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The N-Methylated Peptide SEN304 Powerfully Inhibits Aβ(1−42) Toxicity by Perturbing Oligomer Formation

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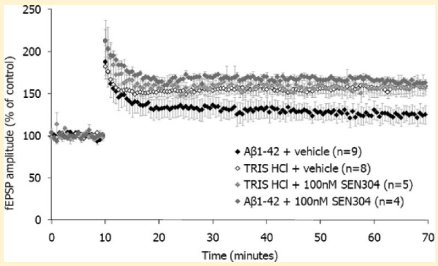
ABSTRACT: Oligomeric forms of β-amyloid (Aβ) have potent neurotoxic activity and are the primary cause of neuronal injury and cell death in Alzheimer's disease (AD). Compounds that perturb oligomer formation or structure may therefore be therapeutic for AD. We previously reported that D-[(chGly)-(Tyr)-(chGly)-(chGly)-(mLeu)]-NH_2 (SEN304) is able to inhibit Aβ aggregation and toxicity, shown primarily by thioflavin T fluorescence and MTT (Kokkoni, N. et al. (2006) N-Methylated peptide inhibitors of β-amyloid aggregation and toxicity. Optimisation of inhibitor structure. *Biochemistry 45*, 9906−9918). Here we extensively characterize how SEN304 affects Aβ(1−42) aggregation and toxicity, using biophysical assays (thioflavin T, circular dichroism, SDS-PAGE, size exclusion chromatography, surface plasmon resonance, traveling wave ion mobility mass spectrometry, electron microscopy, ELISA), toxicity assays in cell culture (MTT and lactate dehydrogenase in human SH-SHY5Y cells, mouse neuronal cell death and synaptophysin) and long-term potentiation in a rat hippocampal brain slice. These data, with dose response curves, show that SEN304 is a powerful inhibitor of Aβ(1−42) toxicity, particularly effective at preventing Aβ inhibition of long-term potentiation. It can bind directly to Aβ(1−42), delay β-sheet formation and promote aggregation of toxic oligomers into a nontoxic form, with a different morphology that cannot bind thioflavin T. SEN304 appears to work by inducing aggregation, and hence removal, of Aβ oligomers. It is therefore a promising lead compound for Alzheimer's disease.



Soluble, oligomeric forms of β-amyloid (Aβ) have potent neurotoxic activity and are the primary cause of neuronal injury and cell death in Alzheimer's disease (AD).[2−6] Plausible mechanisms of oligomer toxicity include links to oxidative stress, metal binding, free radical formation[7] and ion channel formation.[8] Whatever the exact mechanism, an attractive therapeutic strategy is to interfere with oligomer formation and peptide aggregation, because this appears to be the first step in the pathogenic process of amyloidosis which is not associated with some natural biological function.[9] Compounds which reduce oligomer concentration, either by inhibiting Aβ aggregation or by promoting oligomer assembly to nontoxic forms, may therefore be therapeutic for AD.

Many inhibitors of Aβ aggregation are peptides, usually based on the self-recognition element within Aβ(16−20) of KLVFF.[10] While peptides are generally poor drugs *in vivo*, they can be modified to improve their properties. For example, we, and others, have incorporated N-methyl amino acids into peptides to make inhibitors of amyloidosis. These peptides initially correspond to a self-recognition element, i.e., the part of the amyloidogenic peptide responsible for self-assembly. One side of the peptide presents a hydrogen-bonding "complementary" face to the amyloid target, with the other having N-methyl groups in place of backbone NH groups, presenting a "blocking" face. Hughes et al. showed that N-methyl derivatives of Aβ(25−35), an aggregation prone and toxic fragment of Aβ, were able to prevent Aβ aggregation and inhibit toxicity.[11] Meredith and co-workers[12,13] studied N-methylated peptides that prevented Aβ fibrils from forming and broke down preformed fibrils. Cruz et al. used N-methylated inhibitors based on Aβ(36−40) (VGGVV).[14] PPI-1019 D-(H-[(mL)-VFFL]-NH_2) is another effective N-methylated peptide inhibitor of Aβ aggregation and toxicity.[15] N-Methylation confers improved ADMET properties to a peptide in terms of toxicity, solubility, bioavailability, rigidity and resistance to proteolytic degradation.[16−19] Hexapeptides with varying extents of *N*-methylation based on residues 32−37 of Aβ were able to retard β-sheet and fibril formation of Aβ and to reduce Aβ neurotoxicity.[20] A penta-*N*-methylated peptide increased life span and locomotor activity in *Drosophila melanogaster* flies overexpressing human Aβ(1−42), thus showing that N-methylated peptides can work *in vivo*.

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In our previous work, we optimized the structure of an inhibitor of Aβ aggregation and toxicity by starting with KLVFF and varying the side chain identity, chirality, N-terminal acetylation, C-terminal amidation and location of N-methyl groups. The most active compound was found to be D-[(chGly)-(Tyr)-(chGly)-(chGly)-(mLeu)]-NH_2 (SEN304).[1] SEN304 was previously assayed by thioflavin T binding, affinity chromatography, electron microscopy, cell toxicity monitored by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction[1] and long-term potentiation,[21] all at a single concentration and often on Aβ(1−40). Here we extensively characterize the properties, efficacy and mode-of-action of SEN304, using numerous biophysical, cell culture and *ex-vivo* assays. Aβ(1−42) is predominantly used, rather than Aβ(1−40), since this is the more toxic and aggregation prone form. SEN304 is shown to be a highly effective inhibitor of Aβ toxicity that works by perturbing Aβ aggregation.

MATERIALS AND METHODS

Preparation of Aβ(1−42) Peptide. Aβ(1−42) HCl salt peptides were purchased from American Peptide Company or rPeptide as lyophilized powder and stored at −20 °C. A 2 mM stock was prepared by dissolving in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, from Sigma Aldrich) followed by 30 s vortexing and brief 5 s sonication to mix and dissolve. The color of the stock solution would turn from a turbid milky color to a colorless solution over a few hours. The stock solution was sealed with parafilm and stored at 4 °C until required. Prior to experimental use HFIP-treated Aβ(1−42) was aliquoted into protein lo-bind tubes (Eppendorf), lyophilized and stored at −20 °C. The HFIP treatment maintains the amyloidogenic peptide in a monomeric soluble state and allows for experimental reproducibility.[22]

Preparation of Inhibitor Compounds. SEN304 was supplied in lyophilized form at >95% purity by Protein Peptide Research Ltd. SEN304 was dissolved in biotechnology performance certified, spectrophotometric grade dimethyl sulfoxide (DMSO) (Sigma Aldrich) to a stock concentration of 20 mM and stored at −20 °C. Further dilutions in DMSO were made for compound concentrations (10 mM to 2 μM) required in the various assays, and these were stored in 96-well V bottom polystyrene plates (Greiner) at −20 °C.

ThT Methods. The fluorescence emission of ThT is shifted when it binds to β-sheet aggregate structures, such as amyloid fibrils.[23] Stock solutions of ThT (Sigma Aldrich) were freshly prepared every month at a concentration of 2.5 mM in 50 mM glycine (Sigma Aldrich) buffer pH 8.5 and aliquoted into microtubes (813 μL) and stored at −20 °C. When needed, an aliquot was thawed and diluted into 50 mL, 50 mM glycine buffer to make a 40 μM ThT solution. The extent of Aβ(1−42) aggregates in samples at various time points (e.g., 24 and 48 h) were measured by adding 50 μL samples to a black fluorescent plate (Greiner) followed by 50 μL of a 40 μM ThT solution. The plate was shaken for 30s and fluorescence intensity monitored at an excitation wavelength of 440 ± 15 nM and an emission wavelength of 485 ± 10 nM using a BioTek Synergy plate reader.

The ThT assay samples were prepared as follows: An aliquot of lyophilized Aβ(1−42) was dissolved into DMSO to a 2 mM concentration. This was added to a column of wells on a 96-well V bottom polystyrene plate. SEN304 was diluted in DMSO from 20 mM stock to make 10 concentrations ranging from 10 mM to 2 μM, prepared on a 96-well V bottom polystyrene plate termed the "master plate" in which each row represented a 10 point concentration range for one compound. The last row contained only DMSO (no compound) as a control for Aβ(1−42) alone. In a 96-well clear bottom plate (Greiner) termed the "daughter plate" 294 μL phosphate buffer 50 mM, pH 7.4, containing 150 mM NaCl was added per well. Next 3 μL SEN304 at various concentrations in DMSO or DMSO alone from the master plate were added to the daughter plate and mixed thoroughly. Three microliters of 2 mM Aβ(1−42) was added to each well and mixed thoroughly. The daughter plate was then sealed with foil seal and placed in a 37 °C incubator. The daughter plate contained 20 μM Aβ(1−42) coincubating with SEN304 (100 μM − 20 nM) in 2% DMSO. After ThT addition, the final concentrations were 10 μM for Aβ(1−42) and 50 μM − 10 nM for SEN304 in 1% DMSO. The inhibitory activity of compounds at a specific time point (e.g., 24 or 48 h) was calculated by the % reduction in the amyloid-bound ThT fluorescence of the Aβ(1−42):compound mixture compared with that of the Aβ(1−42) ThT fluorescence alone (eq 1).

$$\%\text{amyloid} = \frac{\text{Fx} - \text{Fb}}{\text{Fa} - \text{Fb}} \times 100\% \quad (1)$$

where %amyloid is the percent amyloid-bound ThT fluorescence compared to Aβ(1−42) alone, Fx is the ThT fluorescence of the Aβ(1−42):compound mixture, Fa is the ThT fluorescence of the Aβ(1−42) alone and Fb is the background ThT fluorescence with no Aβ(1−42) or compound present (1% DMSO). EC_{50} values were calculated by fitting the data to a 4 parameter dose response model (eq 2) using XLfit.

$$y = \text{bot} + \frac{\text{top} - \text{bot}}{1 + \left(\frac{EC_{50}}{x}\right)^{\text{Hill slope}}} \times 100\% \quad (2)$$

where bot is the lower baseline value, top is the upper baseline value, EC_{50} is the concentration for the midpoint of the transition and Hillslope shows the cooperativity.

Circular Dichroism. Circular dichroism (CD) spectroscopy has been previously applied by several groups[24−26] to study secondary structure transitions or to define the conformational states of both Aβ(1−40) and Aβ(1−42). CD experiments were performed similarly to the method and conditions reported by Bartolini et al.[26] All prepared buffers and solutions were filtered through 0.2 μm Minisart filters (Sartorius AG). 50 μL aliquots of Aβ(1−42) from 2 mM HFIP stock were lyophilized and stored at −20 °C. The Aβ(1−42) peptide was redissolved in 200 μL freshly prepared redissolution buffer (RDB) resulting in a 500 μM concentration. RDB consisted of acetonitrile, 300 μM sodium carbonate and 250 mM sodium hydroxide (all from Sigma Aldrich) at a volume ratio 48.3:48.3:3.4, v/v/v respectively. The resulting alkaline Aβ(1−42) solution was vortexed and sonicated for 10s. CD spectra confirmed that this solution contained nonaggregating Aβ(1−42) secondary structure. The Aβ(1−42) alkaline stock solution (180 μL) was transferred to a 2 mL protein lo-bind tube (Eppendorf) and diluted 10-fold by the addition of 1620 μL phosphate buffered saline (PBS) to a final 50 μM Aβ(1−42) concentration. PBS consisted of 10 mM phosphate buffer, 1 mM NaCl, pH 7.7 (all from Sigma Aldrich). The final 50 μM Aβ(1−42) solution was in 8.7 mM phosphate buffer that contained 10 mM NaCl, 14.5 μM NaOH and 4.8% acetonitrile with a pH of 8.0.

