

Synthesis and Evaluation of Oleanolic Acid-Conjugated Lactoferrin for β -Amyloid Plaque Imaging

Sung-Min Kim,^a Dongkyu Kim,^{†,a} Min Kyung Chae, Il-ha Jeong, Jee-Hyun Cho, Naeun Choi, Kyo Chul Lee,[‡] Chulhyun Lee,^{*} and Eun Kyoung Ryu^{*}

Division of Magnetic Resonance Research, Korea Basic Science Institute, Chungbuk 363-883, Korea

^{*}E-mail: chulhyun@kbsi.re.kr (C. Lee), ekryu@kbsi.re.kr (E.K. Ryu)

[†]Laboratory Animal Center, Daegu-Gyeongbuk Medical Innovation Foundation (DGMIF), Daegu 706-010, Korea

[‡]Molecular Imaging Research Center, Research Institute of Radiological & Medical Science, Korea Institute of Radiological & Medical Science (KIRAMS), Seoul 139-706, Korea

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β -Amyloid accumulation in the brain is a pathological hallmark of Alzheimer's disease (AD). Since early detection of β -amyloid may facilitate more successful and timely therapeutic interventions, many investigators have focused on developing AD diagnostic reagents that can penetrate the blood-brain barrier (BBB). Oleanolic acid (OA) is a substance found in a variety of plants that has been reported to prevent the progression of AD in mice. In this study, we synthesized and evaluated a new radioligand in which OA was conjugated to lactoferrin (Lf, an iron-binding glycoprotein that crosses the BBB) for the diagnosis of AD. In an *in vitro* study in which OA-Lf was incubated with β -amyloid (1-42) aggregates for 24 h, we found that OA-Lf effectively inhibited β -amyloid aggregation and fibril formation. *In vivo* studies demonstrated that ^{123}I -OA-Lf brain uptake was higher than ^{123}I -Lf uptake. Therefore, radiolabeled OA-Lf may have diagnostic potential for β -amyloid imaging.

Key Words : Oleanolic acid, Lactoferrin, Alzheimer's disease, β -Amyloid, Radioligand

Introduction

Alzheimer's disease (AD) is an aging-related neurodegenerative disorder that leads to neuronal dysfunction and death.^{1,2} While the causes of AD are still not fully understood, it is characterized by abundant senile plaques composed of β -amyloid peptides and neurofibrillary tangles.³⁻⁵ The progressive accumulation of β -amyloid aggregates is widely believed to be instrumental in the initial development of neurodegenerative pathology. Further, these plaques are believed to trigger neurotoxicity, oxidative damage, and inflammation that contribute to the progression of AD. Therefore, monitoring β -amyloid plaques is thought to be a key step in the diagnosis and treatment of AD. To this end, radioligands with specific binding affinity to β -amyloid have been developed using positron emission tomography (PET) and single photon emission computed tomography (SPECT).⁶⁻⁸

Oleanolic acid (3 β -hydroxy-olea-12-en-28-oic acid, OA), is a pentacyclic triterpenoid found in a variety of plant species that has been shown to inhibit tumor proliferation through induction of apoptosis.⁹ Interestingly, some terpenoid family members including oleanolic acid (OA) have therapeutic have been shown to prevent AD progression in mice.^{10,11} Therefore, we hypothesized that OA can bind to and perhaps inhibit Alzheimer's-related aggregate formation if it were able to cross the BBB.

Detection of β -amyloid plaques relies on the successful penetration of diagnostic reagents through the blood-brain

barrier (BBB). Many fluorescent dyes for β -amyloid imaging, such as Congo red (CR), chrysamine-G (CG), and thioflavin S have been developed,^{12,13} yet these imaging agents are unsuitable for *in vivo* imaging because of low brain uptake. Lactoferrin (Lf) is an iron-binding glycoprotein belonging to the transferrin family, which has been implicated in neurodegenerative disorders of the brain. The Lf receptor is present on the BBB in various species where it is involved in transport across the BBB both *in vitro* and *in vivo*.^{14,15} Since Lf can cross the BBB naturally, several groups have used Lf-conjugated nanoparticles to deliver substances into the brain *via* receptor-mediated transcytosis.^{16,17}

In the present study, we prepared a β -amyloid imaging reagent, OA-Lf, by conjugating OA and Lf. We demonstrate that OA derivatives can specifically bind to β -amyloid and can be used as new targeting ligands when they are radio-labeled with ^{123}I using SPECT. Finally, we also demonstrate that OA derivatives can inhibit β -amyloid aggregation *in vitro*. Together, these results indicate that OA-Lf represents a promising agent for the diagnosis and treatment of AD.

