Published in final edited form as:

Neurobiol Aging. 2010 October ; 31(10): 1690–1699. doi:10.1016/j.neurobiolaging.2008.09.019.

Candidate anti-A β fluorene compounds selected from analogs of amyloid imaging agents

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Abstract

Alzheimer's disease (AD) is characterized by depositions of β -amyloid (A β) aggregates as amyloid in the brain. To facilitate diagnosis of AD by radioligand imaging, several highly specific small-molecule amyloid ligands have been developed. Because amyloid ligands display excellent pharmacokinetics properties and brain bioavailability, and because we have previously shown that some amyloid ligands bind the highly neurotoxic A β oligomers (A β O) with high affinities, they may also be valuable candidates for anti-A β therapies. Here we identified two fluorene compounds from libraries of amyloid ligands, initially based on their ability to block cell death secondary to intracellular A β O. We found that the lead fluorenes were able to reduce the amyloid burden including the levels of A β O in cultured neurons and in 5xFAD mice. To explain these *in vitro* and *in vivo* effects, we found that the lead fluorenes bind and destabilize A β O as shown by electron paramagnetic resonance spectroscopy studies, and block the harmful A β O-synapse interaction. These fluorenes and future derivatives, therefore, have a potential use in AD therapy and research.

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Disclosure statement

The compounds reported here have been the subject of a patent application. The University of California has entered into an agreement with a co-owning university to manage the patent application. Should the subject of the invention be commercialized and revenue generated, Dr. Jin would receive an inventor share of such revenue directly from the University of California as dictated by the UC Patent Policy.

Keywords

Amyloid- β ; Alzheimer's disease; Oligomer; Small molecules; Amyloid ligand; Electron paramagnetic resonance spectroscopy; Spin-label; Synapse

1. Introduction

AD is characterized by deposition of various A β aggregates forming amyloid in the brain. To facilitate diagnosis of AD, compounds with various chemical core structures have been developed for their use as small-molecule probes (collectively called amyloid ligands here) for *in vivo* detection of A β plaques in patients with AD (Cai et al., 2007). Candidate amyloid ligands can penetrate the intact blood–brain barrier (BBB) and bind to various cerebral A β aggregates with high affinity and selectivity (Cai et al., 2007). However, the functional consequence of binding of these compounds to A β aggregates remains unknown.

A β aggregates induce various degrees of neurotoxicity and have been hypothesized to be causally related to dementia in AD. A major impediment to the development of effective anti-A β compounds for AD therapy is that essentially 100% of large-molecule drugs and >98% of small-molecule drugs fail to cross the BBB (Pardridge, 2007). Because several amyloid ligands display excellent pharmacokinetics properties and brain bioavailability, we consider that they are valuable candidates for the development of AD research and therapeutic agents specifically targeting A β aggregates (Lee, 2002; Maezawa et al., 2008). This approach was first proposed by Lee (2002), who showed that some amyloid ligands arrest amyloid fibril formation *in vitro*. Extending this line of study, we intend to select amyloid ligands that also target A β O at both intraneuronal and extracellular sites and block A β O-induced toxicity. This is because accumulating evidence indicates that A β O, more than A β fibrils, can induce severe neurodegeneration and cognitive deficits (Walsh et al., 2002; Kaye et al., 2004; Klein et al., 2004; Lesne et al., 2006; Maezawa et al., 2006; LaFerla et al., 2007). Importantly, we previously showed that some amyloid ligands bind A β O with high affinities, suggesting their potential use in anti-A β O therapy (Maezawa et al., 2008). In addition, data obtained from using human tissue and animal models support that A β oligomerization initiates within neurons, indicating the importance of compounds targeting intraneuronal A β O (Walsh et al., 2002; LaFerla et al., 2007). Here we identified two lead fluorene compounds code-named K01-162 and K01-186 based on their ability to block cell death secondary to intracellular A β O. These compounds bind and destabilize A β O, and are capable of penetrating the brain and reducing the cerebral amyloid burden in APP transgenic mice, therefore have a potential use in AD therapy and research.

