Bivalent Ligand Containing Curcumin and Cholesterol as a Fluorescence Probe for Aβ Plaques in Alzheimer's Disease

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Bivalent ligand containing curcumin and cholesterol as fluorescence probe for Aβ plaques in Alzheimer's disease

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Abstract

A recently developed bivalent ligand **BMAOI 14 (7)** has been evaluated for its capability to label and detect aggregated β-amyloid (Aβ) peptide as a fluorescent probe. This probe contains curcumin as the Aβ recognition moiety and cholesterol as an anchorage to the neuronal cell membrane/lipid rafts. The results demonstrate that 7 binds to the monomers, oligomers as well as fibrils of Aβ42 with low micromolar to submicromolar binding affinities. This chemical probe also has many of the required optical properties for use in imaging and can rapidly cross the blood-brain barrier (BBB) *in vivo*. Furthermore, **7** specifically binds to Aβ plaques in both AD human patients and APP transgenic mouse brain tissues. Collectively, these results suggest that **7** is a strong candidate as an Aβ-imaging agent and encourage further optimization of **7** as a new lead to develop the next generation of Aβ-imaging probes.

Keywords

Bivalent ligands; fluorescent probes; Aβ plaques; Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) is a devastating neurodegenerative disease and is the most common cause of dementia. One of the pathological hallmarks is the presence of β-amyloid (Aβ) plaques in the brain of AD patients with the major components being Aβ40 and Aβ42 peptides.[1] Clinical diagnosis of late-stage AD is based on cognition and behavioral tests of patients and definitive diagnosis is only achieved by postmortem examination to show the presence of Aβ plaques and neurofibrillary tangles, another pathological hallmark of AD. Even though the etiology of AD still remains elusive, the Aβ hypothesis has gained extensive attention and continues to evolve.[1] Numerous studies have established a correlation of the Aβ aggregates (oligomers and fibrils) and cognitive impairment associated

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Author contributions

Chemical synthesis, TEM, fluorescence polarization, fluorescence, and HPLC studies were completed by K.L. and J.C.; Animal studies using B6C3F1 mice were performed by T.L.G. and W.R.; Aβ plaque staining was completed by H.G. L. and S.S.; Fluorescence microscope was performed by X.L.W.; Aβ staining experiment design and data analysis were completed by X.W.Z.; Experiment design, data analysis, writing, and editing were completed by S.J.Z.

with AD.[1–3] Therefore, Aβ represents an attractive target to develop labeling and imaging probes to help monitor the progression of the disease as well as to achieve the early detection of AD, thus significantly reducing the social and economic burden caused by this disease.

Many probes have been developed to date for the specific imaging of Aβ plaques by employing techniques such as magnetic resonance imaging (MRI),[4,5] positron emission tomography (PET),[6] single photon emission computed tomography (SPECT),[7] and multiphoton microscopy.[8,9] Although studies employing these probes produced promising results in *in vitro*, *ex vivo* and small animal experiments, further clinical development is limited due to several factors associated with these techniques. These shortcomings include poor spatial resolution, low sensitivity, exposure to radioactivity, short-lived isotopes, and invasive methodology, among others. In search of new chemical probes to overcome these problems, fluorescent probes have gained growing interest in this field as non-invasive alternatives for labeling and imaging Aβ plaques. Ideally, a fluorescent probe should have the following properties to be useful in clinics: 1) specificity to Aβ plaques; 2) high binding affinity to aggregated Aβ; 3) ability to rapidly cross the blood-brain barrier (BBB); 4) emission wavelength above 450 nm to minimize background fluorescence and a large Stokes shift; and 5) a significant change in fluorescence properties upon binding to aggregated Aβ.[10,11] Several fluorescent probes have been developed to meet some of these properties as the proof-of-principle of this methodology (Figure 1) and studies of these fluorescent probes demonstrated promising results in labeling and imaging Aβ plaques, thus attesting to the clinical application of these probes.[10–15]

Recently, we reported the rational design and development of bivalent multifunctional Aβ oligomerization inhibitors (BMAOIs) as potential AD treatments by incorporating the cell membrane/lipid rafts anchorage into molecular design.[16] These BMAOIs contain curcumin as Aβ recognition and the multifunctional moiety on one end and cholesterol as cell membrane/lipid rafts anchorage on the other end. We envisaged that such BMAOIs would chaperone the multifunctional moiety, which is curcumin here, in close proximity to CM/LR in which Aβ aggregates and oxidative stress are produced to increase its accessibility to interfere with these multiple processes, thus improving its clinical efficacy. One compound with a 21-atom spacer, **BMAOI 14 (7)**, was discovered to have both favorable pharmacological properties and the ability to bind Aβ oligomers (AβOs) (Figure 2). As curcumin derivatives have been developed as PET[17] and fluorescent probes[10] and the fact that **7** bears the intrinsic fluorescence of the curcumin fluorophore in the molecule, this compound may be explored as a potential fluorescent probe to label and detect Aβ plaques. Herein, we present results to show that **7** possess both ideal optical properties and Aβ binding affinities that meet many of the required properties for use as a fluorescent probe. In addition, staining of Aβ plaques in human and in transgenic mouse brain tissue and rapid BBB penetration of this compound are also confirmed.

