

Orally bioavailable small molecule drug protects memory in Alzheimer's disease models

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ABSTRACT

Oligomers of beta-amyloid (Aβ) are implicated in the early memory impairment seen in Alzheimer's disease before to the onset of discernable neurodegeneration. Here, the capacity of a novel orally bioavailable, central nervous system-penetrating small molecule 5-aryloxy pyrimidine, SEN1500, to prevent cell-derived (7PA2 [conditioned medium] CM) Aβ-induced deficits in synaptic plasticity and learned behavior was assessed. Biochemically, SEN1500 bound to Aβ monomer and oligomers, produced a reduction in thioflavin-T fluorescence, and protected a neuronal cell line and primary cortical neurons exposed to synthetic soluble oligomeric Aβ_{1–42}. Electrophysiologically, SEN1500 alleviated the in vitro depression of long-term potentiation induced by both synthetic Aβ_{1–42} and 7PA2 CM, and alleviated the in vivo depression of long-term potentiation induced by 7PA2 CM, after systemic administration. Behaviorally, oral administration of SEN1500 significantly reduced memory-related deficits in operant responding induced after intracerebroventricular injection of 7PA2 CM. SEN1500 reduced cytotoxicity, acute synaptotoxicity, and behavioral deterioration after in vitro and in vivo exposure to synthetic Aβ and 7PA2 CM, and shows promise for development as a clinically viable disease-modifying Alzheimer's disease treatment.

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1. Introduction

One of the major diseases of the nervous system associated with age is Alzheimer's disease (AD). Recent theories of the development of neuropathology and behavioral decline in AD provide a persuasive argument that an early stage involves the formation of extracellular soluble β-amyloid peptide (Aβ) into oligomers (Verdile et al., 2004; Walsh and Selkoe, 2004a, 2004b). As a consequence of this approach, memory loss, 1 of the major early symptoms of AD, has been proposed to be the result of synaptic dysfunction related to extracellular Aβ oligomer formation (Haass and Selkoe, 2007; Shankar et al., 2008), which might precede the development of discernible neuropathology. Support for the contention that initial accumulation of extracellular oligomeric Aβ detrimentally affects memory before the onset of quantifiable histopathology has come from several

sources. Cultured cells that express human Aβ precursor protein (7PA2 [conditioned medium] CM) secrete oligomers of human Aβ (Podlisny et al., 1995) and these Aβ oligomers are similar to those found in human cerebrospinal fluid (Walsh et al., 2002). Secretions from the cultured cells contain natural dimers, trimers, and tetramers of Aβ that are composed of N- and C-terminally heterogeneous human Aβ peptides, including the Aβ_{1–40} and Aβ_{1–42} species that occur in human brain and extracellular fluids (Podlisny et al., 1995, 1998; Walsh et al., 2002, 2005). Using long-term potentiation (LTP), a well established model of synaptic plasticity which provides an experimental representation of memory processes at the cellular level, it has been shown that LTP induction in the hippocampus is detrimentally affected by exposure to these Aβ oligomers in normal rats and mice. Aβ oligomers have been found to significantly inhibit hippocampal LTP in vitro (Walsh et al., 2000) and in vivo (Walsh et al., 2002), and it has been suggested that this inhibition effect might mimic an early manifestation of the memory loss seen in AD (Ondrejcek et al., 2010). Moreover, using the alternating-lever cyclic-ratio (ALCR) schedule of behavioral analysis, it has been shown that

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intracerebroventricular (i.c.v.) injection of A β oligomers in the rat produces acute cognitive deficits, and that A β oligomers are necessary and sufficient to disrupt memory-related behavior in freely moving intact animals (Cleary et al., 2005; Poling et al., 2008; Townsend et al., 2006).

Consequently, contemporary research findings suggest that extracellular formation of A β oligomers at an early stage of disease development warrants consideration relative to processes underlying AD. From a therapeutic perspective, the progressive accumulation of A β assemblies has long been considered fundamental to development of the neurodegenerative pathology seen in AD (Tanzi et al., 2004), and preventing the aggregation of A β has been an appealing therapeutic approach. Targeting the effects of A β oligomers, and the oligomer assembly mechanism, is of interest because it is likely to offer a better safety profile than other pharmacotherapies (Amijee and Scopes, 2009). Unfortunately, nonpeptide small molecule inhibitors of A β aggregation have been extremely scarce, and the majority of compounds claimed to be A β aggregation inhibitors are unsuitable from a pharmacotherapeutic perspective because of their biologic profile, poor oral bioavailability, and short half-life (Rishton, 2008). However, 1 nonpeptide small molecule, RS-0406, inhibits A β toxicity and A β _{1–42} aggregation (Nakagami et al., 2002) and has been shown to protect primary hippocampal neurons against A β _{1–42}-induced cytotoxicity, to rescue A β _{1–42}-impaired LTP (Walsh et al., 2005), and to arrest A β oligomer-induced behavioral deterioration in the rat when administered intracerebroventricularly (O'Hare et al., 2010). Taking these findings into account, our group has modified the RS-0406 chemical structure to improve its potency, specificity, and oral bioavailability. In the current series of experiments a novel derivative of RS-0406, the 5-aryloxypyrimidine SEN1500 (Fig. 1), was investigated relative to A β _{1–42} aggregation and A β _{1–42}-induced deficits in cell viability, depression of LTP in vitro and in vivo after exposure to aggregated A β _{1–42} and A β oligomers, and the effects of oral administration on cognitive impairment after i.c.v. injections of 7PA2 CM-derived A β in normal freely moving rats.

2. Methods

2.1. Compound

SEN1500 (2-Fluoro-5-[5-(3-morpholin-4-ylphenoxy)-pyrimidin-2-ylamino]benzonitrile, 98.9% purity) was synthesised by Oxygen Healthcare Limited (Ahmedabad, India).

2.2. SEN1500 effects on A β binding, aggregation, and cell viability

Recombinant A β _{1–42} (rPeptide, Bogart, GA, USA) was prepared for amyloid aggregation and toxicity assays by dissolving A β _{1–42} HCl salt in hexafluoroisopropanol with brief sonication and vortexing. This solution of the A β _{1–42} peptide in hexafluoroisopropanol was stored at 4 °C at 2 mM. When required, an aliquot of the stock solution was freeze-dried and dissolved in dimethyl sulfoxide (DMSO) to 200 times the required final assay

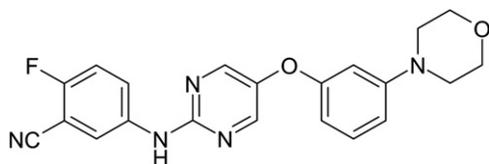


Fig. 1. Chemical structure of SEN1500 (2-Fluoro-5-[5-(3-morpholin-4-ylphenoxy)-pyrimidin-2-ylamino]benzonitrile). Purity 98.9%.

concentration. For thioflavin-T and cell viability assays, a 20 mM stock solution was prepared in DMSO and aliquots of this solution were used to prepare further stock solutions in DMSO, ranging in concentration from 3 μ M to 10 mM. These stock solutions were prepared for use as required and stored at –20 °C (maximum of 3 freeze-thaw cycles).