The method described above was used with SEN304 dissolved in the PBS buffer at 56 μM or 112 μM followed by 0.2 μm filtering. The 10-fold dilution of Aβ(1−42) in RDB into PBS containing compound resulted in final equimolar concentrations of SEN304. To follow Aβ(1−42) conformational changes, a CD Jasco J-810 Spectropolarimeter was used. CD spectra were recorded at 37 °C in the spectral range 190−260 nm, by using 0.1 cm path length quartz cell (110-QS from Hellma) with an internal volume of ∼400 μL. CD spectra were recorded with scans every 5 min at 0.5 nM intervals with 2 nM bandwidth and 20 nm/min scan speed Spectral amplitudes were converted to molar ellipticity $[\theta]$ after subtracting buffer blank control spectra using eq 3:

$$[\theta] = \frac{\mathrm{CD}}{[\mathrm{peptide}] \times 10 \times l \times n} \quad (3)$$

where CD is ellipticity in millidegrees, l is the path length in cm and n is the number of amino acid residues.

SDS-PAGE. HFIP treated Aβ(1−42) was lyophilized and resuspended into DMSO at 2 mM concentration. Five microliters Aβ(1−42) was added to a lo-bind eppendorf and then 5 μL of DMSO or 5 μL of SEN304 (in DMSO at 200× the final concentration) was added. Incubation was initiated by addition of 990 μL dilution buffer, comprising 50 mM NaPi, 100 mM NaCl, pH 7.4. The incubating Aβ(1−42) concentration was 10 μM. At various time points (0 h, 2 h, 6 h, 24 h, and 48 h), the incubating mixture was vortexed and samples removed (for ThT, size exclusion chromatography (SEC) and SDS-PAGE), added to new lo-bind eppendorfs, snap frozen over dry ice and stored at −20 °C until required.

Novex 10−20% 1 mm 12 well precast tricine gels were used in a XCell SureLock Mini-Cell containing 1× Tricine SDS running buffer (all from Invitrogen). The samples containing Aβ(1−42) were prepared in the following manner before loading onto the gel: After thawing, the 100 μL samples were first centrifuged at 16000g for 10 min to remove large Aβ(1−42) aggregates. Thirty microliters supernatant was removed and added to a fresh lo-bind eppendorf containing 30 μL of 2× tricine SDS sample buffer (Invitrogen). After a brief vortex, these samples were boiled for 2 min and stored on ice. Fifteen microliter aliquots were placed on the gel as well as a 10 μL 1:10 dilution of Mark12 (Invitrogen) in one of the wells as a protein molecular weight ladder. Samples were subject to electrophoresis at 125 V for 80 to 100 min.

Silver Staining. Gels were silver stained using the SilverXpress Silver Staining kit (Invitrogen). The gel was removed from its plastic precast case and placed in a staining tray containing 200 mL fixing solution (50% (v/v) methanol and 10% (v/v) acetic acid) for 10 min. The fixing solution was decanted and the gel incubated in two 30 min 100 mL changes of sensitizing solution. Once sensitized, the gel was washed twice for 5 min in ultra pure water. 100 mL of staining solution was added to the gel and incubated for 15 min followed by two further 5 min washes in ultra pure water. 100 mL developing solution was added and incubated for 3−15 min until the desired staining intensity was reached when 5 mL stopping solution was given to stop further stain development. Finally, the stopping solution was removed and the gel put through three 10 min washes in ultra pure water. The gel was documented by placing between two sheets of transparency and scanning at 600 dpi using an Epson 1260 flatbed scanner.

Luminescent Staining. The gel was removed from its plastic precast case and placed in a staining tray containing 100 mL of fixing solution (50% (v/v) methanol and 7% (v/v) acetic acid) for 30 min. This step was repeated once more with fresh fix solution. The fixing solution was decanted and 60 mL of SYPRO Ruby gel stain (Invitrogen) was added and the gel agitated on an orbital shaker overnight. The gel was transferred to a clean container and washed in 100 mL of washing solution (10% (v/v) methanol and 7% (v/v) acetic acid) for 30 min. This transfer step helps in minimizing background staining irregularities and stain speckles on the gel. Before imaging, the gel was rinsed in ultra pure water a minimum of two times for 5 min. The gel was documented using a UV transilluminator coupled to a CCD camera bio imaging system.

Size Exclusion Chromatography (SEC). Aβ(1−42) species of different molecular weights were physically separated by running on a BioSep SEC-S 2000 7.8 × 300 mm column (Phenomenex). The Aβ(1−42) containing incubation mixtures for running on the SEC column were prepared similarly to SDS-PAGE. Prior to injection, the samples were centrifuged at 16000g for 10 min to remove any large Aβ(1−42) aggregates. The supernatant sample of 25 to 100 μL was injected onto the column and eluted with 50 mM NaPi, 100 mM NaCl pH 7.4 at 1 mL/min. The Aβ(1−42) peptide was detected at 214 nm. The approximate molecular weight sizes of the Aβ(1−42) species observed on a SEC trace were determined by running a gel filtration standard (Bio-Rad) containing the proteins thyroglobulin (670 kDa), γ-globulin (158 kDa), BSA (67 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), aprotinin (6.5 kDa) and vitamin B_{12} (1.35 kDa).

Electron Microscopy (EM). 10 μM Aβ(1−42) alone or with SEN304 [0.5 mM, 50 μM and 5 μM] stocks were left at room temperature (37 °C) for 4 days and 10 μL was taken out each day. 7 μL drops of the peptide solution samples were placed side by side on a clean strip of parafilm with 7 μL drops of 2% uranyl acetate (w/v in water). Carbon-coated copper grids (400 mesh/inch) were glow discharged for 30 s to render them hydrophilic. The grids were inverted on the peptide solution protein drops for 30 s, blotted by touching the grid edge to filter paper and negatively stained by inverting them on the uranyl acetate drop for 30 s. The grids were allowed to dry for 2 h before viewing by transmission electron microscopy (TEM-Philips-Tecnai10). Electron micrographs were recorded using an FEI Eangle 4k CCD Camera.

Cell Culture. Human neuroblastoma cells (SH-SY5Y, from ECACC) were maintained in media containing F-12 Ham's nutrient mixture supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (L-Q), 1% penicillin/streptomycin antibiotics (P/S) and 1% nonessential amino acids (n-aa) (all components from Invitrogen) and grown in a 5% CO_2 atmosphere at 37 °C in 175 cm^2 flasks (Becton Dickinson). When required, nearly confluent (90−95%) cells were harvested from flasks by trypsinisation and resuspended in low serum Opti-Mem media (Invitrogen) supplemented with 1.5% FBS, 1% L-Q, 1% P/S and 1% n-aa. The cells were counted and plated in 96-well polystyrene plates (Becton Dickinson) with approximately 30 000 cells per 50 μL of medium per well. Plates were incubated overnight at 37 °C to allow cells to adhere to the plastic.

Preparation of SEN304 Dose Response Incubated with and without Aβ(1−42) on Cells. Lyophilized Aβ(1−42) was dissolved in DMSO to a concentration of 2 mM. SEN304 was diluted in DMSO from 20 mM stock to make 10 concentrations ranging from 10 mM to 2 μM. These were prepared on a 96-well V bottom polystyrene plate in which

each row represented a 10 point concentration range for a compound. The last row contained DMSO (no compound) as a control for the Aβ(1−42) toxicity on the cells. In a daughter plate 294 μL Opti-Mem supplemented with 1.5% FBS, 1% L-Q, 1% P/S and 1% n-aa was added per well. The SEN304:Aβ mixture was prepared and added to cells using a program written on the Biotek precision 2000 liquid handling instrument. First, 3 μL of SEN304 from the master plate was added to the daughter plate and mixed thoroughly, followed by 3 μL of 2 mM Aβ(1−42) to the daughter plate. 50 μL of this mixture from the daughter plate was added to the plate of cells in the same corresponding columns. Columns 1 and 12 of the 96-well plate were prepared to contain 1% DMSO as live and dead cell control. The preparation of the daughter plate and transfer to cells was repeated 3 times. The final concentrations of SEN304 on the cells ranged from 50 μM to 10nM with a fixed Aβ(1−42) concentration of 10 μM in 1% DMSO. To assess the toxicity of SEN304 alone, the above protocol was repeated, but 3 μL DMSO was added instead of 2 mM Aβ(1−42). The cell plates were incubated for 24 h at 37 °C, 5% CO_2 before assaying for cell viability. Ten minutes prior to assessing cell viability, 2 μL 0.5% (v/v) Triton X-100 was added to eight live cell control wells (1% DMSO) to lyse the cells and produce a dead cell control.

MTT Cell Viability. The MTT cell viability assay is extensively used in studies measuring Aβ toxicity.[27,28] The MTT CellTiter 96 Proliferation assay (Promega) is an *in vitro* assay measuring the conversion of yellow MTT tetrazolium component of the CellTiter 96 dye solution in to a blue formazan product by mitochondrial dehydrogenases in living SH-SY5Y cells. The formazan product becomes solubilized and the absorbance is read at 570 nm. The color change from yellow to blue that takes place is calculated as a measure of the % of MTT reduced, where the percentage of cellular MTT reduction provides an indication of cell condition or health.

After the cell plates had been incubated for 24 h and dead cell controls prepared, 15 μL of MTT dye was added to each well followed by 4 h incubation at 37 °C, 5% CO_2. 100 μL of stop/solubilization solution was then added to each well and the plates stored in a humidified container overnight. The absorbance was measured at 570 nm with 650 nm as a reference using a BioTek Synergy plate reader. % MTT reduction was calculated as

$$\%\text{MTT reduction} = \frac{x - A}{B - A} \times 100\% \quad (4)$$

where x is the ΔOD absorbance (ΔOD = 570−650 nm) value in each well under study, A is the mean absorbance of the negative control (dead cells) and B is the mean absorbance of the positive control (live cells in 1% DMSO).

Lactate Dehydrogenase Toxicity. The MTT assay measures viable cells by their metabolic activity in converting cell permeable dyes into measurable colored products. Another means is to detect nonviable cells by measurement of the leakage of components from the cytoplasm into the surrounding culture medium.

The CytoTox-ONE (Promega) assay is a fluorescent measure of the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. LDH released into the culture medium is measured by supplying lactate, NAD^+, and resazurin as substrates in the presence of diaphorase, resulting in the conversion of resazurin into resorufin. The generation of the fluorescent resorufin product is proportional to the amount of LDH released and therefore dead cells.