2. Materials and methods

2.1. Compounds

The syntheses of compounds tested here (styrylbenzene, benzothiazole, fluorene, stilbene, and biphenyl) have been reported (Lee et al., 2003; Kung et al., 2001, 2002; Zhuang et al., 2001a,b, 2005).

2.2. Cell culture models showing intraneuronal A β O

MC65 cells and U18666A-treated neurons were described previously (Hong et al., 2007; Jin et al., 2004; Maezawa et al., 2006, 2008). For details, please see Supplementary data.

2.3. Mice and intracerebroventricular infusion of compounds

The line Tg6799 5xFAD mice co-express human APP695 with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) mutations and human PS1 harboring M146L and L286V mutations (Oakley et al., 2006). For intracerebroventricular infusion, miniosmotic pumps (Alzet) were loaded either with 100 μ M K01-162, K01-182 or equivalent amount of DMSO solvent (mock) in 100 μ l of artificial cerebrospinal fluid. The procedure for implantation of the pumps was the same as previously described (Dolev and Michaelson, 2004). Mice were infused at a flow rate of 0.25 μ l/h for 2 weeks. This amounted to ~400 ng/g of brain/day. At the conclusion of the infusion, the mice were sacrificed and their brains were cut in half sagittally. The left hemispheres were snap frozen for biochemical assays. The right hemispheres were fixed in 4% paraformaldehyde for immunohistochemical studies. All animal procedures were approved by the UC Davis Animal Care and Use Committee. All surgical procedures were performed with care to minimize pain and discomfort.

2.4. Western blotting and immunohistochemistry

Cultured cells or 1-mm thick cryosections of frozen mouse brains were lysed in radioimmune precipitation assay (RIPA) buffer with protease inhibitor mixture solution, sonicated briefly, and incubated for 20 min at 4 °C. The RIPA-soluble fractions were obtained from the supernatants after centrifuge at 100,000 $\times$ g. Western blots and immunofluorescent staining were performed according to our published protocols (Hong et al., 2007; Jin et al., 2004; Maezawa et al., 2006). For each mouse, a 20- μ m thick coronal section containing hippocampus and subiculum at 2mm rostral to the posterior end of the cerebrum and a second section at 100 μ m further rostral were immunostained and photographed for quantification of amyloid load using the Photoshop and Image J softwares.

2.5. Preparation of A β O from synthetic A β 1–42 peptide

Solutions of seedless, unaggregated A β and A β O (including biotinylated A β O) were prepared according to established protocols (Hong et al., 2007; Maezawa et al., 2008).

2.6. Surface plasmon resonance spectroscopy (SPR)

The analysis of the compound binding to the A β O (made of A β 1–42) on SPR sensor chip was performed following our previously published protocol (Maezawa et al., 2008). For details, please see Supplementary data.

2.7. Detection of synapse-bound A β O

This is performed according to our previously published protocol (Maezawa et al., 2008) modified from Lacor et al. (2004). For details, please see Supplementary data.

2.8. Electron paramagnetic resonance spectroscopy (EPR) of spin-labeled A β

Site-specific incorporation of a nitroxide spin label at position 2 of A β was achieved by synthesis of A β 1–40 containing a cysteine substitution at residue 2. For details, please see Supplementary Fig. 1 and Supplementary Methods.

The spin-labeled and unlabeled A β 1–40 peptides were completely dissolved in hexafluoroisopropanol (HFIP) at room temperature for 1–3 h and lyophilized. The resulting A β film was dissolved with DMSO and stored at –80 °C. To generate spin-labeled A β O, a mixture of 10 μ M of labeled and 30 μ M of unlabeled A β 1–40 in PBS was incubated for 6 h at 4 °C. The resulting A β O were confirmed by AFM and their cytotoxicity to N2A neuroblastoma cells (Hong et al., 2007) was found comparable to the unlabeled A β O.