2.3. Surface plasmon resonance studies with SEN1500

A Biacore T-100 protein interaction analysis system (GE Healthcare) equipped with 4 flow cells on a sensor chip was used for real-time binding studies. HBS-EP buffer, which contained 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.15 M NaCl, 3 mM ethylenediaminetetraacetic acid and 0.5% of Tween was used as the assay running buffer and also for sample preparation. A streptavidin (SA) chip (GE Healthcare) was used to immobilize biotinylated A β _{1–42} monomer or oligomers on different flow cells. N-terminus biotinylated A β _{1–42} was freshly prepared in 50% DMSO and pulse injected onto the SA surface until the required immobilization level of approximately 1300 response units was reached to give a monomer surface. A β _{1–42} oligomers were prepared using the method described by Kaye et al. (2003) to facilitate more reproducible biotinylation than with alternative oligomeric preparations, but modified in that a 1:10 ratio of biotinylated A β _{1–42} to non-biotinylated A β _{1–42} was used. When prepared, these A β oligomers were injected over a flow cell at 2 μ L/min for 15 minutes to achieve full saturation of the flow cell surface. As a control, 1 mM biotin was injected onto another flow cell to allow saturation of the SA chip with biotin. The binding of SEN1500 in the flow phase, onto immobilized A β _{1–42} monomer or oligomer, was measured by response units. The response units elicited by SEN1500 injected onto the biotin control flow cell was set as the reference response, which represented the refractive index signals caused by solvent in the injected samples and was subtracted from the response units elicited by the same compounds injected onto the A β _{1–42} flow cells. To perform the binding studies, 120 μ L of SEN1500 ranging from 0.5 μ M to 30 μ M prepared in HBS-EP buffer containing 5% DMSO were injected and passed onto the sensor chip for 4 minutes at 30 μ L/min, and responses were recorded. After each injection, HBS-EP with 5% DMSO buffer was passed over the chip for 10 minutes at 30 μ L/min, to allow the bound compound to dissociate from the immobilized A β _{1–42} monomer, and the dissociation curves were obtained. The response elicited by injecting HBS-EP buffer alone with 5% DMSO was used as the blank. To establish the negative control condition, an analogue of SEN1500 which was inactive in the fibrillogenesis and cell viability assays was used, and was found not to bind to immobilized A β _{1–42}. After the dissociation phase, 15 μ L of the regeneration solution, 1 M NaCl in 20 mM NaOH was injected and passed over the chip for 1 minute at 30 μ L/min to remove residual bound compound from immobilized A β _{1–42}. Biacore T-100 control and evaluation software (version 2.0.2) was employed to record the changing responses, plot the binding curves, analyze the data, and calculate the K_D of SEN1500 according to a 1:1 binding model for binding to A β _{1–42} monomer.

2.4. Thioflavin-T assay

Activity in inhibiting 10 μ M A β _{1–42} aggregation was assessed using a thioflavin-T fluorimetric assay. SEN1500 was incubated in 50 mM NaPi, 150 mM NaCl with 20 μ M A β _{1–42} at a final DMSO concentration of 2% for 24 hours at 37 °C, pH 7.4. A 50 μ L aliquot was taken and dispensed into a black 96-well plate. An equal volume (50 μ L) of thioflavin-T (40 μ M) (in Glycine buffer [50 μ M] and NaOH pH 8.5) was added to each well. The plate was shaken and fluorescence and recorded using the top reader (Biotek Synergy) setting

(10 times at 1 ms), with excitation and emission filters of 440 (± 15) and 485 (± 10) nm, respectively. Fluorescence readings from equivalent experiments were averaged and percent of amyloid formation was determined as follows:

$$\text{Percent of amyloid formed} = \frac{[F(\text{sample}) - F(\text{blank})] \times 100\%}{[F(\text{amyloid alone}) - F(\text{blank})]}$$

2.5. Cell viability assay for amyloid toxicity using MTT reduction

The activity of compounds in protecting the neuroblastoma cell line SH-SY5Y (HPA Culture Collection, Salisbury, UK) from a toxic insult of 10 μM $\text{A}\beta_{1-42}$ was assessed using inhibition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction as a measure of cell viability. SH-SY5Y cells were grown in F12 media supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin and 1% nonessential amino acids (all from Invitrogen, Paisley, UK). For testing the SH-SY5Y, cells were plated in 96-well plates at a density of 30,000 cells per well in Opti-Mem (Invitrogen), containing 2% FBS, 1% L-glutamine, 1% penicillin/streptomycin and 1% nonessential amino acids. The concentrations of SEN1500 on the cells ranged from an approximate 15 nM to 50 μM with a final concentration of $\text{A}\beta_{1-42}$ of 10 μM and final DMSO concentration of 1%. Cell plates were incubated for 24 hours before performance of the MTT assay (Shearman et al., 1995). Briefly, 15 μl of MTT dye (R&D Systems, Abingdon, UK) was added to each well and the plates were incubated in 5% CO_2 at 37 $^\circ\text{C}$ for 4 hours. One hundred microliters of Stop/solubilization solution (R&D Systems) was then added to each well and the plates were left overnight in a humidified box at room temperature. The plates were then shaken and the absorbance was recorded at both 570 nm and 650 nm using a Biotek Synergy plate reader. ΔA values were calculated by subtracting absorbance at 650 nm from absorbance at 570 nm, to reduce nonspecific background absorbance. ΔA values from equivalent experiments were averaged and percentage of cell viability was determined as follows:

$$\text{Percent of cell viability} = \frac{[\Delta A(\text{sample}) - \Delta A(\text{dead cell control})] \times 100\%}{[\Delta A(\text{live cell control}) - \Delta A(\text{dead cell control})]}$$

where live cell controls were treated with 1% DMSO in Opti-Mem, and dead cell controls with 0.1% Triton X-100 added to cells.

2.6. Synaptophysin assay

The loss of synapses and the reduction in synaptophysin levels are features of AD that strongly correlate with cognitive decline, and the amount of synaptophysin in neuronal cultures can be measured as a surrogate marker of synaptic function. It has been shown that low concentrations of synthetic $\text{A}\beta_{1-42}$ cause a deficit in synaptophysin levels in cortical/hippocampal neurons in a dose-related manner (Bate et al., 2008). Cortical neurons were prepared from the brains of Day 15 C56 black 6 mouse embryos. Dissected tissue was chilled in ice-cold Ham's F12 plus glutamax containing 5% fetal calf serum, nonessential amino acids, 0.35% glucose, 0.025% trypsin, 0.2 mg/mL papain, 0.2 mg/mL collagenase type IV, and DNase 1. The cortical tissue was placed in fresh medium at 37 $^\circ\text{C}$ and triturated, followed by incubation for 30 minutes at 37 $^\circ\text{C}$ with gentle agitation every 5 minutes. Cells were passed through a 70 μm nylon cell strainer (BD Falcon) and twice centrifuged for 5 minutes at 170g, then resuspended by very gentle agitation in Ham's F12 plus glutamax containing 5% fetal calf serum, nonessential amino acids, and 0.35% glucose at 37 $^\circ\text{C}$. Viable cells were counted (refractory to

trypan blue) and dispensed into 96-well plates previously coated with poly-L-lysine. Cells were plated at approximately 35,000 cells per well in 100 μl of neurobasal medium and incubated at 37 $^\circ\text{C}$ in 5% CO_2 . After 4 hours the medium was gently aspirated and replaced with neurobasal medium containing B27 supplements. Cells were incubated for 6 days, with a 50% replacement every 2 days, before being assayed against test compounds. For assay, cell media was aspirated and replaced with 50 μl of fresh neurobasal medium containing B27 supplements. Test compounds were incubated with newly made synthetic $\text{A}\beta_{1-42}$ at twice the final concentration for 30 minutes at room temperature before diluting this mixture 1:1 onto the cells, giving a final well volume of 100 μl . Cell plates were then returned to the incubator for 24 hours, the media was then aspirated and the cells washed with phosphate-buffered saline (PBS). Assay samples were prepared by aspirating primary cell experimental media and washing the cells 3 times with PBS. The final buffer added was 70 μl of 150 mM NaCl, 10 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid, 0.2% sodium dodecyl sulfate and broad spectrum protease inhibitor mix containing AEBSF, Aprotinin, Leupeptin, Bestatin, Pepstatin A, and E-46 (Sigma). Cells were frozen and thawed 3 times, and after each freezing the cell plate was floated in a sonicating water bath at 37 $^\circ\text{C}$. Cells were sonicated as they thawed for 30 seconds and the content of each well was removed to a centrifuge tube. Samples were centrifuged at 16,000g and nuclei and cellular debris was removed. Levels of synaptophysin were then measured using a sandwich enzyme-linked immunosorbent assay. Briefly, the capture antibody used was a mouse monoclonal anti-synaptophysin antibody (MAB368, Chemicon), adsorbed in carbonate buffer for 1 hour at 37 $^\circ\text{C}$ onto Nunc Maxisorb immunoplates. Wells were washed in PBS with 0.2% Tween between each addition, then blocked for 1 hour at room temperature with PBS and Tween supplemented with 5% nonfat milk powder. Fifty microliters of the cell extract sample was added to each well and incubated for 2 hours at room temperature. Rabbit polyclonal anti-human synaptophysin (ab53166; Abcam) diluted in PBS and Tween at 1:2000 was added to each well and incubated for 1 hour at room temperature. After washing, 1 in 1000 biotinylated anti-rabbit antibody (IgG; Dako) was added and incubated for 1 hour at room temperature. Bound antibodies were detected with 1 in 2000 extravidin-alkaline phosphatase (Sigma) and 1 mg/mL 4-nitrophenol phosphate in diethanolamine buffer. Absorbance was measured at 450 nm.

