

5. Translational Research on Antiatherosclerotic Effects

The purpose of lowering serum cholesterol levels is to inhibit atherogenesis and to circumvent the cardiovascular and cerebrovascular events. The WHHL rabbit contributed to prove the effects of cholesterol-lowering therapies on delaying the progression of atherosclerosis. Statin treatment resulted in a decrease in serum total cholesterol levels by 20–30%, and the cross-sectional narrowing of the coronary arteries was significantly decreased [41–45].

In several clinical studies, the incidence of cardiovascular events was significantly reduced in the statin-treated groups despite little or no improvement in coronary stenosis on evaluation by coronary angiography [55]. The WHHL rabbit contributed to the clarification of this paradoxical mechanism [42–45]. On the administration of statin to 10-month old WHHL rabbits for one year, in which coronary atherosclerosis had already developed to a mature stage, statin treatment showed not only the prevention of further progression of the coronary atherosclerotic lesions, but also various stabilizing effects on coronary plaques, such as reductions in the contents of macrophages and extra cellular lipids in lesions, and increase in the contents of collagen fibers and preservation of the smooth muscle cells in lesions. Thus it was clarified that, statin administration makes atherosclerotic lesions more stable, that is, less likely to rupture. With this study, it was confirmed that the stabilization of atherosclerotic lesions is important for the prevention of coronary events. Nowadays, more than 40 million patients worldwide are prescribed statins. Another type of cholesterol synthesis inhibitor, squalene synthesis inhibitors, that act downstream of the cholesterol synthesis pathway, also showed similar hypocholesterolemic and atheroma-stabilizing effects in WHHLMi rabbits [56].

Using WHHLMi rabbits, antiatherosclerotic effects have also been evaluated with other compounds such as omega-3 fatty acids, which decrease serum triglyceride levels by changing the composition of fatty acids [57–61]; antioxidants, such as probucol, vitamin C, and vitamin E [62–65]; agents that regulate the function of macrophages [66, 67]; drugs that inhibit the rennin-angiotensin pathway [68–71]. Interestingly, antiatherosclerotic effects of antihypertensive agents were unequal in WHHL or WHHLMi rabbits. Angiotensin converting enzyme (ACE) inhibitors and angiotensin-II receptor blockers (ARBs) showed antiatherogenic effects [69–72], but calcium antagonists and beta-blockers were not effective [73, 74]. Systolic blood pressure in WHHL and WHHLMi rabbits is 100–120 mmHg, which is slightly higher than normal [75]. This may be why calcium antagonists and beta-blockers did not show distinct antiatherosclerotic effects. In contrast, antihypertensive effects of ACE inhibitors and ARBs are mediated by suppressing the effects of angiotensin II. Angiotensin-II stimulates atherogenesis by impairing the function of arterial endothelial cells, proliferation of arterial smooth muscle cells, and inflammation [76]. These pleiotropic effects of angiotensin-II are considered to be mediated by reactive oxygen species. Thus, the WHHL rabbit

is indispensable for studies on the antiatherosclerotic effects of the various compounds.

6. Imaging Technology for Evaluation of Atherosclerotic Lesions

Although it is important to evaluate drug efficacy in clinical use, it is difficult to evaluate atheroma-stabilizing effects of drugs in clinical practice. With coronary angiography, it is possible to see the degree of stenosis but difficult to evaluate the severity of lesions, if the lesions are spread and extended in the coronary arteries, or if the coronary arteries are expanded due to the outward remodeling of the vessels. Furthermore, it is very important to develop noninvasive technologies and equipment to detect dangerous lesions, that is, vulnerable plaques that are prone to rupture, not only for the diagnosis but for the prevention of cardiovascular events. As vulnerable plaques that cause cardiovascular events, soft-type plaques rich in macrophages and large lipid droplets covered with a thin fibrous cap are important. To detect such soft-type plaques, computed tomography (CT) [77], positron emission tomography (PET) [77], CT plus PET [78], magnetic resonance (MRI) [78, 79], and intravascular ultrasound (IVUS) [80] have been applied to WHHLMi rabbits. One successful example was evaluation of the antiatherosclerotic effect of probucol, a potent antioxidant, in WHHLMi rabbits by imaging with CT plus PET [81]. Ogawa et al. demonstrated clearly that imaging with CT plus PET is powerful technology to detect antiatherosclerotic effects of compounds. Once imaging technologies for the evaluation of atherosclerotic lesions are established, they can be used not only for the assessment of drug effects, but also for the detection of dangerous coronary lesions that could lead to cardiovascular events such as acute coronary syndromes and consequently the prevention of ischemic heart diseases.

7. Perspectives

To overcome cardiovascular diseases, many research issues remain unresolved, despite diligent studies for the development of diagnostic methods and lipid-lowering agents. Particularly important is clarifying the mechanism of the disruption of coronary lesions (arterial plaque rupture and the following formation of a thrombus), which depress the trigger for the onset of acute coronary syndromes, and establishment of treatments. Still no suitable animal model, which is compatible with the study of human acute coronary syndromes, has been developed. To develop a suitable animal model for human acute coronary syndromes, trial studies/experiments such as the enhancement of vulnerable coronary lesions, and application of physical pressure to coronary lesions, are currently underway with WHHLMi rabbits. To destabilize coronary lesions, serial selective breeding with new criteria such as the formation of vulnerable plaques is also ongoing, in parallel with the development of genetically modified WHHLMi rabbits overexpressing matrix metalloproteinases (MMPs), and so forth. The established strain would be a subject of analyses for the identification of

the genes/loci responsible for the phenotype established. In the near future, with advances in gene-targeting technologies by using ES or iPS cells capable of germ-line transmission, in combination with the nuclear transfer technique, more precise manipulation of the rabbit genome may also be available. Since the lesion composition and severity of coronary lesions differ even in WHHLMI rabbits, despite no difference in the serum cholesterol levels, it will be important to explore marker proteins and/or risk factors affecting coronary lesions. Once markers and risk factors relating to vulnerable coronary atheromas are found, the mechanism of cardiovascular events may be clarified. Such findings would contribute to the development of new clinical diagnostics and thence to the prevention of cardiovascular events.

In conclusion, selecting appropriate animal model is important in translational research. WHHL and WHHLMI rabbits have contributed to development of hypocholesterolemic and antiatherosclerotic compounds and medical devices, such as imaging technologies for atherosclerosis, and diagnostic techniques for acute coronary syndromes, in addition to elucidation of the mechanisms of atherogenesis and coronary plaque rupture. These studies are helpful for progression of therapeutics.

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A novel bifunctional chelating agent based on bis(hydroxamamide) for ^{99m}Tc labeling of polypeptides

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This paper describes the synthesis and biological evaluation of a novel bifunctional chelating agent (BCA) based on bis(hydroxamamide) for ^{99m}Tc labeling of polypeptides. We successfully designed and synthesized $\text{C}_3(\text{BHam})_2\text{-COOH}$ as a new BCA. $\text{C}_3(\text{BHam})_2\text{-COOH}$ formed a stable ^{99m}Tc complex and enabled us to prepare ^{99m}Tc -labeled polypeptides by using a 2,3,5,6-tetrafluorophenol (TFP) active ester of $\text{C}_3(\text{BHam})_2\text{-COOH}$. $^{99m}\text{Tc}\text{-C}_3(\text{BHam})_2\text{-HSA}$ prepared with $\text{C}_3(\text{BHam})_2\text{-TFP}$ was stable in both murine plasma and an excess of L-cysteine without any dissociation of ^{99m}Tc from polypeptides. Furthermore, the blood clearance of $^{99m}\text{Tc}\text{-C}_3(\text{BHam})_2\text{-HSA}$ in mice was similar to that of $^{125}\text{I}\text{-HSA}$, suggesting that $\text{C}_3(\text{BHam})_2\text{-COOH}$ retained stable binding between ^{99m}Tc and the polypeptides *in vivo*. When $^{99m}\text{Tc}\text{-C}_3(\text{BHam})_2\text{-NGA}$ was injected into mice, the radioactivity showed high hepatic uptake early on and a rapid clearance from the liver, indicating that $\text{C}_3(\text{BHam})_2\text{-COOH}$ did not affect the pharmacokinetics of polypeptides *in vivo* and gave radiometabolites, which displayed a rapid elimination from the liver. Such characteristics would render $\text{C}_3(\text{BHam})_2\text{-COOH}$ attractive as a new BCA for ^{99m}Tc labeling of polypeptides.