Experimental

All chemicals, including OA, Lf, and 6-(fluorescein-5(6)-carboxamido)hexanoic acid (FITC), were purchased from Sigma-Aldrich (St. Louis, MO, USA). The iodogen tube for the radioiodination reaction was purchased from Pierce (USA). Mass spectra were obtained by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry by the Korea Basic Science Institute (KBSI,

^aThese authors contributed equally to this work.

Korea). Radioactivity was determined with a Cobra II gamma scintillation counter (Packard Canberra Co., MA, USA). The animal experiments were carried out according to a protocol approved by the KBSI Committee (KBSI-AEC1115).

Preparation of OA Derivatives.

OA-Lf: OA (1 mg) dissolved in dimethyl sulfoxide (DMSO, 500 μ L) was added to 500 μ L Lf solution (2 mg/mL, phosphate buffered saline, PBS). After that, 4 equivalents of *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluorophosphate (HBTU) and hydroxybenzotriazole (HOBT) were added, and the solution was incubated in the basic condition (diisopropylethylamine, DIEA) at room temperature overnight. OA-Lf was then purified using a size-exclusion chromatography (PD-10 column) and washed with PBS buffer. The molecular mass of the product was measured by MALDI-TOF mass spectrometry.

OA-FITC: Amine modification of OA (OA-NH₂) was accomplished by activation of the OA carboxyl group (20 mg, 0.04 mmol) with HBTU (50 mg, 0.13 mmol), HOBT (18 mg, 0.13 mmol), and DIEA in anhydrous *N,N*-dimethylformamide (DMF) for 20 min at room temperature, followed by addition of mono-*N*-Boc-ethylenediamine (26 mg, 0.13 mmol). The reaction was continued overnight at room temperature. The crude mixture was purified using silica column chromatography, and eluted with ethyl acetate/hexane (2:3, v/v). Deprotection of the *N*-Boc amine was accomplished using trifluoroacetic acid in methylene chloride. The OA-NH₂ (5 mg, 0.01 mmol) and FITC (8 mg, 0.02 mmol) solutions were added to a vial to which 1 mL of DMF was added, followed by DIEA before stirring overnight. The final product was purified by reverse-phase high-performance liquid chromatography with a liner gradient over 30 min of CH₃CN from 40% to 100% at a flow rate of 2.5 mL/min. The molecular mass of the products were measured by MALDI-TOF.

Lf-FITC: Lf (1 mg) and FITC (2 mg, 5 μ mol) were added to a vial. DMF (1 mL) followed by DIEA was added to the vial and stirred overnight. Lf-FITC was purified using a spin filter (cutoff: 30 K), and the molecular mass was measured by MALDI-TOF.

OA-Lf-FITC: OA-Lf and FITC (2 mg, 5 μ mol) were added to a vial. DMF (1 mL) and DIEA were added to the vial and stirred overnight. OA-Lf-FITC was purified, and the molecular mass was confirmed by MALDI-TOF.

Synthesis of Lf and OA-Lf Radioligands (¹²⁵I-Lf, ¹²⁵I-OA-Lf). Lf and OA-conjugated Lf were labeled with ¹²⁵I by the iodogen tube method.¹⁸ In brief, 0.3–0.4 mg of protein (Lf or OA-Lf) was dissolved in 1 mL of 0.1 M PBS (pH 7.4) in an iodogen tube. [¹²⁵I]NaI (185 MBq) was added to the tube. After incubation for 60 min at room temperature, the mixture was purified by PD-10 column. The total synthesis time was 70 min, and radiochemical yield was 15–20% overnight.