2.7. 7PA2 cells

7PA2 cells are stably transfected Chinese hamster ovary (CHO) cells incorporating cDNA for amyloid precursor protein (APP751) specific for the familial AD mutation Val717Phe (Shankar et al., 2011; Walsh et al., 2002). The cells were grown to just below confluence in Dulbecco's modified Eagle's medium, containing 10% FBS and 200 $\mu\text{g}/\text{mL}$ G418, briefly washed in Dulbecco's phosphate buffered saline and incubated at 37 $^\circ\text{C}$ with 5% CO_2 for 18 hours with sufficient volume of Dulbecco's modified Eagle's medium to just cover the cells. After incubation the medium was centrifuged at 3000g for 15 minutes and either used directly or snap frozen and stored at -20 $^\circ\text{C}$. Using enzyme-linked immunosorbent assay, the concentration of total $\text{A}\beta$ in the 7PA2 CM used in the LTP and behavioral studies was in the range of 2–5 nM.

2.8. In vitro electrophysiology

Evoked extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded from 400- μm transverse hippocampal slices taken from male Sprague-Dawley rats. After a 1-hour recovery period at room temperature in artificial cerebrospinal fluid (aCSF), slices were transferred to an interface chamber warmed to 30 ± 1 $^\circ\text{C}$

and perfused with aCSF. Schaffer collateral fibers were stimulated every 20 seconds with a concentric bipolar electrode (FHC, Bowdoinham, ME, USA) and evoked fEPSPs were recorded from the stratum radiatum of area CA1 of the hippocampus. Stimulation intensity was set to evoke fEPSPs of 40%–50% of the maximum amplitude. A minimum 10-minute stable baseline period was recorded; thereafter, administration of test substances in aCSF for a 30-minute application period commenced 10 minutes before, and continued during 3 periods of high frequency stimulation (HFS; 100 Hz for 1 second) at 10-minute intervals. The A β manipulation consisted of a 24-hour agitation at 37 °C of 1 mL of 33 μ M A β _{1–42} in tris-HCl with 5 μ L DMSO or 100 μ M SEN1500 added. This was diluted to 33 mL with aCSF directly before application onto the slice. The final concentrations of SEN1500 and A β _{1–42} were 3 μ M and 1 μ M respectively. The 7PA2 manipulation consisted of 7PA2 CM, or wild type (CHO) CM, with 10 μ L DMSO, or 60 μ M SEN1500 added and equilibrated for 1 hour at room temperature, then diluted to 20 mL with aCSF directly before application on the slice, giving final concentrations of 3 μ M SEN1500 and 0.05% DMSO. fEPSPs were recorded for 80 minutes after the final HFS stimulation, and the final 10 minutes of recording (30 sweeps) was selected for group comparisons of fEPSP amplitude using the Student unpaired *t* test.

2.9. *In vivo* electrophysiology

Extracellular fEPSPs were recorded from the hippocampal stratum radiatum of adult male Sprague-Dawley rats anaesthetized initially with 5% isoflurane in oxygen followed by intraperitoneal injection of 25% urethane at 1.25 g/kg; small additional doses of urethane (approximately 1/10 initial dose) were administered intravenously (i.v.) as required during the course of the experiment. Core body temperature was maintained at 37 °C using a homeothermic blanket and polyethylene catheters were inserted into the femoral artery and vein for monitoring blood pressure and for i.v. drug administration, respectively. The head was secured in a stereotaxic frame before lowering a concentric bipolar stimulating electrode and carbon fiber recording electrode (FHC) vertically into the hippocampus. Stereotaxic coordinates (Paxinos and Watson, 1998) were: recording electrode, bregma –4.25 mm, 2.6 mm lateral to midline and 2.25 mm below the pial surface; stimulating electrode, bregma –3.25 mm, 2.4 mm lateral to midline and 2.25 mm below the pial surface. Subsequently, a preloaded stainless steel i.c.v. catheter (30 gauge) was lowered into the lateral ventricle: bregma 0.5 mm, 1.5 mm lateral to midline and 3.6 mm below the pial surface at a 15° angle rostral from vertical. Electrical stimulation of the Schaffer collateral pathways every 30 seconds evoked fEPSPs which were amplified and filtered using Neurolog equipment (Digitimer; gain 0.5–1 K, bandpass settings 10 Hz–5 kHz) and recorded to PC using a Micro 1401 interface and Spike2 software (CED). Stimulation intensity was set to produce fEPSPs of 30%–40% of the maximum amplitude and stable output was obtained for the last 10 minutes before commencing the full experiment protocol. This consisted of recording output for 2 minutes before administration of the first dose of SEN1500 (0.1, 0.3, or 1 mg/kg i.v.), followed 8 minutes later by i.c.v. administration of 10 μ L of 7PA2 CM or CHO CM (control; 2.5 μ L/min). After an additional 20 minutes, a second administration of SEN1500, at the same dose as the first was given 8 minutes before HFS (3 periods of 10 trains of 10 pulses, intervals: interperiod 30 seconds; intertrain 50 ms; interpulse 5 ms) of the Schaffer collateral pathway. The 7PA2 CM manipulation consisted of 10 μ L 7PA2 CM or CHO CM equilibrated for 1 hour at room temperature, then preloaded into the i.c.v. cannula approximately 1 hour before administration, and injected over 4 minutes at 2.5 μ L/min. Field potentials were recorded for 90 minutes after HFS. The final 10 minutes of recording (20 sweeps) was selected for intergroup comparisons using

Kruskal–Wallis 1-way analysis of variance on ranks with a Student–Newman–Keuls post hoc procedure. In all electrophysiological studies, differences were considered significant when $p < 0.05$.