Keywords: ^{99m}Tc ; bifunctional chelating agent; polypeptide; hydroxamamide

Introduction

Many polypeptides such as antibodies, single-chain Fv fragments, diabodies, affibodies, minibodies, and bioactive peptides have been used as scaffolds of radiolabeled probes for targeted imaging of sites of tumors, infection, and thrombosis.^{1–10} Among radionuclides for radiolabeling of these polypeptides, ^{99m}Tc is ideal for scintigraphic imaging because of its excellent physical properties, low cost, and ready availability.^{11,12} Generally, polypeptides do not possess binding sites to form ^{99m}Tc chelates of high stability *in vivo*. To prepare ^{99m}Tc -labeled peptides for application *in vivo*, therefore, one must incorporate appropriate chelating agents into polypeptide molecules.

^{99m}Tc labeling using bifunctional chelating agents (BCAs), which possess both a binding site for polypeptides and a site for complexation with ^{99m}Tc , is required. It has been reported that tetradentate ligands with N_3S or N_2S_2 (containing one or two thiol groups)^{13–15} and hydrazino nicotinamide (HYNIC) (thiol-free chelating agent)^{16–19} serve as a BCA for ^{99m}Tc labeling. However, some N_3S or N_2S_2 ligands require harsh ^{99m}Tc complexation (elevated temperatures or high pH) to prepare ^{99m}Tc chelates with high radiochemical yields.^{13–15} When $^{99m}\text{Tc}\text{-HYNIC}$ -labeled polypeptides were administered *in vivo*, they showed not only localized radioactivity in target tissues but also strong, persistent radioactivity in non-target tissues.¹⁷

To improve BCAs, we have developed 4'-aminomethyl-*N,N'*-trimethylene bisbenzohydroxamamide [$\text{C}_3(\text{BHam})_2\text{-NH}_2$], which has $\text{C}_3(\text{BHam})_2$ for chelating with ^{99m}Tc and a primary amino group for binding with polypeptides (Figure 1).^{20–24} A recent report showed that the ^{99m}Tc complexes of benzohydroxamamide

(BHam) possessed square base pyramid coordination geometry, and the equatorial plane was formed by two-amine nitrogen and two-oxime oxygen atoms in a trans-orientation, whereas the oxo core of the Tc(V) occupied the apical position.²⁵ Indeed, $\text{C}_3(\text{BHam})_2\text{-NH}_2$ provided stable ^{99m}Tc -labeled antibodies in mild conditions to image tumor sites in tumor-bearing mice.²³ However, as $\text{C}_3(\text{BHam})_2\text{-NH}_2$ cannot directly conjugate to polypeptides, *N*-(6-maleimidocaproyloxy)succinimide (EMCS) and 2-iminothiolane (2-IT) were essential for the preparation of ^{99m}Tc -labeled polypeptides by using $\text{C}_3(\text{BHam})_2\text{-NH}_2$ as a BCA. When $\text{C}_3(\text{BHam})_2\text{-NH}_2$ is applied to ^{99m}Tc labeling for lower molecular weight polypeptides, the incorporation of spacers (EMCS and 2-IT) between $^{99m}\text{Tc}\text{-C}_3(\text{BHam})_2$ and polypeptides may affect the pharmacokinetics and bioactivity of the polypeptides.^{26,27}

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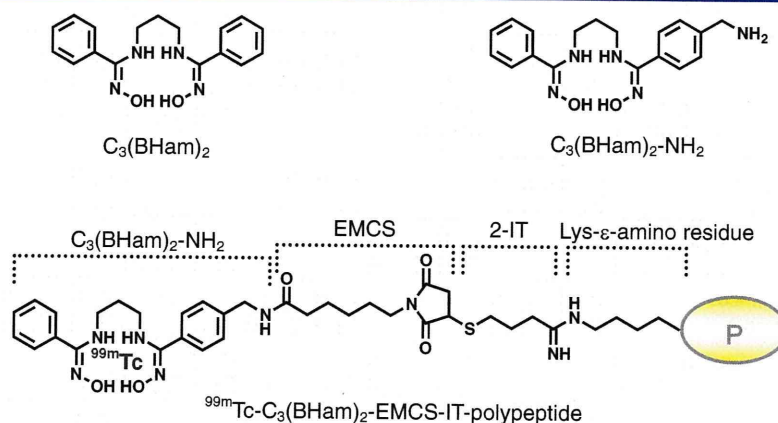


Figure 1. Chemical structure of $C_3(\text{BHAM})_2$, $C_3(\text{BHAM})_2\text{-NH}_2$, and $^{99\text{m}}\text{Tc-C}_3(\text{BHAM})_2\text{-EMCS-IT-polypeptide}$.

The objective of this study was to develop a novel BCA based on $C_3(\text{BHAM})_2$, which can be introduced into polypeptides without a long linker such as EMCS and 2-IT. We designed and synthesized 4'-carboxyl-*N,N'*-trimethylene bisbenzohydroxamamide [$C_3(\text{BHAM})_2\text{-COOH}$], which has carboxylic acid for binding with the lys- ϵ -amino group of polypeptides. Next, using $C_3(\text{BHAM})_2\text{-COOH}$, we investigated the labeling of $^{99\text{m}}\text{Tc}$ with human serum albumin (HSA) as a model polypeptide and evaluated the stability of $^{99\text{m}}\text{Tc-C}_3(\text{BHAM})_2\text{-HSA}$ *in vitro* and *in vivo*. Furthermore, we used galactosyl-neoglycoalbumin (NGA) as a model polypeptide and evaluated the pharmacokinetics of radiometabolites formed in the liver after the injection of $^{99\text{m}}\text{Tc-C}_3(\text{BHAM})_2\text{-NGA}$ into mice.

Materials and methods

Reagents and chemicals

Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on a Varian Gemini 300 (300 MHz) (Varian Medical Systems, Inc., California, USA). Electron impact mass spectra (EI-MS) and fast atom bombardment mass spectra (FAB-MS) were obtained with a JEOL IMS-DX300 mass spectrometer (JEOL Ltd., Tokyo, Japan). $\text{Na}[^{125}\text{I}]$ (3.7 GBq/mL, 0.01 N NaOH solution) was obtained from MP Biomedicals. $[\text{Pertechnetate } (^{99\text{m}}\text{TcO}_4^-)]$ (111 MBq/mL) was purchased from Nihon-Medi-Physics (Tokyo, Japan). Size-exclusion HPLC (SE-HPLC) was performed using a TSK G3000SW (7.8 \times 300 mm) column (Tosoh Corporation, Tokyo, Japan), eluted with 0.1 M phosphate buffer (PB) (pH 7.0) containing 0.3 M sodium chloride at a flow rate of 0.5 mL/min. Reversed-phase HPLC (RP-HPLC) was performed with a Cosmosil 5 $\text{C}_{18}\text{-MS}$ column (4.6 \times 150 mm, Nacalai Tesque, Kyoto, Japan) at a flow rate of 1 mL/min with a gradient mobile phase from 85% A (PB, 0.01 M, pH 7.4) and 15% B (acetonitrile) to 20% A and 80% B in 10 min. Cellulose acetate electrophoresis (CAE) was run on Separax SP (Joko, Tokyo, Japan) at a constant current of 0.8 mA/cm for 30 min in 0.072 M veronal buffer (pH 8.6). The distance migrated by HSA was determined by Ponceau 3R staining.

Synthesis of benzohydroxamamide

To a solution of hydroxylammonium chloride (10.5 g, 145.2 mmol) and NaHCO_3 (12.3 g, 146.0 mmol) in H_2O (40 mL) was gradually added a solution of benzonitrile (15.3 g, 145.2 mmol) in EtOH (100 mL), and the reaction mixture was stirred at 80 $^\circ\text{C}$ for 4 h. EtOH was removed *in vacuo*, and the mixture was extracted

with ethyl acetate. After drying of the organic layer on Na_2SO_4 , evaporation gave 16.6 g of BHAM (83.1%). ^1H NMR ($\text{DMSO-}d_6$) δ : 5.81 (s, 2H, $-\text{NH}_2$), 7.36–7.38 (m, 3H, aromatic), 7.66–7.69 (m, 2H, aromatic), 9.64 (s, 1H, $-\text{NOH}$).

Synthesis of *O*-carbethoxybenzohydroxamamide (**1**)

To a solution of BHAM (19.0 g, 140.0 mmol) in dry acetone (90 mL) was gradually added a solution of ethyl chlorocarbonate (16.8 g, 156.0 mmol) in acetone (30 mL) in an ice bath for 1 h. After a 5% NaOH solution (126 mL) was added, the mixture was stirred at room temperature for 1 h. The precipitate formed was filtered, and the residue was recrystallized from H_2O –acetone (4:3) to give 15.9 g of **1** (54.9%). ^1H NMR (CDCl_3) δ : 1.37 (t, 3H, $-\text{CH}_3$), 4.33 (q, 2H, $J=7.2$ Hz, $-\text{CH}_2$), 5.11 (s, broad, 2H, $-\text{NH}_2$), 7.41–7.48 (m, 3H, aromatic), 7.68–7.71 (m, 2H, aromatic).

