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

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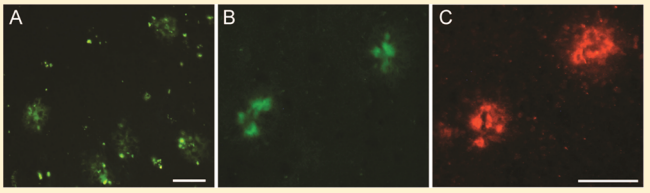
ABSTRACT: A recently developed bivalent ligand BMAOI 14 (7) has been evaluated for its ability to label and detect aggregated β-amyloid (Aβ) peptide as a fluorescent probe. This probe contains curcumin as the Aβ recognition moiety and cholesterol as an anchor to the neuronal cell membrane−lipid rafts. The results demonstrate that 7 binds to the monomers, oligomers, and 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 in vivo. Furthermore, 7 specifically binds to Aβ plaques in both Alzheimer's disease human patients and Aβ precursor protein 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 for the development of the next generation of Aβ imaging probes.



KEYWORDS: *Bivalent ligands, fluorescent probes, Aβ plaques, Alzheimer's disease*

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 achieved only by postmortem examination to show the presence of Aβ plaques and neurofibrillary tangles, another pathological hallmark of AD. Even though the etiology of AD 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 with AD.[1−3] Therefore, Aβ represents an attractive target for developing 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 because of several factors associated with these techniques. These shortcomings include poor spatial resolution, low sensitivity, exposure to radioactivity, short-lived isotopes, invasive methodology, among others. In the search for new chemical probes for overcoming these problems, fluorescent probes have attracted interest in this field as noninvasive alternatives for labeling and imaging Aβ plaques. Ideally, a fluorescent probe should have the following properties to be useful in clinics: (1) specificity for Aβ plaques, (2) high binding affinity for aggregated Aβ, (3) ability to rapidly cross the blood−brain barrier (BBB), (4) an emission wavelength of >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 yielded 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 (CM−LR) anchor into molecular design.[16] These BMAOIs contain curcumin as the Aβ recognition and the multifunctional moiety on one end and cholesterol as the CM−LR anchor on the other end. We envisaged that such BMAOIs would chaperone the multifunctional moiety, which is curcumin here, into the proximity of CM−LR in which Aβ aggregates and oxidative stress are

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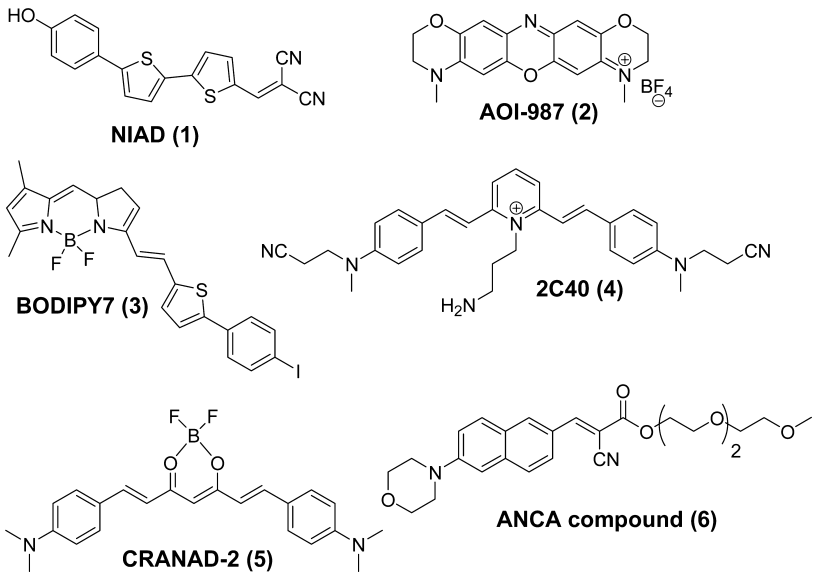

Figure 1. Representative fluorescence probes that stain Aβ plaques.