RESULTS AND DISCUSSION

To determine the binding affinity of **7** to Aβ, we evaluated changes in fluorescence polarization values of this probe at different BMAOI 14:Aβ ratios for monomeric, oligomeric, and fibrillary Aβ species, respectively. Aβ42 is chosen to evaluate the binding of probe as it is the major and most sticky amyloid peptide found in AD plaques.[18–21] The formation of different Aβ42 species was confirmed by transmission electron microscope (TEM) as shown in Figure 3A. As shown in Figure 3B, the apparent binding constants (K_d) of **7** for monomers, oligomers, and fibrils of Aβ42 are 2.03, 2.17, and 0.83 uM, respectively. Compound **7** binds to Aβ42 with micromolar affinity and favors Aβ2 fibrils over monomers and oligomers. Interestingly, another BMAOI with also a 21-atom spacer but a different

connectivity at curcumin (**8**, Figure 2), did not show favorable binding to any form of the Aβ42 species under identical experimental conditions (data not shown). This result is also consistent with our previously reported results showing that **7** is able to reduce the levels of AβOs and protect MC65 cells from AβO-induced cytotoxicity while **8** does not. Next, we tested the binding affinity of **7** to bovine serum albumin (BSA) as low BSA binding is often suggested as one of the required properties for ideal fluorescent probes.[10] As shown in Figure 3C, the binding affinity of **7** to BSA (K_d ~ 71 μM) is significantly less than the binding affinity to Aβ42, thus suggesting that the interference from serum albumin will be minimal for this probe.

To examine the change of fluorescence properties upon binding to Aβ, we compared the fluorescence properties of free compound **7** in aqueous solution to its fluorescence properties in the presence of Aβ42 fibrils as this chemical probe will be used to detect Aβ plaques. As shown in Figure 4, upon binding to Aβ42 fibrils, the intensity of the emission spectra of **7** was significantly increased (4.5 fold increase at Aβ42 = 5 μM) with a excitation of 430 nm. A blue shift in the emission spectra of 10 nm was also observed upon association with Aβ42 fibrils. Taken together, the results suggest that **7** indeed possesses the desired optical properties as a useful fluorescence probe.

An appropriate fluorescent probe must cross the BBB and be able to selectively bind to Aβ plaques. Compound **7** has been shown to have the potential to cross the BBB in a caco-2 assay.[16] Here, we further assessed the BBB permeability of **7** using female B6C3F1 mice combined with HPLC analysis. As shown in Figure 5, compound **7** was detected in the brain tissue of B6C3F1 mice (n=3) at as early as 30 minutes after intravenous (i.v.) administration through the tail-vein of B6C3F1 mice (5 mg/kg dose). The compound remained in the brain tissue of B6C3F1 mice for over two hours as the detection is evident at 30, 60, and 120 minutes, but disappeared by 3 hrs of i.v. administration. The results indicate that **7** can in fact rapidly cross the BBB and reach the brain tissue and become metabolized in a reasonable time window (~ 3 hrs) that allows clinical operation. While **7** appears to violate the empirical rules[22] of BBB permeability with a molecular weight >1000 Da and being more lipophilic, there are exceptions that compounds with molecular weight more than 1000 Da can efficiently cross the BBB and reach brain tissue, such as natural products.[23] The *in vivo* experiment results clearly demonstrate that this molecule efficiently and rapidly cross the BBB, likely due to the unique structure of this probe as bivalent ligands containing cholesterol to associate with cell membrane/lipid rafts have been shown to efficiently cross the membrane system via internalization.[24,25]

Finally, to assess the binding ability of this compound to Aβ plaques in situ, **7** was applied to hippocampal sections from cases of AD as well as from TgCRND8 transgenic mice, a widely used mouse model of AD.[26,27] In AD brain, fluorescent microscopy revealed Aβ plaques readily bind **7** (Figure 6A). In the transgenic mice, **7** also strongly and specifically bound to the Aβ plaques (Figure 6B), and specific monoclonal antibodies against ADDLs labeled the same plaques in adjacent serial sections (Fig 6C). These findings are very important such that this compound is specific for human disease, which allows for development for use as a therapeutic approach and viable for translational research. Furthermore, its comparable reliability in a mouse model of AD will allow for its continued and expanded experimentation for disease evaluation and treatment options necessary for development of a clinical benchmark.

