

FEATURED ARTICLE

Preclinical and clinical biomarker studies of CT1812: A novel approach to Alzheimer's disease modification

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Abstract

Introduction: Amyloid beta ($A\beta$) oligomers are one of the most toxic structural forms of the $A\beta$ protein and are hypothesized to cause synaptotoxicity and memory failure as they build up in Alzheimer's disease (AD) patients' brain tissue. We previously demonstrated that antagonists of the sigma-2 receptor complex effectively block $A\beta$ oligomer toxicity. CT1812 is an orally bioavailable, brain penetrant small molecule antagonist of the sigma-2 receptor complex that appears safe and well tolerated in healthy elderly volunteers. We tested CT1812's effect on $A\beta$ oligomer pathobiology in preclinical AD models and evaluated CT1812's impact on cerebrospinal fluid (CSF) protein biomarkers in mild to moderate AD patients in a clinical trial (ClinicalTrials.gov NCT02907567).

Methods: Experiments were performed to measure the impact of CT1812 versus vehicle on $A\beta$ oligomer binding to synapses in vitro, to human AD patient *post mortem* brain tissue ex vivo, and in living APP_{Swe}/PS1dE9 transgenic mice in vivo. Additional experiments were performed to measure the impact of CT1812 versus vehicle on $A\beta$ oligomer-induced deficits in membrane trafficking rate, synapse number, and protein expression in mature hippocampal/cortical neurons in vitro. The impact of CT1812 on cognitive function was measured in transgenic Thy1 huAPP^{Swe/Lnd+} and wild-type littermates. A multicenter, double-blind, placebo-controlled parallel group trial was performed to evaluate the safety, tolerability, and impact on protein biomarker expression of CT1812 or placebo given once daily for 28 days to AD patients (Mini-Mental State Examination 18–26). CSF protein expression was measured by liquid chromatography with tandem mass spectrometry or enzyme-linked immunosorbent assay in samples

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drawn prior to dosing (Day 0) and at end of dosing (Day 28) and compared within each patient and between pooled treated versus placebo-treated dosing groups.

Results: CT1812 significantly and dose-dependently displaced A β oligomers bound to synaptic receptors in three independent preclinical models of AD, facilitated oligomer clearance into the CSF, increased synaptic number and protein expression in neurons, and improved cognitive performance in transgenic mice. CT1812 significantly increased CSF concentrations of A β oligomers in AD patient CSF, reduced concentrations of synaptic proteins and phosphorylated tau fragments, and reversed expression of many AD-related proteins dysregulated in CSF.

Discussion: These preclinical studies demonstrate the novel disease-modifying mechanism of action of CT1812 against AD and A β oligomers. The clinical results are consistent with preclinical data and provide evidence of target engagement and impact on fundamental disease-related signaling pathways in AD patients, supporting further development of CT1812.

KEYWORDS

Abeta oligomers, Alzheimer's disease, clinical trial, sigma-2 receptor, synapse

1 | INTRODUCTION

Alzheimer's disease (AD) afflicts six million people in the United States, yet disease-modifying therapies remain an unmet medical need. Investigational therapeutics are directed against a variety of potential targets,¹ but the amyloid hypothesis remains a compelling rationale for AD drug development.² More than 200 human mutations that cause early-onset familial AD have been identified, almost all conferring a single phenotype, increased amyloid beta (A β) protein concentration or ratio.^{3,4} The only protective mutation that both significantly lowers AD incidence and also exhibits strong functional evidence of protection, A673T (the Icelandic mutation), also impacts the A β protein,⁵ resulting in significantly lowered binding affinity of A β oligomers at synaptic receptors.⁶ Previous therapeutics have not effectively targeted A β oligomers,⁷ one of the most toxic structural forms of the A β protein.⁸ While monomeric A β binds to many receptors, the oligomeric form of A β has been shown to bind specifically and saturably to a single receptor site composed of LiIRB2, Nogo receptor, and cellular prion proteins^{9–11} on neuronal synapses, where they induce changes consistent with a toxic disease-related ligand. After oligomer binding, synaptic protein expression and the number of spines and synapses are reduced,^{12–16} synaptic plasticity processes (long-term potential and depression) are disrupted,^{14,17–25} and the process of new memory formation fails.^{2,8,10,11,26} Importantly, many preclinical studies suggest that after removal or reduction of the toxic oligomer species, synaptic protein expression, synapse number, and behavioral deficits can recover to normal levels.^{12,27}

Here, we report the discovery and continued development of drug candidate CT1812, a brain penetrant small molecule that targets A β oligomers via a novel mechanism.^{26,28} The first selective sigma-2

receptor antagonist to reach clinical trials,²⁹ CT1812 is shown here to specifically displace A β oligomers bound to neuronal receptors. The sigma-2 receptor complex plays a major role in cellular damage response mechanisms, with its constituent proteins regulating processes as diverse as autophagy, cholesterol synthesis and progesterone signaling, lipid membrane-bound protein trafficking, and receptor stabilization at the cell surface.^{30–37} The sigma-2 receptor complex has previously been shown to be a key regulator of A β oligomer receptors.^{26,28} In the present study, we report evidence supporting a hypothesized mechanism of CT1812 action: when CT1812 binds specifically to the sigma-2 receptor ligand binding site and allosterically modulates the sigma-2 receptor complex, it causes destabilization of the neighboring A β oligomer receptor binding sites, resulting in displacement of A β oligomers from their receptors (in other words, an increase in their “off rate”). This lowers A β oligomer affinity for synaptic receptors, mimicking the effect of the protective Icelandic mutation.⁶ This disruption of oligomer binding has previously been associated with an improvement in membrane trafficking, neuronal surface expression of synaptic proteins, prevention of spine loss, and improvement in cognitive deficits in animal models of AD.^{26,28} The present study reports preclinical data demonstrating the novel mechanism of action of CT1812 *in vitro* and *in vivo* in an AD transgenic mouse model, and preliminary cerebrospinal fluid (CSF) biomarker data from the completed phase 1b/2a trial (NCT02907567), supporting its disease-modifying mechanism in AD patients.²⁹

CT1812 is an investigational therapeutic that has not been approved for any use by the US Food and Drug Administration. The risk-benefit profile of CT1812 will be evaluated after adequate placebo-controlled clinical trials have been completed.

RESEARCH IN CONTEXT

- 1. Systematic review:** The authors engage in routine surveillance of relevant publications via PubMed and direct review of new issues of journals that publish research related to Alzheimer's disease (AD) or any mechanisms and research techniques used by the authors of this article. The articles are extensively referenced throughout.
- 2. Interpretation:** These results are consistent with previously published preclinical evidence and demonstrate CT1812's target engagement and fundamental impact on disease-related signaling pathways in patients with AD.
- 3. Future directions:** These results support further development of CT1812, which is currently in multiple phase 2 trials in patients with AD.

(Figure 1A, $K_i = 8.5$ nM), sigma-1 receptors with low affinity ($K_i = 63$ nM), and is 100-fold selective for interacting with sigma-2 receptors versus 72 other drug targets (Cerep/Eurofins; Figure S1 in supporting information). CT1812 effectively prevented ($EC_{50} = 6.7$ μ M) and reversed ($EC_{50} = 0.36$ μ M) synthetic A β oligomer-induced membrane trafficking deficits in vitro in primary hippocampal/cortical cultures (21DIV, Figure 1B) and prevented human AD patient brain-derived oligomer-induced trafficking deficits (Figure 1C). CT1812 prevented binding of A β oligomers to synaptic receptor sites on neurons (A β oligomer binding $K_d = 0.43$ μ M, plus CT1812 = 1.12 μ M, Figure 2A, B, D), and also displaced already-bound A β oligomers (A β oligomer binding $K_d = 0.61$ μ M, plus CT1812 = 1.16 μ M, Figure 2C, E). CT1812 did not block the formation of A β oligomers or disrupt their formation at concentrations up to 20 μ M (Figure S2 in supporting information), suggesting that its ability to stop A β oligomer binding and trafficking deficits results from CT1812 direct binding to sigma-2 receptors.

2 | RESULTS

2.1 | CT1812 displaces and prevents A β oligomer binding

Previously published studies that described unbiased proprietary library screening to identify compounds that block A β oligomer-induced toxicity of intracellular lipid membrane trafficking rates revealed compound potency correlated with sigma-2 receptor affinity.^{26,28} Medicinal chemistry optimization resulted in the design of CT1812. CT1812 binds sigma-2 receptors with high affinity

2.2 | CT1812 restores disease-related protein expression and synapse number in vitro

A β oligomers induce reversible spine retraction in vitro³⁸ and cause a corresponding loss of synapses and synaptic proteins.^{13,26} The effect of CT1812-induced displacement of A β oligomers on oligomer-induced spine and synapse loss in cultured rat neurons was investigated using the cytoskeleton binding protein drebrin (expressed postsynaptically) as a marker of synapse number. Addition of A β oligomers to neuronal cultures for 3 hours caused a significant loss (mean \pm standard error of the mean 12.8% \pm 2.6%, $P < 0.01$, two-tailed Student's *t* test, Figure 2F top, G) of drebrin-immunoreactive synaptic puncta