After the cell plates had been incubated for 24 h and dead cell controls prepared, the plate was equilibrated to room temperature for 30 min. 100 μL of CytoTox-ONE dye was added to each well followed by 10 min incubation at room temperature and then 50 μL of stop solution added per well. The fluorescence was measured at an excitation frequency of 545 ± 20 nm and an emission frequency of 620 ± 7.5 nm using a BioTek Synergy plate reader. % LDH released was calculated as

$$\%\text{LDH released} = \frac{x - N}{D - N} \times 100\% \quad (5)$$

where x is the fluorescence value in each well under study, N is the mean fluorescence of the negative control (media alone, i.e., no cells), and D is the mean fluorescence of the positive control (dead cells).

ELISA. In this assay antibodies with the same epitope were used for both capture and detection of oligomeric Aβ. Monomeric Aβ peptide does not react with the detection antibody because the epitope is engaged by the capture antibody.[29] The incubation mixtures for ELISA were prepared in a similar fashion to the ThT assay. HFIP treated Aβ(1−42) was lyophilized and resuspended into DMSO at 200 x final Aβ incubation concentration. Five μL of Aβ(1−42) were added to each well of a 96-well polypropylene block (Thermo-Fisher) and then 5 μL of DMSO or 5 μL of compounds (in DMSO at 200× the final incubation concentration) were added. The incubation was initiated by addition of 990 μL of dilution buffer comprising 50 mM Tris-HCl, 100 mM NaCl, pH 7.4 in each well. The incubating Aβ(1−42) concentrations assayed ranged from 20 to 250 nM. After 30 min incubation 10 μL 10% (v/v) Tween 20 (Sigma Aldrich) was added to each well and mixed thoroughly to inhibit further oligomerization.[29] A monomeric Aβ(1−42) control was prepared by initiating incubation with the addition of dilution buffer containing 0.1% Tween 20. Twenty-five or 100 μL sample was assayed using the ELISA depending on the incubating Aβ(1−42) concentration (e.g., 10 μL for 25 M and 25 μL for 250 nM).

The Aβ oligomeric ELISA was carried out as previously reported.[29] 50 μL of 5 μg/mL capture antibody 6E10 or 4G8 (Signet) was applied in 10 mM sodium phosphate, pH 7.5, to each well of Maxisorp ELISA plates (Nunc). Plastic sealer film was applied, and the antibody adsorbed overnight at 4 °C. The plate was blocked by addition of 200 μL of TBST (20 mM Tris−HCl, 34 mM NaCl, pH 7.5 + 0.1% (v/v) Tween 20) + 2 mg/mL fatty acid−free bovine serum albumin (BSA) at room temperature for 1−2 h. The wells were washed three times with TBST with an electronic 8 channel pipettor (Thermo-Fisher). 25−100 μL of the sample to be assayed containing Aβ(1−42) was added to each well and the plate was sealed and incubated at room temperature for 2h. The plate was washed three times, and 50 μL of 1 μg/mL of biotinylated detection monoclonal antibody bio-6E10 or bio-4G8 (Signet) in TBST+BSA was added. The sealed plate was incubated for 1h at room temperature and then washed three times. 50 μL of 3:5000 dilution ExtrAvidin peroxidase conjugate (Sigma Aldrich) in TBST+BSA was added to each well and the plate incubated for 1h at room temperature. After washing three times, 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Sigma Aldrich) was added and the plate shaken at room temperature for 2−30 min, depending on the concentration of

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Aβ(1−42) peptide in the assay. The absorbance at 450 nm was determined on a BioTek Synergy plate reader after stopping the reaction with 100 μL of Stop reagent for TMB substrate (Sigma Aldrich). The inhibitory activity of compounds was calculated by the % reduction in the ELISA absorbance of the Aβ(1−42):compound mixture compared with that of the Aβ(1−42) oligomer absorbance alone.

$$\%\text{oligomeric A}\beta = \frac{x}{O} \times 100\% \quad (6)$$

where % oligomeric Aβ is the percent reduction in the oligomeric Aβ(1−42) signal, x is the ELISA absorbance signal of the Aβ(1−42):compound mixture and O is the ELISA absorbance signal of the oligomer Aβ(1−42) alone.

Surface Plasmon Resonance (SPR). A Biacore T-100 (GE Healthcare) equipped with four flowcells on a sensor chip, was used for real-time binding studies. HBS-EP buffer which contains 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.05% of surfactant P20 was used as assay running buffer and also for sample preparation. A streptavidin (SA) chip (GE Healthcare) was used where biotinylated Aβ(1−42) (rPeptide) was immobilized onto one flowcell to a binding level of ∼1300 reponse units (RU). Biotinylated Aβ(1−42) was freshly prepared in 50% DMSO at 1 μM and pulse injected onto the SA surface until the required immobilization level was reached to create a monomeric Aβ(1−42) surface. On another flowcell, biotinylated Aβ(1−40) was freshly prepared in 50% DMSO at 1 μM and pulse injected onto the SA surface until the required immobilization level was reached to create a monomeric Aβ(1−40) surface. On a third flowcell Aβ(1−42) oligomers at 10 μM were immobilized onto the streptavidin surface. These oligomers were prepared to a 1:9 biotinylated:unbiotinylated ratio using the oligomer formation method described by Kayed et al.[30] As a control, a fourth flowcell was left blank. The binding of SEN304 in the flow phase onto immobilized Aβ(1−42) monomer, Aβ(1−40) monomer or Aβ(1−42) oligomers was measured by response units. The response units elicited by SEN304 injected onto the blank control flow cell was set as the reference response, which represented the refractive index signal cause by solvent in the injected samples and was subtracted from the response units elicited by the same compound injected onto the Aβ flowcells. To perform the binding studies, 180 μL of SEN304 ranging from 20 nM to 10 μM concentration, prepared in HBS-EP buffer containing 5% DMSO, were injected and flowed onto the sensor chip for 4 min at 45 μL/min, and the response recorded. After each injection, HBS-EP + 5% DMSO buffer was flowed over the chip for 4 min, at 45 μL/min, to allow the bound compound to dissociate from the immobilized Aβ, and dissociation curves were obtained. The response elicited by injecting HBS-EP buffer alone with 5% of DMSO was used as the blank. After the dissociation phase, 30 μL of the regeneration solution, 1 M NaCl in 20 mM NaOH, was injected and flowed over the chip for 1 min at 30 μL/min to remove residual bound compound from the immobilized Aβ. The Biacore T-100 control and evaluation software (version 2.0.3) was used to record the changing responses, plot the binding curves, analyze the data and calculate the K_D of SEN304 according to a 1:1 binding model or using steady state affinity analysis.

Traveling Wave (T-Wave) Ion Mobility Mass Spectrometry. Aβ(1−42) was solubilized at a concentration of 27.5 μM with 0.5% (v/v) acetonitrile in H_2O in the presence or absence of SEN304 (100 μM final concentration). Both solutions were incubated at ambient temperature with aliquots being analyzed after 24 h.

Mass spectrometric analysis was performed by direct infusion electrospray ionization (ESI) using gold coated nanospray emitter tips (Proxeon, Odense, Denmark) into a Synapt G1 HDMS instrument (Waters, Manchester, UK). Dilution to a final concentration of 13.75 μM was performed immediately prior to analysis with water/acetonitrile/formic acid 50/50/0.2. The capillary voltage, cone voltage and source temperature were typically set at 1.8 kV, 40 V and 90 °C respectively. The quadrupole was set to transmit species between 100 and 4500 m/z and ToF pusher operating at a 64 μs interval. The nitrogen gas pressure in the T-Wave cell was approximately 4.4×10^{-1} Torr and traveling wave speed set to 300 m/s with the wave height ramped from 4.9 to 16.0 V across the entire data collection.

Cortical Neuronal Cultures. Cortical neuronal cultures were prepared from the brains of day 15.5 embryos from mice. Neuronal precursors were plated at a density of 2×10^5 cells/well in 48 well plates (precoated with poly-L-lysine) in Ham's F12 (PAA) containing 5% fetal calf serum (FCS) for 2 h. Cultures were then shaken (600 rpm for 5 min) and nonadherent cells were removed by 2 washes in phosphate buffered saline (PBS). The remaining neuronal precursors were grown in neurobasal medium (NBM) containing B27 components (PAA) for 10 days. Immunostaining showed that 97% of cells were neurofilament positive. Less than 3% of cells stained positive for GFAP (astrocytes) or F4/80 (microglial cells). To assess their effect upon synapses neurons were incubated with peptides for 24 h and membrane extracts collected.

Cell Extracts. Treated neurons were washed three times with PBS and homogenized in a buffer containing 150 mM NaCl, 10 mM Tris−HCl, 10 mM EDTA, 0.2% SDS, mixed protease inhibitors (AEBSF, aprotinin, leupeptin, bestain, pepstatin A and E-46) (Sigma) and a phosphatase inhibitor cocktail including PP1, PP2A, microcystin LR, cantharidin and p-bromotetramisole (Sigma) at 10^6 cells/mL. Nuclei and cell debris was removed by centrifugation (300g for 5 min).

Synaptophysin ELISA. The amount of synaptophysin in neuronal extracts was measured by ELISA.[31,32] Briefly, the capture mAb was 0.5 μg/mL anti-synaptophysin MAB368 (Millipore). Samples were added for 1 h and bound synaptophysin was detected using a rabbit polyclonal anti-synaptophysin (Abcam). Bound antibodies were visualized with a biotinylated antirabbit IgG (Dako) followed by incubation with extravidin conjugated to alkaline phosphatase and subsequent addition of 1 mg/mL 4-nitrophenol phosphate. Absorbance was measured on a microplate reader at 405 nm and the synaptophysin content of samples was calculated. Samples were expressed as "units synaptophysin" where 100 units was defined as the amount of synaptophysin in an extract from 10^6 vehicle treated cells.

Synaptic Vesicle Recycling. The fluorescent styryl dye FM1−43 (Biotium) is taken up into synaptic recycling vesicles and can be used to determine synaptic activity.[33] Neuronal cultures that had been treated for 24 h were incubated with 1 μg/mL FM1−43 and 1 μM acetylcholine for 10 min. The cells were then washed 5 times in ice cold PBS and solubilized in ice cold methanol at 1×10^6 neurons/mL and shaken. Cell extracts were transferred into Sterlin 96 well black microplates and fluorescence was measured using excitation at 480 nm and measuring emission at 625 nm. Background fluorescence was

subtracted. Samples were expressed as "% fluorescence" where 100% fluorescence was defined as the amount of fluorescence in a methanol extract from vehicle treated cells.