In summary, we demonstrate that a bivalent ligand containing curcumin and cholesterol, **7**, can bind to various Aβ42 species with micromolar binding affinity and has appropriate fluorescence properties for labeling and imaging Aβ plaques in situ. We also demonstrate that this chemical probe can rapidly cross the BBB and reach the brain tissue in B6C3F1

mice. In addition, this compound can be cleared out in a reasonable time window. Furthermore, compound **7** can specifically label the Aβ plaques in human AD brain and in the brain of TgCRND8 transgenic mice with high contrast. Collectively, the results from this study suggest that **7** and this type of bivalent molecules hold the promise as fluorescent probes for Aβ-imaging. Further development and optimization of **7** as lead compound may provide useful diagnostic agents for AD.

METHODS

Preparation of Aβ42 oligomers and fibrils

Aβ42 oligomers and fibrils were prepared based on reported procedures.[28] Briefly, Aβ42 peptide (1 mg, American Peptide Inc., CA) was first dissolved in hexafluoroisopropanol (HFIP, 0.5 mL) and incubated at room temperature for 1 h, HFIP was then removed by flow nitrogen to give a clear film, and then further dried by vacuum. HFIP treated Aβ42 was then dissolved in DMSO to 5 mM and stored at −80 °C as stock solution.

For the preparation of Aβ42 monomer—Aβ42 stock solution in DMSO was diluted to 100 μM by ddH2O, and use ddH2O to make the working solution for binding experiments.

Aβ42 oligomer—Aβ42 stock solution was diluted to 100 μM by F12 medium. The mixture was incubated for 24 hrs at 4 °C. The formation of Aβ42 oligomers was confirmed by TEM. Then, this oligomeric Aβ solution was diluted with ddH_2O to make the working solution for binding experiments.

Aβ42 fibril—Aβ42 stock solution in DMSO was diluted to 100 μM by 10 mM HCl. The mixture was incubated for 24 hrs at 37 °C. Then, ddH_2O was used to prepare the working solution for binding experiments.

TEM analysis

An aliquot of Aβ42 sample of various species (5 μL) were adsorbed onto 200-mesh carbon and formavar-coated grids (Electron Microscopy Sciences) for 20 min, washed for 1 min in distilled H_2O. The samples were negatively stained with 2% uranyl acetate (Electron Microscopy Sciences) for 5 min and washed for 1 min in distilled H_2O. The samples were air-dried overnight and viewed with a Jeol JEM-1230 TEM equipped with a Gatan UltraScan 4000SP 4K × 4K CCD camera (100 kV).

Fluorescence polarization (FP) assays

The apparent binding constant (K_d) was measured by FP with different species of Aβ42 at various concentrations in the presence of 7 (1 μM). The final volume is 200 μL. After incubation at room temperature for 3 hrs, the FP was examined by Flexstation 3, at excitation/emisstion: 445/535 nm. Then, K_d was calculated based on the following one site binding equation from Prism 5 software: $Y = Bmax*X/(K_d + X)$ where Y is the fraction of bound Aβ; X is the concentration of 7.

Fluorescence studies of 7 with aggregated Aβ fibrils

The fluorescence change of 7 (1 μM) was examined without or with the presence of pre-aggregated Aβ42 fibrils (0.625, 1.25, 2.5, 5 μM) at excitation of 445 nm and emission from 480 to 700 nm.

BBB penetration assay in B6C3F1 mice

Female B6C3F1 mice (8–10 weeks old) were used for this assay. Both control and treatment group contained 3 mice. Briefly, B6C3F1 mice were given 7 (5 mg/kg, dissolved in cremophor EL/EtOH/H2O: 5%/5%/90%) via i.v. injection through tail-vein. Then mice were sacrificed after 10, 30, 60, 120, and 180 min and brain tissue were collected and rinsed with cold PBS. Brian tissue (100 mg) were diluted with water (300 μL) and homogenized with Dounce homogenizer. The homogenate was mixed with acetonitrile (700 μL) and centrifuged. The supernatants were evaporated under N_2 stream and the residue was dissolved in 50 μL acetonitrile:water (70:30). 20 μL sample was subjected to HPLC analysis using a C18 reverse-phase column (250 × 4.6 mm, Agilent, USA). Mobile phase: MeOH/CH_3CN with 0.1% TFA = 70/30 (v/v). Flow rate: 1 mL/min. Wavelength: 353 nm. The standard of BMAOI 14 was prepared by mixing of 7 (3 μM) with the brain tissue of untreated B6C3F1 mice and extracted following aforementioned procedure.