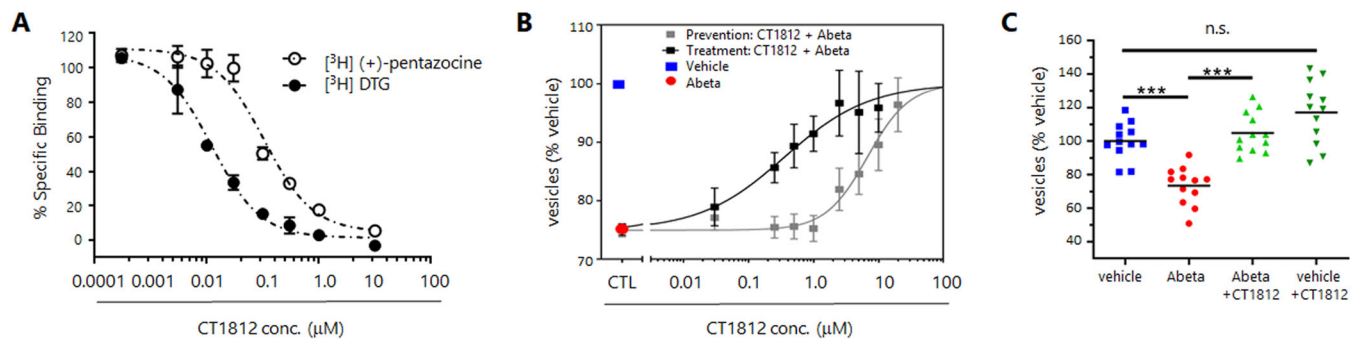


FIGURE 1 CT1812 is a selective sigma-2 receptor ligand that prevents and treats amyloid beta (A β) oligomer-induced membrane trafficking deficits. A, CT1812 binds sigma-2 receptors with a 8.5 nM K_d and sigma-1 receptors with a 63 nM K_d as measured in radioligand displacement studies ($n = 2$; means \pm standard deviation [SD]). B, Effects of synthetic A β oligomers on vesicle trafficking in primary cultures of neurons and glia. Quantification is shown of the amount of formazan within intracellular vesicles in A β oligomer-treated (red dot) or vehicle-treated (blue square) conditions, and the dose-dependent restoration of trafficking rate to normal vehicle-treated values after CT1812 addition 1 hour before addition of A β oligomers (gray squares, $EC_{50} = 6.7$ μ M, $n = 11$ experiments) or 1 hour after addition of A β oligomers (black squares, $EC_{50} = 0.36$ μ M, $n = 12$ experiments). Vesicle trafficking was assayed 24 hours after addition of A β oligomers (1.75 μ M, total peptide; means \pm standard error of the mean). N represents the number of replicate experiments from separate cell culture preparations. C, Human Alzheimer's disease brain derived A β oligomers (1-hour treatment) caused a 27 \pm 11% (SD; $P < 0.001$, $n = 3$) decrease in trafficking rate in cultured neurons and glia. Pretreatment of cultures for 1 hour with 15 μ M CT1812 before addition of human derived A β oligomers blocked the trafficking deficits ($P < 0.001$ for A β oligomers vs. A β oligomers + CT1812, analysis of variance with Tukey's post hoc test, $n = 3$ experiments). Each point represents results from quadruple replicates from A β oligomer preparations isolated from three separate donor brains with the mean of all the points represented by the horizontal lines

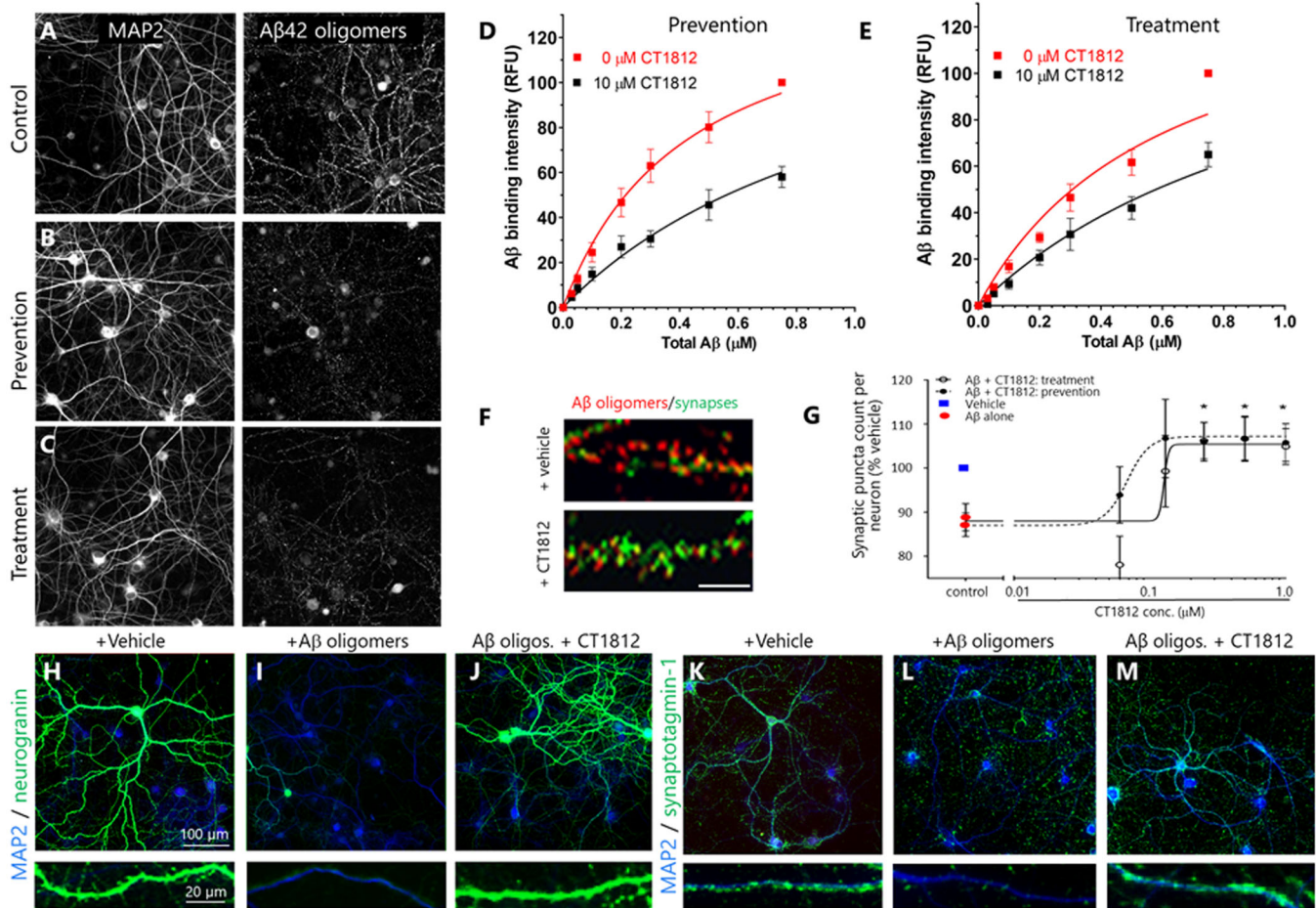


FIGURE 2 CT1812 prevents amyloid beta ($A\beta$) oligomer binding to and displaces bound oligomers from neuronal receptors, leading to restoration of synaptic density and protein expression in cultured mouse neurons. A, $A\beta$ oligomers (750 nM, > 80% of Kd) bind specifically and saturably to a single receptor site on some, but not all, neurons in mature (> 21 DIV) primary hippocampal and cortical neuronal cultures. B, CT1812 [10 μ M] prevents $A\beta$ binding by $59 \pm 8\%$ (standard error of the mean [SEM]) when added 1 hour prior to addition of $A\beta$ oligomers. C, CT1812 [10 μ M] displaces $A\beta$ oligomer binding by $42 \pm 9\%$ (SEM) when added 1 hour after addition of $A\beta$ oligomers. Scale bar = 20 microns. D, CT1812 prevents binding and more than doubles the Kd of oligomer binding, from 430 nM (at 0 μ M CT1812) to 1120 nM (at 10 μ M CT1812; $n = 6$; means \pm SEM). E, CT1812 displaces binding and nearly doubles the Kd of oligomer binding, from 610 nM (at 0 μ M CT1812) to 1160 nM (for 10 μ M CT1812; $n = 6$; means \pm SEM). F, top, $A\beta$ oligomers bind to receptors at neuronal synapses (red, 6E10 immunodetection), resulting in a significant loss of synapses (green, drebrin immunodetection, $12.8 \pm 2.6\%$ [SEM] decrease in synapse density, $P < 0.01$ vs. vehicle-treated, Student's t test [G]; the blue square represents synapse density in vehicle-treated neurons). F, bottom, CT1812 displaces bound $A\beta$ oligomers and restores synaptic numbers to normal in a dose-dependent manner. Scale bar = 5 microns. G, CT1812 restores synapse number to normal whether added before (prevention, $EC_{50} = 68$ nM) or after (treatment, $EC_{50} = 127$ nM) $A\beta$ oligomers ($*P < 0.05$ vs. $A\beta$ oligomers alone for both conditions, Student's t test, means \pm SEM). Treatment with CT1812 restores synaptic protein expression in cultured rat neurons. H, Neurogranin is expressed at high levels in postsynaptic dendrites and synapses in a subset of neurons in primary hippocampal/cortical cultures. I, Addition of $A\beta$ oligomers (0.75 μ M, 24 hours) causes a 28% loss of neurons expressing high levels of neurogranin ($P = 0.014$). J, Treatment with 4.8 μ M CT1812 1 hour after addition of $A\beta$ oligomers restores the expression of neurogranin to normal levels ($A\beta$ oligomers vs. $A\beta$ oligomers + CT1812 $P = 0.031$, vehicle vs. $A\beta$ oligomers + CT1812, n.s., analysis of variance [ANOVA] with Tukey's post hoc test, $n = 20$). K, Synaptotagmin-1 is expressed in presynaptic terminals. L, Addition of $A\beta$ oligomers (3.5 μ M, 48 hours) cause a 37% loss of synaptotagmin-1 presynaptic terminals in primary hippocampal/cortical cultures ($P = 0.0068$, $N = 24$). M, Treatment with 4.8 μ M CT1812 1 hour after addition of $A\beta$ oligomers blocks these losses and restores expression of synaptotagmin-1 levels (vehicle vs. $A\beta$ oligomers + CT1812, n.s.). H–M, ANOVA with Tukey's post hoc test, $n = 20$. For all cell culture experiments, n represents the number of replicate experiments from separate cell culture preparations, and data points represent means \pm SD or SEM as noted

per neuron compared to vehicle treatment. This is similar to the degree of synapse loss seen using ultrastructural stereology methods in *post mortem* hippocampus from humans diagnosed with mild cognitive impairment.³⁹ CT1812 prevented oligomer-induced synapse loss in a dose-dependent manner ($EC_{50} = 68$ nM, $P < 0.05$, paired,

Student's t test of treated vs. vehicle, Figure 2F bottom, G). Furthermore, addition of CT1812 to cultures 1 hour after the addition of $A\beta$ oligomers resulted in a concentration-dependent increase of synaptic number to normal levels ($EC_{50} = 127$ nM, Figure 2G). Examination of synaptic protein expression corroborated these results. Neurogranin is