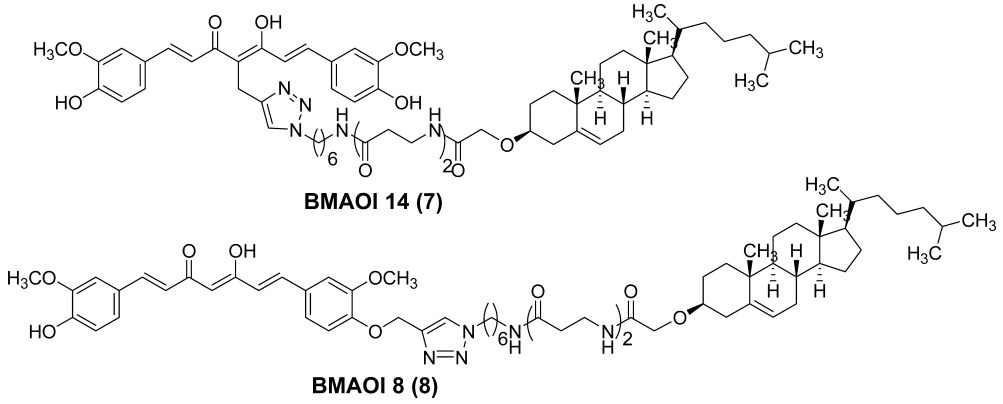

Figure 2. Chemical structures of BMAOI 14 (**7**) and BMAOI 8 (**8**).

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).[16] As curcumin derivatives have been developed as PET[17] and fluorescent probes[10] and because of 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 for labeling and detecting Aβ plaques. Herein, we present results to show that **7** possesses 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 transgenic mouse brain tissue and rapid BBB penetration of this compound are also confirmed.

RESULTS AND DISCUSSION

To determine the binding affinity of **7** for Aβ, we evaluated changes in fluorescence polarization values of this probe at different BMAOI 14:Aβ ratios for monomeric, oligomeric, and fibrillary Aβ species. Aβ42 was chosen for the evaluation of the binding of the 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 microscopy (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 μM, respectively. Compound **7** binds to Aβ42 with a micromolar affinity and favors Aβ2 fibrils over monomers and oligomers. Interestingly, another BMAOI with a 21-atom spacer but a different connectivity at curcumin [**8** (Figure 2)] did not exhibit 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** for bovine serum albumin (BSA) as weak 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** for BSA ($K_d \sim 71$ μM) is significantly lower than the binding affinity for Aβ42, thus suggesting that the interference from serum albumin will be minimal for this probe.

To examine the change in the 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, when **7** binds

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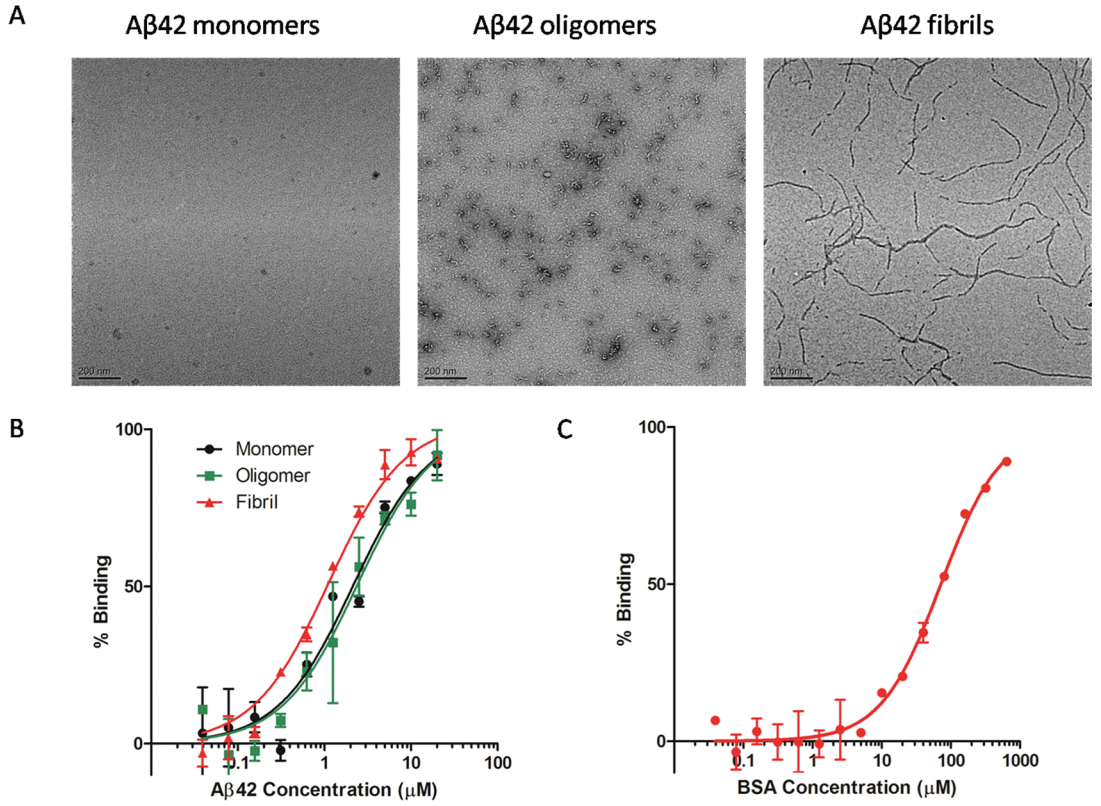