Synthesis of 3-phenyl- Δ^2 -1,2,4-oxadiazolin-5-one (**2**)

To a solution of **1** (15.8 g, 75.7 mmol) in 5% NaOH solution (80 mL) was added excess acetic acid (26.3 mL, 460 mmol). The precipitate formed was filtered to give 10.7 g of **2** (87.5%). ^1H NMR ($\text{DMSO-}d_6$) δ : 7.56–7.67 (m, 3H, aromatic), 7.81–7.85 (q, 2H, $J=6.3$ Hz, aromatic).

Synthesis of potassium 3-phenyl- Δ^2 -1,2,4-oxadiazolin-5-one (**3**)

To a solution of **2** (7.50 g, 46.3 mmol) in MeOH (20 mL) was added a solution of KOH (2.60 g, 46.3 mmol) in MeOH (30 mL). After the mixture was stirred at room temperature for 1 h, evaporation gave 8.97 g of **3** (96.8%). ^1H NMR ($\text{DMSO-}d_6$) δ : 7.37–7.42 (m, 3H, aromatic), 7.76–7.80 (m, 2H, aromatic).

Synthesis of 3-phenyl-4-(3-bromopropyl)- Δ^2 -1,2,4-oxadiazolin-5-one (**4**)

To a solution of **3** (9.28 g, 32.8 mmol) in DMF (31 mL) was added 1,3-dibromopropane (19.9 g, 98.4 mmol) in DMF (25 mL). The reaction mixture was stirred at room temperature for 3 days. After the filtrating precipitate of potassium bromide was filtered and the solvent was removed, the residue was purified by silica gel chromatography (ethyl acetate/hexane = 1:5) to give 4.56 g of **4** (49.1%). ^1H NMR (CDCl_3) δ : 2.22–2.26 (m, 2H, $-\text{NCH}_2\text{CH}_2\text{CH}_2\text{Br}$), 3.35 (t, 2H, $-\text{NCH}_2\text{CH}_2\text{CH}_2\text{Br}$), 3.86 (t, 2H, $-\text{NCH}_2\text{CH}_2\text{CH}_2\text{Br}$), 7.58–7.63 (m, 5H, aromatic).

Synthesis of 4-carboxybenzohydroxamide methyl ester (BHam-COOMe, **5**)

The reaction mixture of 4-cyanobenzoic acid methyl ester (10.0 g, 61.8 mmol), NaHCO₃ (5.35 g, 66.7 mmol), and hydroxylammonium chloride (4.30 g, 61.8 mmol) in MeOH (120 mL) was stirred at room temperature for 30 min. The mixture was then stirred at 80–90 °C for 3 h. After it had cooled to room temperature, 200 mL of water was added to produce a precipitate. **5** was obtained after washing the precipitate with ether in a yield of 68.6% (8.24 g). ¹H NMR (CDCl₃) δ: 3.94 (s, 3H, -CH₃), 4.89 (s, broad, 2H, -NH₂), 7.72 (t, 2H, aromatic), 8.89 (t, 2H, aromatic).

Synthesis of 3-(4-carboxylphenyl)-Δ²-1,2,4-oxadiazolin-5-one methyl ester (**6**)

To a suspension of **5** (4.96 g, 25.5 mmol) in 1,4-dioxane (35 mL) was added 1'-carbonyldiimidazole (5.30 g, 30.6 mmol). The mixture was stirred at 110 °C for 30 min. The solvent was removed, the residue was dissolved in water, and 3 N HCl was added to produce a precipitate. The precipitate was washed with water, ether, and ethyl acetate to give 4.91 g of **6** (87.5%). ¹H NMR (DMSO-*d*₆) δ: 3.90 (s, 3H, -CH₃), 7.96 (d, 2H, *J* = 8.7 Hz, aromatic), 8.15 (t, 2H, aromatic).

Synthesis of potassium 3-(4-carboxylphenyl)-Δ²-1,2,4-oxadiazolin-5-one methyl ester (**7**)

To a solution of **6** (8.62 g, 39.2 mmol) in MeOH (23 mL) was added a solution of KOH (2.25 g, 40.0 mmol) in MeOH (28 mL). After the reaction mixture was stirred at room temperature for 1 h, evaporation of the solvent gave 9.78 g of **7** (96.6%). ¹H NMR (DMSO-*d*₆) δ: 3.87 (s, 3H, -CH₃), 7.93 (t, 2H, aromatic), 8.00 (t, 2H, aromatic).

Synthesis of methyl 4-(4,5-dihydro-5-oxo-4-(3-(5-oxo-3-phenyl-1,2,4-oxadiazol-4-yl)propyl)-1,2,4-oxadiazol-3-yl)benzoate (**8**)

To a solution of **4** (1.15 g, 4.06 mmol) in DMF (20 mL) was added a solution of **7** (1.11 g, 4.30 mmol) in DMF (20 mL) at room temperature. The reaction mixture was then stirred at 40 °C for 7 days. The solvent was evaporated, water was added, and the mixture was extracted with ethyl acetate. The organic layer was dried over Na₂SO₄ and filtered. The residue was purified by silica gel chromatography (ethyl acetate/hexane = 1:2) to give 850 mg of **8** (49.6%). ¹H NMR (DMSO-*d*₆) δ: 1.77 (m, 2H, -CH₂CH₂CH₂-), 3.55–3.62 (q, 4H, *J* = 7.2 Hz, -CH₂CH₂CH₂-), 3.93 (s, 3H, -CH₃), 7.56–7.60 (m, 4H, aromatic), 7.64–7.69 (m, 1H, aromatic), 7.75 (d, 2H, *J* = 7.8 Hz, aromatic), 8.12 (d, 2H, *J* = 8.1 Hz, aromatic). EI-MS calc C₂₁H₁₈N₄O₆ (M⁺): *m/z* 422, found: 422.

Synthesis of C₃(BHam)₂-COOH

8 (850 mg, 2.01 mmol) was added to 5 mL of 1 N NaOH, and the mixture was heated with stirring at 90–100 °C for 1.5 h, then allowed to cool to room temperature. The pH was adjusted to 3 with 1 N HCl. The mixture was neutralized with 1 N NaOH, and the solvent was removed *in vacuo*. The residue was purified by silica gel chromatography (MeOH/CHCl₃/acetic acid = 10:50:1) to give 620 mg of C₃(BHam)₂-COOH (61.5%). ¹H NMR (DMSO-*d*₆) δ: 1.57 (m, 2H, -CH₂CH₂CH₂-), 3.00–3.08 (m, 4H, -CH₂CH₂CH₂-), 7.53–7.62 (m, 7H, aromatic), 8.05 (d, 2H, *J* = 9.0 Hz, aromatic), 8.92 (s, 1H, -COOH). FAB-MS calc C₁₈H₂₁N₄O₄ (MH⁺): *m/z* 357, found: 357.

Preparation of ^{99m}Tc-C₃(BHam)₂-COOH

A solution of C₃(BHam)₂-COOH in DMSO (0.1 M) was prepared. This solution (5 μL) was added to H₂O (495 μL). A solution of

stannous tartrate (375 μL, 3 × 10⁻⁴ M) in H₂O and Na^{99m}TcO₄ (74 MBq/mL, 125 μL) was then added. After incubation for 15 min, radiochemical yields of ^{99m}Tc-C₃(BHam)₂-COOH were determined by RP-HPLC, CAEP, and TLC.

Preparation of ^{99m}Tc-C₃(BHam)₂-TFP

2,3,5,6-Tetrafluorophenol (TFP) (2 mg) was added to a solution of ^{99m}Tc-C₃(BHam)₂-COOH in saline (200 μL). Next, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (4 mg) was added, the reaction mixture was stirred at room temperature for 30 min, DMSO (60 μL) was added, and the mixture was purified using Sep-Pak. The Sep-Pak was replaced with water. The reaction mixture (260 μL) was applied, the column was washed with water (6 mL) and diethylether (1 mL), and ^{99m}Tc-C₃(BHam)₂-TFP was eluted with acetonitrile (1 mL). After evaporation of the acetonitrile, the radioactivity in the residue was analyzed by SE-HPLC and CAEP.

Preparation of ^{99m}Tc-C₃(BHam)₂-HSA

To 200 μL of HSA solution (10 mg/mL in 0.1 M carbonate buffer, pH 9.5) was added an equal volume of ^{99m}Tc-C₃(BHam)₂-TFP prepared above, and the reaction mixture was incubated for 1 h at room temperature. ^{99m}Tc-C₃(BHam)₂-HSA was then purified by the centrifuged column procedure using a Sephadex G-50 column equilibrated and eluted with PB (0.1 M, pH 7.4). The radiochemical yield was assessed by SE-HPLC and CAEP.