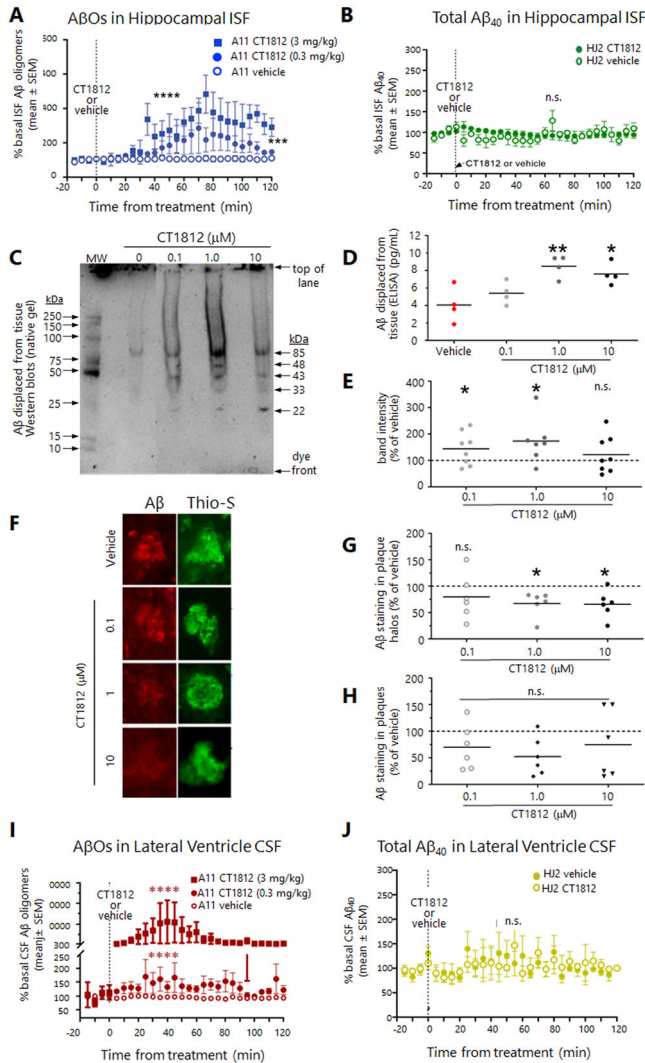


FIGURE 3 CT1812 treatment displaces amyloid beta (Aβ) oligomers from mouse hippocampal receptors and *post mortem* Alzheimer's disease human patient tissue and facilitates their clearance into cerebrospinal fluid (CSF). A, B, Microimmuno-electrodes (MIE) coated with oligomer-specific antibody A11 (A) or mAβ₄₀ antibody HJ2 (B) in 12-month-old transgenic hAPP/PS1 mice detect soluble Aβ in the hippocampal interstitial fluid (ISF). A, After intravenous injection of 0.3 or 3 mg/kg CT1812 (vertical dashed line), soluble Aβ oligomers are significantly elevated in hippocampal ISF in CT1812-treated mice ($n = 7$) compared to vehicle-treated mice ($n = 4$, means ± standard error of the mean [SEM]; *** $P < 0.001$, **** $P < 0.0001$, two-way analysis of variance [ANOVA] with Sidak's post hoc test for effect of drug vs. vehicle). B, CT1812 treatment does not cause a change in total Aβ levels in the hippocampus ($n = 5$) compared to vehicle treatment ($n = 4$, means ± SEM). C–E, Aβ oligomers displaced from *post mortem* brain tissue sections from 8 AD patients were quantified by enzyme-linked immunosorbent assay (ELISA; D) and native western blots (C [representative individual patient], E), and show the expected dose-dependent increase in concentration in CT1812-treated compared to the vehicle-treated condition. For western blots, the sum of the intensity of discrete Aβ protein bands at 22, 33, 43, 48, and 85 Kd was quantified for each donor tissue section (C). In (D) and (E), values for Aβ measured by ELISA for each brain tissue section and for intensity of western blot bands for each brain tissue section normalized to vehicle-treated controls (dashed line)

expressed in dendrites and postsynaptic terminals (Figure 2H), while synaptotagmin-1 is expressed in presynaptic terminals (Figure 2K) of mature primary hippocampal/cortical neurons *in vitro*. Aβ oligomer treatment led to a 28% loss of neurons expressing high levels of neurogranin (Figure 2I) and a 37% loss of synaptotagmin-1 presynaptic terminals (Figure 2L). Treatment with CT1812 blocked this loss and restored expression of both proteins to control levels (Figure 2J, M). This suggests that CT1812-mediated displacement of Aβ oligomers from neuronal synapses stops both downstream oligomer-induced synaptic protein downregulation and synapse loss, facilitating synaptic recovery from toxic oligomer insult.

2.3 | CT1812 displaces Aβ oligomers and facilitates clearance into CSF *in vivo* and in AD patient brain tissue

To confirm the displacement of Aβ oligomers from cells observed *in vitro* using an *in vivo* mouse model of AD, we adapted a novel microimmuno-electrode (MIE) technology previously used to detect rapid kinetic changes in total Aβ concentration in brain interstitial fluid (ISF)⁴⁰ (Figure S3A–E in supporting information). Using electrodes coated with the oligomer-specific antibody A11⁴¹ placed in the hippocampus, Aβ oligomers were measured in ISF of 12-month-old APP^{Swe}/PS1E9 transgenic mice.⁴² A single dose of CT1812 (0.3 μM, or 3.0 μM *i.v.*) resulted in a rapid and significant increase in Aβ oligomer levels in hippocampal ISF relative to predose baseline (Figure 3A, vertical dashed line, see Figure S3F). In contrast, no increase was observed in the ISF of vehicle-treated transgenic mice. The rapid elevation of Aβ oligomers after drug administration declined to baseline levels within the timeframe of the recording (120 minutes). In contrast, total Aβ levels (primarily monomer) in the hippocampal ISF were not affected by the administration of CT1812 (Figure 3B), thus CT1812 selectively

from the same donor are shown, with the mean of all the points represented by the horizontal lines (* $P < 0.05$, ANOVA with Tukey's post hoc test for each treatment vs. vehicle treated controls). F (representative individual patient), G, H, Aβ oligomers (red) located in a 2 μM halo surrounding compact thioflavin-S positive plaques (green) are displaced from frozen *post mortem* human AD brain tissue sections ($N = 5$ patients) by CT1812 in a dose-dependent manner normalized to vehicle treated brain sections (dashed line) from the same individual with the mean of all the points represented by the horizontal lines (* $P < 0.05$, ANOVA with Tukey's post hoc test for each CT1812-treated vs. vehicle-treated controls). H, Aβ intensity within plaques does not change. Scale bar = 20 μM. I, J, Microimmuno-electrodes coated with oligomer-specific antibody A11 (I) or pan-Aβ antibody HJ2 (J) in 12-month-old transgenic hAPP/PS1 mice detect soluble Aβ in the lateral ventricle CSF. I, After intravenous injection of 0.3 ($n = 7$) or 3 ($n = 5$) mg/kg CT1812 (vertical dashed line), soluble Aβ oligomers are significantly elevated in lateral ventricle CSF compared to vehicle-treated mice ($n = 5$, means ± SEM). J, CT1812 treatment ($n = 5$) did not cause a change in Aβ monomer levels in the CSF compared to vehicle treatment ($n = 4$ means ± SEM). *** $P < 0.001$, **** $P < 0.0001$, two-way ANOVA Sidak's post hoc test for effect of drug vs. vehicle

reduces A β oligomer extracellular concentrations without affecting A β monomer levels.

To assess whether CT1812 displacement of A β oligomers in AD model systems in vitro and in vivo may also occur in AD patients, we conducted ex vivo binding experiments in 10 μ M-thick *post mortem* neocortical tissue sections obtained from patients with AD (Clinical Dementia Rating [CDR] = 3, n = 8 patients, Figure 3C-E). After incubation of adjacent sections in identical volumes containing increasing concentrations of CT1812 or vehicle, the supernate was removed and the levels of A β displaced from the tissue sections quantified by enzyme-linked immunosorbent assay (ELISA); the A β remaining in the tissue sections was quantified via immunofluorescent microscopy. Analysis of the displaced material by ELISA for total A β showed that ascending concentrations of CT1812 increased the amount of A β released from the human brain tissue (Figure 3D): non-denaturing western blots confirmed this material to be A β oligomers (Figure 3C, E, Figure S4 in supporting information). Additionally, CT1812 induced a concentration-dependent decrease in A β oligomer immunofluorescence intensity in the 2 μ m region containing high concentrations of oligomers surrounding dense, thioflavin-S labeled plaque cores^{43,44} (Figure 3F, G) with similar but not statistically significant changes occurring within the plaque itself (Figure 3H). These results demonstrate that CT1812 displaces prebound A β oligomers from AD patient brain tissue.

Finally, the fate of A β oligomers displaced by CT1812 was determined by placing the A11-coated MIE in the lateral ventricle of transgenic APP_{Swe}/PS1dE9 mice to measure A β oligomers in the CSF. A significant, dose-dependent rise in A β oligomer levels was detected in CSF after drug administration (Figure 3I) and remained elevated for up to 2 hours, suggesting that displacement of A β oligomers in the brain may lead to increased clearance into the CSF. In contrast, A β monomer levels in the CSF were not affected by administration of CT1812 (Figure 3J), indicating that CT1812 selectively facilitates clearance of oligomers, but not monomers, from the brain into the CSF.

2.4 | CT1812 improves cognitive performance in transgenic mice

To assess whether this evidence of synapse preservation by CT1812 was associated with functional behavioral improvement, we evaluated changes in cognitive deficits in an aged transgenic mouse model of AD.^{26,45,46} Transgenic Thy1 huAPP^{Swe/Lnd+} male mice, aged 3.5 to 4.5 months, or wild-type (WT) littermates, were administered vehicle or CT1812 10 mg/kg once daily by oral gavage for 9 to 10 weeks (n = 12 for each group except for the transgenic CT1812-treated group, which was n = 13). Treatment of WT mice with CT1812 did not significantly alter motor behavior and did not change cognitive performance compared to that of WT vehicle-treated animals. As expected, significant deficits in transgenic vehicle-treated animals compared to WT vehicle-treated animals were observed in the Activity Chamber test measuring exploration, the Y maze measuring spatial working memory, and the Fear Conditioning assays measuring aversive associative learning and memory. Significant improvements in spatial learning and memory

were observed in transgenic animals treated with CT1812 compared to those treated with vehicle in the Water Maze (Figure 4A), the Y-Maze (Figure 4B), and in contextual fear conditioning (Figure 4C). In addition to improving spatial learning and memory, significant improvements were observed in hyperactivity as assessed via the Activity Chamber assay, and cue-dependent learning and memory as assessed in the Fear Conditioning assay. In WT mice, CT1812 had no effect on performance in any of the cognitive tests (Figure 4A-C).