Figure 3. Compound 7 binds to Aβ42 monomers, oligomers, and fibrils, but not BSA. (A) The monomers, oligomers, and fibrils of Aβ42 were prepared according to established protocols and confirmed by TEM. (B) Compound 7 (1 μM) was incubated with different forms of Aβ42 at the indicated concentrations for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

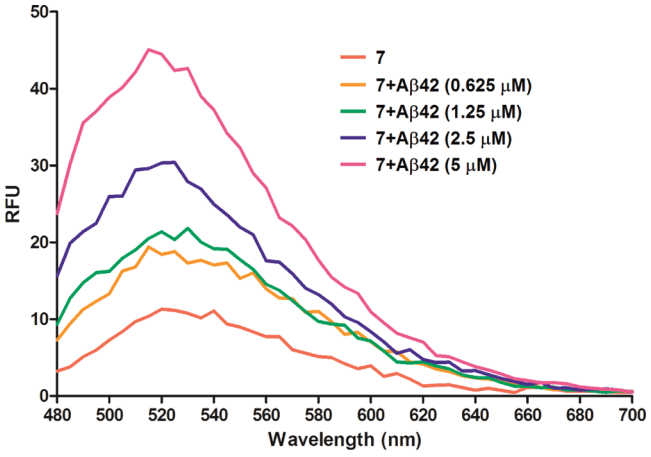

Figure 4. Fluorescence emission of compound 7 (1 μM) before and after it is mixed with Aβ42 fibrils.

to Aβ42 fibrils, the intensity of the emission spectra of 7 was significantly increased (4.5-fold increase at 5 μM Aβ42) with an 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 of 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) as soon as 30 min after intravenous (iv) administration through the tail vein of B6C3F1 mice (dose of 5 mg/kg). The compound remained in the brain tissue of B6C3F1 mice for >2 h as detection is evident at 30, 60, and 120 min but disappeared after iv administration for 3 h. 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 h) that allows clinical operation. While 7 appears to violate the empirical rules[22] of BBB permeability with a molecular mass of >1000 Da and seems to be more lipophilic, there are exceptions as there are compounds with molecular masses of >1000 Da can efficiently cross the BBB and reach brain tissue, such as natural products.[23] The results of the in vivo experiment clearly demonstrate that this molecule efficiently and rapidly crosses the BBB, likely because of the unique structure of this probe as bivalent ligands containing cholesterol to associate with CM−LR have been shown to efficiently cross the membrane system via internalization.[24,25]

Finally, to assess the ability of this compound to bind 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 (Figure 6C). These findings are very important in showing that this compound is specific for human disease, which allows for its development for use as a therapeutic