Preparation of ^{99m}Tc-C₃(BHam)₂-NGA

Galactosyl-neoglycoalbumin was synthesized by conjugation of cyanomethyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-*D*-galactopyranoside, synthesized according to the procedure of Lee *et al.*,²⁸ with HSA. The phenol-sulfuric acid reaction indicated that 25 galactose units were attached to each HSA molecule.²⁹ ^{99m}Tc-C₃(BHam)₂-NGA was prepared similar to ^{99m}Tc-C₃(BHam)₂-HSA, and radiochemical yield was assessed by SE-HPLC and CAEP.

Preparation of ¹²⁵I-HSA

To 200 μL of HSA solution (4 mg/mL in 0.1 M PB, pH 7.4) was added 1 μL of Na¹²⁵I (3.7 GBq/mL) solution and 10 μL of chloramine-T solution (2 mg/mL in 0.1 M PB, pH 7.4). The reaction mixture was incubated for 10 min. A solution of NaHSO₃ (1 mg/mL, 6 μL) was added, and the mixture was purified by the centrifuged column procedure using a Sephadex G-50 column equilibrated and eluted with PB (0.1 M, pH 7.4). The radiochemical yield of ¹²⁵I-HSA was assessed by CAEP and TLC.

Stability of ^{99m}Tc-C₃(BHam)₂-HSA *in vitro*

^{99m}Tc-C₃(BHam)₂-HSA (50 μL, 8.3 kBq) was diluted 20-fold with 0.1 M PB (pH 7.4, 200 μL) or freshly prepared murine plasma, and the solution was incubated at 37 °C. After 1, 3, 6, and 24 h of incubation, the radioactivity of ^{99m}Tc-C₃(BHam)₂-HSA was analyzed by CAEP. To a solution of ^{99m}Tc-C₃(BHam)₂-HSA (12.2 kBq) in 0.1 M PB (pH 7.4, 135 μL) was added L-cysteine (15 μL, 5 × 10⁻⁶ to 5 × 10⁻² M). After incubation for 1 h at 37 °C, the radioactivity of the reaction mixture was analyzed by SE-HPLC. ^{99m}Tc-cysteine was prepared by incubating for 1 h at room temperature after mixing a solution of 0.1 M L-cysteine (100 μL) with an aqueous solution of stannous tartrate (75 μL, 3 × 10⁻⁴ M) and Na^{99m}TcO₄ (25 μL, 3.7 MBq).

Experiments in vivo

Animal experiments were conducted in accordance with our institutional guidelines and approved by the Nagasaki University Animal Care Committee. Biodistribution experiments were performed by intravenously administering $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ (37 MBq/mL in 66 mM PB, pH 7.4) or $^{125}\text{I-HSA}$ (37 kBq/mL in 66 mM PB, pH 7.4) to 5-week-old male ddY mice (20–25 g). Groups of four to five mice each were administered 100 μL of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ prior to sacrifice at 5, 10, 30, and 60 min postinjection by decapitation. Tissues of interest were removed and weighed, and radioactivity was measured with an auto well gamma counter. Data in

the biodistribution experiments were analyzed using the unpaired *t*-test. Differences were considered statistically significant when the *p* value was less than 0.05.

The concentration of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ was adjusted to 90 $\mu\text{g/mL}$ with PB (66 mM, pH 7.4). Biodistribution experiments were performed by intravenously administering $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ to 6-week-old male ddY mice (25–30 g). Groups of five to eight mice each were administered 9 μg of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ prior to sacrifice at 5, 10, 30, 60, 180, and 360 min postinjection by decapitation. Tissues of interest were removed and weighed, and radioactivity was measured with an auto well gamma counter.

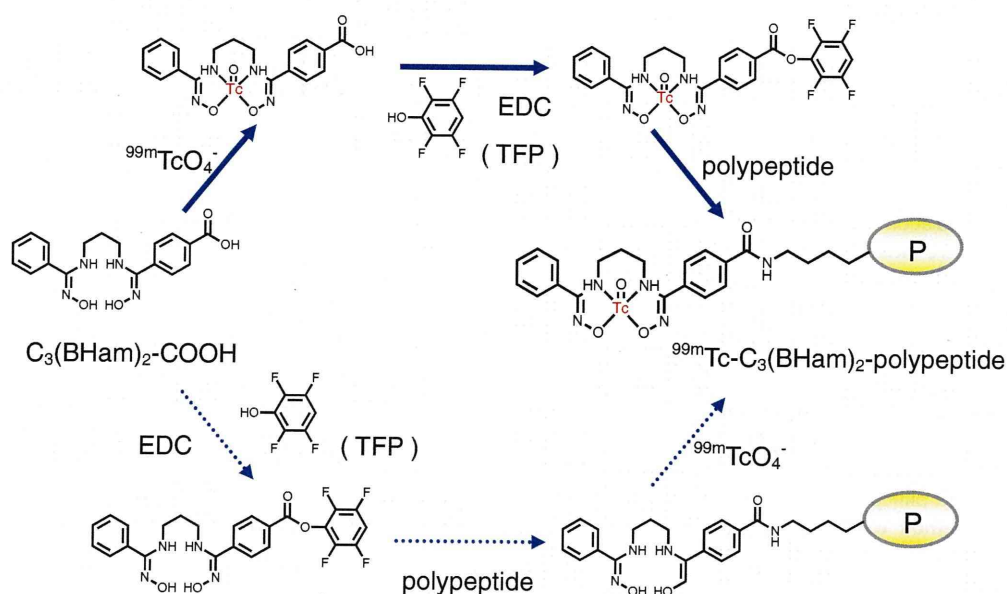
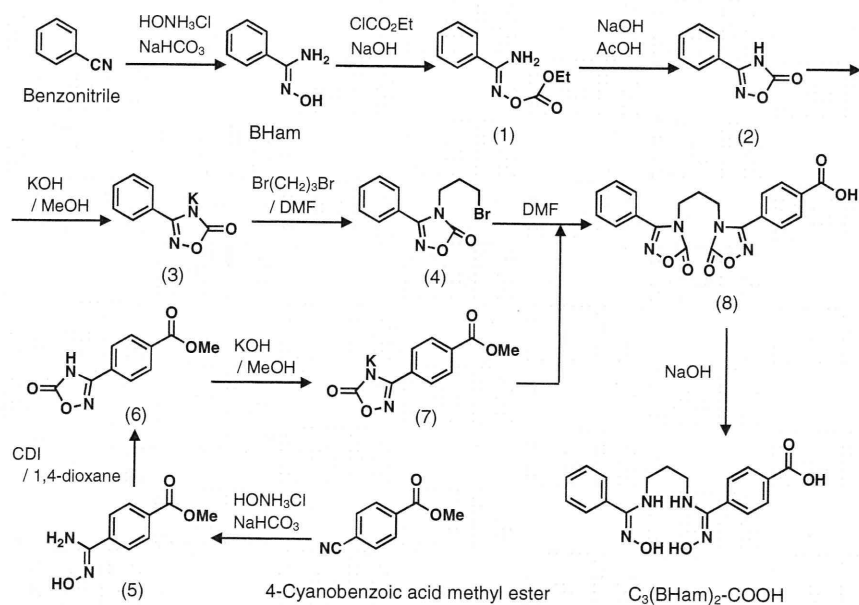


Figure 2. Two schemes for ^{99m}Tc labeling of polypeptides using $\text{C}_3(\text{BHam})_2\text{-COOH}$.



Scheme 1. Synthesis of $\text{C}_3(\text{BHam})_2\text{-COOH}$.

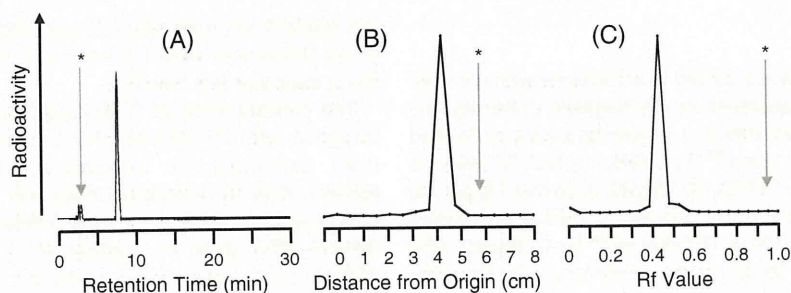


Figure 3. Radiochromatograms of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-COOH}$ obtained by RP-HPLC (A), CAEP (B), and TLC (C). Arrows with single asterisks show $^{99m}\text{TcCO}_4^-$.