Previous studies of eight other sigma-2 receptor antagonists closely related to CT1812 in Thy1 huAPP^{Swe/Lnd+} mice treated daily for 4 weeks to 6 months indicated that behavioral improvements were observed as long as brain concentrations of drug were above a theoretical 80% receptor occupancy threshold concentration,²⁶ but that plaque number and A β monomer concentration (measured by ELISA) did not change. PK measurements of brain concentrations of CT1812 after treatment of Thy1 huAPP^{Swe/Lnd+} mice with 10 mg/kg p.o. for 9 to 10 weeks confirmed that this estimated 80% receptor occupancy level was achieved (Figure 4D, brain concentration 24 hours after last dose = 19.9 ng/mL, 84.4% receptor occupancy, greater than four times the sigma-2 receptor Ki value). Taken together, these studies indicate that threshold brain concentrations of CT1812 effectively improved cognitive deficits found in the huAPP^{Swe/Lnd+} mouse model of AD.

2.5 | Clinical biomarker evidence of CT1812-mediated synapse protection and disease modification

After completion of first-in-man clinical studies (NCT02570997²⁹), CT1812 was advanced into clinical trials in AD patients. Mild-to-moderate AD patients with Mini-Mental State Exam (MMSE) scores of 18 to 26 were randomized to receive one of three doses of CT1812 (90, 280, 560 mg) or placebo once daily for 28 days (N = 19, 4-5/group) in a double-blind phase 1b/2a clinical trial to determine safety and tolerability (trial COG0102, for full description, see clinicaltrials.gov NCT02907567). The randomized patients, 10 male and 9 female, had a mean age of 70.2 years (standard deviation [SD] 9.2) and body mass index of 24.75 kg/m² (SD 2.73). CSF concentrations at baseline and Day 28 confirmed no presence of CT1812 in the placebo group or at baseline in any dose group. Day 28 mean CSF concentrations of CT1812 rose in a dose-dependent manner (1.15 ng/mL [SD 0.53] in the 90 mg treatment group, 2.84 ng/mL [SD 0.69] in the 280 mg treatment group, and 4.96 [SD 8.49] in the 560 mg treatment group). As expected in a study of limited treatment duration, change in exploratory measures of cognitive function from baseline was similar in CT1812-treated and placebo groups. CSF samples collected from each patient at baseline and at the end of study were used for protein measurements using targeted (i.e., ELISA, western blot, liquid chromatography with tandem mass spectrometry [LC-MS/MS]) and nontargeted (i.e., unbiased LC-MS/MS proteomics) methods; missing samples and variable sample volumes reduced the number of matched baseline and day 28 patient CSF samples available for subsequent analysis.

Protein gel electrophoresis (western blot) was used to measure A β oligomer concentration in CSF samples from each patient taken at Day

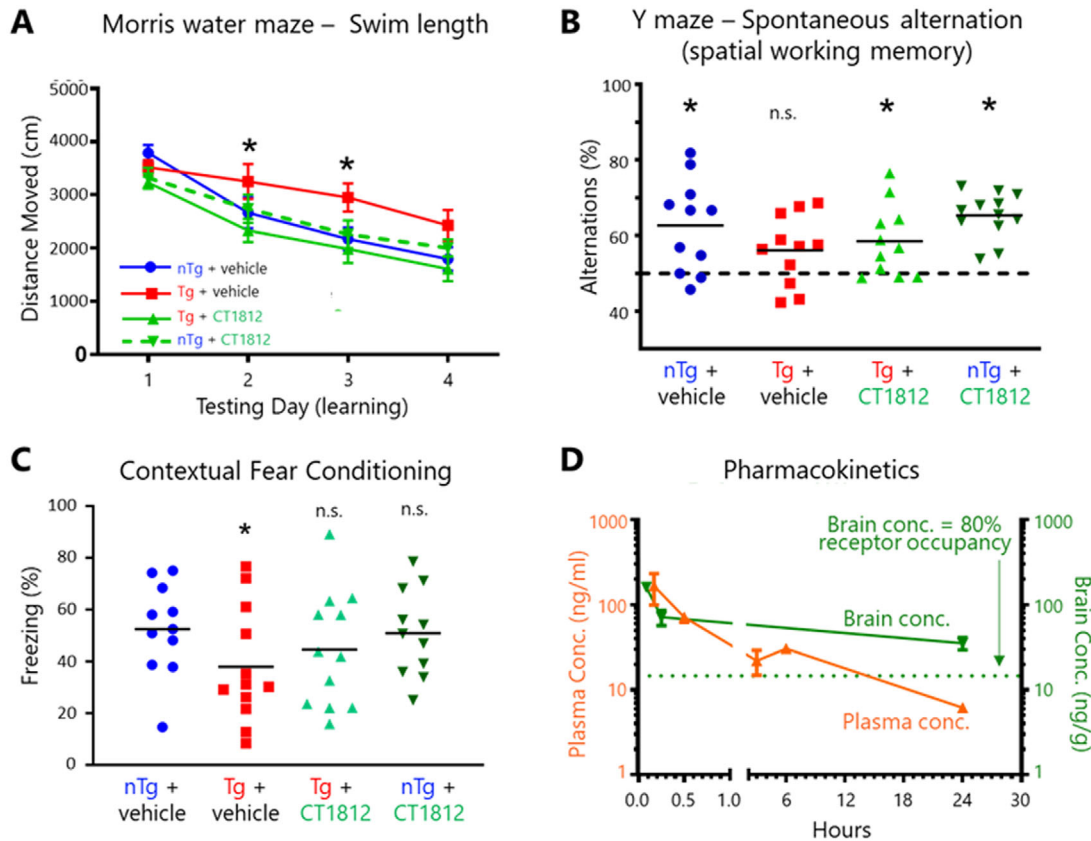


FIGURE 4 Treatment with CT1812 improves learning and memory deficits in transgenic Alzheimer's mice. A, Transgenic Thy-1 huAPP^{Swe}/Ldn+ male mice treated with CT1812 (Tg + CT1812) learn the Morris water maze task significantly better than do transgenic vehicle-treated mice (Tg + vehicle; $P = 0.016$, two-way repeated measures analysis of variance, Bonferroni post hoc $*P < 0.5$; mean \pm standard error of the mean). CT1812 treatment does not affect non-transgenic animal performance (nTg + CT1812). B, Transgenic mice treated with CT1812 remember previous arms entered in the Y maze task significantly better ($P = 0.013$, Student's *t* test) than chance (dashed line), but transgenic vehicle-treated animals do not (nTg + vehicle, $62.7 \pm$ standard deviation [SD] 12.2%; Tg + vehicle, $56.1 \pm$ SD 9.2%; Tg + CT1812, $58.5 \pm$ SD 9.4%; nTg + CT1812, $65.3 \pm$ SD 6.0%). C, Transgenic mice show deficits in the Contextual Fear Conditioning test ($P = 0.037$, Student's *t* test), while transgenic and nontransgenic mice treated with CT1812 do not (nTg + vehicle, $52.5 \pm$ SD 5.4%; Tg + vehicle, $37.9 \pm$ SD 6.4%; Tg + CT1812, $44.6 \pm$ SD 6.5%; nTg + CT1812, $50.9 \pm$ SD 5.1%). Each data point in (B) and (C) represents an individual mouse with the mean of all the points represented by the horizontal lines. D, Plasma and brain concentration of CT1812 after a single p.o. dose of 10 mg/kg ($n = 3$ mice per time point, mean \pm SD)

0 and 28 and percent change for each patient was calculated. Due to the small number of AD patients in each dosing group, CT1812 dose groups were pooled for comparisons to placebo. After 28 days of treatment, CSF A β oligomer concentration ($n = 3$) in placebo-treated patients trended lower from levels at their own baseline, while levels in CT1812-treated patients ($n = 10$) increased significantly compared to placebo-treated patients (Figure 5A and Figure S5 in supporting information). This finding is consistent with preclinical studies indicating that CT1812 displaces and clears toxic A β oligomers from the brain into CSF (Figure 3I) and provides supporting evidence of clinical target engagement. In contrast, A β 40 and 42 monomer levels measured with ELISA were not different between Day 0 and Day 28, or between treatment groups in this trial (Figure S5B, C).

The concentrations of several synaptic and axonal proteins are elevated in CSF of AD patients compared to age-matched cognitively normal individuals as a result of central nervous system synaptic damage due to the disease;⁴⁷⁻⁵¹ some markers exhibit greater sensitivity to

disease-related changes than do others. We measured concentrations of synaptic and axonal proteins in CSF samples from AD patients in trial COG0102 using ELISA (neurogranin and neurofilament light [NFL]) or targeted LC-MS/MS (synaptotagmin-1, SNAP-25). At 28 days, CSF concentrations of neurogranin and SYT-1 decreased in CT1812-treated AD patients relative to placebo-treated AD patients CSF (Figure 5B, neurogranin, $n = 5$ placebo, 11 treated; $P = 0.050$ analysis of covariance; Figure 5C, synaptotagmin-1, $n = 4$ placebo, 9 treated; $F_{1,12} = 8.8$, $P = 0.011$, linear mixed model), consistent with preclinical evidence of CT1812-mediated facilitation of synaptic recovery from insult with toxic A β oligomers. CSF concentrations of NFL and SNAP-25 did not change to a significant degree in CT1812-treated AD patients versus placebo (Figure S5D, G).