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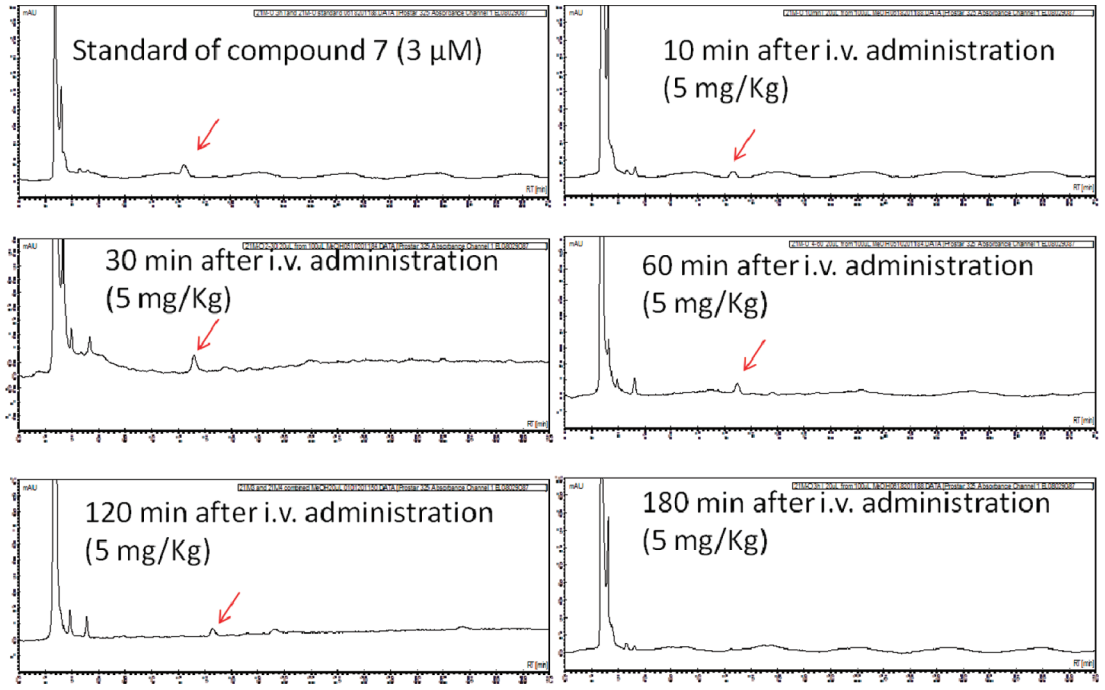

Figure 5. Compound 7 can cross the BBB of B6C3F1 mice. Compound 7 (5 mg/kg) was given to B6C3F1 female mice (n = 3) by iv administration via the tail vein. Then the animals were sacrificed at the indicated time intervals, and brain tissue was collected and analyzed by HPLC using a C18 column.

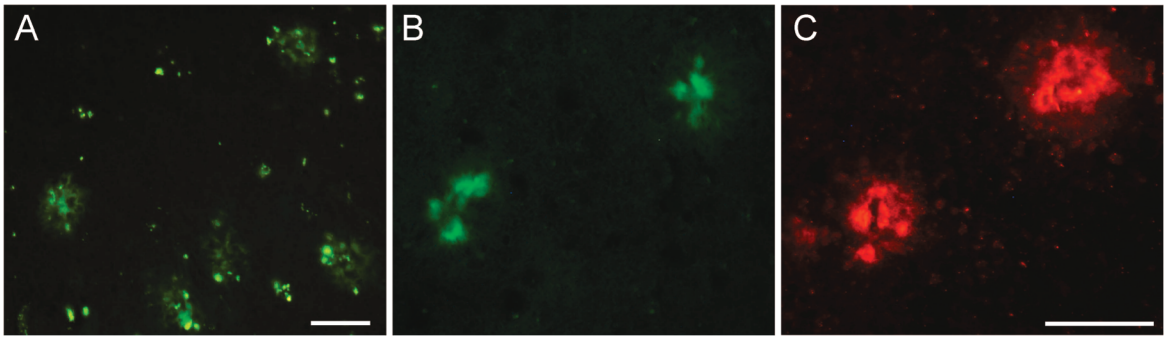

Figure 6. (A) Compound 7 stains the Aβ plaques in the brain tissues of human AD. (B) The brain tissue of TgCRND8 mice was stained with 7 (10 μM) according to the established protocol and viewed using fluorescent microscopy. (C) An adjacent section of the brain tissue of TgCRND8 mice was stained with anti-ADDL antibodies using Alexafluor 568, exhibiting red fluorescence.

approach and makes it viable for translational research. Furthermore, its comparable reliability in a mouse model of AD will allow for its continued and expanded experimentation in 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 molecule hold promise as fluorescent probes for Aβ imaging. Further development and optimization of 7 as a lead compound may provide useful diagnostic agents for AD.