2.10. Behavioral testing

Eighty-four male Sprague-Dawley rats (weighing 220–250 g at the beginning of the experiment) were maintained at 90% of their free-feeding body weights and housed individually, with water available ad libitum in the home cage. The temperature in the vivarium was maintained at 23 °C under a 12-hour light/12-hour dark cycle (lights on at 8:00 AM). The rats were trained and tested in 2-lever rat test chambers (Med Associates Inc.) enclosed in sound attenuating compartments. Reinforcers were 45 mg sucrose pellets (BioServ), which were delivered into a tray situated midway between the operant levers. A training procedure closely approximating that previously reported (Cleary et al., 2005; Reed et al., 2011) was employed. Briefly, behavioral sessions were conducted 7 days a week and the rats were trained to press both levers for food reinforcement. Over approximately 20–30 training sessions, the ALCR schedule was introduced and the response requirement per reinforcer was slowly increased. Under this assay, rats must alternate to the other lever after pressing the currently correct lever a sufficient number of times to obtain a reinforcer. However, the exact number of lever presses required for each reinforcer changes, increasing from 2 responses per food pellet up to 56 responses per food pellet over 6 cycles; 1 complete cycle requires alternating responses of 2, 6, 12, 20, 30, 42, 56, 56, 42, 30, 20, 12, 6, and 2. This provides a measure of what might be termed short-term memory, and generates data on 2 types of errors. Lever switching errors occur when a subject fails to alternate levers after being reinforced, and incorrect lever perseveration errors occur when a subject continues to press the incorrect lever after making a lever switching error. This type of error indicates disruption of well-learned behaviors, or reference memory. When responding under the ALCR schedule reflected no change in trends among subjects, all rats were anesthetized with fentanyl citrate (0.4 mL/kg), placed in a stereotaxic frame, and fitted with a permanent indwelling cannula (23 gauge) aimed at the lateral ventricle. Half of the rats received left lateral ventricle cannula implants and the other half received right lateral ventricle cannula implants. With the incisor bar set 3 mm below the interaural line, stereotaxic coordinates for implantation were: bregma –1.0 mm, –1.5 mm lateral to midline and 3 mm below the surface of the skull (Paxinos and Watson, 1998). All rats were allowed 7 days for recovery before experimental testing, and cannula placement was verified by observation of vigorous drinking (>5 mL per 20 minutes) after i.c.v. injection of 5 μ L angiotensin II (0.5 μ g/mL). After recovery from surgery, rats were randomly assigned to 1 of 6 groups. Group 1 received oral treatment with the vehicle (maple syrup) used for suspension of SEN1500 plus i.c.v. CHO CM, to establish overall baseline performance; group 2 received oral treatment with 20 mg/kg SEN1500 plus i.c.v. CHO CM, to control for any adverse effect of the drug; group 3 received oral treatment with vehicle plus i.c.v. 7PA2 CM, to establish the geodetic datum against which drug effects could be measured; groups 4, 5, and 6 received oral treatment with 1, 5, or 20 mg/kg SEN1500, respectively, plus i.c.v. 7PA2 CM, to investigate dose-response effects. Oral dosing was carried out 1.5 hour before, and again 30 minutes before i.c.v. injections. Subjects were placed in the operant chambers for collection of ALCR data 2 hours after i.c.v. injections (Cleary et al., 2005). Oral treatments were performed by suspending SEN1500 in maple syrup, which the rats drank readily from a syringe. Data were analyzed by 1-way analysis of variance, followed by Fisher's least significant differences post hoc test.

3. Results

3.1. SEN1500 binding to $A\beta_{1-42}$ monomer and oligomers, inhibition of $A\beta_{1-42}$ aggregation, and protection of neuronal cells exposed to $A\beta_{1-42}$

Surface plasmon resonance analysis verified the binding of SEN1500 to $A\beta_{1-42}$ monomer and oligomers. Immobilized $A\beta_{1-42}$ on the Biacore chip was characterized using 6E10, 4G8, A11, and OC anti- $A\beta$ antibodies. For the $A\beta_{1-42}$ monomer surface 6E10 and 4G8 showed strong binding responses whereas A11 and OC ($A\beta$ oligomer and $A\beta$ fibril specific, respectively; Glabe, 2008) showed no binding, supporting the integrity of this surface. For the $A\beta_{1-42}$ oligomer surface, the antibodies A11 and OC showed strong binding responses but only weak signals were apparent with 6E10 and 4G8. SEN1500 bound to monomeric $A\beta_{1-42}$ in a concentration-related manner (Fig. 2A) with a K_D of 3.4 μ M, which suggests that its mode of action involves direct interaction with $A\beta_{1-42}$. SEN1500 also bound to $A\beta_{1-42}$ oligomers (Fig. 2B) in a concentration-related manner. Although binding of SEN1500 to $A\beta_{1-42}$ oligomers was in the same concentration range as to $A\beta_{1-42}$ monomers, the oligomeric preparation bound to the Biacore chip surface was heterogeneous in nature and it was therefore not possible to calculate a precise K_D value. Incubation of SEN1500 (10 μ M) with $A\beta_{1-42}$ (10 μ M) gave a 41% reduction in thioflavin-T fluorescence, demonstrating inhibition of the $A\beta_{1-42}$ aggregation process. In the cell viability MTT assay, SEN1500, protected the SH-SY5Y neuronal cell line exposed to an insult of $A\beta_{1-42}$ (10 μ M); [half maximal inhibitory concentration] IC_{50} = 26 μ M (Fig. 3A). By comparison, RS-0406 had a submaximal effect showing approximately 40% protection at 40 μ M in the MTT assay and approximately 35% inhibition at 10 μ M in the thioflavin-T assay. Using cortical/hippocampal neurons derived from e15 mouse embryos, $A\beta_{1-42}$ caused a deficit in synaptophysin with an IC_{50} = 281 nM. SEN1500 blocked the $A\beta_{1-42}$ -induced lowering of synaptophysin levels in cortical neurons with an IC_{50} = 0.43 μ M (Fig. 3B).

3.2. Pharmacokinetic profile of SEN1500

Key pharmacokinetic parameters for SEN1500 in rats are summarized in Table 1, which shows that SEN1500 was well absorbed after oral administration and also penetrated the brain (see Supplementary data, Tables S1–S7 and Fig. S1). Oral bioavailability was 42% and after a 2 mg/kg i.v. dose of SEN1500 the mean brain:plasma ratio over the first 3 hours was 0.77:1. SEN1500 had a low clearance rate (6 mL/min/kg) and a [peak concentration] C_{max} that equated to 9.88 μ M of SEN1500. Based on the percent plasma protein (94.0) and brain homogenate binding (97.8) of SEN1500, it was predicted that a 20 mg/kg oral dose would provide approximately 600 nM and approximately 200 nM free drug in the plasma and brain respectively, at the [time to peak concentration] T_{max} . These data indicate that SEN1500 has a suitable profile for evaluation in in vivo efficacy studies using oral administration.

3.3. Inhibitory effect of $A\beta_{1-42}$ on LTP in vitro is attenuated by SEN1500

In vitro LTP of fEPSPs in the CA1 region of hippocampal slices was reduced after application of $A\beta_{1-42}$, with control LTP of $166.8 \pm 11.0\%$ compared with LTP of $124.9 \pm 5.8\%$ after $A\beta_{1-42}$ application ($p < 0.05$). The $A\beta_{1-42}$ -induced reduction in LTP was attenuated by coinubation of $A\beta_{1-42}$ with SEN1500; under these conditions LTP of $151.0 \pm 9.4\%$ was observed (Fig. 4). SEN1500 was also effective in reducing the deficit in LTP caused by application of 7PA2 CM to hippocampal slices (Fig. 4). After HFS of the Schaffer collaterals, the