In situ Detection of Amyloid Plaques. We used transgenic APPswe/PS1 mice, which are known to develop amyloid plaques. Transgenic mice were injected with a chimeric amyloid precursor protein (APP, APPSwe695) and a mutant

human presenilin 1 (A246E variant) obtained commercially from the Jackson Laboratory (strain type B6C3-Tg(APP695)3DboTg(PSEN1)5Dbo/J). These transgenic mice, which are known to develop amyloid deposits, were mated with the control normal mice (C57BL/6) in-house. The animals were genotyped for the expression of both transgenes by polymerase chain reaction (PCR) using a sample of mouse tail DNA. Brain tissue obtained from the mice was enucleated, post-fixed in 4% paraformaldehyde overnight, and subsequently cryoprotected in 30% sucrose in phosphate buffer. Frozen sections were cut at a thickness of 30 μ m with a freezing microtome. The sections were incubated with 10 μ g/mL of FITC-conjugated Lf, OA, and OA-Lf for 2 h. After thorough rinsing in PBS containing 0.3% Tween-20 and the sections were observed under a fluorescence microscope. Thioflavin S (Sigma-Aldrich, USA) was used as a detection marker for amyloid plaques.

Disaggregation with Lf or OA-Lf. β -Amyloid (1–42) aggregates were prepared according to a method reported in the literature.^{19,20} Briefly, β -amyloid (1–42) peptides (0.5 mg) were dissolved in sodium phosphate buffer (10 mM) and stirred gently for 3 days. To test the ability of OA to disaggregate amyloid plaques, β -amyloid (1–42) aggregates were incubated with 50 pM or 1 nM of Lf, OA, or OA-Lf at room temperature for 24 h. Then the mixture was washed with PBS. The disaggregation ability was observed using a microscope (Olympus Inc. Japan) at 200 \times and 300 \times magnification. Images of each sample were processed using image analysis software (Metamorph, Molecular Devices Inc., Sunnyvale, CA, USA).

In vivo Distribution of the Radioligands. Animal experiments were performed using male mice (BALB/c nu/nu, 5 weeks old, 20–25 g) that were maintained under specific pathogen-free conditions for the biodistribution studies. The mice were injected with the radioligands (2.5–3.0 MBq) in 0.2 mL of saline via the tail vein. The mice were sacrificed at 2, 30, and 60 min after injection (n = 3). Samples (blood, heart, lung, liver, spleen, kidney, muscle, thyroid, and brain) were collected and weighed, after which radioactivity was determined with a γ -counter. The data are expressed as percent of injected dose per gram of tissue (%ID/g).

Results and Discussion

This study aimed to investigate whether an OA-Lf-conjugated compound would be efficacious as an imaging agent for brain β -amyloid plaques. The toxicity of such reagents is a major concern for their utility *in vivo*. Therefore, it is imperative that diagnostic imaging reagents be developed that can translocate into the brain *in vivo* and also have low toxicity. We designed and prepared a new amyloid plaque imaging reagent based on the OA found in natural products (Fig. 1).

In order to assess whether OA could specifically stain amyloid plaques, we synthesized OA conjugated to the fluorescent FITC (OA-FITC). We compared plaque-associated fluorescence uptake of OA-FITC and thioflavin S (another