Staining of human brain tissue and transgenic TgCRND8 mice brain tissue sections

Hippocampal and cortical brain tissue was obtained at autopsy from cases of Alzheimer disease (n=6) from the University Hospitals of Cleveland, using approved IRB protocols and fixed in either formalin or methacarn. Brain tissue from TgCRND8 (n=3) and wild-type mice (n=3) age of 7 months was dissected following perfusion, cut sagitally and fixed in routine buffered formalin. Samples were embedded in paraffin and 6 μm sections were cut. Following deparaffinization with 2 changes of xylene, sections were rehydrated through a graded series of ethanol and brought to water. A 2 mM stock solution of 7 was made in DMSO and diluted to 10 μM with a buffer of 0.1 M TBS (pH 7.4), 3% BSA, and 0.5% Tween 20 just prior to use. The compound was applied to the tissue sections and incubated for 1 hr at 37 °C. After rinsing with TBS, the sections were coverlipped using fluorogel with 0.1% N-propyl gallate added. Images were obtained under fluorescent microscopy with FITC filter set on a Zeiss Axiovert. Negative controls consisted of unstained tissue sections. Immunocytochemistry was also used to determine the location of the amyloid plaques on adjacent serial sections using NU2 monoclonal antibody specific for ADDLs (gift of William Klein and Sergio Ferreira) using previously described method.[29] Alexafluor 568 conjugated secondary antibody was used and viewed under fluorescent microscopy.

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ABBREVIATIONS

Aβ	amyloid-β
AβOs	amyloid-β oligomers
AD	Alzheimer's disease
APP	Aβ precursor protein
BBB	blood-brain barrier
BMAOIs	bivalent multifunctional Aβ oligomerization inhibitors
BSA	Bovine serum albumin
CHO	Chinese hamster ovary

CM/LR	cell membrane/lipid rafts
DMSO	dimethyl sulfoxide
FITC	Fluorescein isothiocyanate
FP	Fluorescence polarization
HFIP	Hexafluoroisopropanol
HPLC	high performance liquid chromatography
IRB	Institutional review board
MRI	magnetic resonance imaging
PBS	phosphate buffered saline
PET	positron emission tomography
SPECT	single photon emission computed tomography
TBS	Tris buffered saline
TEM	transmission electron microscope

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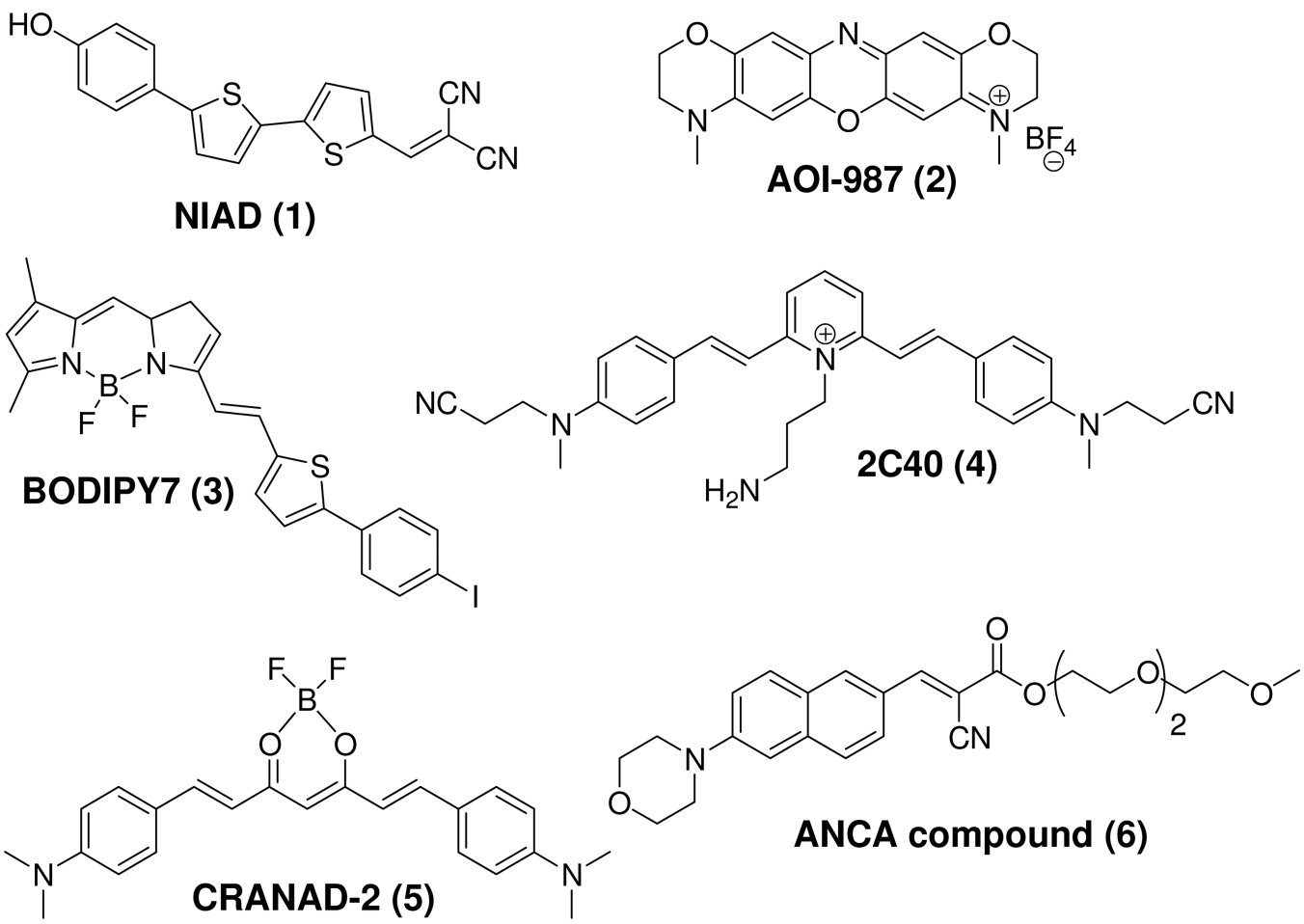