Results and discussion

In the present study, in order to introduce $^{99m}\text{Tc-C}_3(\text{BHam})_2$ into polypeptides, we designed and synthesized $\text{C}_3(\text{BHam})_2$ which has a carboxylic acid for binding to the lys- ϵ -amino group of polypeptides. As shown in Figure 1, we tried two methods of preparing ^{99m}Tc -labeled polypeptides by using $\text{C}_3(\text{BHam})_2\text{-COOH}$ as a BCA. Considering the half-life of ^{99m}Tc , and convenience of labeling experiments or radiation exposure, a method of labeling polypeptides with ^{99m}Tc after conjugation with a BCA is preferred. Therefore, first of all, we tried to synthesize a TFP active ester of $\text{C}_3(\text{BHam})_2\text{-COOH}$ in order to conjugate polypeptides with a BCA before ^{99m}Tc labeling (Figure 2, the scheme shown with a dotted line). We performed the reaction of $(\text{BHam})_2\text{-COOH}$ with TFP under several different conditions. However, all the reactions gave multiple products, and we could not obtain $\text{C}_3(\text{BHam})_2\text{-TFP}$. This may be one of the reasons why $\text{C}_3(\text{BHam})_2\text{-TFP}$ reacts with an active secondary amino group or hydroxy group in the hydroxamamide scaffold. As we had difficulty producing $\text{C}_3(\text{BHam})_2\text{-TFP}$, we used an alternative method to prepare ^{99m}Tc -labeled polypeptides, through the conjugation of polypeptides with $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-TFP}$ after active esterification of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-COOH}$ prepared by labeling $\text{C}_3(\text{BHam})_2\text{-COOH}$ with ^{99m}Tc (Figure 2). Previous papers have reported that ^{99m}Tc -labeled polypeptides were successfully prepared using N_2S_2 and N_3S type ligands as BCAs by such a pre-chelating method without a loss of bioactivity.^{13,14,30} Furthermore, as the chemical form of ^{99m}Tc -labeled polypeptides is the same even using different methods, in the present study, we synthesized ^{99m}Tc -labeled polypeptides through the latter approach, and evaluated the utility of $\text{C}_3(\text{BHam})_2\text{-COOH}$ as a BCA for ^{99m}Tc labeling.

$\text{C}_3(\text{BHam})_2\text{-COOH}$ was synthesized by using benzonitrile and 4-cyanobenzoic acid methyl ester as the starting materials according to the route shown in Scheme 1. Compounds **4** and **6** were synthesized as reported previously. **6** was converted to its potassium salt (**7**). **7** was reacted with **4** to give **8**. **8** was hydrolyzed in a 5% NaOH solution to give $\text{C}_3(\text{BHam})_2\text{-COOH}$ in a total yield of 5.7%.

The radioactivity of the reaction mixture of $\text{C}_3(\text{BHam})_2\text{-COOH}$ and ^{99m}Tc was analyzed by RP-HPLC. The radioactivity of $^{99m}\text{TcCO}_4^-$ at 3 min disappeared, and a new peak derived from $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-COOH}$ was detected at a retention time of 7 min (Figure 3(A)). A new peak was detected 4 cm (anode) from the origin in the analysis by CAEP (Figure 3(B)) and at $R_f = 0.4$ in the analysis by TLC (Figure 3(C)). The results suggested that $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-COOH}$ was produced, because radioactivity of $^{99m}\text{TcCO}_4^-$ was detected at 6 cm (anode) by CAEP and at $R_f = 1.0$ by TLC. The radiochemical yield of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-COOH}$ was over 93% in the RP-HPLC, TLC, and CAEP analyses. These results

indicated that $\text{C}_3(\text{BHam})_2\text{-COOH}$ can function as the coordinating site of ^{99m}Tc in mild labeling conditions.

After the active esterification of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-COOH}$ with TFP using EDC, we analyzed the radioactivity eluted from the Sep-Pak column by RP-HPLC. The peak for $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-TFP}$ was observed at a retention time of 14 min, which was later than that

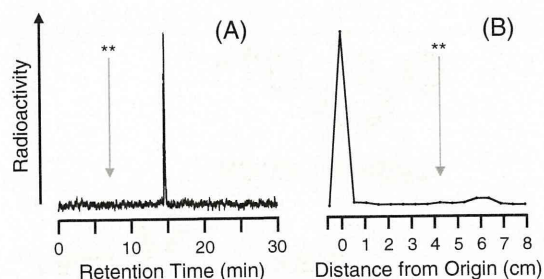


Figure 4. Radiochromatograms of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-TFP}$ obtained by RP-HPLC (A) and CAEP (B). Arrows with double asterisks show $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-COOH}$.

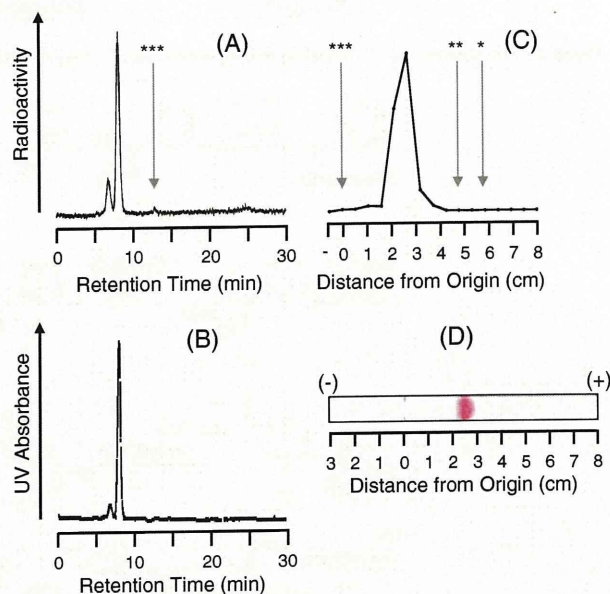


Figure 5. SE-HPLC profiles of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ (radioactivity, A) and unmodified HSA (UV absorbance 254 nm, B). A typical radioactivity profile of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ (C) and Ponceau 3R staining of unmodified HSA analyzed by CAEP (D). Arrows with single, double, and triple asterisks show $^{99m}\text{TcCO}_4^-$, $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-COOH}$, and $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-TFP}$, respectively.

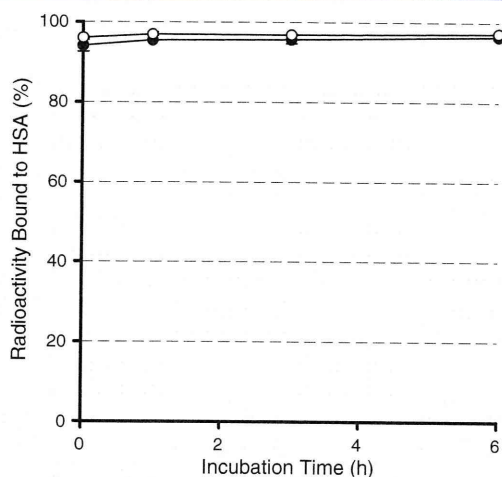


Figure 6. Percent radioactivity in HSA fractions following incubation of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ in phosphate buffer (●) and murine plasma (○) at 37 °C. Each value was determined by CAEP.

of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-COOH}$ (Rt = 7 min) (Figure 4(A)). In the analysis by CAEP, almost all radioactivity existed at the origin different from the site where the radioactivity of $^{99m}\text{TcO}_4^-$ and $\text{C}_3(\text{BHam})_2\text{-COOH}$ was detected, suggesting the production of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-TFP}$ (Figure 4(B)). The recovery rate of radioactivity was ca. 50% after purification by Sep-Pak, and the rest remained in the Sep-Pak column.

We performed by gel filtration chromatography after the conjugation of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-TFP}$ with HSA and analyzed the radioactivity eluted from the gel filtration column by SE-HPLC and CAEP (Figure 5). In the SE-HPLC analysis, the peaks of radioactivity at retention times of 7 (dimer) and 8 min (monomer) corresponded with the peaks of UV (254 nm) for unmodified HSA (Figure 7(A, B)). In the CAEP analysis, over 95% of radioactivity was detected at the same position as HSA stained with Ponceau 3R (Figure 5(C, D)). The radiochemical yield and purity were

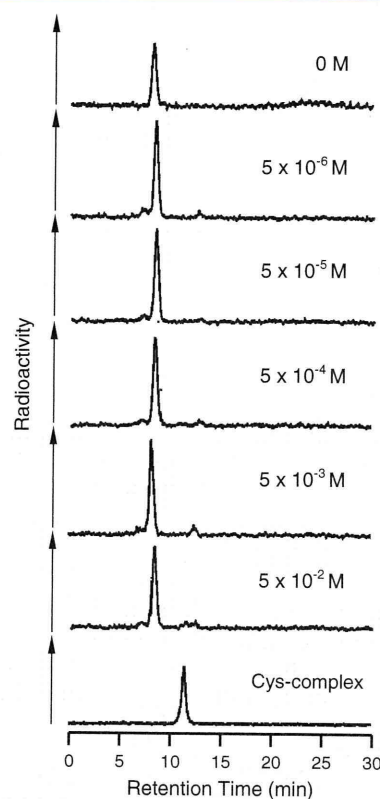


Figure 7. SE-HPLC profiles of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ after incubation with L-cysteine for 1 h at 37 °C.

ca. 75% and >95%, respectively. The results suggested that $\text{C}_3(\text{BHam})_2\text{-COOH}$ may serve as a BCA for the labeling of polypeptides with ^{99m}Tc .