EPR measurements were carried out in a JEOL X-band spectrometer fitted with a loop-gap resonator. To measure EPR spectra, 9 μ l of spin-labeled oligomer solution was combined with 1 μ l of 100 μ M compound in DMSO (or 1 μ l of DMSO vehicle control), incubated for 1 h at 4 °C, and then an aliquot (~5 μ l) transferred to a sealed quartz capillary and then placed in the resonator. Spectra were acquired at room temperature (20–22 °C) by signal-averaging three 60-s scan over a field of 100G at a microwave power of 2 mW and a modulation amplitude optimized to the natural line width of the individual spectrum (typically 0.5 G).

2.9. Atomic force microscopy (AFM) and electron microscopy (EM)

AFM and EM were performed as previously described (Hong et al., 2007).

2.10. Horse radish peroxidase (HRP) inhibition assay

The assay for the antioxidative effect of compounds was performed according to published protocols (Mishra et al., 2006) with modification. Briefly, in a 100 μ l of solution, HRP (100 ng) was mixed with hydrogen peroxide (0.03%) in the absence or presence of different concentrations of compounds (16–1000 nM). The reaction was initiated by adding 50 μ l of 20 μ M *o*-phenylenediamine dihydrochloride (OPD) substrate. After 5 min incubation at room temperature, the reaction was stopped by adding H₂SO₄ and absorbance at 490 nm was measured.

2.11. Statistics

We examined the statistical significance of differences between groups by applying one-way analysis of variance (ANOVA) with post hoc Tukey test, using the SigmaStat 3.1 (Systat Inc. Point Richmond, CA) program.

3. Results

3.1. The lead fluorenes K01-162 and K01-186 attenuate intracellular A β O and protect A β O-induced toxicity

Because the primary goal of our compound development is neuron protection, we employed our previously developed neuronal culture-based assay called MC65 protection assay for primary screening (Maezawa et al., 2006; Hong et al., 2007). The death of MC65 neuroblastoma cells is the result of the intracellular accumulation of A β O which are immunoreactive to the oligomer-specific antibody A11 (Kayed et al., 2003). By selecting compounds that block this death, previously we showed that this method preferentially identifies those with good permeation properties and capable of interacting with A β O (Hong et al., 2007). Using this method, we screened amyloid ligands of various pharmacophore scaffolds and their derivatives, including those of styrylbenzene, benzothiazole, fluorene, stilbene, and biphenyl (Cai et al., 2007; Kung et al., 2001, 2002; Lee et al., 2003; Zhuang et al., 2001a,b, 2005). Results from selected compounds are summarized in Supplementary Table 1. While the application of IBOX (EC₅₀ = 230 nM) and IMPY (EC₅₀ = 260 nM), two thioflavin-like brain permeable amyloid ligands (Zhuang et al., 2001a; Kung et al., 2002), may be limited due to their narrow therapeutic window, the two fluorenes K01-162 and K01-186 (Lee et al., 2003) (Fig. 1A) are promising due to their highest MC65 protective effect and low toxicity. K01-162 showed full MC65 protection at 125 nM, an EC₅₀ of 80 nM, and no cytotoxicity up to 50 μ M (Supplementary Table 1). K01-186 has the highest MC65 protective effect (EC₅₀ = 50 nM) and is a potent anti-oxidant (Bordwell et al., 1988; see below).

Corresponding to their cytoprotective effect, K01-162 and K01-186 reduced the level of intracellular A β O that are immunoreactive to the A11 antibody (Fig. 1B). A11 specifically

recognizes the conformation of prefibrillary oligomers that may cause toxicity by increasing membrane conductance (Kayed et al., 2003,2004). Western blot analysis using anti-A β 3-8 antibody 6E10 showed that both K01-162 and K01-186 substantially reduced the levels of SDS-stable A β trimer and larger aggregates (Fig. 1C). The effect on A β dimer was not certain because it co-migrated with the putative CTF Δ 31, a caspase cleavage product of C99 (Maezawa et al., 2006). The MC65-inactive fluorene analogs AD27 and K01-182 did not reduce the level of intracellular A β O. Thus, the ability of the compound to block MC65 death appears to correlate with its ability to reduce the level of intracellular A β O.