Long-Term Potentiation. Male Sprague−Dawley rats (200−250 g) were humanely killed by cervical dislocation. Transverse hippocampal slices (400 μm) were cut in chilled (<4 °C) aerated aCSF using a Leica VT1000S microtome and maintained in aCSF at room temperature for at least 1h prior to electrophysiological recording (aCSF [mM]: NaCl 127, KCl 1.6, KH_2PO4 1.24, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaCHO_3$ 26, D-glucose 10). Slices were transferred to an interface chamber and perfused (1.5−3.0 mL min^{-1}) with warmed aCSF (~30 °C). Schaffer collaterals were stimulated (7−28 V, 0.1 ms pulse width, 0.05 Hz) with a concentric bipolar electrode and extracellular fEPSPs were recorded from the stratum radiatum of the hippocampal CA1 region using an Axoclamp 2A amplifier and Axon software (pClamp 8.2) with microelectrodes filled with 4 M NaCl (resistance 2−4 MΩ). Stimulation parameters were set to yield fEPSPs of approximately 50% of maximum amplitude. A 10 min baseline period was recorded followed by administration of test substances 10 min prior to HFS (1 s; 100 Hz train) and subsequent wash. fEPSPs were recorded for 60 min post-HFS. Differences were calculated using Student's t test with $p < 0.05$ considered significant.

Lyophilized Aβ(1−42) was prepared and dissolved to a 33 μM stock solution in Tris-HCl in 1 mL. SEN304 was prepared as a 165 μM stock solution in DMSO. Both solutions were stored at −20 °C until required. Twenty μL DMSO was added to the Aβ(1−42) (1 mL) and was agitated at 37 °C for 24 h using an orbital shaker. In some experiments, in place of DMSO, 20 μL of 165 μM SEN304 was added to the Aβ(1−42) (1 mL) and agitated as described above. On the day of the experiment the mixture was diluted to 33 mL in aCSF immediately prior to use. The final concentrations of Aβ(1−42), SEN304 and DMSO were 1 μM, 100 nM and 0.06% respectively. Vehicle control comprised of 20 μL of DMSO in tris-HCl (1 mL), agitated at 37 °C for 24 h as above.

RESULTS

Thioflavin T. The aggregation of Aβ(1−42) alone, measured using the amyloid binding dye ThT, showed that by 24 to 48 h incubation, the aggregation process had reached a plateau with little further increase in ThT fluorescence (not shown). SEN304 was coincubated with Aβ(1−42) for 24 h at 37 °C at a range of concentrations, prior to a sample being taken and mixed with ThT for fluorescence measurements. SEN304 showed a reduction in % ThT fluorescence as concentration of compound increased, suggesting that SEN304 inhibits Aβ(1−42) aggregation and fibril formation (Figure 1). Fitting the data to a 4 parameter dose response model (eq 1) using XLfit gave an EC_{50} value of 1.8 ± 0.3 μM.

Circular Dichroism. CD analysis of Aβ(1−42) confirmed that Aβ(1−42) is initially unfolded. The peptide converts to a species with high β-sheet content upon incubation at 37 °C for about 240 min (Figure 2A). This transition is consistent with the conformational changes that are usually required for aggregation of peptides into amyloid fibrils. A clear isodichroic point is observed at 208 nm suggesting a two-state transition from random coil to β-sheet conformation (Figure 2A). By plotting the absolute molar ellipticity at 218 nm against time, a sigmoidal curve was obtained that was characterized by three kinetic steps: a lag phase, an exponential growth phase and a plateau phase (Figure 2B), consistent with a nucleation-dependent polymerization model. During the lag phase the initial Aβ(1−42) conformation did not change. An increase in β-sheet content was then observed. The length of the lag phase is known to depend on experimental conditions, such as peptide concentration, ionic strength of buffer, temperature and the acetonitrile cosolvent content.[26] If SEN304 affects the aggregation pathway, a change should be observed in the kinetics.[26] If the compound lengthens the lag phase, then the compound is able to stabilize non-β sheet soluble monomer/small oligomers and delay the conformation change. If the exponential phase is slowed, the inhibitor interferes with β-sheet formation and aggregation. Further assembly of species that have already adopted high β-sheet contents are unlikely be detectable by CD.

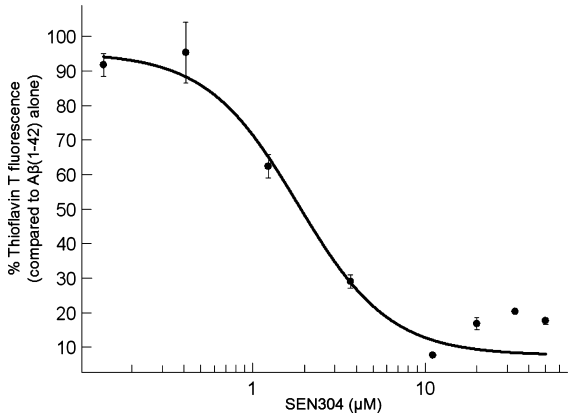

Figure 1. Dose response curve for SEN304 measured with the ThT assay. SEN304 was coincubated with 10 μM Aβ(1−42) at 37 °C for 24 h prior to ThT measurements. The % ThT fluorescence was calculated by normalizing to 100% using the Aβ(1−42) alone control. Data was fitted to a 4 parameter dose response model (eq 2) and their EC_{50} calculated using XLfit. Each point was in triplicate.

The time taken for Aβ(1−42) to convert to β-sheet generally increased in the presence of equimolar SEN304, though with sizable variability (Figure 2B), reflecting the stochastic nature of the initiation of aggregation, leading to variable lag times. SEN304 induced a slight change in the CD spectra (Figure 2C). The CD spectrum at onset of incubation was indistinguishable to Aβ(1−42) alone at onset, representing a disordered, unfolded state. At the end of incubation, both CD spectra showed β-sheet formation. However, the minimum with SEN304 had slightly shifted to ~215 nm compared to ~218 nm for Aβ(1−42) alone, suggesting that SEN304 causes a subtle change in β-sheet structure. The CD spectrum of SEN304 alone showed no secondary structure (not shown).

SDS-PAGE. The aggregation of Aβ(1−42) was followed by observing the separation of different sized oligomers on Tricine SDS-PAGE gels. At various time intervals (0, 1, 2, 6, 24, and 48 h) aliquots of 10 μM Aβ(1−42) were taken and snap frozen over dry ice. These samples were thawed, centrifuged and the supernatant placed under electrophoresis followed by silver stain to visualize Aβ(1−42).

Aβ(1−42) in nanogram quantities can be detected by the silver stain. At the early time points (0, 2 and 6h) banding is characteristic of the protein sizes monomer (~4.5 kDa), trimer (~13.5 kDa) and tetramer (~18 kDa) (Figure 3A). After 24 h, the trimer and tetramer bands were not visible and the monomer bands were fainter, probably due to less Aβ(1−42) running down the gel. ThT data showed a large signal for ThT

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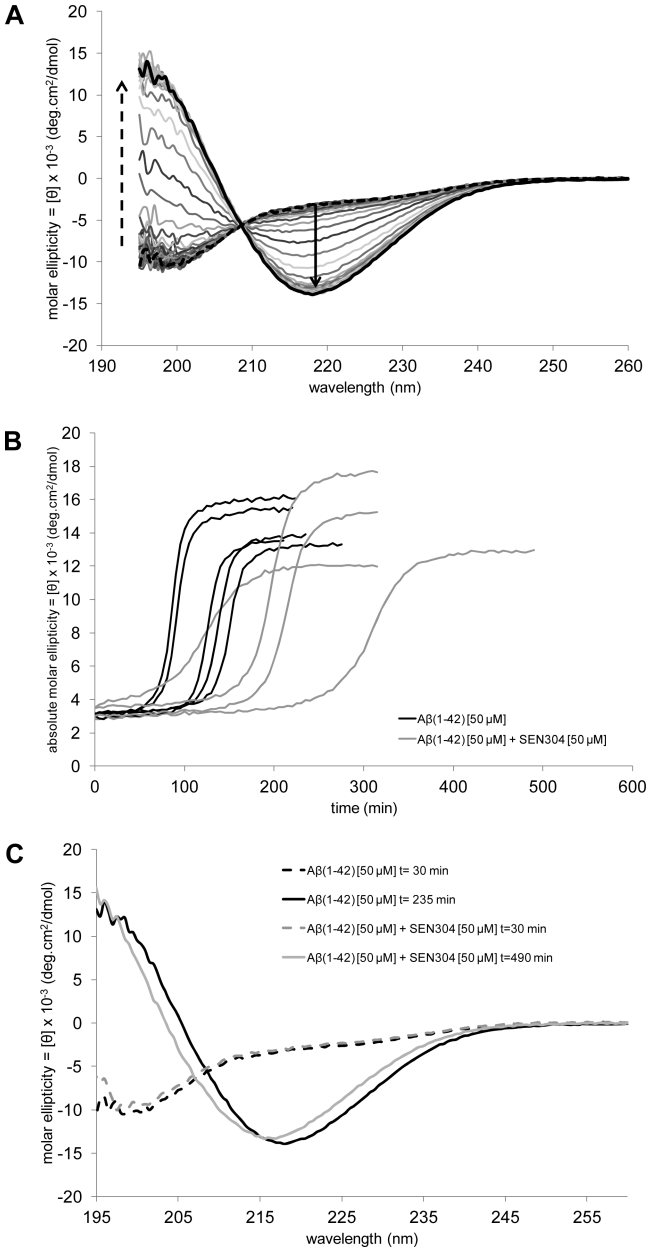

Figure 2. (A) CD kinetic study of 50 μM Aβ(1−42) pH 8, 10 mM NaCl, 37 °C. Overlaid CD spectra (range 195−260 nm) recorded at 15 min time intervals. First spectrum recorded after 30 min, final spectrum after 235 min. The increase in the β-sheet content is indicated by the increase of a negative minimum at 218 nm (black solid arrow) and a positive maximum at 195 nm (black dotted arrow). (B) Time courses plotting absolute molar ellipticity against incubation time, Aβ(1−42) [50 μM, pH 8, 10 mM NaCl, 37 °C] was incubated with (gray) and without (black) 50 μM SEN304. (C) Overlaid CD spectra at onset of Aβ(1−42) incubation with and without compounds after 30 min (dashed lines) and at end of incubation (time shown in legend) (solid lines).

binding to Aβ(1−42) fibrils after 24 h (Figure 3B). The large Aβ(1−42) aggregates formed after 24 h would be pelleted prior to loading onto the gels, accounting for the faint monomer band.

Following the same experimental conditions as in the ThT assay, Aβ(1−42) was incubated with different concentrations of SEN304 for various time intervals. Samples were taken for SDS-PAGE and ThT fluorescence (Figure 3). There was little difference between 3 μM SEN304, 0.3:1 molar ratio, coincubated with Aβ(1−42) compared to Aβ(1−42) alone, except for a more intense band seen at the 24 h time point (Figure 3A, solid gray). When equimolar SEN304 and Aβ(1−42) was present, a monomer band was observed even at the 24 and 48 h time points. However, the diffuse trimer and tetramer bands were less intense (Figure 3A, dashed gray). When incubated in 5-fold excess, (50 μM SEN304), no trimer and tetramer bands could be seen, only monomer (Figure 3A, dotted gray). The intensities of the monomer bands were faint, probably due to Aβ(1−42) being pelleted and less Aβ(1−42) being loaded onto gel.