Figure 1.
Representative fluorescence probes that stain Aβ plaques.

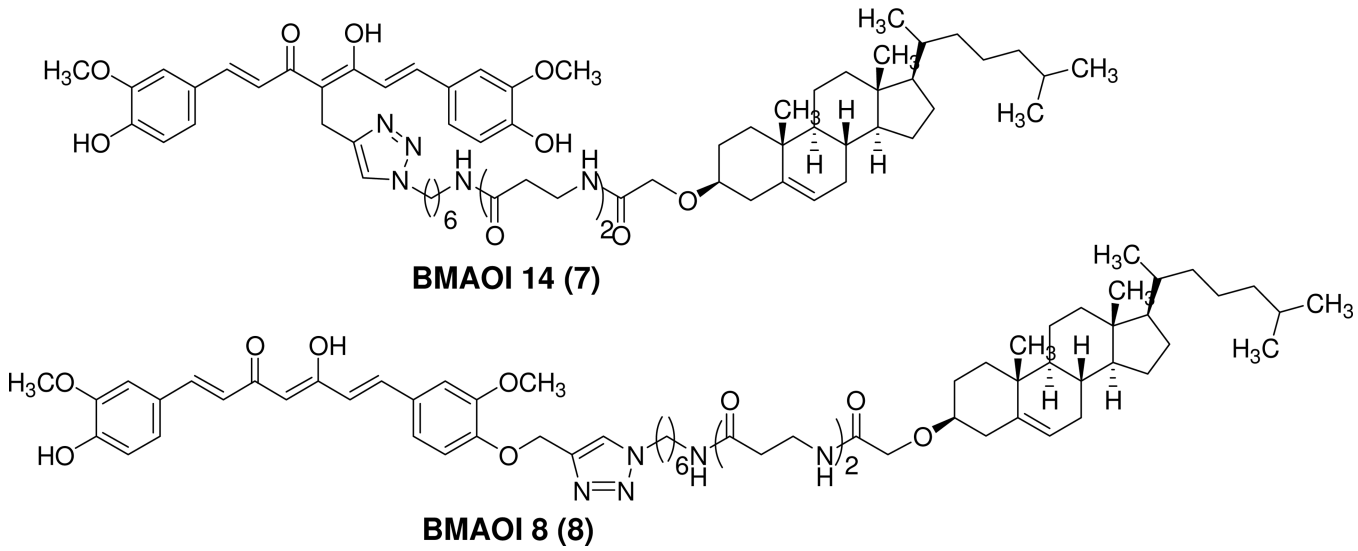

Figure 2.
Chemical structures of BMAOI 14 (7) and BMAOI 8 (8).