We further tested the ability of K01-162 and K01-186 to reduce intraneuronal A β O in primary neuronal cultures. Previously we showed that the intraneuronal accumulation of A β O composed of A β 42 can be induced in APP-expressing primary cortical neurons or N2A neuroblastoma cells when treated with U18666A, a class 2 amphiphile that directly inhibits the function of NPC1 protein (Niemann-Pick type C disease protein 1) (Jin et al., 2004). The intraneuronal A β 42 deposits are mainly localized to endosomes (Jin et al., 2004), consistent with the previously identified sites of A β oligomerization (Takahashi et al., 2004) and the location of intraneuronal A β in early AD brains and the brains of transgenic animals (Cataldo et al., 2004; Takahashi et al., 2004; LaFerla et al., 2007). As shown in Fig. 1D, intraneuronal A11-immunoreactive A β O were substantially attenuated by treatment with K01-162 and K01-186 (100nM to 10 μ M). Western blot analysis using 6E10 demonstrated that U18666A-treated APP-expressing neurons contained two major SDS-stable A β O bands at 56 kDa and 80 kDa (Fig. 1E). The 56 kDa band corresponds in size with A β 42 12-mer oligomers recently shown to correlate with cognitive deficits in Tg2576 mice, dubbed A β *56 (Lesne et al., 2006). Treatment with K01-162 and K01-186 substantially reduced the intensities of the two A β O bands. Due to the inherent toxicity of the U18666A, the neuroprotective effect of K01-162 and K01-186 in this culture system could not be assessed.

3.2. K01-162 attenuates amyloid load in vivo

To provide a proof of principle that fluorenes thus selected have anti-A β properties *in vivo*, we tested K01-162 in a model of cerebral A β amyloidosis called 5xFAD mice (Oakley et al., 2006). In these mice, A β 42 amyloid deposition begins by 2 months, especially in hippocampus and subiculum, and increases sharply between 2 and 4 months. We infused K01-162 intracerebroventricularly into 3-month-old 5xFAD mice for 2 weeks. This course did not cause apparent toxicity and significantly reduced the amyloid load in hippocampus to ~50% of the mock-treated level (Fig. 2A and B). While the immunostain-detectable amyloid load may roughly represent the insoluble fibrillar aggregates in the brain, we found that K01-162 infusion also reduced the level of RIPA buffer solubilized A β species that may better represent the smaller aggregates such as oligomeric A β (Oddo et al., 2006; Cheng et al., 2007), but did not affect the transgenic expression of the human amyloid- β precursor protein (hAPP) (Fig. 2C). Western blot showed a collectively ~50% reduction of the SDS-stable, mainly 9.7, 15.3, and 21.6 kDa oligomeric species, which may correspond to dimeric, trimeric, and tetrameric A β (Fig. 2C and D). Thus, K01-162 can reduce the brain amyloid burden that exists in both fibrillar and RIPA-soluble, non-fibrillar forms. In contrast, the similarly infused MC65-inactive fluorene K01-182 showed no such effect.

3.3. K01-162 and K01-186 directly interact with A β O and inhibit A β O binding to synapses

Previously we showed that A β fibrils and A β O may share common binding pockets for some amyloid ligands, therefore it is possible that the lead fluorenes, being able to inhibit A β O-induced cell death and reduce brain A β O levels, directly interact with A β O, in addition to binding to fibrils. To examine the binding of compounds to A β O, we employed our previously reported SPR method, which allows for the qualitative and quantitative measurements of interactions between the small-molecule compounds in the flow phase and

the A β O immobilized on the sensor chip (Maezawa et al., 2008). The method is described in details in the Supplementary data. The advantage of this method includes the highly sensitive real-time detection of interactions and not requiring a labeling procedure which might change the property of the compounds. The SPR result showed that K01-162 and K01-186 bind directly to A β O with a K_D of 19.0 μ M and 20.4 μ M, respectively, comparable to that of Congo red (CR) (Maezawa et al., 2008) (Fig. 3A). In contrast, the MC65-inactive fluorenes AD27 and K01-182 failed to show any binding.