Next, we tested the stability of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ in PB or murine plasma at 37 °C by CAEP (Figure 6). The amount of radioactivity bound to HSA was over 95% until 6 h. We also

Table 1. Biodistribution of radioactivity after injection of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ in mice

Tissue	Percentage of injected dose per tissue				
	5 min	10 min	30 min	1 h	3 h
Blood ^a	35.60 (2.69)	32.92 (2.01)	31.42 (1.47)	27.94 (3.17)	19.54 (2.62)
Liver	7.39 (1.60)	8.72 (1.09)	8.07 (0.70)	7.28 (1.07)	5.68 (0.62)
Kidney	1.68 (0.41)	1.47 (0.29)	1.88 (0.21)	1.76 (0.29)	1.31 (0.08)
Intestine	2.04 (0.45)	2.62 (0.35)	4.81 (0.39)	7.27 (1.18)	11.56 (0.78)
Spleen	0.33 (0.01)	0.35 (0.05)	0.32 (0.06)	0.30 (0.04)	0.21 (0.03)
Stomach	0.51 (0.10)	0.63 (0.02)	1.00 (0.24)	1.02 (0.10)	0.95 (0.11)
Lung	3.50 (0.47)	3.65 (0.72)	2.62 (0.38)	3.13 (0.44)	1.80 (0.52)

Each value represents the mean (SD) for four to five animals.

^aExpressed as% injected dose per gram.

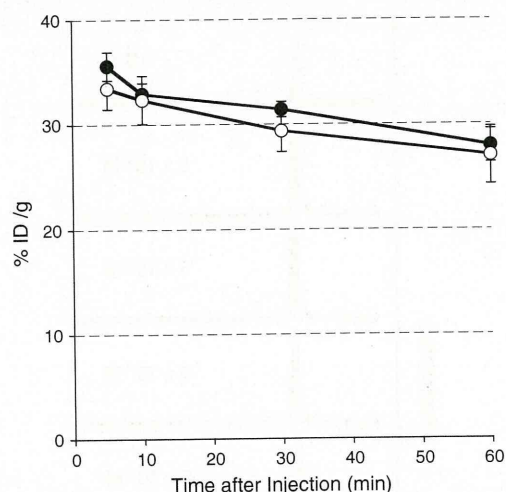


Figure 8. Blood clearance of radioactivity after injection of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ (●) and $^{125}\text{I-HSA}$ (○) in mice.

analyzed the radioactivity of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ by SE-HPLC after incubation with excess L-cysteine (Figure 7). No marked change in the peak of radioactivity was observed at 5×10^{-5} M, 5×10^{-4} M, 5×10^{-3} M, or 5×10^{-2} M of L-cysteine. These results suggested that ^{99m}Tc stably binds to HSA via $\text{C}_3(\text{BHam})_2\text{-COOH}$.

Furthermore, we determined the biodistribution of radioactivity after the injection of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ into mice (Table 1). At 5-min postinjection, 36% ID/g was observed in the blood, and at 180 min, 20% ID/g. The profile of radioactivity was similar to that for $^{125}\text{I-HSA}$ (Figure 8). The results suggested that $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ may reflect the pharmacokinetics of HSA without the dissociation of ^{99m}Tc from HSA, and in addition, $\text{C}_3(\text{BHam})_2\text{-COOH}$ serves as a BCA, which can give stable ^{99m}Tc -labeled polypeptides. As no marked increase in radioactivity was detected in the stomach and lungs, the reoxidation to $^{99m}\text{TcO}_4^-$ or production of ^{99m}Tc -colloid did not seem to occur, supporting the notion that $\text{C}_3(\text{BHam})_2\text{-COOH}$

serves as a BCA that produces stable ^{99m}Tc -labeled polypeptides *in vivo*.

When radiolabeled polypeptides were administered *in vivo*, persistently high levels of radioactivity were observed in the liver and kidney where catabolism of the parent proteins and peptides occurs, which compromises the diagnostic accuracy of these radiopharmaceuticals. Previous investigations regarding the radiometabolites produced in the liver and kidney indicated that slow elimination rates are responsible for the persistent localization of radioactivity in the lysosomal compartment of hepatic and renal cells.^{31–34} Several recent studies suggested that an experimental system using NGA, which is incorporated by hepatic parenchymal cells via receptor-mediated endocytosis immediately after its administration, is suitable for investigating radiometabolites formed in the liver.^{31–34} To investigate the pharmacokinetics of the radiometabolites produced in the metabolic tissues after the injection of ^{99m}Tc -labeled polypeptides by using $\text{C}_3(\text{BHam})_2\text{-COOH}$ as a BCA, NGA was selected as a model polypeptide, $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ was prepared, and the biodistribution in mice was evaluated. The biodistribution of radioactivity after the injection of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ is summarized in Table 2. At 5-min postinjection, more than 89% of the radioactivity was accumulated in the liver. The radioactivity was rapidly eliminated from the liver by hepatobiliary excretion. At 6-h postinjection, the radioactivity retained in the liver was just 7% of the injected dose. No marked accumulation in the blood or the other tissues was observed.

We compared the clearance of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ from the liver with that of $^{99m}\text{Tc-(HYNIC-NGA)(tricine)}_2$ (Figure 9).¹⁷ Radioactivity in the liver cleared faster after the injection of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ than that of $^{99m}\text{Tc-(HYNIC-NGA)(tricine)}_2$, indicating that ^{99m}Tc -labeled polypeptides prepared with $\text{C}_3(\text{BHam})_2\text{-COOH}$ produce radiometabolites, which show rapid elimination from the liver. We have not determined the radiometabolites produced in the liver after the injection of ^{99m}Tc -labeled polypeptides by using $\text{C}_3(\text{BHam})_2\text{-COOH}$ as a BCA. Previous studies using ^{111}In -labeled NGAs with cDTPA or SCN-Bz-EDTA or $^{99m}\text{Tc-HYNIC-NGA}$ showed that radiolabeled NGAs generated lysine-adducts ($[^{111}\text{In}]$

Table 2. Biodistribution of radioactivity after injection of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ in mice

Tissue	Percentage of injected dose per tissue					
	5 min	10 min	30 min	1 h	3 h	6 h
Blood ^a	1.35 (0.28)	0.89 (0.39)	1.84 (0.39)	1.49 (0.48)	0.98 (0.42)	0.75 (0.31)
Liver	89.43 (4.15)	80.52 (7.71)	59.96 (2.79)	32.66 (2.87)	14.64 (3.05)	7.12 (0.62)
Kidney	0.36 (0.11)	0.33 (0.04)	0.45 (0.09)	0.53 (0.13)	0.50 (0.13)	0.36 (0.01)
Intestine	0.93 (0.03)	2.28 (0.34)	22.33 (1.87)	43.66 (6.16)	62.44 (4.05)	27.74 (11.02)
Spleen	0.19 (0.07)	0.16 (0.03)	0.18 (0.03)	0.14 (0.01)	0.13 (0.06)	0.11 (0.03)
Stomach	0.41 (0.13)	0.51 (0.11)	0.58 (0.16)	0.74 (0.28)	0.51 (0.22)	0.27 (0.09)
Lung	0.24 (0.13)	0.17 (0.03)	0.19 (0.05)	0.21 (0.03)	0.21 (0.01)	0.12 (0.03)

Each value represents the mean (SD) for five to eight animals.

^aExpressed as % injected dose per gram.

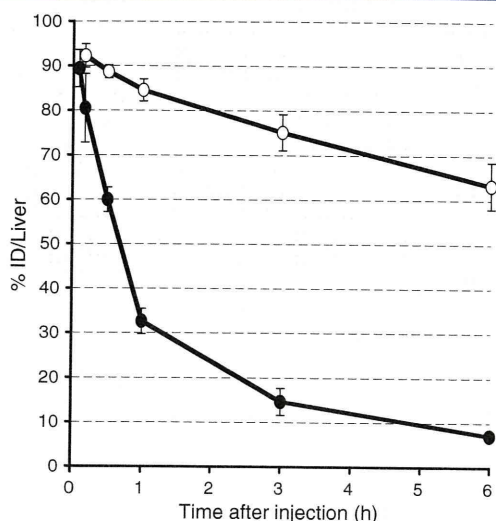


Figure 9. Elimination of radioactivity from the liver after injection of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ (●) and $^{99m}\text{Tc-(HYNIC-NGA)(tricine)}_2$ (○) (data from Abrams *et al.*¹⁶) in normal mice.