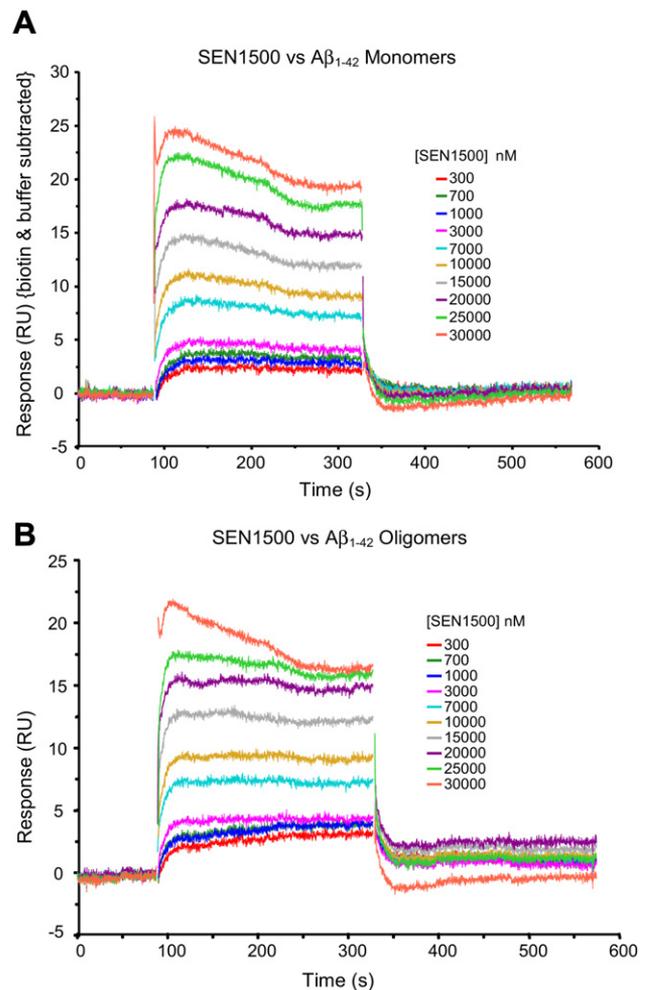


Fig. 2. Surface plasmon resonance (SPR) curves for binding of SEN1500 to $A\beta_{1-42}$ monomer and oligomers. (A) SPR data for $A\beta_{1-42}$ monomer: N-terminus biotinylated $A\beta_{1-42}$ freshly prepared in 50% dimethyl sulfoxide and immobilized on streptavidin chip surface. (B) $A\beta_{1-42}$ oligomers prepared from biotin- $A\beta_{1-42}$: $A\beta_{1-42}$ (1:10) and immobilized on streptavidin chip surface. Concentration of SEN1500 ranged from 0.3 to 30 μ M.

control LTP of fEPSPs amounted to an increase of $163.0 \pm 6.3\%$ of baseline fEPSP amplitude. In the presence of 7PA2 CM, the amplitude of fEPSPs after HFS was reduced to $93.7 \pm 9.7\%$ of baseline. This inhibition of LTP was attenuated after coinubation of 7PA2 CM with SEN1500, the magnitude of LTP amounting to $156.9 \pm 11.9\%$ ($p < 0.05$).

3.4. Inhibitory effect of 7PA2 CM on LTP in vivo is attenuated by SEN1500

After administration of CHO CM, control LTP of $188.7 \pm 7.0\%$ of baseline fEPSP amplitude was observed. When 7PA2 CM was administered, subsequent HFS produced significantly weaker LTP of $123.1 \pm 4.1\%$ of baseline ($p < 0.05$; Fig. 5). This 7PA2 CM-induced inhibition of LTP was significantly and dose-dependently attenuated by 2 consecutive treatments at each dose of 0.1, 0.3, and 1 mg/kg SEN1500 ($p < 0.05$); under these conditions, LTP of $135.1 \pm 8.2\%$, $143.0 \pm 2.9\%$, and $165.0 \pm 5.7\%$ of baseline fEPSP amplitude was recorded, respectively (Fig. 5). Control LTP obtained in the presence of CHO CM was not significantly affected by SEN1500 at a dose of 0.3 mg/kg ($177.1 \pm 4.5\%$; Fig. 5).

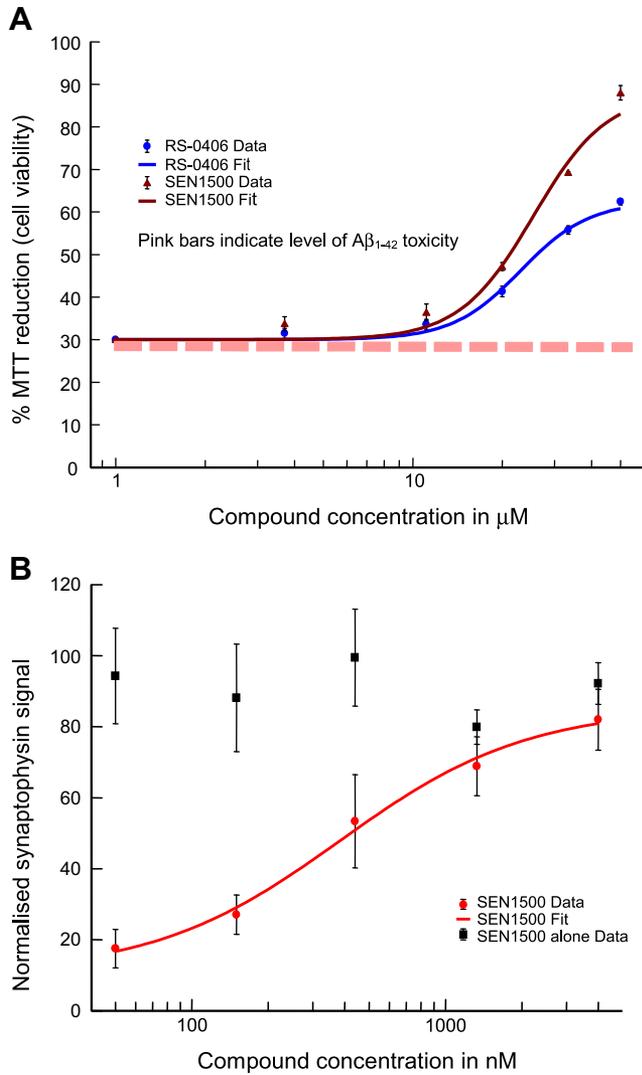


Fig. 3. (A) Dose response curves of SEN1500 and RS-0406 incubated with 10 μM Aβ₁₋₄₂ for 24 hours on SH-SY5Y cells. (B) Dose response curve of SEN1500 incubated with 1 μM Aβ₁₋₄₂ for 24 hours on cortical neurons. SEN1500 was preincubated with 1 μM Aβ₁₋₄₂ for 30 minutes and then application to the cells for 24 hours afforded significant protection against the synaptophysin deficit. Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

3.5. Acute disruptive effect of i.c.v. 7PA2 CM on memory in normal rat is attenuated dose-dependently by previous oral administration of SEN1500

The ALCR schedule revealed a statistically significant overall treatment effect on lever switching errors ($F(5,78) = 2.61$; $p = 0.03$). Fig. 6A shows that the 7PA2 CM group that was pre-dosed orally with vehicle executed significantly more lever switching errors

than either the CHO CM plus vehicle group or the CHO CM group pre-dosed orally with 20 mg/kg SEN1500 (p 's < 0.01). This demonstrates that 7PA2 CM had a significant detrimental effect on accurate responding under the ALCR schedule. The 7PA2 CM injected groups pre-dosed orally with either 5 or 20 mg/kg SEN1500 executed significantly fewer lever switching errors than the 7PA2 CM injected group pre-dosed with vehicle ($p = 0.04$ and 0.01, respectively), indicating a protective effect of SEN1500. At a dose of 1 mg/kg, the effect of SEN1500 was not significant ($p = 0.2$).

The ALCR schedule also revealed a statistically significant overall treatment effect on incorrect lever perseverations ($F(5,78) = 5.98$; $p = 0.0001$). Fig. 6B shows that the 7PA2 CM group pre-dosed with vehicle executed significantly more incorrect lever perseverations than either the CHO CM group pre-dosed with vehicle or the CHO CM group pre-dosed with 20 mg/kg SEN1500 (p 's < 0.0001). This also demonstrates that i.c.v. 7PA2 CM injection had a significant detrimental effect on accurate responding under the ALCR schedule. The i.c.v. 7PA2 CM injected groups pre-dosed orally with 1, 5, and 20 mg/kg SEN1500 executed significantly fewer incorrect lever perseverations than the i.c.v. 7PA2 CM injected group pre-dosed with vehicle ($p = 0.02, 0.002, \text{ and } 0.0002$, respectively), again indicating a dose-dependent protective effect of SEN1500.