What might be the consequence of the compound-A β O interaction? We hypothesize an immediate effect (seconds to minutes) to mask the conformation of A β O required for interaction with cellular targets (Townsend et al., 2006; Hong et al., 2007), and a delayed effect (hours to days) to destabilize the A β aggregates (Hong et al., 2007). To test the former, we determined the compounds' ability to block the strong A β O binding to synapses, which was shown to induce aberrations in synapse composition, shape, and density, and was proposed as a molecular basis for loss of connectivity in AD (Lacor et al., 2004). This binding appears to depend on the oligomer conformation rather than the primary A β sequence (Lacor et al., 2004). Fig. 3B and C show that K01-162 and K01-186 blocked the synaptic binding activity of A β O after a preincubation for 5 min, an effect comparable to that of CR (Maezawa et al., 2008). This effect is not due to damage to synapses by the compounds, because prior incubation of the cultures with the compounds did not block subsequent A β O binding to synapses (data not shown). This result suggests that the interaction of K01-162 and K01-186 with A β O may provide an immediate synapto-protective effect. Significant effects were seen at 5–10 equiv. of compound to A β (molar ratio relative to A β monomeric peptide), which corresponds to a compound:peptide ratio of 0.3–0.6:1 (w/w). This is comparable to the effect of inositol stereoisomers, a group of previously reported promising anti-A β small molecules (McLaurin et al., 2000).

3.4. K01-162 and K01-186 destabilize and affect the aggregation state of A β O

To test the delayed effect of fluorene compounds to destabilize A β O, we performed EPR spectroscopy (Hubbell et al., 2000) to observe the conformational dynamics of A β peptide as a function of oligomeric state and compound binding. EPR signals were analyzed from a paramagnetic nitroxide probe targeted specifically to the second residue of A β O (Supplementary Fig. 1). To alleviate dipolar broadening between nitroxide labels on neighboring peptides, the fraction of spin-labeled A β was diluted down to a 25 mol% fraction with native (unlabeled) A β . A β O thus made containing spin-labeled peptide were shown to have a comparable degree of toxicity to N2A cells (Hong et al., 2007) and a similar particle size distribution as the unlabeled A β O (data not shown). We incubated the spin-labeled A β O with candidate compounds for 1 h and examined the EPR spectra. As shown in Fig. 4, the EPR spectra of samples treated with K01-162 or K01-186 display additional line narrowing compared to the solvent control or the inactive compound K01-182. The decreased line width of spin-labeled A β O in the presence of K01-162 or K01-186 reflects increased disorder of the spin-labeled side chain at position 2 on A β . CR, known to disaggregate A β , also induces a similar increase in the molecular dynamics (Fig. 4). In contrast, the inactive compounds K01-182 (Fig. 4) and AD27 (data not shown) did not alter the spectrum of A β O. The increase in motion likely arises from either a more flexible backbone or an increase in global rotational dynamics, consistent with an increased tumbling rate of a monomeric peptide versus the oligomer species.

