mixed-gender 14–15-month-old mice (n = 6–7 per group) were treated for 28 days with 40 mg/kg/d CLR01 in saline as a vehicle, or with vehicle alone, administered subcutaneously using osmotic minipumps. After treatment, the mice were sacrificed and their brains were analysed by immunohistochemistry for the presence of plaques, tangles and inflammatory markers. Analysis of brain sections from vehicle-treated mice using monoclonal antibody 6E10 showed amyloid plaques deposited predominantly in the subiculum and CA1 regions of the hippocampus (Fig. 4A), as reported previously (Oddo et al., 2003). In addition, the mice showed phosphorylated paired helical filaments detected by the anti-p-tau monoclonal antibody AT8, predominantly in the hippocampal regions CA1 and CA3 (Fig. 4D). Mice treated with CLR01 showed a significant decrease in amyloid β burden of 33.3% in...
the total brain area analysed (per cent burden: vehicle-treated 3.03% ± 0.19% versus CLR01-treated 2.02% ± 0.17%, P < 0.01, Fig. 4A and C), 34.7% in the hippocampus (vehicle-treated 4.18% ± 0.27% versus CLR01-treated 2.73% ± 0.20%, P < 0.05), 49.3% in the perirhinal and entorhinal cortices (vehicle-treated 1.42% ± 0.24% versus CLR01-treated 0.72% ± 0.08%, P < 0.05) and 18.6% in the piriform cortex and amygdala (vehicle-treated 2.37% ± 0.23% versus CLR01-treated 1.93% ± 0.25%). Similarly, a 33.3% reduction in AT8-positive p-tau was observed in the total brain area analysed (% burden: vehicle-treated 4.30% ± 0.32% versus CLR01-treated 2.87% ± 0.21%, Fig. 4E and F), 24.3% reduction in CA1 (vehicle-treated 4.82% ± 0.38% versus CLR01-treated 3.65% ± 0.26%) and a 45.8% reduction in CA3 (vehicle-treated 3.69% ± 0.52% versus CLR01-treated 2.00% ± 0.29%) regions in mice treated with CLR01. In contrast, immunohistochemistry with the anti-tau monoclonal antibody HT7 showed no effect on total tau (Fig. 4G and H).

Compared with vehicle-treated triple-transgenic mice (Fig. 4I), the CLR01-treated triple-transgenic mice showed a 46.2% reduction in the number of microglia per hippocampal area (vehicle-treated 41.79 ± 9.64 versus CLR01-treated 22.5 ± 4.12, P < 0.05; Fig. 4J and K) and in the microglial stained area (data not shown). Similarly, a 43.9% reduction in microglial stained area was found in the cortex of CLR01-treated mice relative to vehicle-treated mice (data not shown). In comparison, there was essentially no difference between vehicle- and CLR01-treated wild-type mice in the level of microgliosis (vehicle-treated

**Figure 4** CLR01 decreases amyloid β protein and p-tau deposition and ameliorates microgliosis in transgenic mouse brain.

Triple-transgenic mice were treated with 40 μg/kg/d CLR01 or vehicle. (A, D, G and I) Vehicle-treated transgenic mouse hippocampus. (B, E, H and J) CLR01-treated transgenic mouse hippocampus. (A and B) Transgenic mouse brain stained with monoclonal antibody 6E10 showing amyloid plaque deposition. (C) Per cent amyloid β (Aβ) burden was quantified by calculating the total 6E10-stained area divided by the total area measured. (D and E) Transgenic mouse brain showing AT8-positive neurofibrillary tangles in the CA1 region. (F) Per cent aggregated p-tau load was quantified by calculating the total AT8-stained area divided by the total area. (G and H) Transgenic mouse brain stained with monoclonal antibody HT7 for total tau. (I and J) Transgenic mouse brain showing Iba1-positive microglia in the subiculum and CA1 region. (K) Number of stained microglia in a 1.14 mm² area of hippocampus per treatment condition. Scale bars: bar in B applies to both A and B; bar in J applies to D–J. *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle-treated mice.

Amyg = amygdala; Ent = entorhinal; Hippo = hippocampus; Peri = perirhinal; Pir = piriform cortices; WT = wild-type.
32.67 ± 4.16 versus CLR01-treated 33.73 ± 4.81, Fig. 4K). In contrast to the effect on microglia, CLR01 treatment had little or no impact on the number or staining level of astrocytes in transgenic or wild-type mice in either the hippocampus or cortex (data not shown).