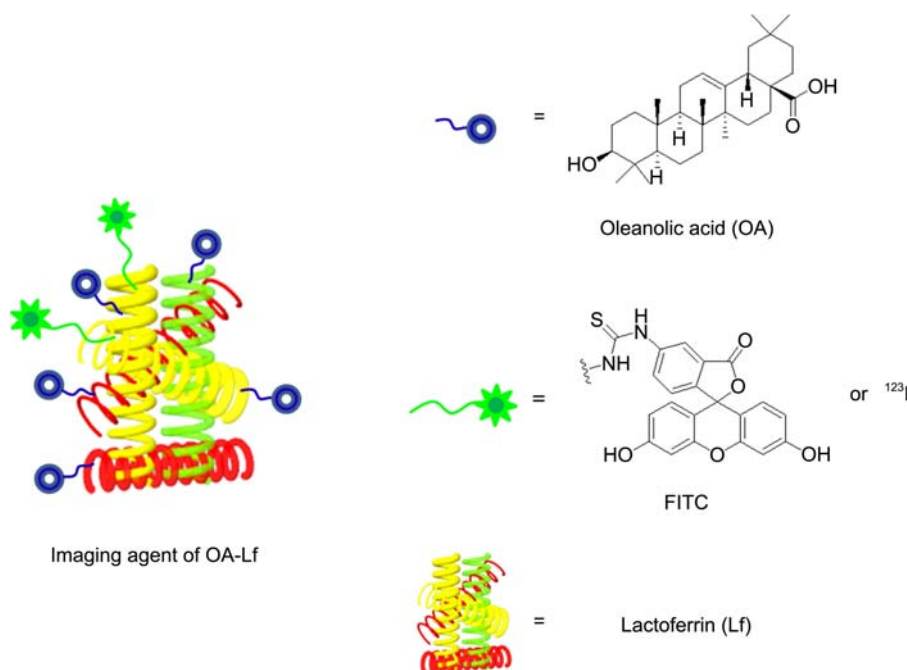


Figure 1. Diagram of the OA-Lf imaging agent.

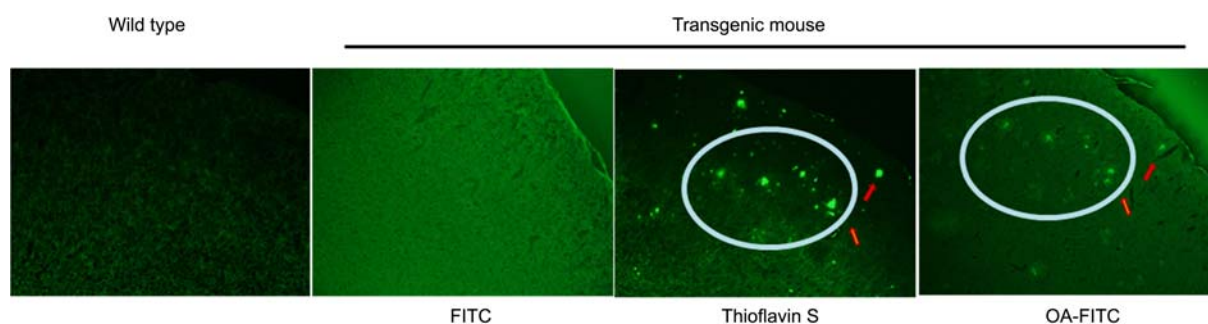


Figure 2. Amyloid accumulation of OA in brain sections from transgenic mice. FITC (10 $\mu\text{g/mL}$), OA-FITC (10 $\mu\text{g/mL}$), or thioflavin S (10 mg/mL) were incubated with the transgenic and wild-type mice brain sections.

dye used to stain amyloid plaques) in sections of transgenic AD mouse brain (APPswe/PS1 mice; Fig. 2). We stained alternating sections with OA-FITC and thioflavin S so that the regions stained by these two reagents could be compared. Thioflavin S stained a majority of the cortex and the hippocampal region. The regions labeled with OA-FITC were spatially correlated with the thioflavin S-stained plaques, although they displayed low fluorescent intensity due to the lower incubation concentration of OA-FITC (10 $\mu\text{g/mL}$)

compared to thioflavin S (10 mg/mL). However, transgenic brain tissue sections treated with only FITC solution did not show any positive fluorescence staining. Moreover, no fluorescent signals were observed in wild-type brain tissues exposed to both FITC and OA-FITC, indicating that this staining was in fact specific to amyloid plaques *in vitro*.