Data from unbiased LC-MS/MS proteomics measurements of CSF were evaluated for effects on synaptic proteins (Figure 5D). Of the 3160 proteins detected in the CSF of this patient cohort, the abundance of 315 proteins was significantly different between

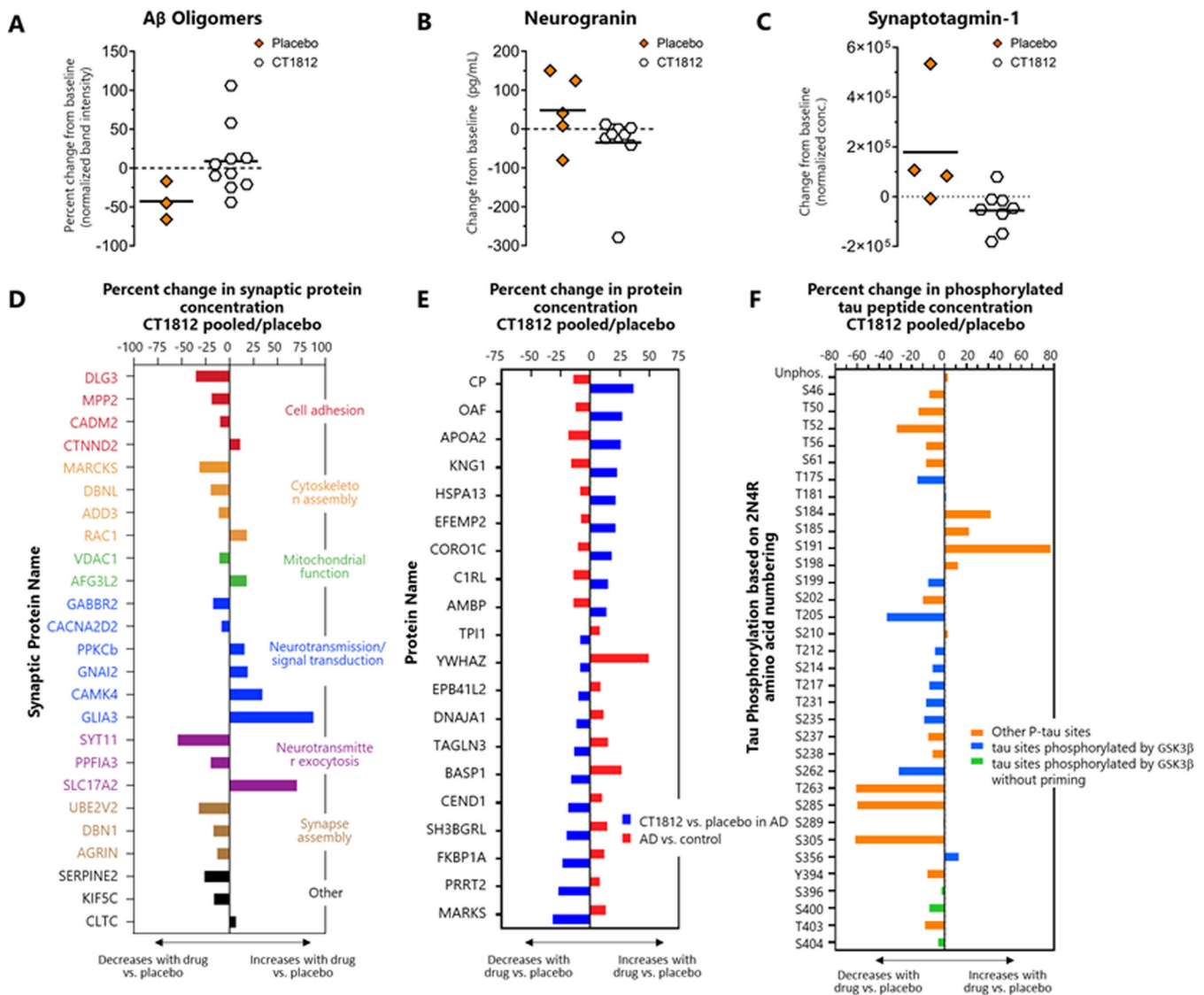


FIGURE 5 CT1812 treatment significantly impacts cerebrospinal fluid (CSF) biomarkers of target engagement and disease-related biology. Protein concentrations in CSF samples from Alzheimer's disease (AD) patients in a 28-day phase 1b/2a clinical trial of CT1812 vs. placebo were measured via enzyme-linked immunosorbent assay (ELISA), liquid chromatography tandem mass spectrometry (LC-MS/MS), or gel electrophoresis. A, After 28 days the concentration of amyloid beta (Aβ) oligomers measured via western blot in CT1812-treated AD patient CSF increases compared to the patient's own baseline and vs. placebo ($P = 0.014$, Student's t test, $n = 3$ placebo, 10 CT1812-treated), providing supporting evidence of clinical target engagement. At day 28 of the study, the concentration of synaptic damage proteins neurogranin (B, measured by ELISA) and synaptotagmin-1 (C, measured by LC-MS/MS) decreases compared to the patient's own baseline and vs. placebo ($P = 0.050$ analysis of covariance, $n = 5$ placebo, 11 CT1812-treated; and $F_{1,12} = 8.8$, $P = 0.011$, $n = 4$ placebo, 9 CT1812-treated, respectively) providing evidence of a positive effect on synapses in patients with AD. A, B, and C, Data from individual patients are displayed with the treatment group mean represented by the horizontal lines. These changes are corroborated (D) by significant expression changes ($P < 0.05$) in multiple synaptic proteome proteins in the CSF measured by unbiased LC-MS/MS in AD patients treated for 28 days with CT1812 vs. placebo. E, CSF proteomics identifies a subset of proteins significantly altered with CT1812 that have been reported to be significantly regulated in AD.⁹² Data are expressed as mean % change in protein concentration in CSF from CT1812 treated ($n = 11$) vs. placebo ($n = 4$; blue) and AD vs. control to illustrate how these proteins are altered in AD relative to cognitively normal age-matched controls. All analytes shown are significantly regulated ($P < 0.05$) in AD patients vs. control⁵³ and in the present study. F, After 28 days of treatment, the abundance of six tau phosphorylation peptides decreased by 30% (the threshold for noise distribution), or more after treatment with CT1812 compared to placebo (T52, T205, S262, S263, S285, S305) while one site increased more than 30% (S191), but the concentration of unphosphorylated tau did not change, providing evidence of CT1812 impact on pathological disease signaling. Colored bars represent amino acid sites within tau that are phosphorylated by GSK3β with priming (blue bars) or without priming (green bars)^{93,94}

CT1812- and placebo-treated patients (analysis of variance [ANOVA] followed by Fisher's least significant difference [LSD]; $P < 0.05$). Pathway analysis using three independent bioinformatics platforms (IPA Canonical Pathway [v51963813], Metacore [v19.4 build 69900], STRING [v11]) indicates that CT1812 significantly ($P < 0.05$) impacts synaptic-related pathways including N-methyl-D-aspartate receptor trafficking, glycogen kinase synthase kinase-3 β (GSK3 β), and Wnt signaling, as well as cytoskeletal reorganization. Given the loss of synapses in AD patients along with the preclinical evidence that CT1812 rescues synapse number, we queried which proteins in the synaptic proteome⁵² were altered by CT1812 treatment. Twenty-five proteins in the synaptic proteome⁵² were identified to be differentially expressed (ANOVA followed by Fisher's LSD; $P < 0.05$) in CT1812-compared to placebo-treated patient CSF. To understand the functions of these proteins with respect to specific synaptic function in more detail, network analysis was performed. The highest scoring network, Cell Morphology, Cellular Assembly and Organization, Cellular Development, comprised 14 out of 25 of the synaptic proteins, a significantly greater number than expected by random chance (Figure S6A in supporting information, IPA Score = 26; $P < 1 \times 10^{-25}$). This indicates that these proteins may play a role in dendritic branching, cytoskeletal remodeling, and neurotransmission (Figure S6B). This provides evidence supporting CT1812's positive effect on synapses in patients with AD.

Out of 520 CSF proteins significantly altered in AD patients versus age-matched controls ($P < 0.05$) in a recent study,⁵³ 334 were detected in the COG0102 CSF proteomics dataset. Of those 334 proteins, a subset of 20 moved in the opposite direction (i.e., reversed AD-related changes) and were also significantly different in CT1812-treated compared to placebo (Figure 5E). Several of these proteins are involved in key biological pathways known to be disrupted in AD, including cholesterol transport (apolipoprotein A2 [APOA2]), oxidative stress (ceruloplasmin [CP]), complement (complement C1r subcomponent-like protein [C1RL]), and synaptic transmission (14-3-3 protein beta/alpha [YWHAZ]). This provides supporting evidence for a broad improvement of disease-related signaling by CT1812 in patients with AD.

One of the hallmarks of AD is hyperphosphorylated tau protein, which comprises neurofibrillary tangles (NFTs).⁵⁴⁻⁵⁸ We evaluated AD patient CSF for concentrations of unphosphorylated and phosphorylated tau protein fragments taken at baseline and after 28 days of CT1812 or placebo treatment via LC-MS/MS (Figure 5F); phosphorylated peptide fragments representing 33 distinct phosphorylation sites on tau were detected. The abundance of six phosphorylation sites decreased by 30% or more (Figure 5F)^{59,60} after treatment with CT1812 compared to placebo while one site increased more than 30% (the threshold for noise distribution⁶¹), but the concentration of unphosphorylated tau did not change. In agreement with these results, ELISA measurements of the change from baseline of phospho tau (p-tau) (181) and total tau (t-tau) was similar in CT1812-treated and placebo groups (Figure S5E, F). Additionally, LC-MS/MS measurements of concentrations of kinases that phosphorylate tau, such as GSK3 β , trended lower by 25% in CT1812-treated patients (not significantly different from placebo-treated patients, $P = 0.098$). These

results suggest that CT1812 may act upstream of tau kinases to reduce their concentration and resulting activity, while having no impact on tau expression or regulation. To identify phosphorylation sites within tau that might covary in abundance in response to CT1812 treatment, correlation analyses were performed and a heatmap was generated. The abundance of 21 pairs of p-tau sites was found to be significantly correlated with one another (Figure S7 in supporting information), supporting the hypothesis that CT1812 may act upstream of tau kinases/phosphatase to impact tau phosphorylation at specific amino acid sites. Taken together, these clinical biomarker data support a potential therapeutic impact of CT1812 in patients with AD.

3 | DISCUSSION

3.1 | CT1812 displaces A β oligomers in several preclinical models of AD

The A β oligomer hypothesis remains a compelling rationale for AD drug development programs.² Supporting evidence for this comes from the genetic determinants of autosomal dominant AD and AD complicating Down syndrome,⁶² the protective effect of the Icelandic mutation of the amyloid precursor protein^{5,6} animal data indicating the toxic effects of A β oligomer species on synaptic function^{8,63} and its reversibility,²⁷ and the observation that A β accumulation alone in cognitively and clinically normal individuals predicts symptomatic AD with high likelihood.⁶⁴ Drug development programs targeting A β include active and passive A β immunotherapeutics⁷ and secretase inhibitors that decrease A β peptide generation,⁶⁵ but none effectively or selectively target A β oligomers.