METHODS

Preparation of Aβ42 Oligomers and Fibrils. Aβ42 oligomers and fibrils were prepared using reported procedures.[28] Briefly, the Aβ42 peptide (1 mg) (American Peptide Inc.) was first dissolved in hexafluoro-2-propanol (HFIP, 0.5 mL) and incubated at room temperature for 1 h. HFIP was then removed by a flow of nitrogen to give a clear film and then further dried by vacuum. HFIP-treated Aβ42 was then dissolved in DMSO to a final concentration of 5 mM and stored at −80 °C as a stock solution.

For the preparation of the Aβ42 monomer, an Aβ42 stock solution in DMSO was diluted to 100 μM with ddH_2O, and ddH_2O was used to make the working solution for binding experiments.

For the Aβ42 oligomer, an Aβ42 stock solution was diluted to 100 μM with F12 medium. The mixture was incubated for 24 h 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.

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For Aβ42 fibrils, an Aβ42 stock solution in DMSO was diluted to 100 μM with 10 mM HCl. The mixture was incubated for 24 h at 37 °C. Then, ddH_2O was used to prepare the working solution for binding experiments.

TEM Analysis. An aliquot of an Aβ42 sample of various species (5 μL) was adsorbed onto 200-mesh carbon and formavar-coated grids (Electron Microscopy Sciences) for 20 min and washed for 1 min with 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 transmission electron microscope 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 h, the FP was examined by Flexstation 3, with excitation and emission at 445 and 535 nm, respectively. Then, K_d was calculated on the basis of the following one-site binding equation from Prism 5: $Y = B_{max}X/(K_d + X)$, where Y is the fraction of bound Aβ and X is the concentration of **7**.

Fluorescence of 7 with Aggregated Aβ Fibrils. The fluorescence change of **7** (1 μM) was examined in the absence or presence of preaggregated Aβ42 fibrils (0.625, 1.25, 2.5, and 5 μM) with 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 groups contained three mice. Briefly, B6C3F1 mice were given **7** (5 mg/kg, dissolved in a 5:5:90 cremophor EL/EtOH/H_2O mixture) via iv injection via the tail vein. Then mice were sacrificed after 10, 30, 60, 120, and 180 min, and brain tissue was collected and rinsed with cold PBS. Brain tissue (100 mg) was diluted with water (300 μL) and homogenized with a Dounce homogenizer. The homogenate was mixed with acetonitrile (700 μL) and centrifuged. The supernatants were evaporated under a N_2 stream, and the residue was dissolved in 50 μL of an acetonitrile/water mixture (70:30). A 20 μL sample was subjected to HPLC analysis using a C18 reverse-phase column (250 mm × 4.6 mm) (Agilent). The mobile phase was a MeOH/CH_3CN mixture with 0.1% TFA (70:30, v/v). The flow rate was 1 mL/min. The wavelength was 353 nm. The standard of BMAOI **14** was prepared by mixing **7** (3 μM) with the brain tissue of untreated B6C3F1 mice and extracted following the aforementioned procedure.

Staining of Human Brain Tissue and Transgenic TgCRND8 Mouse Brain Tissue Sections. Hippocampal brain tissue and cortical brain tissue were obtained at autopsy from cases of Alzheimer's disease (n = 6) from the University Hospitals (Cleveland, OH) using approved IRB protocols and fixed in either formalin or Methacarn. Brain tissue from TgCRND8 (n = 3) and wild-type (n = 3) mice (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 two changes of xylene, sections were rehydrated through a graded series of ethanol and brought to water. A 2 mM stock solution of **7** was created in DMSO and diluted to 10 μM with a buffer that consisted 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 h at 37 °C. After being rinsed with TBS, the sections were covered with cover slips using fluorogel with 0.1% *N*-propyl gallate added. Images were obtained via fluorescence microscopy with an 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 the NU2 monoclonal antibody specific for ADDLs (gift of W. Klein and S. Ferreira) using a previously described method.[29] The Alexafluor 568-conjugated secondary antibody was used and viewed via fluorescence microscopy.

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Author Contributions

Chemical synthesis, TEM, fluorescence polarization, fluorescence, and HPLC studies were conducted 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.L.S. Fluorescence microscopy was performed by X.W. Aβ staining experimental design and data analysis were completed by X.Z. Experiment design, data analysis, writing, and editing were completed by S.Z.

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Notes

The authors declare no competing financial interest.

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, hexafluoro-2-propanol; 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 microscopy

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