In order to increase brain uptake of OA *in vivo*, we conjugated OA to Lf (OA-Lf), a compound known to promote brain uptake. Conjugation of the OA-Lf was confirmed

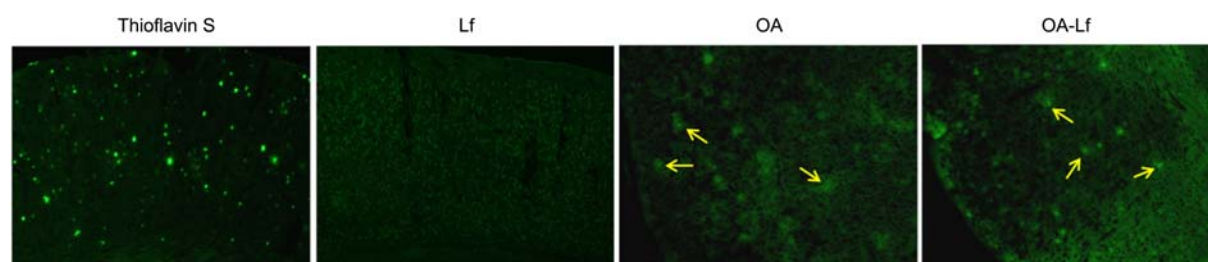


Figure 3. Amyloid accumulation of OA-Lf in transgenic mice. Thioflavin S, FITC conjugated Lf, OA, and OA-Lf were incubated with the transgenic mice brain sections.

using MALDI-TOF, which showed that approximately 4.5 OA molecules were bound to each Lf molecule. OA-Lf positively stained the amyloid plaques in the transgenic mice brain sections. The thioflavin S and OA plaque staining results were comparable, indicating that OA-Lf was selectively bound to the amyloid sites (Fig. 3). However, Lf showed non-specific binding in the brain, which means that OA confers binding specificity, rendering OA-Lf a useful diagnostic tool. Therefore, these data demonstrate that OA may be used as a plaque detection agent *in vivo*.

We next sought to test whether OA could interfere with β -amyloid aggregation. We used an optical microscope and examined the amount of plaque aggregation that occurred in the presence or absence of OA. β -Amyloid (1-42) aggregates were incubated in PBS with either 50 pM or 1 nM each of Lf, OA, or OA-Lf for 24 h. After this incubation, disaggregation was evaluated optically. Disaggregation was significantly increased in the group treated with 1 nM OA. Furthermore, OA-Lf was more efficient at disaggregating fibrils than was OA alone. In contrast, fibrils were not affected by Lf treatment (Fig. 4). Our results demonstrate that OA decreased β -amyloid aggregation in a time- and dose-dependent manner, suggesting that OA could be used as a new targeting ligand as well as a therapeutic agent for AD.

We used the radioisotope ^{123}I to evaluate the uptake and clearance of our chemical compounds in the brain. ^{123}I has an appropriate half life time ($t_{1/2} = 13.2$ h) for determining long-term retention and clearance of radioligand in biological models using SPECT. Moreover, the synthesis condi-

tions required to radiolabel a protein are milder than other radioisotopes, making it amenable for *in vivo* use, which means that ^{123}I labeling with protein is required no chelators or high reaction temperature. Initial brain uptake (2 min after the injection of radioligands) is an important time frame for determining whether radioligands (^{123}I -Lf and ^{123}I -OA-Lf) will cross the BBB. In our study, radioligands were injected into wild type mice and uptake was determined by performing biodistribution studies (Fig. 5). The organs of interest (brain, blood, heart, lung, liver, spleen, kidney, muscle, and thyroid) were collected and weighed, and the radioactivity in each sample was counted.

Both radioligands showed similar patterns of organ uptake. However, ^{123}I -OA-Lf showed increased uptake in the spleen compared to ^{123}I -Lf. There was also a slight time-dependent increase in radioligand thyroid uptake, suggesting that the radioligands underwent *in vivo* deiodination (^{123}I -Lf, 3.84 ± 0.17 , 5.80 ± 0.99 , and $7.92 \pm 4.61\%$ ID/g; ^{123}I -OA-Lf, 4.16 ± 1.04 , 5.73 ± 1.22 , and $6.15 \pm 1.52\%$ ID/g at 2, 30, and 60 min, respectively). Initially, the brain uptake of ^{123}I -OA-Lf was higher than that of ^{123}I -Lf (^{123}I -Lf, 0.50 ± 0.11 , 0.30 ± 0.13 , and $0.25 \pm 0.16\%$ ID/g; ^{123}I -OA-Lf, 0.79 ± 0.32 , 0.35 ± 0.07 , and $0.31 \pm 0.05\%$ ID/g at 2, 30, and 60 min, respectively; Fig. 6).