The ThT data (Figure 3B) demonstrated an inhibition of ThT fluorescence with increasing SEN304 concentrations. When Aβ(1−42) was incubated with equimolar or above concentrations of SEN304, low ThT fluorescence was observed. This suggests that no Aβ(1−42) fibrils were present or that ThT could not bind the Aβ(1−42):SEN304 complex. The latter may be the case, since if few large Aβ(1−42) aggregates had formed, the monomer together with diffuse trimer and tetramer banding pattern would have been observed at the earlier time points in SDS-PAGE, not just a faint monomer band (Figure 3A, gray dotted)

While it is tempting to interpret the trimer and tetramer bands as direct detection of oligomers, it is possible that these species are artifactual, only forming in the presence of SDS, and therefore may not be present in aqueous solution.[34] A more cautious interpretation is that the gels simply report on the presence of Aβ species that are sufficiently small to run on a gel. The centrifugation step will pellet any large Aβ aggregates. The gels therefore suggest that SEN304 induces Aβ aggregation to species that are larger than for Aβ alone.

Size Exclusion Chromatography. SEC can be used to observe Aβ aggregation as it separates species by size. Standards were loaded onto a Biosep-2000 column, to determine molecular weights with elution time of a protein. This calibration curve (not shown) gave a line of best fit of log molecular weight = −0.524 × elution volume + 8.5296.

Initial SEC traces were produced to observe Aβ(1−42) aggregation in isolation. 100 μL of 1 μM Aβ(1−42) in 1% DMSO was injected onto the column after various time intervals from initiation of aggregation (0, 2, 6 24 and 48 h) (Figure 4A). The SEC traces show a peak eluting at ∼9 mL, estimated to be about 6.5 kDa using the above equation. This can be attributed to monomer/dimer Aβ(1−42) species (LMW), as the molecular weight of Aβ(1−42) is 4.5 kDa. The difference between the observed apparent molecular weight and those of the monomer or dimer is presumably due to shape differences between the Aβ(1−42) and the proteins used to make the calibration curve. Over time this peak gradually decreased in intensity and by 24 h had disappeared. As the LMW peak decreased, smaller, broader peaks eluting between 5 and 7 mL were detected, representing larger Aβ(1−42) species, such as protofibrils, of approximately 400 kDa. After 24 h, nothing was detected using SEC. This could be due to formation of fibrils which had been pelleted in the centrifugation step prior to injecting onto the column.

10 μM SEN304 was coincubated with 10 μM Aβ(1−42) at different incubation time intervals. At each time point a 100 μL sample was briefly centrifuged and injected onto the column (Figure 4B). The SEC traces showed only one peak eluted at ∼9 min representing LMW size Aβ(1−42) species. No peaks were observed representing large aggregates eluting in the void

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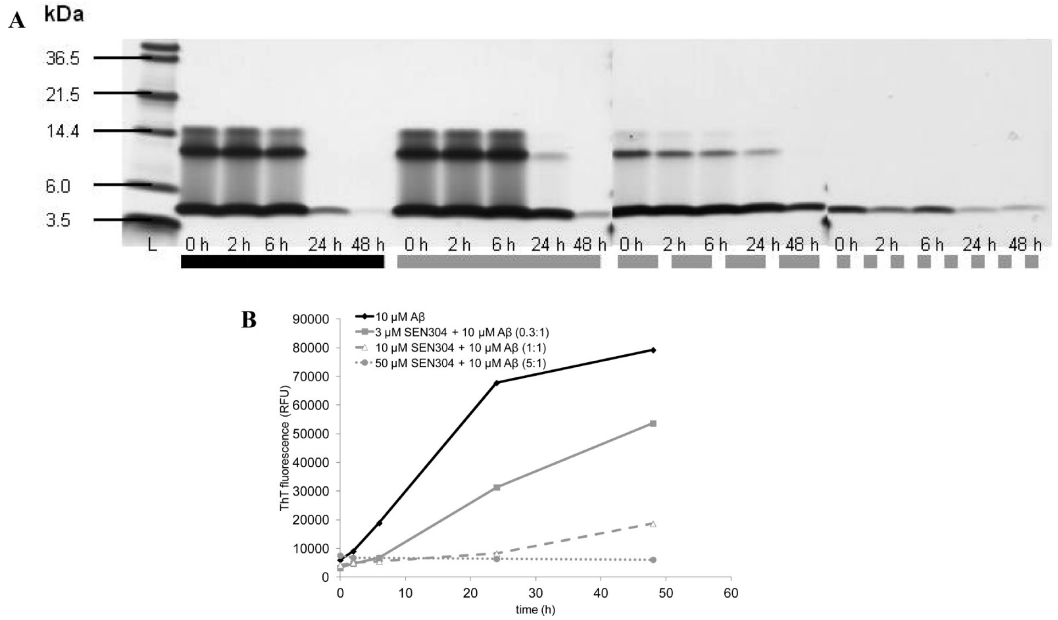

Figure 3. Effects of SEN304 on Aβ(1−42) aggregation observed using SDS-PAGE and silver stained tricine gels, and ThT fluorescence. (A) Gel show the banding for the different size Aβ(1−42) species after 0, 2, 6, 24, and 48 h. Black denotes 10 μM Aβ(1−42) samples, solid gray 3 μM SEN304 + 10 μM Aβ(1−42), dashed gray 10 μM SEN304 + 10 μM Aβ(1−42) and dotted gray 50 μM SEN304 + 10 μM Aβ(1−42). Lane L represents the ladder for the Mark12 protein molecular weight standard. (B) ThT fluorescence for each of the samples prior to loading on gel to measure Aβ(1−42) aggregation. (Negligible) error bars represent SEM for one sample in quadruplicate.

volume of the column. There was a difference between the LMW peak observed with SEN304 present compared to Aβ(1−42) alone. The peak height was not as tall (~100 mAu) with SEN304, compared to ~160 mAu with Aβ(1−42) alone at the 0 h time point. The peak in the SEN304 SEC traces also decreased more slowly as even after 48 h incubation a peak was still observed. In the case of Aβ(1−42) alone, no peak was seen after 6 h incubation. SEN304 therefore slows aggregation, prevents formation of the 'protofibrillar' species and promotes formation of large aggregates. The lower amplitude peak at ~9 min and the absence of peaks at ~6 min indicate the absence of soluble large oligomers with SEN304, and probably also rapid formation of insoluble species.

Sandwich Enzyme-Linked Immunosorbant Assay (ELISA) for Oligomeric Aβ. Recent work using dot blot analysis with Aβ oligomer specific antibodies has shown that compounds exist that can efficiently inhibit fibril formation or oligomerization, but not both.[35] A second technique that can be used to evaluate the ability of compounds to inhibit formation of Aβ oligomers is single-site ELISA.[29] This method employs an antibody, usually monoclonal, against a specific epitope to capture Aβ peptide and detect with a labeled antibody to the same epitope. Monomer peptide is also captured, but it is not detected because the single epitope site is occluded by the capture antibody. This assumes that oligomers retain equivalent binding sites and that binding of the capture antibody does not affect subsequent binding of the detection antibody. Aβ multimers large enough to overcome the steric hindrance between antibodies, >30−50 kDa for Aβ, will bind the detection antibody and produce a signal.[36] Small Aβ oligomers (tetramers and smaller), are captured but not detected. The same size selectivity was observed for the A11 oligomer-specific antibody when used in direct detection of nitrocellulose or ELISA plate adsorbed oligomers.[30]

Using the same antibody for capture and detection was sensitive in determining oligomeric Aβ species at nanomolar concentrations. Initially, both 6E10 and 4G8 Aβ specific antibodies were used (Figure 5A). The specificity of 6E10 is for amino acid residues 1−17 and 4G8 for 17−24. SEN304 showed a dose dependent inhibition of oligomer formation using either Aβ specific antibody in the ELISA. The EC_{50} for SEN304 was estimated to be 0.5 μM using a 4 parameter sigmoidal dose−response model (eq 2) using XLfit. Further ELISA experiments were carried out using the 4G8 antibody as it was observed to give less background signal when samples containing no Aβ(1−42) were used.

SEN304 was incubated at various concentrations with two different concentrations of Aβ(1−42) (250 nM and 25 nM), to observe whether the effect of SEN304 on oligomer formation was sensitive to Aβ(1−42) concentrations (Figure 5B). SEN304 showed inhibitory action of oligomer formation at both 250 nM and 25 nM Aβ(1−42). The EC_{50} for SEN304 was ~400 nM when incubated with 250 nM Aβ(1−42) and ~200 nM when incubated with 25 nM Aβ(1−42), calculated from a 4 parameter sigmoidal dose−response model (eq 2) using XLfit.

Surface Plasmon Resonance. We used SPR to demonstrate that SEN304 binds directly to both monomeric Aβ(1−40) and Aβ(1−42), and oligomeric Aβ(1−42), that have been immobilized onto streptavidin-coated chips. The immobilized Aβ surfaces were characterized using the A11, OC, 6E10, 4G8 and 12F4 antibodies: A11 and OC (Aβ oligomer and Aβ fibril specific respectively) showed a binding response to the oligomeric Aβ(1−42). However, no binding of these oligomer/fibril specific antibodies was observed to the monomeric Aβ surfaces (data not shown). SEN304 bound to the Aβ surfaces in a concentration related manner (Figure 6). The K_D values for binding to Aβ(1−42) monomer was calculated to be 2.5 μM using steady state affinity analysis or 900 nM when applying the 1:1 binding model. The K_D values

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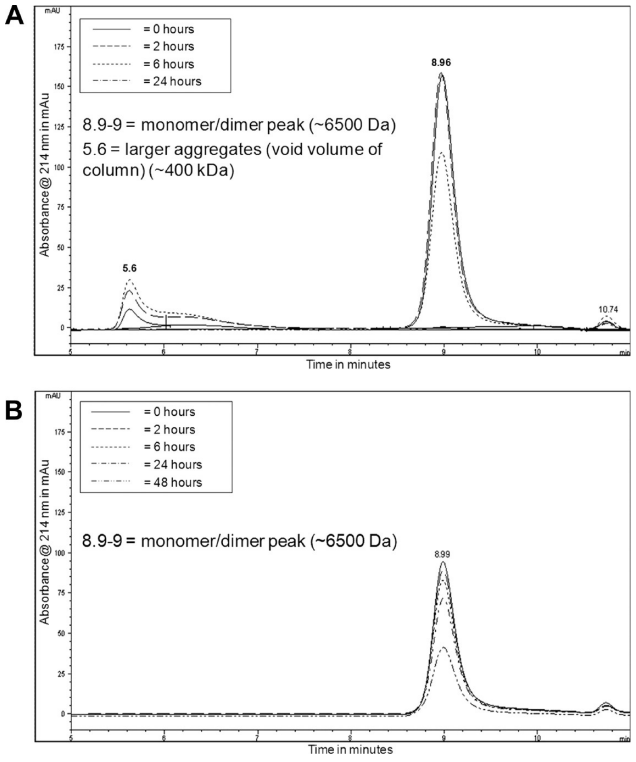

Figure 4. (A) SEC traces for 100 μL 10 μM Aβ(1−42) loaded onto Biosep-SEC S-3000 column after various incubation times (0, 2, 6, and 24 h). Peptides were eluted in NaPi/NaCl buffer at 1 mL/min and detected at 214 nm. The peak eluting at ∼9 min represents ∼6.5 kDa (monomer/dimer Aβ(1−42) size) and the broad smaller peaks at ∼6 min represents larger aggregates eluting in the void volume of the column. (B) SEC traces for 10 μM Aβ(1−42) incubated with 10 μM SEN304. 100 μL was loaded onto Biosep-SEC S-3000 column after various incubation times (0, 2, 6, 24, and 48 h). Peptides were eluted in NaPi/NaCl buffer at 1 mL/min and detected using 214 nm wavelength. The peak eluting at ∼9 min represents ∼6.5 kDa (monomer/dimer Aβ(1−42) size).