DTPA-lysine, [^{111}In]SCN-Bz-EDTA-lysine, and $^{99m}\text{Tc-HYNIC-lysine}$) as the major final radiometabolites in murine hepatocytes.^{31,32,35,36} As $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-COOH}$ was also conjugated to the ϵ -amine residues of NGA, it was speculated that a lysine adduct of $^{99m}\text{Tc-C}_3(\text{BHam})_2$ may be produced in the liver as the final radiometabolite. The rapid elimination of radioactivity after the injection of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ may reflect the rapid clearance of the final radiometabolites from the lysosomal compartment of hepatocytes. Although ^{99m}Tc -labeled polypeptides showed high and persistent levels of radioactivity in the liver and kidney, the present results observed for $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ suggested that $\text{C}_3(\text{BHam})_2\text{-COOH}$ is a useful BCA for labeling with ^{99m}Tc to reduce the non-specific accumulation in the liver observed for $^{99m}\text{Tc-HYNIC}$ -polypeptides. Low molecular weight polypeptides including single-chain Fv fragments, diabodies, affibodies, minibodies, and bioactive small peptides are attractive scaffolds of ^{99m}Tc -labeled probes for targeted imaging because of the pharmacokinetic properties of these molecules. As ^{99m}Tc has an appropriate half-life to label such polypeptides, the application of $\text{C}_3(\text{BHam})_2\text{-COOH}$ as a BCA to low molecular weight polypeptides is expected in the future.

In conclusion, we successfully designed and synthesized $\text{C}_3(\text{BHam})_2\text{-COOH}$ as a new BCA for ^{99m}Tc labeling of polypeptides. $\text{C}_3(\text{BHam})_2\text{-COOH}$ formed a stable ^{99m}Tc complex and enabled us to prepare ^{99m}Tc -labeled polypeptides by using a TFP active ester of $\text{C}_3(\text{BHam})_2\text{-COOH}$. $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ existed stably in murine plasma and an excess of L-cysteine without any dissociation of ^{99m}Tc from polypeptides. Furthermore, as the blood clearance of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-HSA}$ in mice was similar to that of $^{125}\text{I-HSA}$, $\text{C}_3(\text{BHam})_2\text{-COOH}$ retained stable binding between ^{99m}Tc and polypeptide *in vivo*. When we determined radioactivity after the injection of $^{99m}\text{Tc-C}_3(\text{BHam})_2\text{-NGA}$ into mice, we found high liver uptake early on and rapid clearance from the liver, indicating that $\text{C}_3(\text{BHam})_2\text{-COOH}$ did not affect the pharmacokinetics of polypeptides *in vivo* and gave radiometabolites which showed rapid elimination from the liver. Such characteristics would render $\text{C}_3(\text{BHam})_2\text{-COOH}$ attractive as a new BCA for ^{99m}Tc labeling of polypeptides.

Acknowledgements

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Conflict of Interest

The authors did not report any conflict of interest.

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^{18}F -Labeled Phenyldiazenyl Benzothiazole for in Vivo Imaging of Neurofibrillary Tangles in Alzheimer's Disease Brains

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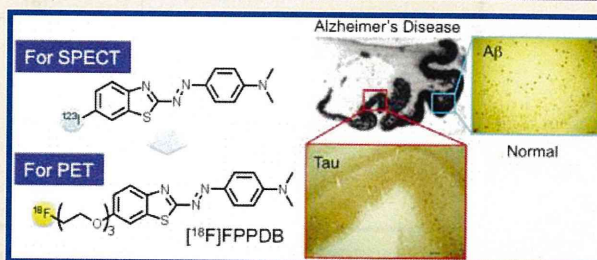
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Supporting Information

ABSTRACT: We synthesized and evaluated (*E*)-4-((6-(2-(2-fluoroethoxy)ethoxy)ethoxy)benzo[*d*]thiazol-2-yl)-diazenyl)-*N,N*-dimethylaniline (FPPDB) as a probe for the imaging of neurofibrillary tangles (NFTs) in patients with Alzheimer's disease (AD). In assays using thioflavin S (ThS) as a competitive ligand, FPPDB competed with ThS well and showed high affinity for both tau and $A\beta_{1-42}$ aggregates ($K_i = 13.0$ and 20.0 nM, respectively). The results of saturation binding assays also verified that FPPDB bound to both tau and $A\beta_{1-42}$ aggregates with high affinity ($K_d = 44.8$ nM and $B_{max} = 45.8$ pmol/nmol protein for tau aggregates and $K_d = 45.4$ nM and $B_{max} = 38.9$ pmol/nmol protein for $A\beta_{1-42}$ aggregates). Furthermore, [^{18}F]FPPDB substantially labeled NFTs and senile plaques in AD brain sections but not control brain sections. In biodistribution experiments using normal mice, [^{18}F]FPPDB displayed higher uptake (4.28% ID/g at 2 min postinjection) into and washout (2.53% ID/g at 60 min postinjection) from the brain with time. On the basis of the chemical structure of FPPDB, further increases in selective binding to tau aggregates may lead to the development of more useful probes for the imaging of NFTs in AD brains.

KEYWORDS: Alzheimer's disease (AD), neurofibrillary tangles (NFTs), imaging, benzothiazole, PET



Alzheimer's disease (AD) is a progressive neurodegenerative brain disorder associated with cognitive decline, disorientation, and language impairment and is characterized by the presence of senile plaques (SPs) composed of β -amyloid ($A\beta$) peptides and neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein.¹ At present, the clinical diagnosis of AD depends on medical history and neuropsychological findings, and the early cognitive and behavioral symptoms of AD are often indistinguishable from normal signs of aging. Because a definite diagnosis of AD is based on the postmortem histopathological examination of SPs and NFTs, useful methods of evaluating the histopathological changes in vivo are strongly needed. The formation of SPs is considered an initial manifestation of AD. Therefore, considerable effort has focused on the development of imaging probes targeting SPs for positron emission tomography (PET) and single photon emission computed tomography (SPECT). Among them, PET/SPECT probes such as IMPY,² SB-13,³ PIB,⁴ AZD2184,⁵ FDDNP,⁶ BAY94-9172,⁷ AV-45,⁸ and GE-067⁹ have been tested clinically and demonstrated the potential

utility of the in vivo imaging of SPs in the brain. Many other compounds with structural similarities have also been reported.

Since the accumulation of NFTs is highly correlated with symptoms of AD^{10,11} and the detection of NFTs in the brain should lead to the early diagnosis of AD and the evaluation of severity and staging, the development of NFT-selective binding probes is needed. However, there have been few reports on the development of PET/SPECT imaging probes targeting NFTs.

A previous paper has reported quinoline and benzimidazole derivatives as candidate probes for the imaging of NFTs in AD brains.^{12,13} However, these derivatives showed affinity for both NFTs and SPs, suggesting that they may not be NFT-selective tracers. Therefore, to investigate the selective binding affinity for both NFTs and SPs, we recently developed radioiodinated compounds based on another chemical structure, a phenyldiazenyl benzothiazole (PDB) scaffold (Figure 1).¹⁴ All of the PDB derivatives displayed high affinity for tau aggregates. PDB-

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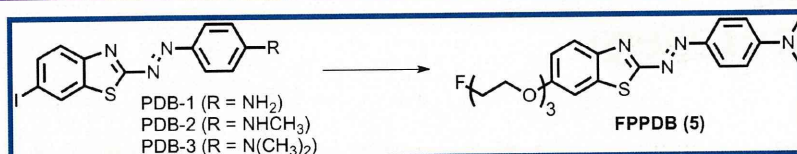
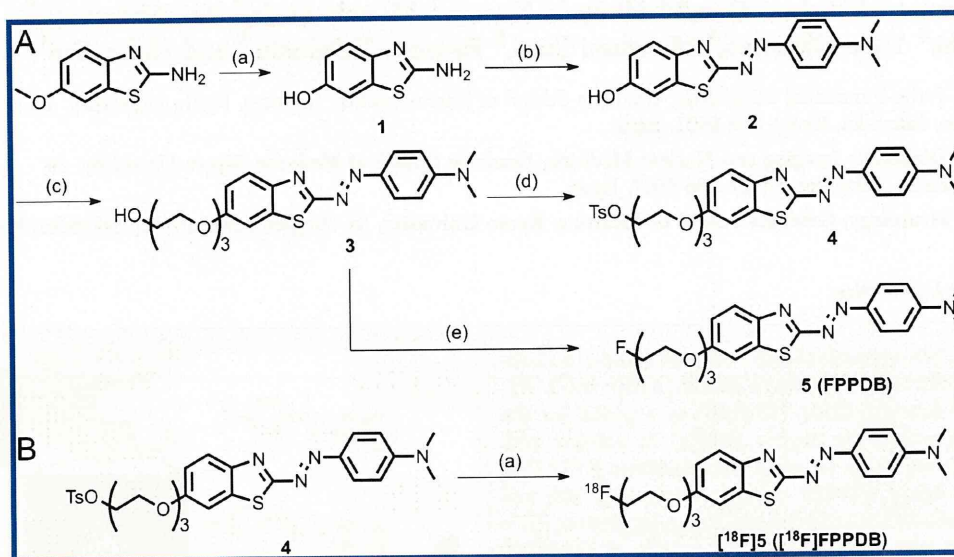


Figure 1. Chemical structure of iodinated and fluoro-pegylated PDB derivatives.