β -Amyloid SPECT imaging studies have shown that both β -amyloid plaque deposition and cognitive impairment are detectable before the onset of AD symptoms. Therefore, new radioligands are needed to diagnose and monitor AD. In order for a new radioligand to be effective for AD diagnosis,

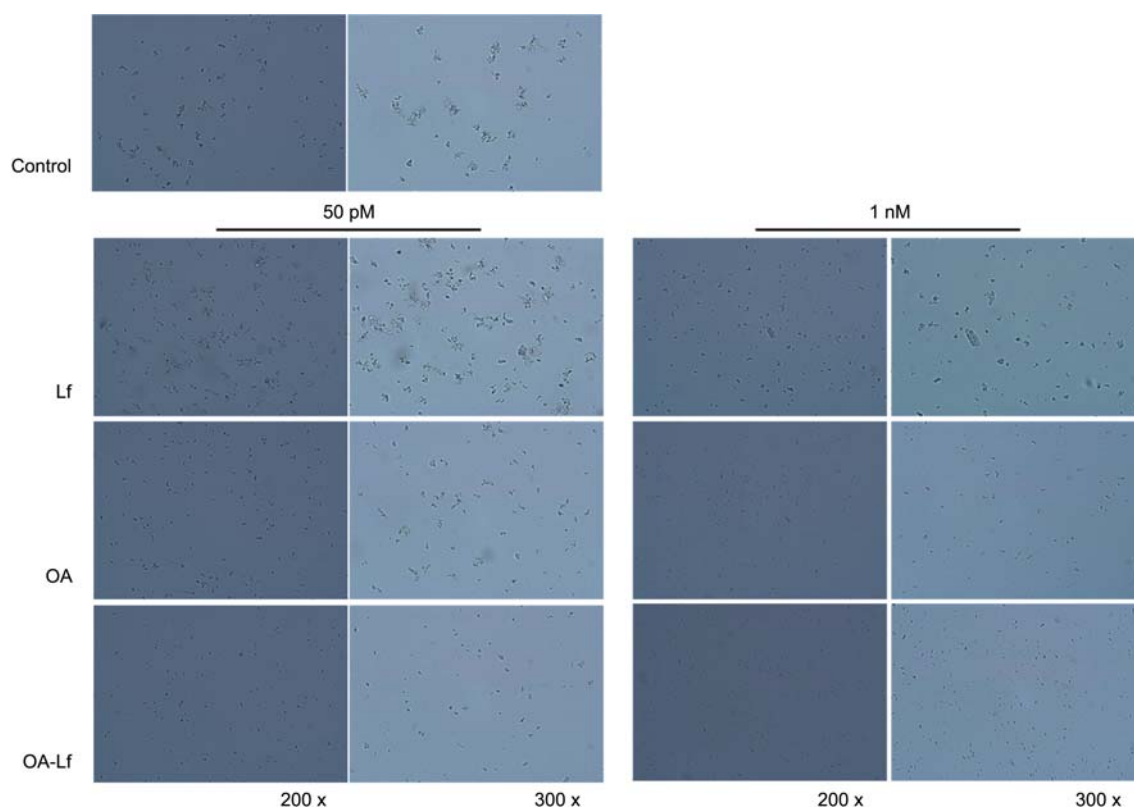


Figure 4. Disaggregation of OA derivatives using microscopic analysis. β -Amyloid (1-42) aggregates (0.5 mg/mL) were incubated with 50 pM or 1 nM of vehicle, Lf, OA, or OA-Lf for 24 h. The magnification was 200 \times and 300 \times .