The above EPR results suggest that K01-162 and K01-186 destabilize A β O. Indeed, direct visualization of A β O by atomic force microscopy (AFM, Fig. 5) revealed that K01-162, K01-186 and CR treatment resulted in apparently much less (estimated 60% less) detectable A β O particles, likely due to the compound's ability to depolymerize A β O into smaller units not detectable by AFM. However, due to the uneven distribution of the particles on the AFM

mica, the number of detectable particles was not quantified. Instead, quantitation of the sizes of detectable A β particles clearly showed that K01-162 and K01-186 were able to block the progressive polymerization of preformed A β O to larger aggregates that were observed in mock-treated samples (Fig. 5B). There was also a trend that K01-162 and K01-186 reduced the size of A β O to a more homogeneous population, as indicated by the left shift and sharper peak of the size distribution curve (Fig. 5C). Furthermore, this size analysis likely underestimated the destabilizing effect of fluorenes on A β O, considering the ~60% less detectable particles in K01-162- and K01-186-treated samples as noted above. In contrast, the MC65-inactive fluorene AD27 showed no such effect. Similar results were also obtained when particles were visualized by electron microscopy (EM, Supplementary Fig. 2). The same conclusion reached by both AFM and EM reduces the possibility that our results were due to compound-altered binding of A β O particles to the AFM mica or EM grids. Taken together, these results indicate that K01-162 and K01-186 are able to destabilize and disaggregate A β O.

3.5. K01-186 is a potent antioxidant

We found that a previously identified potent antioxidant has the same structure as K01-186 (identified as 2,7-(Me₂N)₂FIH₂; Bordwell et al., 1988). The presence of two dimethylamino functions at C2 and C7 of its fluorene ring stabilizes the cation radical intermediate in the oxidative process and is responsible for its antioxidative effect. Confirming this effect, we found that K01-186 was able to inhibit the horse radish peroxidase (HRP)-catalyzed oxidation reaction (Mishra et al., 2006) with an IC₅₀ around 500 nM, ten fold higher than the EC₅₀ (50 nM) of its MC65 protective effect (Supplementary Fig. 3). K01-162 as well as a few inactive compounds did not show significant antioxidative effect, indicating that the antioxidative effect is not required for the MC65 protective effect of fluorenes.

4. Discussion

Our approach was initiated based on the hypothesis that intraneuronal accumulation of A β and its aggregation might be the first step of the amyloid cascade, and that intraneuronal A β O are highly toxic (LaFerla et al., 2007). Using our unique MC65 model, we quickly selected compounds that reduce the level of intracellular A β O and block A β O-related cell death. Mounting evidence also indicates that extracellular soluble A β O substantially damage neurons (Walsh et al., 2002; Kaye et al., 2004; Klein et al., 2004; Lesne et al., 2006). Our data demonstrate that the two lead fluorenes also interact with soluble A β O and block their harmful binding to synapses (Lacor et al., 2004). Furthermore, they are able to destabilize A β O, which may alter the aggregation into lesser toxic or more degradable forms and may explain their ability to reduce the level of A β O in cultured neurons and in 5xFAD mouse brains. In addition, K01-186 is a potent antioxidant. Considering that oxidative damage induced by A β or non-A β causes may contribute causally to AD-related pathology (Lin and Beal, 2006), the dual benefits of anti-oxidation and anti-A β provided by K01-186 might be preferable for treating AD patients.

We provide the initial proof of principle that the fluorenes have an *in vivo* effect. A short 2-week intraventricular infusion resulted in ~50% reductions in the levels of RIPA-soluble A β O and histologically quantified A β load in 5xFAD mice, a line with perhaps the most robust production of A β 42. RIPA-solubilized samples would contain membrane-associated intraneuronal and matrix-bound extracellular A β , in addition to soluble extracellular A β O. Similarly RIPA-solubilized brain samples from J20 (Cheng et al., 2007), ARC (expressing Arctic-mutant human APP) (Cheng et al., 2007), and 3xTg-AD (Oddo et al., 2006) mice contained the putative A β *56 that was first shown in Tg2576 mice to be closely linked to memory deficits (Lesne et al., 2006). Interestingly, although we detected A β *56 in U18666A-treated cultured cortical neurons, we did not detect A β *56 in the 3-month-old

5xFAD mice despite their high A β 42 levels. Instead, the 5xFAD samples displayed smaller aggregates corresponding to dimers, trimers and tetramers (Fig. 2C), suggesting that either they are the native assemblies in 5xFAD brains or the SDS-stable subunits of larger A β O that are assembled differently from A β *56 and therefore SDS-unstable. In any event, we found that fluorene compound treatment reduced the cumulative levels of these small aggregates in 5xFAD mice (Fig. 2D) and the level of A β O including A β *56 in primary cortical neurons (Fig. 1E). This is important in light of the observation that therapeutic interventions that reduce A β fibrils at the cost of augmenting A β O could be harmful (Cheng et al., 2007).