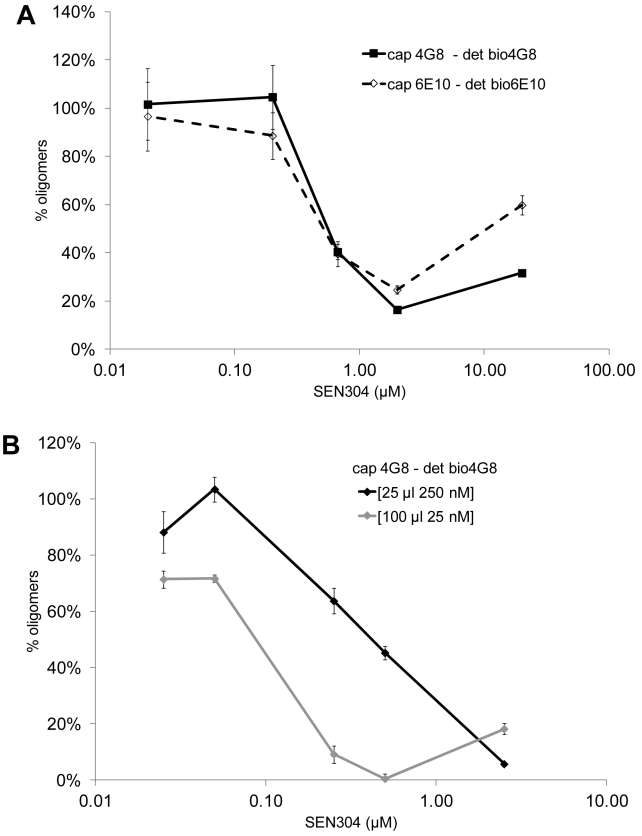

Figure 5. (A) Aβ(1−42) oligomer formation incubated with SEN304 and measured using ELISA. The same antibody was used to capture and detect Aβ(1−42) oligomers. The solid line uses the 4G8 antibody and dashed line the 6E10 antibody. After a 30 min incubation, a 25 μL sample containing 200 nM Aβ(1−42) mixed with different concentrations of compound was transferred to the ELISA plate for Aβ(1−42) oligomer measurement. Error bars represent SEM for quadruplicate samples. (B) Aβ(1−42) oligomer formation incubated with SEN304 measured using ELISA. The 4G8 antibody was used to capture and detect Aβ(1−42) oligomers. The black line shows the data when a 25 μL [250 nM] Aβ(1−42) sample and the gray line when a 100 μL [25 nM] Aβ(1−42) sample mixed with different concentrations of compound was transferred to the ELISA plate for Aβ(1−42) oligomer measurement. Error bars represent SEM for quadruplicate samples.

for binding to Aβ(1−40) monomer was 14 μM using steady state affinity analysis or 9 μM with the 1:1 binding model. Due to the heterogeneity of the Aβ(1−42) oligomer surface only the steady state affinity analysis was used to calculate the K_D and this was determined to be 11 μM. These data suggest that the SEN304 mode of action involves direct interaction with Aβ.

Traveling Wave Ion Mobility Mass Spectrometry. To monitor the effect of SEN304 on the aggregation of Aβ(1−42), traveling wave (T-wave) ion mobility mass spectrometry (IM-MS) analysis was conducted. IM-MS gives information on both the size and shape of molecular ions by measuring both the m/z (MS) and the drift time through a N_2 containing mobility cell (IM), giving information on the rotationally averaged collision cross-sectional areas. Molecular ions of the same primary structure but altered conformation therefore have the same m/z but exhibit a change in the time taken to drift through the T-wave mobility cell, with longer drift times equating to larger collision cross sections. As shown in Figure 7A, two distinct conformers of Aβ(1−42) were resolved by ion mobility in the absence of inhibitor, with the compact and extended conformations exhibiting the faster and slower drift times in the T-wave mobility cell respectively. Similarly, IM-MS studies of Aβ(1−40) oligomers were recently found to form both compact and extended states.[37] Incubation of 27.5 μM Aβ(1−42) for a period of 24 h in the presence of 100 μM SEN304 indicates inhibitor binding in both a 1:1 (Figure 7E) and 1:2 (not shown) stoichiometric ratio for Aβ(1−42): SEN304 as determined by the m/z value. However, even in the presence of excess inhibitor, unbound Aβ(1−42) was still observed, exhibiting the faster drift time characteristic of the smaller, more compact form of Aβ(1−42) (Figure 7C,D). The slower drift time of the inhibitor bound Aβ(1−42) was analogous to that observed for the extended nonincubated peptide monomer, indicating preferential inhibitor binding to the more extended species (Figure 7A,E). That the compact form is still observed under conditions of excess inhibitor suggests that the binding of the inhibitor does not itself promote 'unravelling' of the compact to the more extended species and that equilibrium has not been reached. We have therefore demonstrated that Aβ(1−42) adopts at least two distinct conformations, only one of which binds to SEN304. Furthermore, the comparable drift times of the extended forms Aβ(1−42) with and without bound SEN304 indicates

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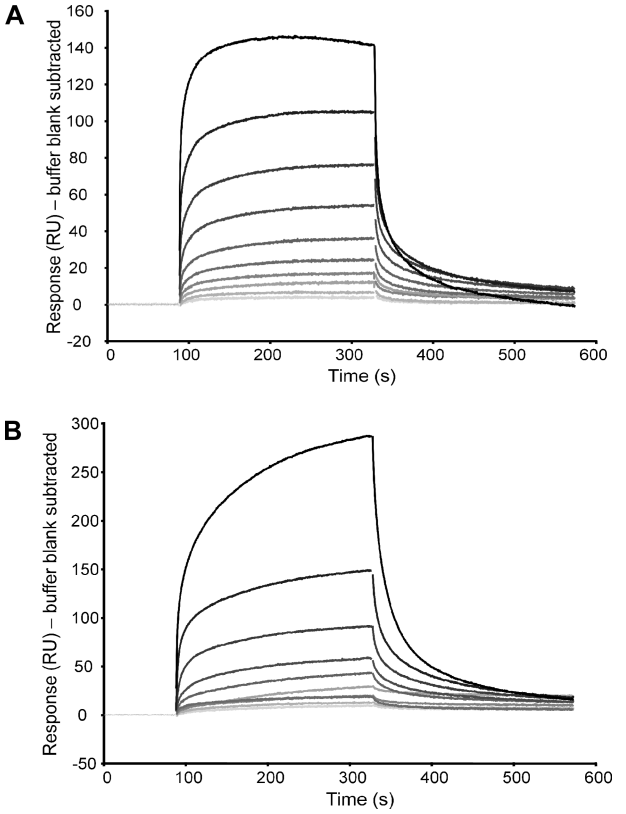

Figure 6. Surface plasmon resonance for SEN304 binding to (A) Aβ(1−42) monomers. (B) Aβ(1−42) oligomers.

that any effect of inhibitor binding on structure results in minimal change in monomer cross-sectional area.

Electron Microscopy. Figure 8 shows EM micrographs of 10 μM Aβ(1−42) after 1 or 2 days aggregation, compared to Aβ(1−42) with 5 μM SEN304. After 1 day SEN304 had caused increased fibril formation with a different morphology of wider fibrils (Figure 8C). After 2 days or longer, much fewer fibrils were seen in the presence of SEN304 (Figure 8D). Our previous work, showed that SEN304 and Aβ(1−40) formed very short (200−300 nm) twisted ribbons composed of two fibers with a helical repeat of 100 nm and a diameter length of 13−23 nm, similar to those seen in Figure 8C.[1]

Cell Toxicity. SEN304 was tested for its effect at alleviating Aβ(1−42) toxicity on SH-SY5Y cells using the MTT assay. Cell viability was measured after treating the cells with a final concentration of 10 μM Aβ(1−42) and 10 point concentration range (2 nM − 50 μM) of SEN304 followed by incubation for 24 h (Figure 9). The EC_{50} of SEN304 was calculated by fitting the data to a 4 parameter dose response model (eq 2) using XLfit (Figure 9), giving an EC_{50} value of 5.0 ± 0.4 μM.

The effect of SEN304 at alleviating Aβ(1−42) toxicity on SH-SY5Y cells was also measured with the LDH assay. Cell viability was measured after treating the cells with a final concentration of 20 μM Aβ(1−42) and an 8 point concentration range (400 nM − 100 μM) of SEN304 followed by incubation for 48 h (Figure 10). The data showing the % LDH released when compounds were incubated with Aβ(1−42) were calculated by normalizing to the Aβ(1−42) alone and background media control values. The % LDH release for SEN304 incubated alone were normalized using the live cell and dead cell control values. SEN304 showed complete reversal of Aβ(1−42) toxicity. The EC_{50} of SEN304 was calculated by fitting the data to a 4 parameter dose response model (eq 2) using XLfit (Figure 10), giving an EC_{50} value of 10.3 ± 0.2 μM. SEN304 incubated alone showed no evidence of toxicity to at least 50 μM with either the MTT or LDH assay (not shown).

Neuronal Toxicity and Synapse Degeneration. The early stages of AD are characterized by memory impairment and subtle behavioral changes, associated with changes in synaptic function and a reduction in the levels of synaptophysin, a presynaptic membrane protein essential for neurotransmitter release and the recycling of synaptic vesicles within the brain.[38] These occur before any gross neurological damage is observed.[39] The loss of synapses and the reduction in synaptophysin levels are features of AD that strongly correlate with cognitive decline.[40] We previously developed an *in vitro* model to examine the effects of Aβ peptides on synapses where the amounts of synaptophysin in neuronal cultures were measured as a surrogate marker of synapse function. The addition of Aβ(1−42) reduced the synaptophysin content of neurons, indicating the loss of synapses in these cultures.[41]

Aβ(1−42) was premixed with 10 μM SEN304 for 30 min and then added to cortical neurones for 24 h. Pretreatment of Aβ(1−42) significantly inhibited the loss of synaptophysin in cortical neurones; it increased the EC_{50} of Aβ(1−42) from 20 nM to ∼1 μM (Figure 11A). The efficacy of SEN304 was also determined; SEN304 gave a dose-dependent increase in synaptophysin in the presence of 100 nM recombinant Aβ(1−42) (Figure 11B). Pretreatment of cortical neurones for 1 h with 10 μM SEN304 (rather than incubation with peptide) did not affect the synapse loss induced by the subsequent addition of recombinant Aβ(1−42) (data not shown). These results suggest that SEN304 affects the inherent toxicity of Aβ(1−42) oligomers rather than exerting an effect upon neuronal sensitivity.