The current study demonstrates that CT1812 displaces A β oligomers from neurons with three independent approaches: in vitro cell culture, in vivo rodent studies (including two different mouse models of AD), and ex vivo human brain CSF and tissue. In cultured rat brain cells in vitro, CT1812 shifted the binding of exogenously applied, synthetic A β oligomers to lower affinity whether added before or after oligomers. Importantly, CT1812-mediated reduction of oligomer affinity phenocopies or mimics the effect of the AD's protective Icelandic mutation on oligomer affinity,⁶ and is the only drug candidate currently in development reported to do so. This is significant because drug candidates with the same mechanism as protective variants are expected to have a higher success rate in the clinic.⁶⁶ CT1812 treatment for 1 hour did not decrease the intensity of pre-bound A β oligomers as completely as it did when added to cultures prior to the oligomers. It is possible that longer treatment with CT1812 can achieve a greater level of displacement. However, incubation with A β oligomers for longer times results in internalization of a portion of the A β oligomer labeling,²⁶ complicating quantification of the compound treatment. Nonetheless, our results demonstrate that CT1812 both displaces already-bound A β oligomers and prevents the binding of A β oligomers to neuronal synaptic receptors; displaced oligomers will not re-bind as long as CT1812 remains present. Thus, CT1812 will likely continue to exert its effect as concentrations of oligomers rise

throughout the course of AD. In separate biochemical assays using an oligomer-specific ELISA, CT1812 does not interact directly with oligomers and did not disrupt preformed oligomers or block oligomer formation (Figure S2). In *post mortem* human AD brain tissue sections, CT1812 caused a concentration-dependent increase in A β displaced from the tissue as measured by A β ELISA, as well as a concentration-dependent increase in several oligomeric species of A β as detected by western blot analysis of the material displaced from the tissue.⁶⁷ This was accompanied by a decrease in A β within the tissue section in the 2 micron oligomer-enriched halo surrounding plaques. While we cannot rule out compound-mediated displacement of oligomers from extrasynaptic sites that are below the level of detection of our imaging methods, oligomers that are dose-dependently displaced by CT1812 are highly likely to originate from synaptic receptor sites; because oligomers are water-soluble, oligomers in the interstitial fluid in these frozen tissue sections should freely diffuse in any aqueous media and be detectable in the vehicle treated condition as well (Figure 3C-E). The transgenic mouse MIE measurements provide additional insight on the selectivity of CT1812's effect on oligomers: a single dose of CT1812 rapidly increased concentrations of A β oligomer, but not A β monomer in the brain. Together, these results demonstrate that CT1812 is a dose-dependent, negative allosteric modulator of A β oligomer binding.

Unexpectedly, these same measurements provided evidence suggesting that CT1812 directly or indirectly facilitates clearance of A β oligomer, but not monomer, out of the brain and into the CSF; it rapidly increases ISF and CSF concentrations of oligomers in a dose-dependent manner, but not monomers. The difference in kinetic rate of increase in A β oligomer concentration between the hippocampus and lateral ventricle may result from different electrode sampling volumes due to diffusion constraints within brain tissue compared to CSF. The basis for this selective clearance of A β oligomers but not monomers is currently unclear. While mechanisms of A β monomer clearance from the brain into CSF have been studied extensively (reviewed in Yuede et al.⁴⁰), oligomer clearance mechanisms are currently unknown. One possible mechanism is suggested by recent studies demonstrating direct interactions of sigma-2 receptor complex component proteins PGRMC1 and TMEM97 with low-density lipoprotein (LDL) receptor, required for ligand-bound LDL receptor internalization,³¹ and sigma-2 receptor-mediated cellular uptake of A β via interactions with apolipoprotein E and the LDL receptor.^{31,68} Previously tested closely related sigma-2 receptor antagonists do not impact monomer or fibril levels in Thy1 huAPP^{Swe/Lnd+} transgenic mice after chronic short-term administration.²⁶ To more fully explore the role of these proteins in AD, future studies (including studies in AD mouse models cross-bred with conditional tissue-specific knockouts of each protein to avoid lethality) are required.

We hypothesize that displacement of toxic A β oligomers from synaptic receptor sites and clearance of oligomer into the CSF underlies the improvement of cognitive performance in transgenic AD mice seen with chronic CT1812 administration. As with other closely related sigma-2 receptor antagonists,²⁶ CT1812 improves transgenic mouse behavior at brain concentrations corresponding to an estimated 80%

receptor occupancy; typical for an antagonist mechanism of action, threshold concentrations of drug are required to block effects. While CT1812's *in vitro* assay EC50 values range from high nanomolar to low micromolar concentrations, behavioral improvement is observed at brain concentrations closer to CT1812's Ki at sigma-2 receptors (8.5 nM). The basis for this difference is unclear but may be due to the short duration and high concentrations of synthetic A β oligomers used in the *in vitro* assays. Once displaced from synaptic receptors, the oligomers are no longer able to harm synapses and are subject to elimination through yet to be defined clearance pathways.

3.2 | Preliminary biomarker evidence for CT1812 target engagement and disease-modification in patients with AD

Protein measurements of CSF samples from the 28-day clinical trial COG0102 provide preliminary evidence of target engagement and potential impact on disease in mild-to-moderate AD patients consistent with preclinical data. CSF concentrations of A β oligomers increase in CT1812-treated patients versus placebo similar to preclinical studies, indicating that CT1812 displaces and clears toxic A β oligomer from the brain into CSF. CSF concentrations of the postsynaptic dendritic protein neurogranin, typically significantly elevated in AD patients,^{51,69-71} are reduced by CT1812 treatment, compared to changes in placebo patients. Additionally, several synaptic proteins are significantly different in CT1812- versus placebo-treated patients; bioinformatics analysis of these changes support preclinical evidence of a positive impact of CT1812 on synaptic pathways.

Analysis of CSF concentrations of phosphorylated tau fragments in AD patients revealed that treatment with CT1812 induced a broad reduction in tau phosphorylation at multiple amino acid sites without affecting unphosphorylated tau concentration. Many of the residues altered by CT1812 treatment have been previously implicated in AD-related changes.⁷²⁻⁷⁷ Data correlating phosphorylation at any given amino acid of tau with disease progression in patients (as well as how closely mouse disease models reflect such changes) are still emerging.

Preclinical evidence demonstrates that A β oligomer binding results in upregulation of tau kinase activity² leading to tau phosphorylation at multiple sites. The observed reduction in GSK3 β as well as tau phosphorylation in CT1812-treated patients provides the first clinical biomarker evidence supporting the A β oligomer hypothesis of AD and further support of CT1812's disease-modifying mechanism of action.

We hypothesize that changes in CSF concentration of a subset of proteins with CT1812 treatment reflect target engagement in the brain. Further clinical studies with longer treatment duration are underway; treatment-related impact on biomarkers in these independent clinical cohorts will provide additional insight on CT1812 target engagement and AD pathobiology as well as insight into how covariates like sex, age, and apolipoprotein E status impact response to drug. The effects of these changes in CSF protein concentrations on cognition and health is unknown and the relative benefit of these effects

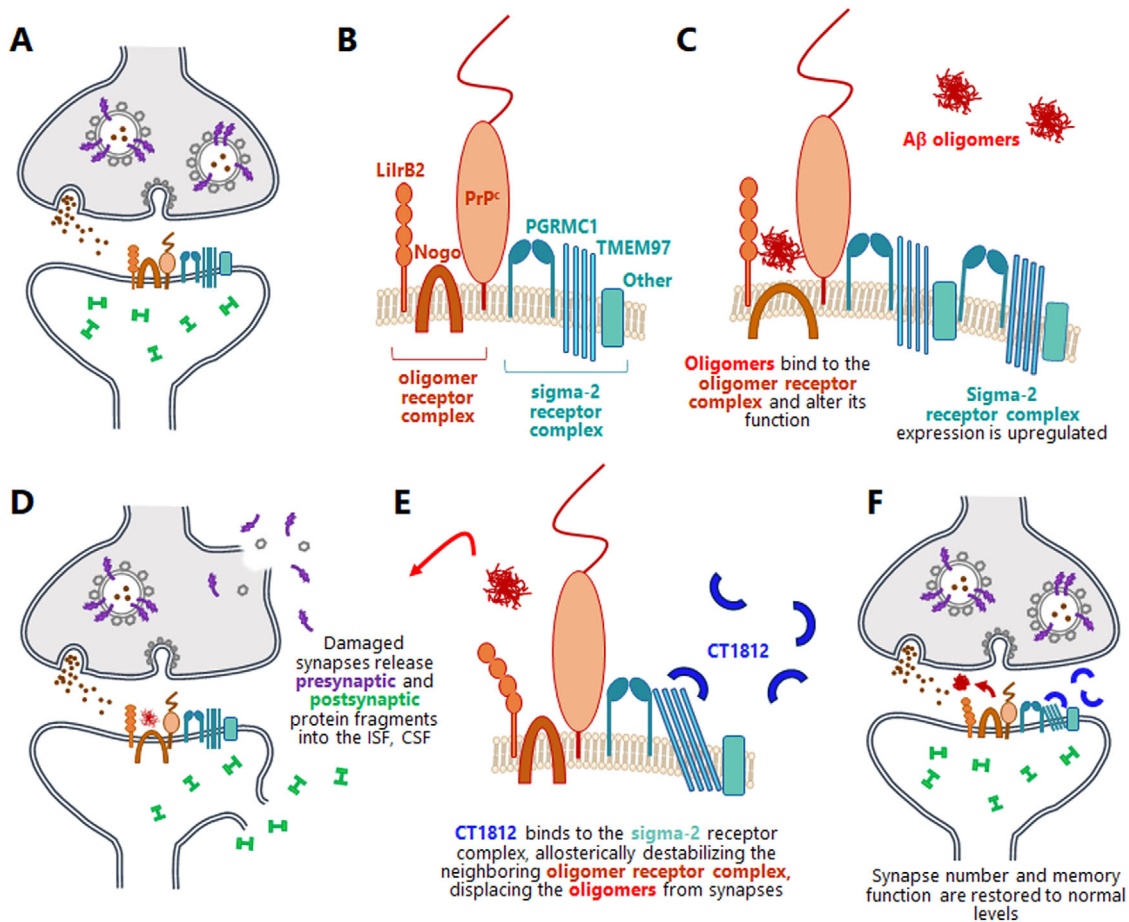


FIGURE 6 Hypothesized mechanism of action of CT1812: synaptoprotective displacement of toxic amyloid beta (A β) oligomers from neurons. A, In the absence of A β oligomers, pre- and post-synaptic proteins such as synaptotagmin-1 and neurogranin are expressed at normal levels. B, The sigma-2 receptor complex tightly regulates the oligomer receptor complex by stabilizing plasma membrane expression of oligomer receptor component proteins. C, Oligomer binding results in changing expression/localization of synapse associated proteins, spine loss, and memory failure, as well as upregulation of the sigma-2 receptor complex. D, Fragmented pre- and post-synaptic proteins such as synaptotagmin-1 and neurogranin move into interstitial fluid and then cerebrospinal fluid. E, CT1812 binds to the sigma-2 receptor ligand binding site within the sigma-2 receptor complex, acting as a negative allosteric modulator of A β oligomer binding. This destabilizes the oligomer binding site and displaces oligomers from synapses without affecting normal synapse protein function. F, Synapse number and memory are restored to normal levels

compared to the risks of the drug are being determined in ongoing and future placebo-controlled clinical trials.