As has been described previously (Hirata-Fukae et al., 2008), we found that the triple-transgenic female mice in our study had more amyloid β pathology than the males. The vehicle-treated females (n = 3) had 204% the amyloid β load of the males (n = 3) in the hippocampus, 394% in the entorhinal/perirhinal cortices and 205% in the piriform cortex/amygdala region. A similar trend was observed with the CLR01-treated mice (n = 4 females, 3 males): 138% in the hippocampus, 164% in the entorhinal/perirhinal cortices and 308% in the piriform cortex/amygdala region. Correspondingly the effect of CLR01 treatment was substantially more pronounced in females than in males. Female mice showed a decrease of 45% in amyloid β load in the hippocampus, whereas males had a decrease of 19%. A similar trend was observed in the entorhinal/perirhinal cortices, 64% decrease in females and 15% decrease in males, whereas in the piriform cortex/amygdala region, the trend was reversed—17% decrease in females and a 45% decrease in males. Consistent with the previous study (Hirata-Fukae et al., 2008), the p-tau load did not differ significantly between male and female triple-transgenic vehicle-treated mice, and CLR01 treatment affected p-tau load to the same extent in both genders.

Because CLR01 may bind to Lys residues in proteins other than amyloid β and tau and affect their activity, we used several criteria, including appetite loss, weight loss (Table 1), lethargy and mortality to explore whether CLR01 treatment had adverse effects on the triple-transgenic mice. We did not observe any adverse effects. To assess potential interactions between CLR01 treatment and general behavioural measures, mouse activity was analysed during a single 7-min monitoring period. We did not observe any significant changes in velocity, path shape or mobility between the CLR01-treated and the vehicle-treated transgenic or wild-type mice (Table 2). Similarly, no effects were observed on habituation rates in any of these measures (Table 2). As hyperactivity and other perturbations of locomotor activity, as well as disruptions of habituation, have commonly been observed after CNS toxicity (Platel and Porsolt, 1982; Hess et al., 1986), the lack of such behavioural effects suggests that the 28-day CLR01 regimen did not adversely impact the neural systems subserving these traits.

To address further potential concerns regarding toxicity due to the unique mechanism of CLR01, we studied the effect of CLR01 in several in vitro and in vivo systems. Previously, CLR01 showed no toxicity at <400 μM in cell lines and in primary neurons (Sinha et al., 2011, 2012a), a concentration that is 1–3 orders of magnitude higher than needed for inhibition of toxicity in cell culture. Zebrafish treated with CLR01 up to 10 μM dissolved in the water in which the fish developed showed no signs of toxicity (Prabhudesai et al., 2012). In mouse brain slices treated with 2 μM CLR01 alone, no changes were seen in levels of long-term potentiation (Fig. 3B). We also did not find adverse effects by weight, activity or mortality in mice treated subcutaneously with CLR01 doses from 40 μg/kg/d (Tables 1 and 2) up to 1200 μg/kg/d (data not shown) or in mice treated intracerebroventricularly with 10 μM CLR01 (data not shown), suggesting the existence of sufficient therapeutic window for CLR01. Further, we assessed the effect of CLR01 on amyloid β protein precursor processing. In the amyloid β protein precursor sequence, Lys residues exist directly N-terminal to the α-secretase cleavage site and two residues N-terminal to the β-secretase cleavage site (in the triple-transgenic

### Table 1. No toxic effect of CLR01 on weight change (% of pre-surgery baseline)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Day 0 (%)</th>
<th>Day 8 (%)</th>
<th>Day 23 (%)</th>
<th>Day 28 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Vehicle</td>
<td>100.0 ± 0.0</td>
<td>96.7 ± 0.01</td>
<td>97.8 ± 0.01</td>
<td>96.2 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>CLR01</td>
<td>100.0 ± 0.0</td>
<td>95.6 ± 0.05</td>
<td>96.2 ± 0.01</td>
<td>95.8 ± 0.01</td>
</tr>
<tr>
<td>Triple-transgenic</td>
<td>Vehicle</td>
<td>100.0 ± 0.0</td>
<td>95.6 ± 0.02</td>
<td>97.4 ± 0.01</td>
<td>97.6 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>CLR01</td>
<td>100.0 ± 0.0</td>
<td>95.9 ± 0.02</td>
<td>100.4 ± 0.02</td>
<td>97.8 ± 0.03</td>
</tr>
</tbody>
</table>