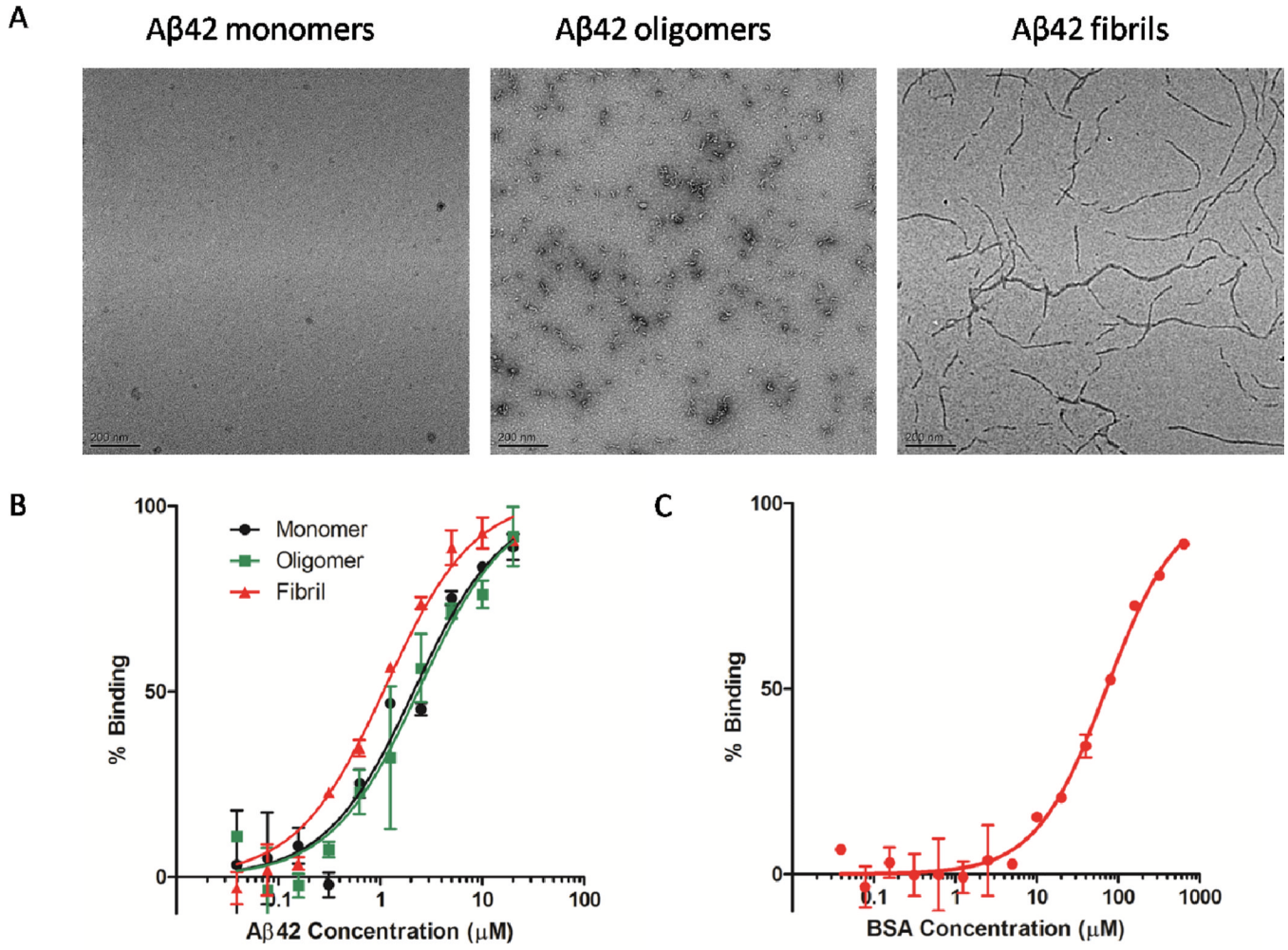

Figure 3.

Compound 7 binds to Aβ42 monomers, oligomers, and fibrils, but not BSA. A. The monomers, oligomers and fibrils of Aβ42 were prepared according to established protocols and confirmed by TEM; B. Compound 7 (1 μM) was incubated with different forms of Aβ42 at indicated concentrations for 3 hrs. Then, fluorescence polarization change of 7 was recorded and the binding affinity was calculated; C. Compound 7 was incubated with BSA as described in B and the binding affinity was calculated.