3.3 | CT1812 for the treatment of AD

The hypothesized disease-modifying mechanism of action of CT1812 is illustrated in Figure 6. In the normal brain (Figure 6A), the sigma-2 receptor complex regulates the plasma membrane surface expression of putative A β oligomer receptor complex component proteins LilrB2, Nogo receptor, and cellular prion protein as well as several other receptors⁷⁸ (Figure 6B). In AD, A β oligomers bind to their receptor, altering its function and/or protein interactions, and resulting in a compensatory upregulation of the sigma-2 receptor complex (Figure 6C).^{79,80} The binding of A β oligomers then leads to synaptic damage and release of presynaptic and postsynaptic pro-

teins such as neurogranin and synaptotagmin-1 into the ISF and CSF (Figure 6D). CT1812 binds to and allosterically modulates the sigma-2 receptor (Figure 6E), causing destabilization of the neighboring A β oligomer receptor binding sites with resultant displacement of A β oligomers from synaptic receptors (i.e., increase in the “off rate”) without affecting normal synaptic protein function. CT1812 is thus a negative allosteric modulator of A β oligomer binding to synaptic receptors. As a result of the removal of toxic oligomers, synapse number and memory are restored to normal (Figure 6F). CT1812 selectively displaces A β oligomers shortly after entering the brain and prevents them from rebinding as long as brain drug levels remain above an estimated 80% receptor occupancy.²⁶ Chronic administration provides sustained protection from the synaptotoxic oligomer assault, facilitating synaptic recovery.

The current studies suggest that CT1812 effectively and selectively targets A β oligomers by a novel mechanism of action not

previously demonstrated for any other therapeutics currently in development. CT1812 is both the first selective sigma-2 receptor antagonist to be used in clinical trials, and the first therapeutic demonstrated to displace toxic A β oligomers from synaptic receptor sites and selectively facilitate their clearance into the CSF. Displacement of oligomers lowers their binding affinity (K_D) to synaptic receptors significantly, thus CT1812 is the only therapeutic currently in development that mimics the effect of the protective Icelandic A673T mutation.⁶ This mechanism is significant, as therapeutics that recapitulate the effects of protective mutations have a track record of clinical success.⁶⁶

Currently, approved AD medications do not stop the disease course, but simply treat symptoms for a limited time. These preclinical results of CT1812 in rodent models of AD demonstrate that CT1812 blocks A β oligomer binding to neurons, reduces synapse loss, restores synaptic protein expression, facilitates oligomer clearance into the CSF, and restores cognitive function. The ability of CT1812 to improve cognitive performance in transgenic mice while not affecting the performance of WT mice supports a specific effect of CT1812 on the pathological pathway of AD: this disease-selective mechanism may provide an advantage over other therapeutic approaches that impact brain function more widely.

Preliminary evidence from the phase 1b/2a clinical trial reported here supports these findings. This study was limited by small patient numbers and short duration of treatment in the COG0102 trial. Larger studies with additional dose levels will be required to establish dose-response relationships with biomarker changes, and distinguish both acute and long-term disease-modifying effects of CT1812 treatment. While not all proteins and signaling pathways known to be dysregulated in AD were affected by treatment with CT1812, several pathways were significantly impacted (including cholesterol transport, oxidative stress, complement, and synaptic transmission; Figure 5E). Together, these results provide encouraging evidence of the impact of CT1812 on multiple aspects of disease in AD patients and support further development of this drug candidate so that the relative benefit and risks of CT1812 can be more thoroughly evaluated.

CT1812 is currently being studied in four randomized, double-blind, placebo-controlled phase 2 studies (6 months–1 year in treatment duration) in patients with mild to moderate AD: SNAP (NCT03522129), SPARC (NCT03493282), SHINE (NCT03507790), and SEQUEL. An additional 18-month study in 540 early AD patients (MMSE 20–30) is being planned in collaboration with the ACTC clinical trial network. The safety and tolerability of CT1812 will continue to be explored in all clinical trials.

4 | MATERIALS AND METHODS

4.1 | Radioligand binding

Radioligand competition assays to determine affinity for sigma-2 and sigma-1 receptors were performed at Eurofins Cerep SA (Le Bois L'évêque, France) in membranes from Jurkat cells (immortalized

human T cells). Assays for sigma-2 receptors used 25 nM [³H]1,3-di(2-tolyl) guanidine in the presence of 1 μ M (+)-pentazocine while assays for sigma-1 receptors used 15 nM [³H](+)-pentazocine. Non-specific binding in both assays was defined in the presence of 10 μ M haloperidol.⁸¹ Estimated percent receptor occupancy for in vivo behavioral studies (see below) was calculated according to the formula $100 \times (\text{concentration}/K_i) / ((\text{concentration}/K_i) + 1)$, where K_i is determined by radioligand competition binding (CT1812 = 8.5 nM).²⁶

4.2 | Neuronal cultures

Mixed neuronal and glia cultures were made from embryonic day 18 (E18) Sprague Dawley rat brains and grown in poly-D Lysine coated plates for 21 days, as previously described.^{26,28} All procedures were approved by the Institutional Animal Care and Use Committee at Cognition Therapeutics and were in compliance with the Office of Laboratory Animals Welfare and the Guide for the Care and Use of Laboratory Animals, Eighth Edition. Embryos from a single rat were pooled to produce a unique dissociated neuronal culture. All analyses described below were performed on at least three unique cultures. Healthy cultures typically contain 20% to 35% MAP2-positive neurons. CT1812 was tested in quadruplicate wells for each concentration in at least three replicate experiments with data from all experiments pooled for analysis with means \pm standard deviation (SD) or standard error of the mean (SEM) as noted.

4.3 | A β oligomer preparation

Synthetic human A β 1-42 peptide was prepared as previously described.^{26,28} Briefly, peptide was treated according to published methods to remove any structural assemblies.^{82,83} An A β monomer film was prepared, dissolved, diluted to 100 μ M, and incubated at 4°C to form oligomers. The resulting preparations were centrifuged to remove any insoluble fibrils, and the supernate added to cultures. All studies using synthetic oligomers were performed with this preparation unless otherwise specified. All lots of A β 1-42 are put through a strict quality control processes, described previously,^{26,28} before use in experiments. As previously reported, oligomers produced via this method typically range from 25–275 kDa when measured via non-denatured western blots (Figure 2²⁶).

Human AD patient- (Braak V/VI) and age-matched control hippocampal brain specimens, less than 24 hours *post mortem*, were obtained from the Brown Brain Bank following previously described procedures.^{26,28} Tissue was prepared as previously described.^{26,28} Briefly, the brain tissue was homogenized and centrifuged, supernate were immuno-depleted and concentrated to obtain the 10–100 kDa size-fractionated oligomers, which were then captured on 6E10-conjugated agarose columns (Pierce). The released material was desalted and stored at –80°C. Use of *post mortem* brain specimens met the exemption criteria for Department of Health and Human Services regulations, as previously explained.^{26,28}

4.4 | In vitro trafficking assay

Vesicular trafficking was measured using an adaptation of a method as previously described.^{26,84} Neurons were treated with compounds and/or A β oligomer preparations (0.086% DMSO in culture media) and incubated for 1 to 24 hours at 37°C in 5% CO₂. For prevention studies, CT1812 was added at indicated concentrations 1 hour prior to addition of A β oligomers. In treatment studies, A β oligomers were added 1 hour prior to addition of CT1812. Tetrazolium salts (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Roche Molecular Biochemicals) were added at a final concentration of 0.75 mM and incubated at 37°C for 60 to 90 minutes. Vesicular formazan remaining in cells was quantified by absorbance spectrometry (590 nm with 690 nm subtracted) after extraction with 1.6% Tween-20. All compounds were tested in quadruplicate wells for each concentration in at least eight replicate experiments with data from all experiments pooled for analysis with means \pm SEM.

4.5 | In vitro A β binding assay

Assessment of A β oligomer binding was performed as previously described.^{26,28} Briefly, cultures were either (for prevention studies) treated with CT1812 for 30 minutes, followed by synthetic A β 1-42 oligomer preparation for 60 minutes, or (for displacement studies) treated with oligomers 30 minutes prior to the addition of CT1812. Cells were then fixed and labeled for immunofluorescent microscopy as previously described.²⁶ All data for A β binding presented represents total fluorescent intensity of A β label in neurite spots per neuron in relative fluorescent units (RFUs). Replications and statistical procedures for the quantification of A β immunofluorescent intensity were previously described.²⁶

4.6 | In vitro synapse counting assay

Synapse number was quantified using quantitative immunofluorescent microscopy of drebrin-labeled puncta (Millipore AB-10140) along MAP2-positive neurites (Millipore AB5543) as previously described.²⁶ Additional immunofluorescent labeling was performed on neuronal cultures for SYT-1 (Synaptic Systems #105 103) and for neurogranin (Abcam ab23570).