4. Discussion

The increasing prevalence of AD as a result of natural aging has presented a significant challenge, and an obvious response to this challenge has been focus on the development of pharmacotherapies to alleviate adverse symptomatology. Currently approved pharmacotherapies for AD consist of acetylcholinesterase inhibitors (AChEIs) and an antagonist of the N-methyl-D-aspartate (NMDA) receptor. AChEIs were designed to promote levels of acetylcholine neurotransmission by preventing the degradation of acetylcholine into choline and choline acetyltransferase. Currently, there are 4 approved for the treatment of mild to moderate AD (Farlow, 2002). However, the use of these in mild cognitive impairment, which precedes AD, has not shown an effect relative to prevention of the inevitable decline into full symptomatology (Raschetti et al., 2007). The NMDA receptor antagonist, memantine, is a noncompetitive, voltage-dependent NMDA receptor antagonist of moderate affinity (Parsons and Gilling, 2007) and is the only non-AChEI treatment approved for use in moderate to severe AD (Shah and Reichman, 2006). The drug is thought to act by blocking the NMDA receptor channel (Lipton, 2004), and this allows for voltage dependent inhibition of Ca²⁺ influx (Danysz and Parsons, 2003; Parsons et al., 1999). In moderate to severe cases of AD, memantine has been shown to be of relatively limited, albeit clinically relevant benefit in slowing behavioral deterioration (Areosa et al., 2005; Reisberg et al., 2003; Tariot et al., 2004). Furthermore, in mild to moderate cases of AD, memantine has recently been reported to be of no clinical benefit (Schneider et al., 2011). At best memantine slows cognitive deterioration but not the progression of AD, with some studies showing that its protective benefits disappear over a 6-month period (e.g., Shah and Reichman, 2006).

Consequently, there has been increased interest in the development of alternative pharmacotherapeutic approaches. For example, tramiprosate is a glycosaminoglycan (GAG) mimetic, GAGs bind to soluble Aβ promoting formation and deposition of fibrillar amyloid, and GAG mimetics compete for GAG binding sites, thereby maintaining Aβ in a nonfibrillar form and inhibiting Aβ deposition (Gervais et al., 2007). However, a recent clinical trial indicated no significant treatment effect of this compound in mild to moderate AD (Aisen et al., 2011). Colostrinin is a proline rich polypeptide complex which inhibits Aβ aggregation and neurotoxicity in vitro (Froud et al., 2010; Janusz et al., 2009),

Table 1
Key pharmacokinetic parameters for SEN1500

Dose	t _{1/2} (min)	CL (mL/min/kg)	V _d (mL/kg)	AUCinf (min/mg/mL)
2 mg/kg, iv	254	6	2164	339,397
Dose	t _{1/2} (min)	C _{max} (ng/mL)	T _{max} (min)	AUCinf (min/mg/mL)
20mg/kg, po	221	3864	80	1,433,138

Key: AUCinf, area under the concentration time-curve from zero to infinity; CL, clearance rate; C_{max}, peak concentration; iv, intravenous; t_{1/2}, half life of the drug; T_{max}, time to peak concentration; V_d, drug clearance based on distribution as mL/kg.

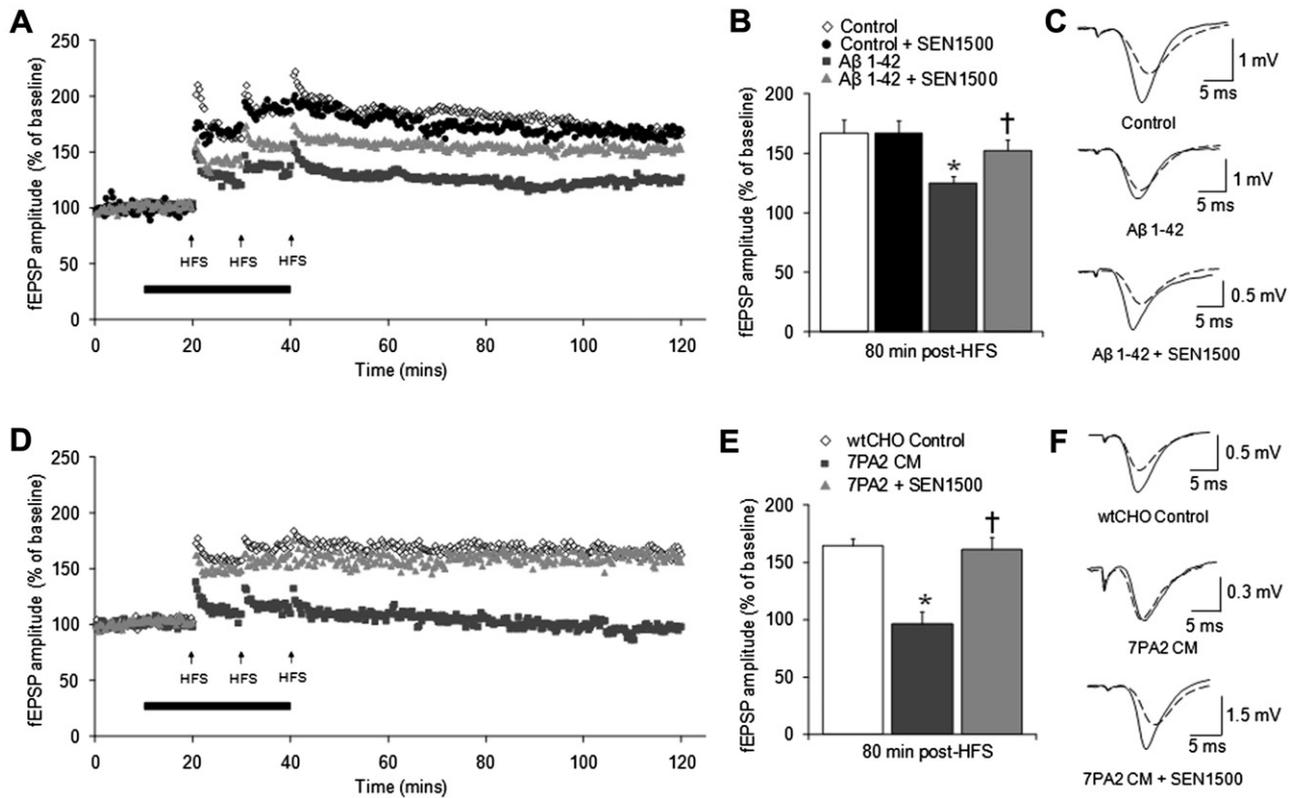


Fig. 4. Synthetic $A\beta_{1-42}$ and 7PA2 conditioned medium (CM)-induced deficits in long-term potentiation (LTP) in vitro. (A) Scatter plot showing the effect of coadministration of SEN1500 (3 μ M) and synthetic $A\beta_{1-42}$ ($n = 6$), $A\beta_{1-42}$ alone ($n = 6$), vehicle control ($n = 4$), and SEN1500 (3 μ M) with vehicle control ($n = 4$) on the enhancement of field excitatory postsynaptic potential (fEPSP) amplitude evoked by high frequency stimulation (HFS) on hippocampal slices. (B) Histograms showing the level of LTP recorded 80–90 minutes post HFS. * $p < 0.05$ compared with control; † $p < 0.05$ compared with $A\beta_{1-42}$. (C) Typical fEPSPs before (dotted line) and 80 minutes after (solid line) HFS for 3 of the treatment groups; traces shown are the average of 10 sweeps. (D) Scatter plot showing the effect of coadministration of SEN1500 (3 μ M) with 7PA2 CM ($n = 4$), 7PA2 CM alone ($n = 4$), and CHO CM alone ($n = 4$) on the enhancement of fEPSP amplitude evoked by HFS on hippocampal slices. (E) Histograms showing the level of LTP recorded 80 minutes post HFS. * $p < 0.05$ compared with CHO CM; † $p < 0.05$ compared with 7PA2 CM; ‡ $p < 0.05$ compared with 7PA2 CM. (F) Typical fEPSPs before (dotted line) and 80 minutes after (solid line) HFS for the 3 treatment groups; traces shown are the average of 10 sweeps.