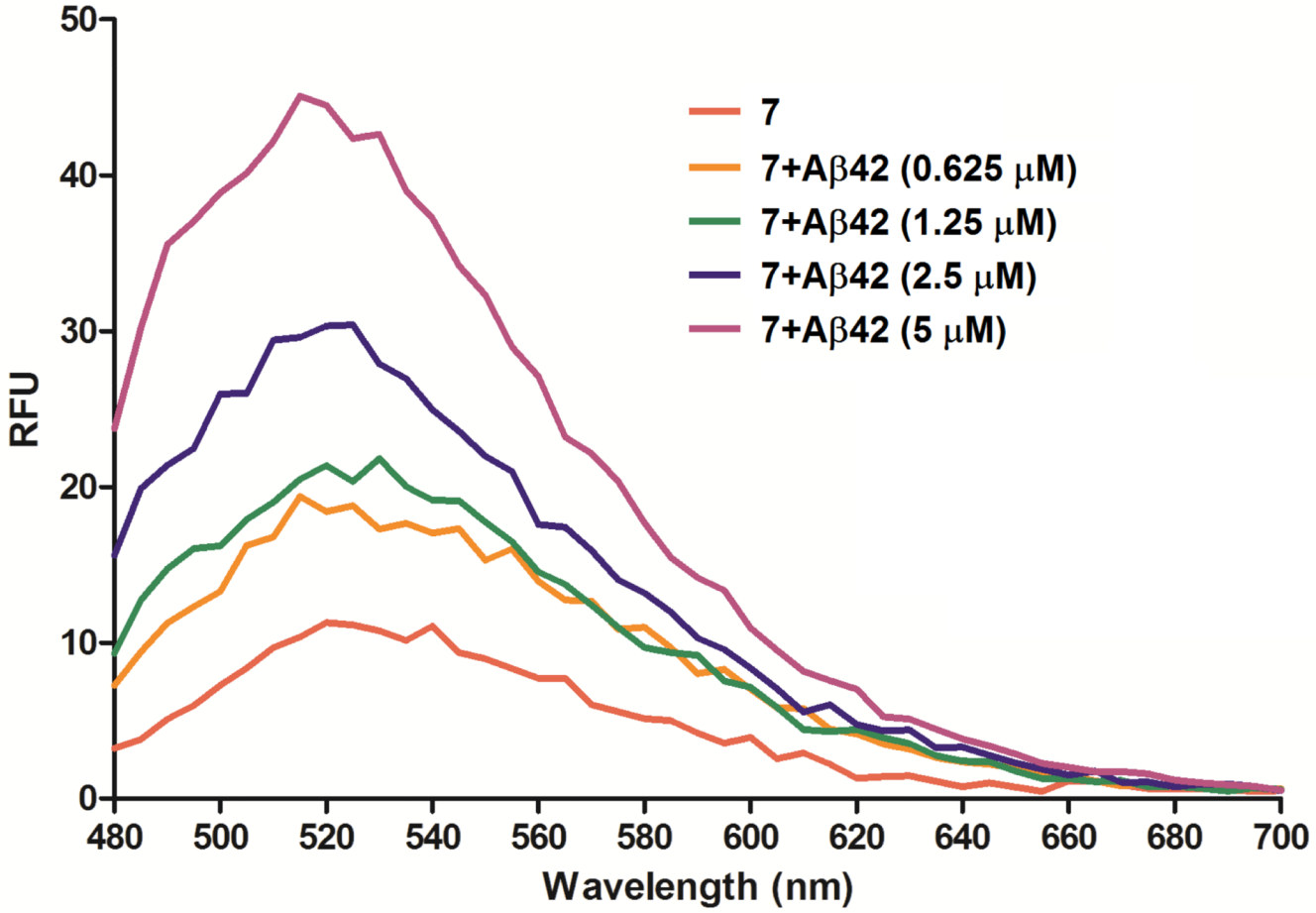

Figure 4.
Fluorescence emission of compound 7 (1 μM) before and after mixing with Aβ42 fibrils.