4.7 | Human tissue ex vivo A β displacement

Displacement of A β from human tissue was assessed as previously described.²⁸ Briefly, brains from human AD patients were obtained through the Massachusetts Alzheimer's Disease Research Center and Massachusetts General Hospital (MGH) Neuropathology Department. Experiments using human brain were reviewed and approved by the Academic and Clinical Central Office for Research and Development medical research ethics committee, a joint office of the University of Edinburgh and National Health Service (NHS) Lothian (approval 15-

HV-016). Parahippocampal gyrus sections (one section per specimen per condition) were incubated with 0.1, 1.0, 10, or 15 μ M CT1812 in phosphate-buffered saline (PBS), or vehicle for 60 minutes as previously described²⁶ and then labeled with an antibody specific to A β (AW-7, gift from Dominic Walsh⁸⁵). Dense core amyloid plaques and NFTs were labeled with 0.05% Thioflavin-S (Sigma Aldrich) in 50% ethanol for 8 minutes before treatment with 80% ethanol for 30 seconds. Images were analyzed in ImageJ⁸⁶ using a custom macro (described in figure S4 in Izzo et al.²⁸). A total of 105 to 279 plaques were analyzed for each treatment from eight different donors and average intensities for each specimen were calculated. The median intensity value was calculated for each specimen and subjected to multivariate correlation analysis as previously described.²⁸ Determination of total A β concentration eluted from human donor brain tissue was performed using a high sensitivity, human Beta Amyloid (1-42) ELISA Kit (Wako, cat # 296-64401) which uses BAN50/BC05 (Fab').

4.8 | Western blot analysis of A β oligomers

Non-denaturing western blot conditions were used to avoid the generation of A β assembly and disassembly artifacts, as previously described.²⁶ Briefly, baseline and end of study CSF samples from AD patients participating in clinical trial COG0102 were collected in polypropylene tubes in 500 μ L and frozen at -80°C until analysis. Upon receipt, the samples were thawed and quickly aliquoted into polypropylene tubes coated with isotonic human serum albumin and refrozen until analysis by native western gels. Because the effects of blood on A β oligomer formation in CSF is unknown, an aliquot of each patient sample was pre-analyzed on a western blot using luminol and horseradish peroxidase to test for the presence of heme. Final western gel analysis was performed on samples excluding those containing hemolyzed blood. Samples were run on Tris-HCl nondenaturing gels, transferred to nitrocellulose, and probed with 82E1 mouse monoclonal A β antibody (IBL America) and visualized by chemiluminescence after detection using a goat anti mouse IgG-horseradish peroxidase secondary antibody (Millipore). All reagents were filtered through 0.1 micron filters to eliminate background debris. Band intensities on the gels were quantified using an Alpha Innotech image system and Alpha View software (ProteinSimple). Each patient's sample at day 28 was normalized as a percent of that patient's own baseline value.

4.9 | Measurement of A β using microimmuno-electrodes

APP/PS1+/- hemizygous mice⁴² were bred to WT C3H/B6 mice (The Jackson Laboratory) and the APP/PS1+/- offspring were used for experiments at 12 months of age. Male and female littermate mice were distributed between experimental groups.

MIEs, which can measure A β every 60 seconds for up to 3 hours, were prepared similar to previously described methods.⁴⁰ Carbon fiber microelectrodes were used as the platform sensor due to their

high signal-to-noise ratio, biological compatibility, and miniature size,⁸⁷ and ability to sensitively detect tyrosine oxidation (66). Electrodes were coated with antibodies specific for A β monomer (HJ2)⁴⁰ or for oligomers (A11)⁴¹ and inserted into the hippocampus to measure brain ISF A β levels or into lateral ventricles to measure CSF A β levels.

Electrodes were pre-calibrated in vitro (see supporting information). In addition to the MIE, a stainless-steel bone screw used as a counter electrode and an Ag/AgCl reference electrode (InVivo Metrics) were implanted into the brains of 12-month-old APP/PS1+/- transgenic mice. All electrodes were stereotaxically implanted under isoflurane anesthesia. Mice were placed on a heating pad to maintain body temperature and repetitive SWV scans were run every 60 seconds for up to 180 minutes using a CH Instruments 830D Electrochemical Analyzer with PicoAmp booster. The stereotax was enclosed in a faraday cage to reduce electrical noise. CT1812 or vehicle were administered intravenously via tail vein injection at the concentrations noted. All procedures were approved by the Institutional Animal Care and Use Committee at Washington University, St. Louis, and were in compliance with the Office of Laboratory Animals Welfare and the Guide for the Care and Use of Laboratory Animals, Eighth Edition.

More detail can be found in the supporting information section.

4.10 | Behavioral studies

All procedures were approved by the Institutional Animal Care and Use Committee at Stanford University and were in compliance with the Office of Laboratory Animal Welfare and the Guide for the Care and Use of Laboratory Animals, Eighth Edition. Testing of male transgenic Thy1 huAPP^{Swe/Lnd+} mice in the Morris water maze, Y-Maze, or fear conditioning tasks was done according to published methods.^{45,46} Studies were designed with four arms: WT, vehicle treated ($n = 12$); WT, CT1812 treated ($n = 12$); transgenic, vehicle treated ($n = 12$); transgenic, CT1812 treated ($n = 13$). All procedures were approved by the Institutional Animal Care and Use Committee at Stanford University and were in compliance with the Office of Laboratory Animals Welfare and the Guide for the Care and Use of Laboratory Animals, Eighth Edition.

To measure brain concentration of drugs, brains were homogenized in 1 mL PBS per gram of brain tissue using a handheld homogenizer. Brain homogenate was then extracted with three volumes of ice-cold methanol on ice for 15 minutes and centrifuged for 15 minutes. The supernates were analyzed by LC-MS/MS. Samples were compared to a calibration curve prepared in a similar manner by spiking blank control homogenate with standards prepared in DMSO and then extracted as above.

4.11 | Clinical trial of CT1812 in mild-to-moderate AD patients

The clinical trial was performed at six sites in Australia and administered by Neuroscience Trials Australia. The study protocol was approved by the Human Research Ethics Committee at the Alfred Hos-

pital, Melbourne, Australia, and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All subjects provided written informed consent before participating. Mild to moderate (MMSE 18–26) AD patients ($N = 19$) were randomized to one of three doses of CT1812 (90, 280, or 560 mg Q.D.) or placebo given orally once daily for 28 days (4 weeks) to determine safety and tolerability (COG0102, for full listing of inclusion and exclusion criteria see ClinicalTrials.gov NCT02907567). Exploratory outcomes included CSF protein expression measured at baseline (Day 0) and end of treatment (Day 28). Missing samples (declined or failed spinal taps) and variable CSF sample volumes reduced the number of matched baseline and day 28 patient CSF samples available for subsequent analysis.

4.12 | ELISA measurement of CSF synaptic proteins

CSF levels of A β 40, A β 42, t-tau, and p-tau were measured using the INNOTEST ELISA (Fujirebio, Ghent, Belgium), following the recommendations by the manufacturer. NfL was measured using an ELISA from Uhman Diagnostics. These analyses were conducted by board-certified laboratory technicians according to Swedish Board for Accreditation and Conformity Assessment (SWEDAC)-approved protocols. Neurogranin CSF concentrations were measured using an in-house ELISA method based on the Ng2 and Ng22 antibodies, as described previously in detail.⁸⁸ Concentrations of SNAP-25 in CSF were measured using immunoprecipitation combined with mass spectrometry (IP-MS) as described previously.⁸⁹

4.13 | LC-MS/MS measurement of CSF synaptic proteins and phosphorylated tau fragments

Synaptotagmin-1 measurements were obtained as part of unbiased LC-MS/MS proteomics analysis of AD patient CSF samples at Caprion Biosciences using published methods.⁹⁰ Additional unbiased proteomics analyses including an enrichment for phospho-proteome were performed on CSF samples at Proteome Sciences using the protocols as previously published^{61,91} with minor modifications. For statistical analysis of unbiased LC-MS-MS proteomics, a linear mixed model was used. Detailed methods can be found in the supporting information section.

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AUTHOR CONTRIBUTIONS

N.I., C.Y., G.L., G.R., H.S., H.L., M.S., T.S., J.C., and S.C. designed the research. S.D., L.S., M.G., and S.C. designed the clinical trial and performed the clinical data analysis. C.Y., K.M.L., L.W., C.L., C.R., R.Y., C.H.,

H.L., M.S., D.C., T.S., and J.C. performed the research. N.I., C.Y., K.M.L., C.L., L.W., K.S., C.W., C.R., R.Y., G.L., G.R., C.H., H.L., M.S., T.S., J.C., D.C., M.H., and S.C. analyzed the data. N.I., S.C., and M.H. wrote the paper.

CONFLICTS OF INTERESTS

N.I., K.M.L., C.L., C.R., R.Y., L.W., G.L., G.R., H.S., M.H., C.W., K.S., and S.C. are employees of Cognition Therapeutics, Inc. C.Y., J.C., H.L., M.S., M.G., and T.S.-J. are paid consultants of Cognition Therapeutics, Inc. L.S. reports grants and personal fees from Eli Lilly; personal fees from Avraham, Ltd; personal fees from Boehringer Ingelheim; grants and personal fees from Merck; personal fees from Neurim, Ltd; personal fees from Neuronix, Ltd; personal fees from Cognition Therapeutics, Inc.; personal fees from Eisai; personal fees from Takeda; personal fees from vTv; grants and personal fees from Roche/Genentech; grants from Biogen; grants from Novartis; personal fees from Abbott; grants from Biohaven, outside the submitted work. S.D. is a member of the Neuroscience Advisory Board for Amgen; Chair of the Medical Scientific Advisory Board for Acumen; Chair of the Drug Monitoring Committee for Biogen; Chair of the Medical Advisory Board for Cognition Therapeutics, Inc.; and Editor for Dementia for UpToDate. H.Z. has served at scientific advisory boards for Cognition Therapeutics, Roche Diagnostics, Wave, and Samumed; has given lectures in symposia sponsored by Biogen and Alzecure; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. T.S. is a scientific advisory board member of Cognition Therapeutics, Inc. and receives collaborative grant funding from two pharmaceutical companies. K.B. has served as a consultant or at advisory boards for Alector, Biogen, Cognition Therapeutics, Lilly, MagQu, Novartis, and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. International Patent WO 15/116923 pertains to the results presented in this paper.

DATA AVAILABILITY STATEMENT

All data needed to evaluate the conclusions in the paper are present in the paper itself and the supporting information or available upon request from the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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