Our approach also benefited from using small libraries of derivatives or analogs of known amyloid-binding compounds with good pharmacokinetics properties and brain bioavailability (Lee, 2002). K01-162 should have excellent brain uptake in addition to very specific binding to cerebral A β aggregates, inferred from previous studies of the fluorene ¹²⁵I-C2f (Lee et al., 2003). ¹²⁵I-C2f has almost the same structure as K01-162 except that the *Br* in K01-162 was replaced by radioactive I-125. We have also measured the oral bioavailability of K01-186 and found that at 1 h after an oral dose of 25 mg/kg to wild-type mice, the brain level of K01-186 was 5.9 μ g/g of brain (Hong and Jin, unpublished data), indicating a robust brain uptake. Taken together with our *in vivo* treatment data, K01-162 and K01-186 are likely to be brain-active compounds. Experiments are underway in our laboratories to determine whether K01-162 and K01-186 applied orally are able to block A β O toxicity *in vivo* by electrophysiological and behavioral studies using APP transgenic mice.

As with protein mis-folding disorders in general, efforts to understand the mechanism of A β toxicity have been frustrated due to the inherent instability of the oligomeric species, which complicates studies due to sample inhomogeneity. This is apparent when A β O preparations are viewed under AFM and EM (Fig. 5 and Supplementary Fig. 2). Here we employed a novel approach using EPR spectroscopy of site-directed spin labels to evaluate compound binding to A β O and the resulting conformational changes in A β O. This method (Hubbell et al., 2000) allows us to examine A β structure and assembly from a diverse set of sample states under physiological conditions. We are able to observe changes in the conformational dynamics of A β induced by compound binding in a real-time manner (Fig. 4). Our success in this approach encourages future dissection of the conformational features of A β O by EPR analysis of spin labels targeted to other locations within the A β sequence. In this regard, inhibitor compounds such as K01-162 and K01-186 will be highly useful for elucidating the molecular properties inherent to the toxic A β conformation. Site-directed spin labeling experiments will also facilitate the testing of models for compound-A β interaction, and provide insights for future compound design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AD	Alzheimer's disease
A β	β -amyloid protein
A β O	A β oligomers
BBB	blood-brain barrier
CR	Congo red

SPR	surface plasmon resonance spectroscopy
EPR	electron paramagnetic resonance spectroscopy
AFM	atomic force microscopy
EM	electron microscopy
RIPA	radioimmune precipitation assay
HRP	horse radish peroxidase

Acknowledgments

We are grateful to Drs. Hank Kung and Mei-ping Kung for providing the tested compounds and Dr. Danielle Harvey for statistical assistance. This work was supported by grants from the Alzheimer Association (IIRG-06-26782), the California State Alzheimer's Disease Research Fund, and the National Institute of Health (AG025500 and AG029246).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.neurobiolaging. 2008.09.019.

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Fig. 1.