and improves cognitive performance in animal models of AD (Popik et al., 1999), but has only demonstrated modest improvements for mild AD in clinical trials (Bilikiewicz and Gaus, 2004). Also, the inositol series of small molecules have been shown to prevent $A\beta$ aggregation and to promote disassembly of plaques (McLaurin et al., 2000; Nitz et al., 2008), with scyllo-inositol (AZD-103) appearing to be the most effective stereoisomer. Scyllo-inositol halts $A\beta$ fibrillogenesis and causes existing fibrils to disassemble in vitro (Sun et al., 2008), it has been found to prevent the behavioral decline caused by $A\beta$ insult and the associated neuropathology, to reduce concentrations of soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in the brain, and to reduce synaptic loss, plaque burden, glial inflammatory reaction, and early mortality (McLaurin et al., 2006). It has also been shown to dose-dependently rescue in vivo LTP from the inhibitory effects of soluble $A\beta$ oligomers (Townsend et al., 2006). Another compound that inhibits $A\beta_{1-42}$ aggregation and toxicity in vitro is RS-0406 (Nakagami et al., 2002). RS-0406 also protects primary hippocampal neurons against $A\beta_{1-42}$ -induced cytotoxicity, rescues $A\beta_{1-42}$ -induced impairment of LTP (Walsh et al., 2005), and arrests 7PA2 CM-induced behavioral deterioration in the rat (O'Hare et al., 2010).

A growing body of evidence points toward the extracellular accumulation of $A\beta$ assemblies being of importance at an early stage of the development of AD. Obviously, pharmacotherapeutic intervention in response to the developmental aspects of any disease is a desirable clinical approach. Therefore, investigating pharmacotherapies that target $A\beta$ oligomers directly, and the

underlying oligomer assembly mechanism is of importance, because it is these drugs that could most likely produce the best clinical outcome for patients diagnosed early with AD. However, nonpeptide small molecule $A\beta$ aggregation inhibitors have proven difficult to find, and problems exist relative to oral bioavailability and sufficient half-life (Rishton, 2008). In animal models the non-peptide small molecule RS-0406 has been shown to inhibit $A\beta$ toxicity and $A\beta_{1-42}$ aggregation (Nakagami et al., 2002), to protect hippocampal neurons against $A\beta_{1-42}$ -induced toxicity, to rescue $A\beta_{1-42}$ -induced impairment of LTP (Walsh et al., 2005), and to arrest 7PA2 CM-induced behavioral deterioration (O'Hare et al., 2010). Unfortunately, RS-0406 only brings about its behavioral effect when administered directly into the ventricles of the brain, and thus is not a realistic candidate for the clinical treatment of AD.

As a consequence, our group modified the RS-0406 chemical structure to produce improvements in its oral bioavailability and potency. The eventual outcome of these modifications resulted in SEN1269 (Scopes et al., 2012), and then the 5-aryloxy pyrimidine SEN1500. In the current study, the ability of SEN1500 to bind to $A\beta_{1-42}$ monomer and oligomers was determined using surface plasmon resonance, and SEN1500 bound to $A\beta_{1-42}$ monomer and oligomers. This is an important finding which suggests that a component of the activity profile of SEN1500 is binding to $A\beta$ monomer to prevent dimerization, and binding to $A\beta$ oligomers to block further multimerization and neutralize the toxic effects of these species. Subsequently, the effects on aggregation might not simply explain its neuroprotective actions. It is possible that