We recently developed another assay to determine synaptic function in cortical neurons.[33] Styryl dyes (synaptogreen/FM1−43 - Biotium) are rapidly taken up by recycling vesicles and can be measured by fluorescence. Thus, the specific fluorescence in neuronal cultures can be seen as a measure of synaptic vesicle recycling and hence a surrogate marker of synaptic function. Fluorescence in cortical neurones was reduced following incubation with recombinant Aβ(1−42) in a dose-dependent manner. The concentration of Aβ(1−42) required to reduce synaptogreen uptake (synaptic vesicle recycling) by 50% was 0.5 nM (Figure 12). Pretreatment of Aβ(1−42) with 10 μM SEN304 significantly increased the amount of synaptogreen in cortical neurones so that the EC_{50} of the SEN304:Aβ(1−42) complex was now 40 nM, an 80-fold increase.

Long-Term Potentiation. AD is considered a product of initial synaptic failure that precedes neuronal degeneration, and Aβ-induced dysfunction of synaptic plasticity appears to contribute to early memory loss.[5,42] Long-term potentiation (LTP), a well-established model of synaptic plasticity, involves a sustained increase in excitatory synaptic transmission, and inhibition of LTP by Aβ may mimic an early manifestation of AD.[43] We have previously shown that SEN304 is able to reverse inhibition of LTP by Aβ(1−40), the shorter, more abundant, but less toxic variant of Aβ.[21] The effects of 1 μM Aβ(1−40) could be prevented by just 1 nM SEN304, an Aβ:inhibitor stoichiometry of 1000:1, in contrast with the approximately equimolar stoichiometries required to show efficacy by biophysical and cell toxicity assays.[1] Here we performed the same experiment using Aβ(1−42).

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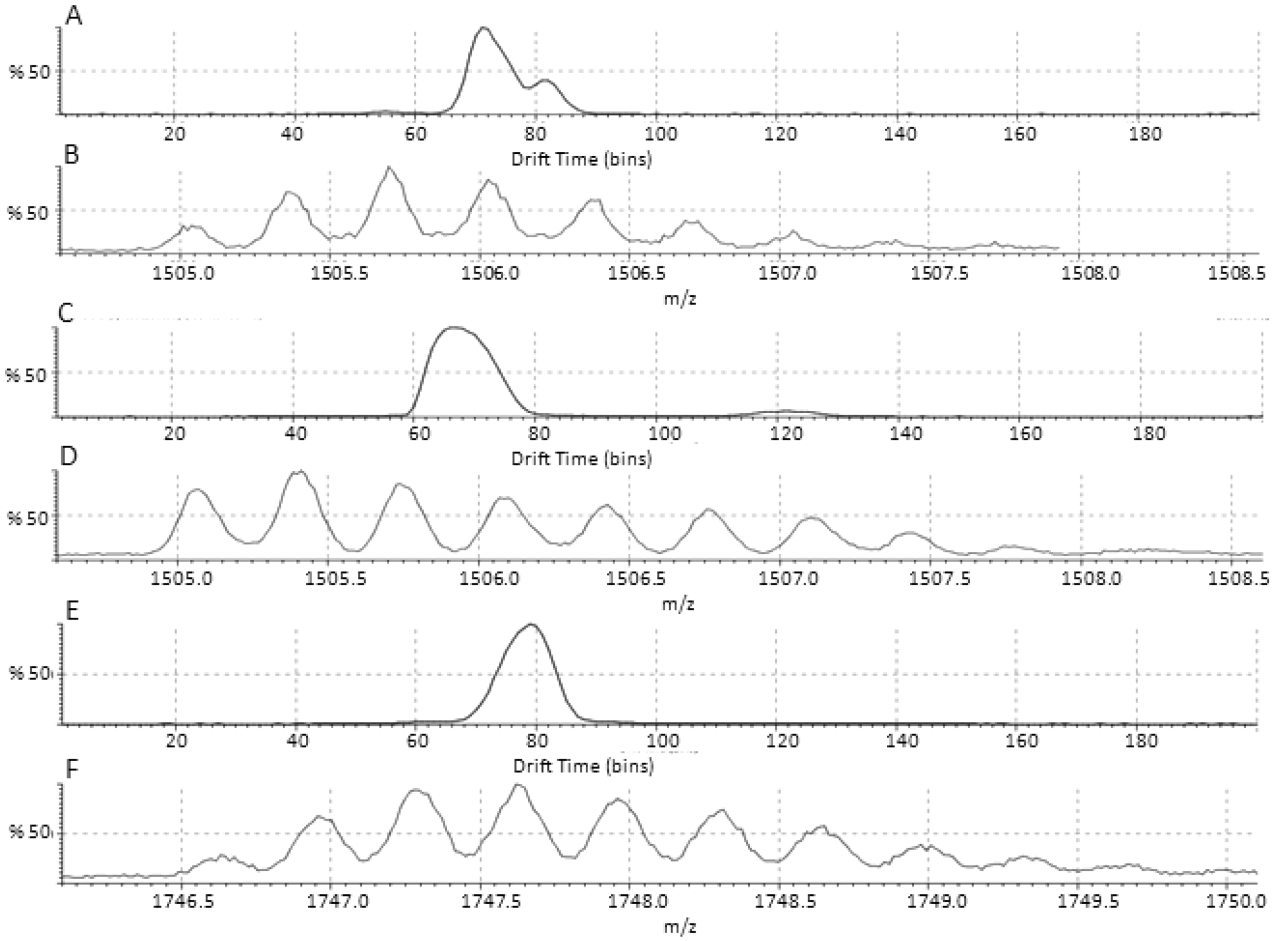

Figure 7. Traveling wave ion mobility mass spectrometry analysis of the triply protonated monomer of Aβ42 after 24 h incubation. (A) Drift time (bins) of the $[M+3H]^{3+}$Aβ42 monomer in the absence of SEN304. (B) $[M+3H]^{3+}$monomer at m/z 1505.09 in the absence of SEN304. (C) Drift time (bins) of the $[M+3H]^{3+}$unbound Aβ42 monomer in the presence of SEN304. (D) $[M+3H]^{3+}$unbound Aβ42 monomer at m/z 1505.09 in the presence of SEN304. (E) Drift time (bins) of the inhibitor bound Aβ42 $[M+3H]^{3+}$monomer in the presence of SEN304. (F) Inhibitor bound Aβ42 $[M+3H]^{3+}$monomer at m/z 1746.63 in the presence of SEN304.

1 μM Aβ(1−42) gave a strong inhibition of LTP, shown by a decrease in fEPSP amplitude, in agreement with previously reported work (Figure 13).[44,45] The mean normalized fEPSP amplitude 60 min post-HFS with Aβ(1−42) was significantly different to vehicle, with a p-value of 0.017 (Table 1). When the experiment was repeated in the presence of 100 nM SEN304, inhibition of LTP by Aβ(1−42) was almost completely abolished (Figure 13). SEN304 had no effect on LTP in the absence of Aβ(1−42) (Table 1). SEN304 can therefore prevent inhibition of LTP by Aβ when present in subequimolar concentrations, when Aβ is in either its 40 or 42 amino acid length forms. The lower stoichiometry in this assay relative to the cell viability, synaptophysin, and synaptic vesicle assays, suggest that SEN304 may bind to an unknown target, in addition to its binding and direct effect on Aβ.

DISCUSSION

The use of a wide range of biophysical and biological assays allows detailed characterization of the effects of SEN304 on Aβ(1−42) and the opportunity to compare different assays. SEN304 is a powerful inhibitor of Aβ(1−42) toxicity, as shown by the MTT, LDH, synapse degeneration and synaptogreen assays in cell culture, and LTP in a brain slice. While data generally fitted well to sigmoidal dose−response curves, EC_{50} values varied substantially. While changes in Aβ(1−42) concentration and conditions that are necessary for each assay undoubtedly lead to some variations in EC_{50}, some values appear to be assay dependent. For example, while most methods (e.g., ELISA, MTT, LDH) have EC_{50} for SEN304 close to the Aβ(1−42) concentration, with LTP a much lower concentration of SEN304 is required, as 100 nM SEN304 almost entirely prevents inhibition of LTP by 1 μM Aβ(1−42) (Figure 12). We previously found that inhibition of Aβ(1−40) by SEN304 was even more sensitive to low concentrations of inhibitor.[21] This is encouraging, as the LTP assay in a hippocampal slice is the closest to *in vivo* conditions of any of the assays that we used here. It may be that inhibition of LTP responds to a subset of Aβ species that are particularly sensitive to SEN304 inhibition, as is potentially indicated by the preferential binding of SEN304 to a distinct Aβ(1−42) conformer observed during IM-MS studies.

Although each of the biophysical assays we used could potentially be criticized in isolation, taken together the results give a comprehensive picture of how SEN304 affects Aβ aggregation. Traveling wave ion mobility mass spectrometry demonstrates that Aβ(1−42) adopts at least two distinct conformations, one of which binds to SEN304 with a 1:1 stoichiometry (Figure 7). The ThT assay showed a dose-related reduction in ThT fluorescence associated with large Aβ(1−42)