### Table 2. No toxicity of CLR01 by behavioural end-points

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Velocity (cm/s)</th>
<th>Velocity habituation (% change)</th>
<th>Mobility (% time)</th>
<th>Mobility habituation (% change)</th>
<th>Meander (cm)</th>
<th>Meander habituation (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Vehicle</td>
<td>4.6 ± 0.6</td>
<td>−24 ± 7</td>
<td>63 ± 6</td>
<td>−33 ± 13</td>
<td>425 ± 39</td>
<td>40 ± 10</td>
</tr>
<tr>
<td></td>
<td>CLR01</td>
<td>5 ± 1</td>
<td>−15 ± 16</td>
<td>65 ± 5</td>
<td>−50 ± 31</td>
<td>418 ± 34</td>
<td>48 ± 20</td>
</tr>
<tr>
<td>Triple-transgenic</td>
<td>Vehicle</td>
<td>5.5 ± 0.7</td>
<td>−24 ± 8</td>
<td>74 ± 1</td>
<td>−25 ± 11</td>
<td>377 ± 32</td>
<td>49 ± 15</td>
</tr>
<tr>
<td></td>
<td>CLR01</td>
<td>5.6 ± 0.7</td>
<td>−9 ± 7</td>
<td>75 ± 4</td>
<td>−8 ± 6</td>
<td>358 ± 30</td>
<td>21 ± 8</td>
</tr>
<tr>
<td>P-value (two-way ANOVA)</td>
<td>Treatment effect</td>
<td>0.62</td>
<td>0.23</td>
<td>0.58</td>
<td>0.99</td>
<td>0.71</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Genotype effect</td>
<td>0.43</td>
<td>0.77</td>
<td>0.083</td>
<td>0.19</td>
<td>0.12</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Interaction effect</td>
<td>0.78</td>
<td>0.80</td>
<td>0.82</td>
<td>0.35</td>
<td>0.85</td>
<td>0.21</td>
</tr>
</tbody>
</table>
mice we used here, the Lys–Met dipeptide at positions 670 and 671 is replaced by Asn–Leu). Additionally, several Lys residues are a few amino acids C-terminal to the γ-secretase cleavage site. Brain extracts from wild-type and transgenic mice treated with either vehicle or CLR01 were analysed by Western blots using antibodies specific for the soluble N-terminal portion of amyloid β protein precursor, or for the C-terminal fragments of amyloid β protein precursor. We found no differences in concentration levels of soluble amyloid β protein precursor, C-terminal fragment-α or C-terminal fragment-β between vehicle- and CLR01-treated mice (Supplementary Fig. 2), supporting CLR01’s putative process-specific mechanism of action.

**CLR01 interacts weakly with major cytochrome P450 isoforms**

The cytochrome P450 family of enzymes catalyses the oxidation of a vast array of endobiotic and xenobiotic molecules to increase their hydrophilicity during phase-I metabolism, accounting for ~75% of the total number of metabolic reactions in the body (Williams et al., 2004; Guengerich, 2008). Thus, inhibition or activation of cytochrome P450 enzymes may cause metabolic toxicity. In addition, drug–drug interactions are an important consideration in the development of new therapeutics and are highly related to the cytochrome P450 system. Induction or inhibition of particular cytochrome P450 isoforms by one drug or food product may affect the rate of metabolism of other substrates (i.e. drugs) of these isoforms. Thus, it is important to evaluate the interaction of molecular tweezers with cytochrome P450 if they are to become drug candidates.

Measurement of the inhibitory potency of CLR01 on five major cytochrome P450 isoforms responsible for 95% of drug metabolism (Williams et al., 2004) yielded the following half-maximal inhibition concentration values, listed in order from the most potently inhibited enzyme to the least: 2C19 (1.5 μM) > 3A4 (1.7 μM) > 2C9 (2.2 μM) > 2D6 (3.6 μM) > 1A2 (>20 μM). Inhibitory potency values <1 μM are expected to cause drug interactions of at least 2-fold, based on comparison of in vivo drug interaction data and primary experimental in vitro results for 44 drugs (Obach et al., 2006). The half-maximal inhibition concentration values for the interaction of CLR01 with cytochrome P450 were above the 1-μM threshold for all the isoforms tested. The in vitro inhibition order is generally expected to line up with the in vivo magnitude of drug–drug interactions involving the substrates for the specific cytochrome P450 isoforms (Obach et al., 2006).