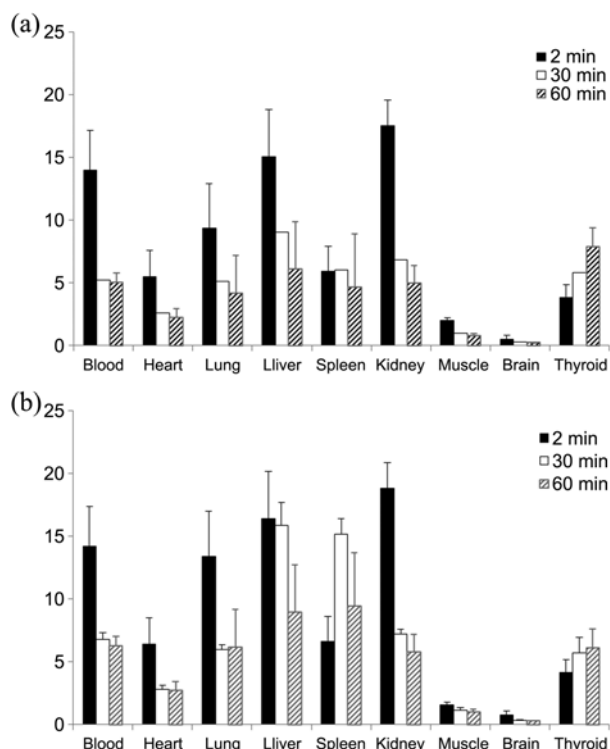


Figure 5. Biological distribution of ^{123}I -Lf (a) and ^{123}I -OA-Lf (b) in normal mice organs at 2, 30, and 60 min ($n = 3$). The data are expressed as percent of injected dose per gram of tissue (%ID/g).

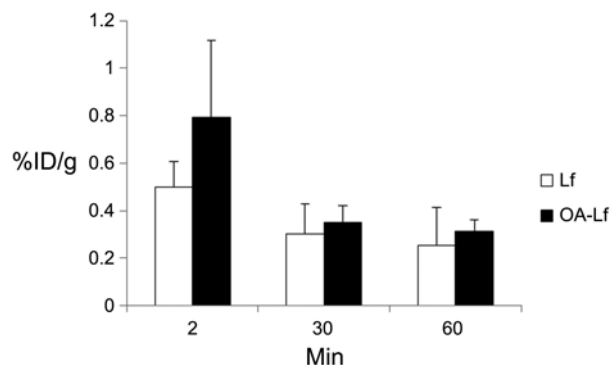


Figure 6. Brain uptake of ^{123}I -Lf and ^{123}I -OA-Lf at 2, 30, and 60 min after injection ($n = 3$). The data are expressed as percent of injected dose per gram of tissue (%ID/g).

it must have high initial brain uptake and desirable pharmacokinetics (such as fast washout). In this study, we demonstrated a high initial brain uptake of ^{123}I -OA-Lf in normal mice (0.79% ID/g) at 2 min. Brain uptake improved to 58% with the OA-conjugated radioligand compared to without OA-radiolabeled Lf. The clearance of ^{123}I -OA-Lf from the brain was also fairly rapid.

Conclusions

In this study, we synthesized and evaluated a new radioligand, OA-Lf, for *in vivo* β -amyloid imaging. We conducted an experiment in which plaques were stained with either thioflavin S, OA, or OA-Lf. We found that OA-Lf binding to

the β -amyloid was highly correlated with thioflavin S staining, suggesting that it was specifically bound to β -amyloid. We also performed a study on the ability of OA to disaggregate amyloid plaques. Significant inhibition of the OA-Lf compound *in vitro* indicated that it selectively promoted β -amyloid disaggregation. OA-Lf was also able to translocate into the brain by penetrating the BBB and binding to the β -amyloid plaque regions. While future studies are required to determine the utility of OA-Lf as a diagnostic reagent using SPECT *in vivo*, this compound represents a promising diagnostic and therapeutic agent in the treatment of AD.

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