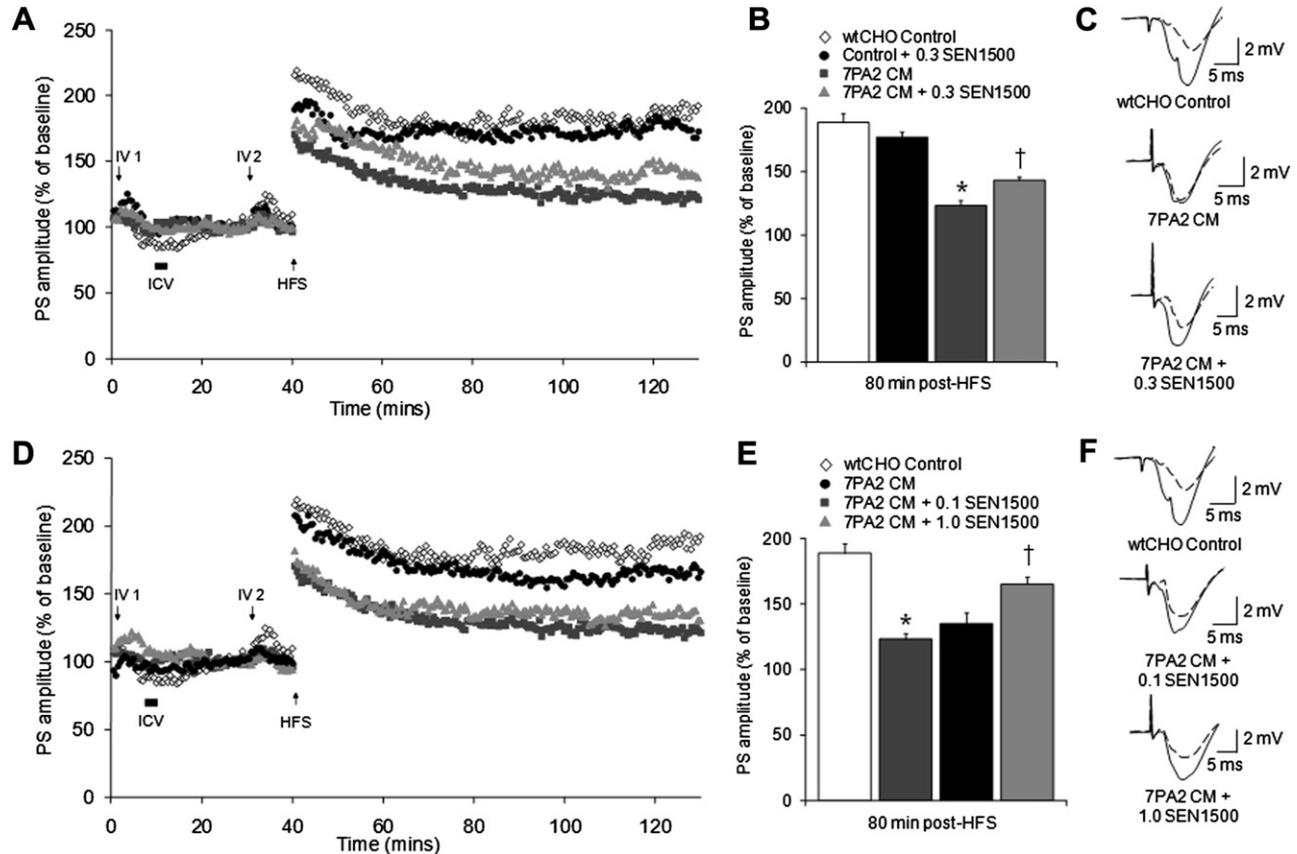


Fig. 5. 7PA2 CM-induced deficits in long-term potentiation (LTP) in vivo. (A) Scatter plot showing the effect of administration of SEN1500 (0.3 mg/kg) with 7PA2 CM ($n = 6$), vehicle with 7PA2 CM ($n = 6$), SEN1500 (0.3 mg/kg) with Chinese hamster ovary (CHO) conditioned medium (CM) ($n = 6$) and vehicle with CHO CM ($n = 6$) on the enhancement of field excitatory postsynaptic potential (fEPSP) amplitude evoked by high frequency stimulation (HFS) in the CA1 region of the hippocampus in an anesthetized rat. (B) Histograms showing the level of LTP recorded 90 minutes post HFS. * $p < 0.05$ compared with wild type (wt)CHO; † $p < 0.05$ compared with 7PA2 CM. (C) Typical fEPSPs before (dotted line) and 80 minutes after (solid line) HFS for the 3 treatment groups; traces shown are the average of 10 sweeps. (D) Scatter plot showing the effect of coadministration of SEN1500 (0.1 mg/kg) with 7PA2 CM ($n = 6$), SEN1500 (1 mg/kg) with 7PA2 CM ($n = 6$), vehicle with 7PA2 CM ($n = 6$), and vehicle with CHO CM ($n = 6$) on the enhancement of fEPSP amplitude evoked by HFS in the CA1 region of the hippocampus in an anesthetized rat. (E) Histograms showing the level of LTP recorded 90 minutes post HFS. * $p < 0.05$ compared with CHO CM; † $p < 0.05$ compared with 7PA2 CM. (F) Typical fEPSPs before (dotted line) and 80 minutes after (solid line) HFS for the 3 treatment groups; traces shown are the average of 10 sweeps.

SEN1500 could also interfere with the interaction of A β with neurons and synaptic structures.

The results from the thioflavin-T assay indicated that SEN1500 blocks A β_{1-42} aggregation, and MTT assay demonstrated that SEN1500 protected neuronal cell lines exposed to A β_{1-42} . These results suggest that mechanistically, SEN1500 acts by binding A β to prevent assembly and fibrillogenesis; this is of importance because the key pathogenic event in AD is believed to be aggregation of the A β peptide into synaptotoxic, prefibrillar oligomers (Verdile et al., 2004; Walsh and Selkoe, 2004a, 2004b). In addition, SEN1500 blocked the A β_{1-42} -induced lowering of synaptophysin levels in cortical/hippocampal neurons derived from e15 mouse embryos. Noteworthy here is the fact that much lower concentrations of A β_{1-42} were effective in causing the deficit in synaptophysin levels (cf., MTT assay) and that correspondingly lower concentrations of SEN1500 were effective in protecting against this. Investigation of the pharmacokinetic profile of SEN1500 indicated that it was well absorbed after oral administration, penetrated the central nervous system, and had a low clearance rate.

With respect to LTP, SEN1500 significantly reduced the depression of fEPSPs induced by aggregated A β_{1-42} and A β oligomers. Coincubation of A β_{1-42} with SEN1500 attenuated the reduction in LTP attributable to A β_{1-42} , and the depression of LTP induced by 7PA2 CM was attenuated after coincubation of 7PA2

CM with SEN1500. These findings indicate that, using the LTP model of cellular mechanisms underlying learning and memory, SEN1500 afforded protection against the deficits induced by A β_{1-42} and 7PA2 CM. Similarly, 7PA2 CM depressed LTP in vivo and this depression of LTP was significantly and dose-dependently attenuated by SEN1500. Taken in conjunction with findings from the biochemical assays above, SEN1500 would appear to be a useful moderator of adverse effects induced by A β . The final phase of this study extended the investigation to effects in freely moving intact animals. Under the ALCR operant assay of behavioral performance, which has been widely used in the assessment of A β effects in the rat, oral pre-dosing with SEN1500 before i.c.v. injection of 7PA2 CM produced a significant overall treatment effect on lever switching errors. This indicated that SEN1500 alleviated 7PA2 CM-induced 'confusion' as to the correct operant lever for appropriate responding. Pre-dosing with SEN1500 also produced a significant and dose-dependent overall treatment effect on incorrect lever perseverations. This indicated that SEN1500 also alleviated 7PA2 CM-induced 'confusion' occurring persistently after an initial error had been made. This is of interest, as perseverative behavior might be an indicator of early AD (Traykov et al., 2005), and the 7PA2 CM injection model employed is argued to involve features relevant to the early stages of AD (e.g., Cleary et al., 2005). By comparison, RS-0406 only arrests A β -induced

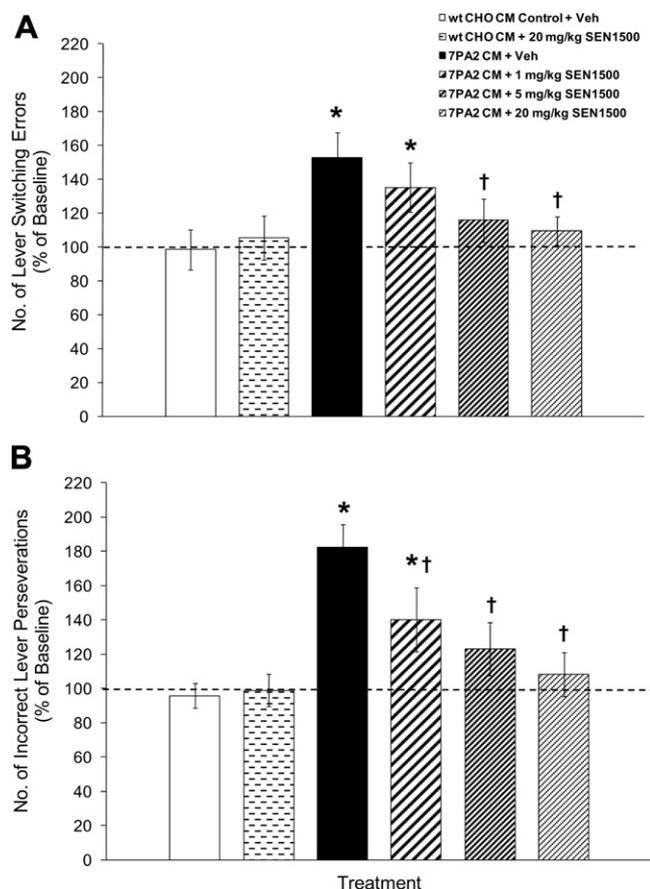


Fig. 6. (A) Effect of oral SEN1500 (1 mg/kg, 5 mg/kg, and 20 mg/kg) treatment on intracerebroventricular (i.c.v.) 7PA2 CM-induced lever switching errors. (B) Effect of oral SEN1500 (1 mg/kg, 5 mg/kg, and 20 mg/kg) treatment on i.c.v. 7PA2 CM-induced incorrect lever perseverations. Data presented as mean \pm standard error of the mean. * $p < 0.05$ compared with wild type (wt) Chinese hamster ovary (CHO) conditioned medium (CM) with vehicle (Veh) and † $p < 0.05$ compared with 7PA2 CM with Veh.

behavioral deterioration in the rat when administered i.c.v. (O'Hare et al., 2010).

In summary, these data indicate that the first orally bioavailable derivative of RS-0406, SEN1500, showed protective effects against A β in all of the assays investigated. Reed et al. (2011) recently reported that the most striking finding of their study was A β oligomers derived from 7PA2 CM demonstrated a behaviorally determined (ALCR schedule) potency of at least 100 times greater than all other forms of A β investigated, regardless of source or form. The current study shows that SEN1500 bound to A β oligomers, and had protective effects against 7PA2 CM-derived A β oligomer insult relative to in vitro LTP, in vivo LTP, and cognitive impairment in intact freely moving rats. These results suggest that SEN1500 might be a realistic pharmacotherapeutic candidate for the disease-modifying treatment of AD.

Disclosure statement

David Scopes, Hozefa Amijee, and Mark Treherne are employees of Senexis Limited and hold share options, Eugene O'Hare holds share options.

All animal experimentation was conducted under UK Home Office license and with the approval of the appropriate institutional ethics committees.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neurobiolaging.2012.10.016>.

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