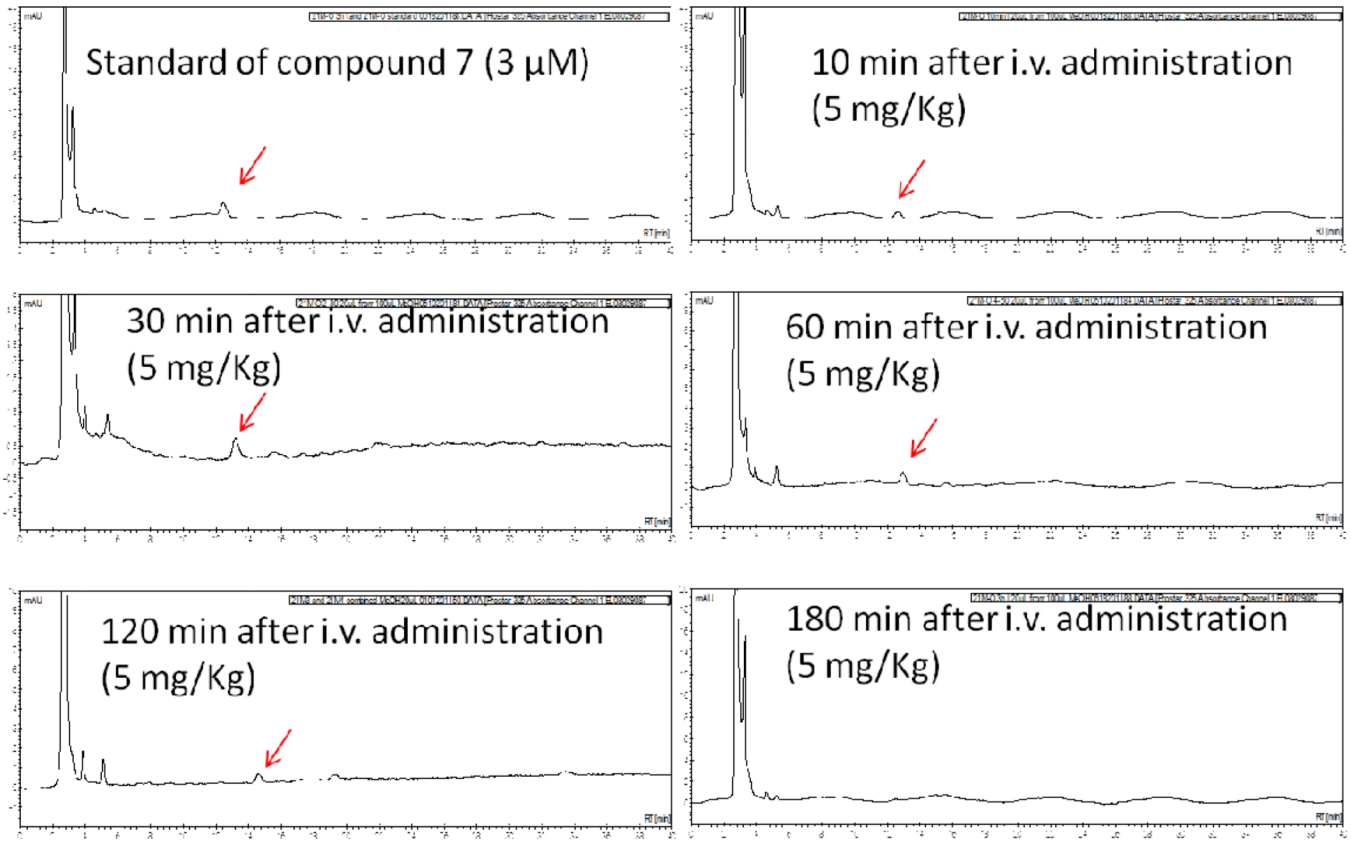

Figure 5.

Compound 7 can cross the BBB of B6C3F1 mice. Compound 7 (5 mg/kg) was given to B6C3F1 female mice (n=3) by i.v. administration through tail-vein. Then the animals were sacrificed at indicated time intervals and the brain tissues were collected and analyzed by HPLC using a C18 column.

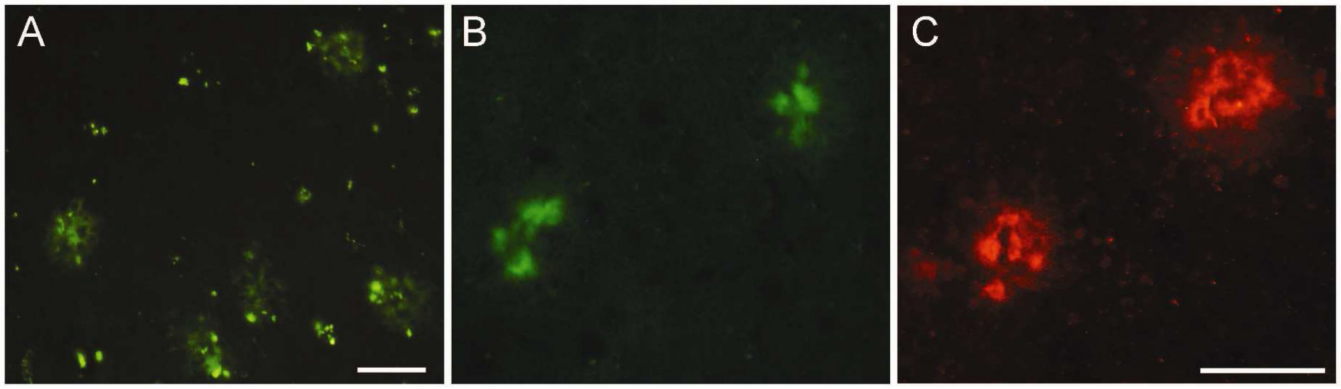

Figure 6.

A. Compound 7 stains the Aβ plaques in the brain tissues of human AD; B. The brain tissue of TgCRND8 mice was stained with 7 (10 μM) according to established protocol and viewed using fluorescent microscopy; C. Adjacent section of the brain tissue of TgCRND8 mice was stained with anti-ADDL antibodies using Alexafluor 568, exhibiting red fluorescence.