We also evaluated induction of the cytochrome P450 system using the pregnane X receptor (PXR) reporter gene assay (Jones et al., 2002). PXR is a nuclear receptor and transcription factor for genes that are highly involved in xenobiotic and endobiotic uptake, metabolism and elimination. It is a key activator of the xenobiotic-inducible cytochrome P450 isoforms 3A, 2B, and 2C and of glutathione S-transferase gene expression. Importantly, the PXR ligand-binding domain is uniquely large allowing the receptor to bind promiscuously to a large variety of structurally diverse molecules in different orientations (Staudinger et al., 2006), making it a robust target whose activation can be used to predict cytochrome P450 induction and potential toxicity of new experimental drugs (Staudinger and Lichti, 2008). To evaluate PXR activation and potential toxicity of CLR01, African green monkey kidney fibroblasts were transfected with plasmids containing luciferase and β-galactosidase reporter genes under control of a PXR response element. Forty-eight hours post-transfection, the cells were treated for 24 h with 10 μM rifampicin, an antibiotic and known PXR ligand, as a positive control, or with different concentrations of CLR01. At concentrations up to 1 μM, CLR01 exhibited luciferase activity similar to that of vehicle alone. At 10 and 50 μM, CLR01 induced a luciferase activity 56.4% and 39.5% the magnitude of rifampicin, respectively (Fig. 5). CLR01 (50 μM) reduced the β-galactosidase

![Figure 5](https://academic.oup.com/brain/article-abstract/135/12/3735/286376/FIG5)  
**Figure 5** Weak induction of the cytochrome P450 system by CLR01. African green monkey kidney cells were treated with 10 μM rifampicin (rif; positive control) or CLR01 for 24 h. Cells transfected with luciferase but not PXR (XREM) were used as a negative control. (A) Luciferase activity was determined using a standard luciferase assay system (Promega). (B) β-galactosidase activity was determined using standard methods by the o-nitrophenolgalactoside assay.
induction by 39.6%. These data demonstrate minimal PXR activation by low micromolar concentrations of CLR01 compared with a known ligand of PXR and a commonly used drug, rifampicin.

The highest plasma concentration found in pharmacokinetic experiments in which CLR01 was administered intravenously at 1 mg/kg (25 times the dose used for the experiments shown in Fig. 4) was <11 μM at time zero, suggesting that toxicity is not anticipated at doses needed for the in vivo effects observed on aggregated amyloid β, p-tau and microgliosis.

Discussion

We have recently reported that CLR01 is a process-specific inhibitor of aberrant assembly and toxicity of amyloidogenic proteins (Sinha et al., 2011). The putative mechanism of action of molecular tweezers and the reason we refer to them as process-specific is their labile moderate-affinity binding to solvent-exposed Lys residues, thereby disrupting a combination of hydrophobic and electrostatic interactions that are key to the aberrant self-assembly process. We previously showed that CLR01 inhibited the assembly and toxicity of multiple disease-associated proteins, disaggregated preformed amyloid β fibrils and stabilized non-toxic assemblies (Sinha et al., 2011). Additionally, by a similar effect on α-synuclein, CLR01 prevented developmental deformities and death and facilitated proteasomal clearance of α-synuclein in a novel zebrafish model (Prabhudesai et al., 2012). Following up on these promising efficacity data and minimal toxicity found for CLR01 in cell culture or in zebrafish, here we report its capability to protect synaptic structure, function and plasticity against amyloid β insults and to ameliorate brain pathology in Alzheimer’s disease transgenic mice.

Based on in vitro experiments using electron microscopy, dot blots with an oligomer-specific antibody, dynamic light scattering and solution-state nuclear magnetic resonance (Sinha et al., 2011), we expected that incubation of amyloid β42 with CLR01 would lead to a rapid modulation of amyloid β22 to a non-toxic state. Using electrophysiological readouts as functional correlates of the toxicity state, we found that CLR01 provided significant relief from amyloid β42–induced toxic effects on basal synaptic activity and long-term potentiation, supporting our prediction. Additionally, comparison of co-application with preincubation of amyloid β and CLR01 before addition to brain slices in long-term potentiation experiments showed that preincubation increased the protective effect without changing the distribution of amyloid β species as analysed by native- and SDS-PAGE western blots (Supplementary Fig. 1). The lack of difference seen by the Western blot analysis compared with the positive difference seen in cell viability (Sinha et al., 2011, 2012a, dendritic spine (Fig. 1) and electrophysiological assays (Figs 2 and 3) suggest that the changes in amyloid β assembly induced by CLR01 are subtle or that CLR01 binding prevents contact with the cellular targets of the toxic amyloid β species. The increased protective effect after preincubation supports our previous findings of rapid disruption of amyloid β2 self-association and its remodelling into non-toxic structures by CLR01 (Sinha et al., 2011), which is common to a number of inhibitors (Liu and Bitan, 2012). One such inhibitor, scyllo-inositol, was shown to inhibit long-term potentiation deficits caused by cell-secreted amyloid β oligomers when preincubated with conditioned media containing these oligomers before application to slices (Townsend et al., 2006), similar to CLR01, whereas post-application of scyllo-inositol after incubation of cells with the conditioned media provided no protection against amyloid β toxicity. Additional comparison of our electrophysiological data with previous literature is provided in the Supplementary material.