Selected fluorene compounds reduce intraneuronal AβO levels. (A) The structures of MC65-active fluorenes and inactive analogs. (B) MC65 cells were treated with indicated compounds (1μM) immediately after the removal of the transgene repressor tetracycline (-TC) to induce AβO accumulation. At 24 h, cells were fixed and immunofluorescently stained with oligomer-specific antibody A11. The cytoprotective compounds K01-162 and K01-186, but not the MC65-inactive K01-182 and AD27, attenuated the intracellular AβO (red fluorescent puncta). (C) Lysates from cultures described in (B) were analyzed by Western blotting using 6E10. K01-162 and K01-186 reduced the levels of AβO including Aβ trimer. (D) Primary cortical neurons expressing APP^{sw} (APP-695 with Swedish mutations) were treated with U18666A (+U) to induce accumulation of A11-immunoreactive AβO (red puncta). -U was the mock treatment control. Arrows point to AβO distributed in perikaryon and in neuritis, which were attenuated by K01-162 and K01-186. (E) Lysates from cultures described in (D) were analyzed by Western blotting which showed that K01-162 and K01-186 reduced the levels of AβO including the putative Aβ*56 (Lesne et al., 2006).

Fig. 2.

The fluorene K01-162 reduces the levels of immunostained Aβ and RIPA-soluble AβO *in vivo*. K01-162, the inactive analog K01-182, and mock solution (DMSO solvent in artificial CSF) were infused into the cerebral ventricle of 3-month-old 5xFAD mice for 2 weeks. (A) The hippocampal sections stained with the anti-Aβ17-24 antibody 4G8 (red) and the neuronal marker NeuN (green). Representative fluorescence micrographs are shown. Scale bar: 500 μm. (B) Amyloid loads quantified as the % of area occupied by the 4G8-immunoreactivities in the defined hippocampal region. Error bars represent standard error. $n = 5$, ANOVA with post hoc Tukey test. (C) Representative Western blot analysis of RIPA-soluble Aβ assemblies using 6E10. The bands for the human amyloid-β precursor protein (hAPP) transgene and endogenous β-actin of samples are also shown to demonstrate equal sample loading. (D) The cumulative intensities of immunoreactive bands between the 6 and 30 kDa ranges on Western blots, normalized to the intensities of their respective β-actin bands, were compared and presented as % of the mock-treatment level. $n = 5$, ANOVA with post hoc Tukey test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 3. K01-162 and K01-186 bind AβO and interfere with AβO binding to synapses. (A) Typical SPR responses following the binding of fluorene compounds to AβO(1–42) on the sensor chip. Shown are the response curves for compounds ranging in concentrations from 0.78 to 50 μM. The calculated dissociation constant (KD) values are indicated. (B) Biotinylated AβO (100 nM total peptide) were incubated with 1 μM of indicated compounds for 5 min before added to cultured mouse hippocampal neurons. AβO were allowed to bind to synapses for 5 min. Bound AβO were visualized with Streptavidin-Alexa596 and demonstrated as red fluorescent puncta. Shown are representative neurons with dendritic puncta of bound AβO. Scale bar: 50 μm. (C) The density of bound-AβO puncta in each condition was quantified

and expressed as mean fluorescence per unit area. $n = 3$, one-way ANOVA with post hoc Tukey test.

Fig. 4.

EPR analysis of spin-labeled A β detects changes in A β O dynamics following fluorene compound treatment. A β O (40 μ M total peptide) containing 25% spin-labeled peptide were incubated with 100 μ M of the indicated compound for 1 h, and then scanned by EPR. Each treatment (red tracing) is compared to the control containing DMSO (black tracing) equivalent to the DMSO present in the compound solutions. Shown are the average tracings from three independent experiments. All samples are compared to the same control spectrum also generated from the average of three experiments. The values on the left report the change in line width for each sample as approximated by the fractional amplitude of its

center ($M_I = 0$) line relative to the control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 5.

K01-162 and K01-186 affect the aggregation state of A β . Pre-made A β O (1–42) (O, 0h) were incubated for 24 h in the absence or presence of indicated compounds and then visualized by AFM. (A) Representative AFM images. Scale bar: 1 μm . (B) The average diameter of detectable A β O in each condition, averaged from 3 to 4 randomly selected 25- μm^2 fields (more than 300 particles in total counted), was determined using the Image J Program. Data are expressed as mean diameter $\pm$ S.E.M. ($n = 4-5$). (C) The diameters of A β particles were stratified into steps of 5 nm increments to reveal the size distribution.