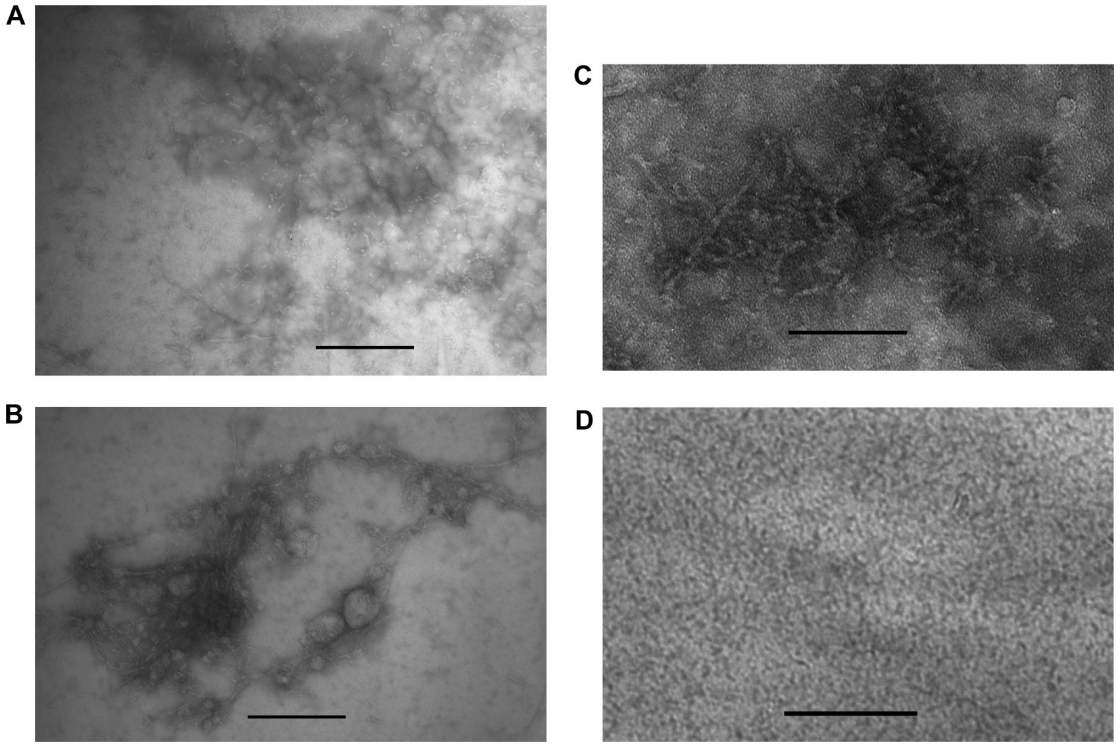

Figure 8. Electron micrographs of 10 μM Aβ(1−42). Scale bar is 300 nm. (A) Aβ(1−42) alone, 1 day after initiation of aggregation. (B) Aβ(1−42) alone, 2 days after initiation of aggregation. (C) Aβ(1−42) with 5 μM SEN304, 1 day after initiation of aggregation. (D) Aβ(1−42) with 5 μM SEN304, 2 days after initiation of aggregation.

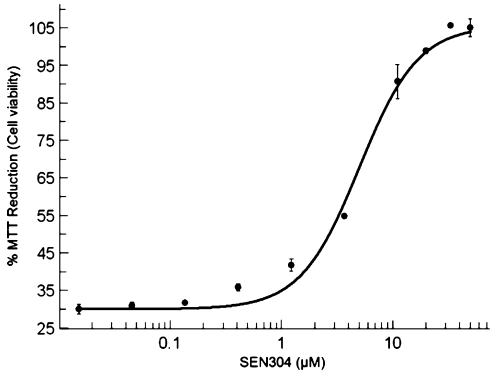

Figure 9. Dose response curve for inhibition of Aβ toxicity with SEN304 on SH-SY5Y cells in the MTT assay. Data were fitted to a 4 parameter dose response model (eq 2) and their EC_{50} calculated using XLfit. EC_{50} = 5.0 ± 0.4 μM.

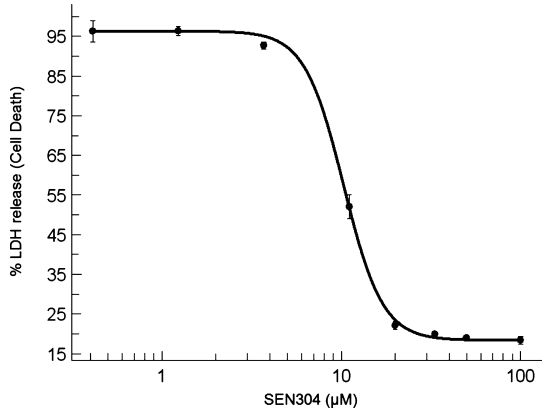

Figure 10. Dose response curve for inhibition of Aβ toxicity with SEN304 on SH-SY5Y cells in the LDH assay. Data were fitted to a 4 parameter dose response model (eq 2) and their EC_{50} calculated using XLfit. EC_{50} 10.3 ± 0.2 μM.

aggregates or fibrils. CD shows that SEN304 inhibits Aβ(1−42) aggregation by delaying the time taken for the Aβ(1−42) to change from an unfolded to β-sheet configuration (Figure 2). Aβ(1−42) remains as monomer for longer in the presence of SEN304, as shown by SDS-PAGE and SEC (Figures 3 and 4). At higher concentrations of SEN304 (5:1 molar ratio), SDS-PAGE indicates that the compound is promoting Aβ(1−42) aggregation as fewer bands are visualized using SDS-PAGE (Figure 4). However, the increase in larger aggregates is not shown by the ThT assay, suggesting that ThT cannot bind to these SEN304 induced larger Aβ(1−42) aggregates. SEN304 binds to both Aβ(1−42) oligomers and monomers (shown by SPR) and reduces the formation of Aβ(1−42) oligomers when measured by ELISA. The SEC, SDS-PAGE and EM results indicate how the oligomer concentration is reduced. Lower levels of monomer are present with SEN304. This is observed clearly with SDS-PAGE, with only a faint monomer band being present when SEN304 is coincubated with Aβ(1−42) at a 5:1 molar ratio (compound:Aβ) (Figure 3). The lower levels of monomer when Aβ is in the presence of SEN304 suggests that the compound inhibits Aβ toxicity by promoting rapid aggregation of Aβ monomers and oligomers, in an alternative aggregation mode (seen in EM), to larger noncytotoxic aggregates that do not bind ThT. It may be the case that at lower SEN304 concentrations, the compound binds to the monomer and/or oligomers and these complexes remain until a concentration is reached that causes these SEN304:Aβ complexes to seed and further promote Aβ aggregation to nontoxic larger aggregates. This would explain the bands

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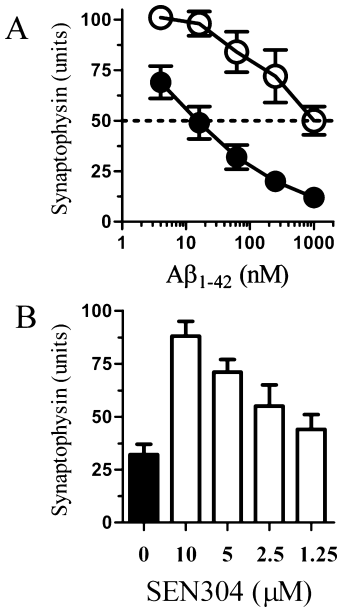

Figure 11. SEN304 reduces Aβ1−42-induced synapse damage. (A) The amount of synaptophysin in cortical neurons incubated for 24 h with different concentrations of Aβ1−42 that had been premixed with a vehicle control (●) or with 10 μM SEN304 (○). Values shown are the mean amount of synaptophysin (units) ± SD from triplicate experiments performed 4 times, n = 12. (B) The amount of synaptophysin in cortical neurons incubated for 24 h with 100 nM Aβ1−42 that had been premixed with a vehicle control (□) or with different concentrations of SEN304 as shown (■). Values shown are the mean amount of synaptophysin (units) ± SD from triplicate experiments performed 3 times, n = 9.

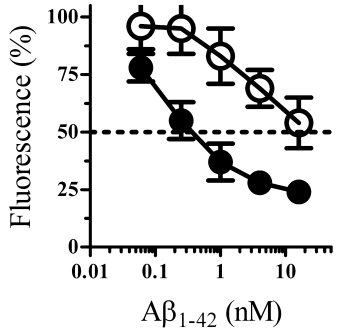

Figure 12. SEN304 reduces Aβ1−42-induced suppression of synaptic vesicle recycling. The fluorescence of neuronal cultures treated for 24 h with different concentrations of Aβ1−42 that had been premixed with a vehicle control (○) or with X μM SEN304 (●). Synaptic vesicle recycling was assessed by the addition of 1 μg/mL FM1−43 and 1 μM ionomycin for 1 min. Values shown are the mean fluorescence ± SD from triplicate experiments performed 3 times, n = 9.

observed in SDS-PAGE and the lower monomer peaks in the SEC results when SEN304 is incubated with Aβ(1−42) in equimolar concentrations. This mode-of-action is similar to resveratrol, which selectively remodels soluble oligomers, fibrillar intermediates and amyloid fibrils into an alternative aggregated species that is nontoxic, has a high molecular weight and is unstructured.[46]

In conclusion, SEN304 is a powerful inhibitor of Aβ toxicity, working by inducing aggregation, and hence removal, of Aβ oligomers. It is therefore a promising lead compound for Alzheimer's disease.

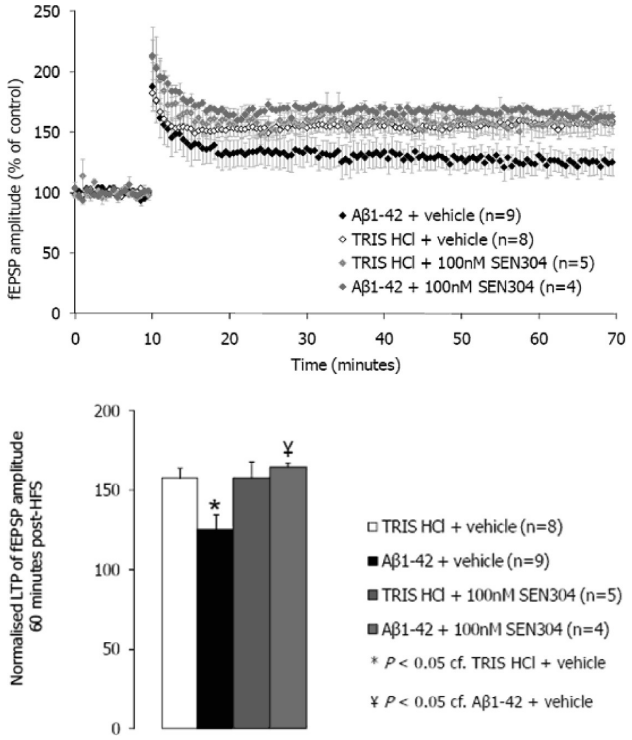

Figure 13. Inhibition of Long-term potentiation by 1 μM Aβ(1−42) is reversed by 100 nM SEN304. Upper figure shows variation of LTP with time; lower figure compares equilibrated values.

Table 1. Long Term Potentiation

condition	Tris HCl + vehicle	1 μM Aβ(1−42) + vehicle	Tris HCl + 100 nM SEN304	1 μM Aβ(1−42) + 100 nM SEN304
mean normalized fEPSP amplitude 60 min post-HFS	157.4	125.0	165.2	162.0
standard error	6.5	9.9	11.2	7.3
t test p-value cf Aβ(1−42)	0.017			0.009

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Notes

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■ ABBREVIATIONS USED

AD, Alzheimer's disease; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; ADMET, absorption distribution metabolism excretion toxicity; DMSO, dimethyl sulfoxide; ThT, Thioflavin T; CD, circular dichroism; HFIP, hexfluoroisopropanol; RDB, redissolution buffer; PBS, phosphate buffered saline; SEC, size exclusion chromatography; EM, electron microscopy; OD, optical density; LDH, lactate dehydrogenase; TBST, 20 mM Tris−HCl, 34 mM NaCl, pH 7.5 + 0.1% (v/v) Tween 20; BSA, bovine serum albumin; CSF, cerebrospinal fluid

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