As a first step in characterizing CLR01 in vivo, we quantified the blood–brain barrier permeability of CLR01 in both wild-type and transgenic mice. Though blood–brain barrier disruption has been reported in Alzheimer’s disease and transgenic mouse models, we found genotype-independent ~2% blood–brain barrier permeability, 1 h post-injection, in both 12-month-old wild-type and triple-transgenic mice, consistent with a previous study of the triple-transgenic mice at 11-months of age (Bourasset et al., 2009). At this age range, the mice display a moderate disease phenotype, which apparently does not affect blood–brain barrier integrity.

Next, we assessed brain amyloid β and p-tau load in a small group of transgenic mice treated continuously for 28 days and found a significant decrease in amyloid β levels (Fig 4). Similar effects were observed on p-tau and microglia, however, because of the small sample size, statistical significance was not reached in all cases. Based on CLR01’s ability to disaggregate preformed fibrils in vitro (Sinha et al., 2011), it is possible that a similar action disaggregated amyloid plaques in the brains of the treated mice into soluble non-toxic structures amenable to clearance and/or degradation. Although additional experiments are needed to establish the mechanism by which CLR01 exerted its beneficial effects in the mice, the clearance hypothesis is supported by our recent in vivo study in zebrafish expressing human α-synuclein that were treated with CLR01 and showed recovery of proteasomally active and increased α-synuclein clearance (Prabhudesai et al., 2012).

Consistent with amyloid β–induced neuroinflammation (Akiyama et al., 2000), the reduction in amyloid β load correlated with a decrease in microgliosis (Fig 4). Although activation of microglia plays a dual role in the brain, phagocytosing deposited amyloid β (Frautschy et al., 1992; Ard et al., 1996) and releasing cytotoxic compounds, such as reactive oxygen and nitrogen species (Colton and Gilbert, 1987; Chao et al., 1992), reducing brain microgliosis is typically considered a beneficial treatment outcome in Alzheimer’s disease (Akiyama et al., 2000).

Although mechanisms of tau toxicity in Alzheimer’s disease are still under discussion, oligomerization and hyperphosphorylation, likely downstream of amyloid β insults, are believed to be involved (Bloom et al., 2005; Gendron and Petrucelli, 2009). We found reduction in levels of p-tau (Fig. 4), which could result from either direct disaggregation by CLR01 as was shown in vitro (Sinha et al., 2011) and eventual clearance downstream of the significant decrease in amyloid β aggregates, or reflect both mechanisms. Answering this question will require additional exploration, yet the data suggest that molecular tweezers’ process-specific mode of action is uniquely suitable to affect both the amyloid β and tau components of Alzheimer’s disease.
pathology, and therefore using these compounds is a promising intervention strategy.

Labile binding to Lys residues with micromolar affinity is a unique mode of action that potentially could disrupt normal protein function and cause side effects. However, the aberrant self-assembly process that leads to the formation of toxic amyloid β and tau oligomers involves many weak intra- and intermolecular interactions (Roberts and Shorter, 2008). Thus, the labile binding of molecular tweezers is predicted to be effective in preventing these weak interactions without substantially disrupting structurally stable proteins. In practice, solvent-exposed Lys residues are commonly used for covalent attachment of biotin, fluorescent dyes or other tags without interfering with biological activity of stably folded proteins. It is therefore plausible that non-covalent interactions with major cytochrome P450 isoforms, CLR01 demonstrates its ability to ameliorate Alzheimer’s disease-associated phenotypes of synaptic dysfunction.

Process-specific modulation of amyloid protein self-assembly is a novel approach towards treatment of amyloidoses. Much work still lies ahead for developing molecular tweezers as therapeutic tools for amyloid-related disease, including addressing additional questions about potential toxicity in more stringent systems and therapeutic effects on disease-associated behavioural deficits. As multiple proteins of unrelated sequences cause amyloidoses, a treatment paradigm that is process-specific is a promising approach to the problem. With brain penetration of ~2% of blood levels, robust stability in plasma and liver microsomes, and weak interaction with major cytochrome P450 isozymes, CLR01 shows a favourable drug profile and is expected to be stable inside the brain. Development of pro-drug derivatives and/or formulations of CLR01 will likely improve further the compound’s pharmacokinetic/pharmacodynamic characteristics and increase the likelihood of it becoming a viable drug candidate.

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Supplementary material

Supplementary material is available at Brain online.

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