Scheme 1. ^a



^a(A) Reagents and conditions: (a) HBr, reflux. (b) *N,N*-Dimethylaniline, NaNO₂, 50% H₂SO₄, concentrated HCl, 0 °C. (c) 2-[2-(2-Chloroethoxy)ethoxy]ethanol, K₂CO₃, DMF, 105 °C. (d) Tosyl chloride, pyridine. (e) Diethylamino sulfur trifluoride, 1,2-dimethoxyethane. (B) Reagents and conditions: (a) ¹⁸F⁻, Kryptofix222, K₂CO₃, acetonitrile, 100 °C, 5 min.

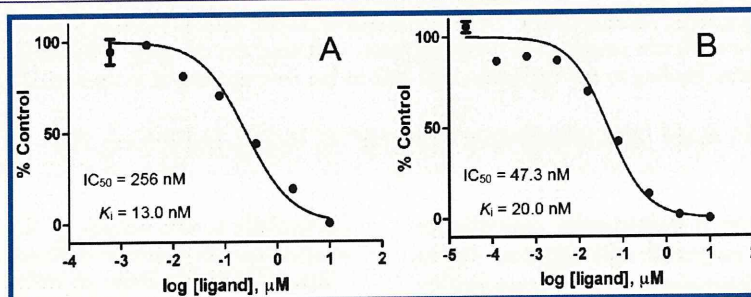


Figure 2. Inhibition curves for the binding of ThS to tau (A) and A β_{1-42} (B) aggregates using FPPDB as a test compound.

3, with a dimethylamino group at position 4 of the phenyl group, showed the highest affinity (17.2-fold that for A β_{1-42} aggregates), indicating it to be a tracer with greater selective binding to tau aggregates than the quinoline and benzimidazole derivatives reported previously. However, ¹²⁵I-labeled PDB derivatives showed a relatively low uptake into and slow washout from the brain, suggesting high nonspecific binding, which would contribute to a high level of background noise. Since the slow washout of [¹²⁵I]PDB derivatives in normal mice prevents imaging *in vivo*, the improved property of the PDB derivatives should make them better candidates for the study of tau aggregates.

Previous studies regarding uptake into and clearance from the brain points to high lipophilicity as one of the reasons for a slow washout.^{15,16} We then planned to develop a novel PDB derivative with less lipophilicity by substituting iodine, which

increases the lipophilicity of a compound, with fluorine for the preparation of PET tracers. Recent reports have introduced a new approach, fluoro-pegylation (FPEG) of the core structure, to labeling with ¹⁸F.^{7,17,18} Because this approach offers a simple and easy way to incorporate ¹⁸F without any increase in lipophilicity, we selected FPEG for ¹⁸F-labeling of the PDB scaffold. In the present study, we designed and synthesized a novel fluorinated ligand, (*E*)-4-((6-(2-(2-(2-fluoroethoxy)ethoxy)ethoxy)benzo[*d*]thiazol-2-yl)diazenyl)-*N,N*-dimethylaniline (FPPDB, Figure 1) with a fluoro-polyethylene glycol side chain instead of iodine at position 6 of PDB-3, and evaluated its potential as a probe for the imaging NFTs in the brains *in vivo*.

A new ligand, FPPDB, was prepared as outlined in Scheme 1A. A methoxy group of 2-amino-6-methoxybenzothiazole was converted to a hydroxy group using a HBr solution, which afforded **1** in a yield of 71.0%. To obtain the backbone

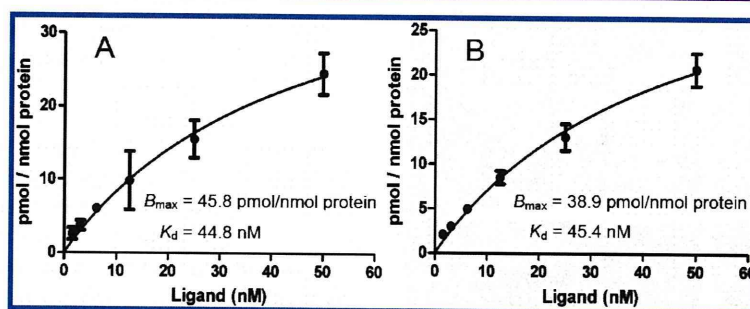


Figure 3. Saturation curves of $[^{18}\text{F}]$ FPPDB for tau (A) and $A\beta_{1-42}$ (B) aggregates.

structure of PDB, we used a diazo coupling reaction with *N,N*-dimethylaniline to afford **2**. Thereafter, 2-[2-(2-chloroethoxy)ethoxy]ethanol was coupled with the OH group of **2** to obtain **3**. Fluorination of **3** to prepare **5** was achieved using diethylamino sulfur trifluoride (DAST). ^{18}F -labeling of **5** was performed on a tosyl precursor (**4**) undergoing a nucleophilic displacement reaction with the fluoride anion (Scheme 1B). Radiolabeling with ^{18}F was successfully performed on the precursor to generate $[^{18}\text{F}]\mathbf{5}$ ($[^{18}\text{F}]$ FPPDB) with a radiochemical yield of 35% and radiochemical purity >99%. The identity of $[^{18}\text{F}]$ FPPDB was verified by a comparison of retention time with the nonradioactive compound.

To evaluate the affinity of FPPDB for both tau aggregates and $A\beta_{1-42}$ aggregates, an assay using thioflavin-S (ThS) as a competitive ligand was carried out. Similarly to iodinated PDB derivatives reported previously, FPPDB competed well with ThS to bind to tau aggregates (Figure 2A). The K_i value of FPPDB for tau aggregates was estimated at 13.0 nM. Although FPPDB exhibited significantly lower affinity than PDB-3 ($K_i = 0.48$ nM), it still maintained high enough affinity for tau aggregates to image NFTs. Because both tau and $A\beta_{1-42}$ aggregates possess a β -sheet structure, it would be important to examine the selectivity of FPPDB. To this end, we determined the affinity of FPPDB for $A\beta_{1-42}$ aggregates using a competitive inhibition assay with ThS. FPPDB also inhibited the binding of ThS to $A\beta_{1-42}$ aggregates (Figure 2B), the K_i value being 20.0 nM. The ratio of the K_i values for tau and $A\beta_{1-42}$ aggregates was 1.54. As compared to PDB-3 (ratio of 17.2),¹⁴ FPPDB exhibited less selectivity between tau and $A\beta_{1-42}$ aggregates. The results suggest that the substituted group at position 6 in the PDB scaffold plays an important role in the binding to β -sheet structures in both tau and $A\beta_{1-42}$ aggregates. The concentration of tau aggregates (~ 150 – 300 pmol mg^{-1} of wet tissue) was reported to be higher than that of $A\beta$ aggregates (~ 9 pmol mg^{-1} of wet tissue) in the frontal and temporal cortices in late-stage AD,^{19,20} so it may be possible for $[^{18}\text{F}]$ FPPDB to show contrast between NFTs and SPs in these areas in vivo. However, to diagnose AD in the early stages, probes with much higher NFT selectivity will be needed.

In competitive inhibition assays, FPPDB competed with ThS to bind to both tau and $A\beta_{1-42}$ aggregates. To verify that FPPDB bound to these aggregates directly, saturation binding assays of $[^{18}\text{F}]$ FPPDB to these aggregates were carried out (Figure 3). A Scatchard analysis revealed the K_d value and B_{max} of FPPDB for both tau and $A\beta_{1-42}$ aggregates to be almost equal ($K_d = 44.8$ nM and $B_{\text{max}} = 45.8$ pmol/nmol tau protein for tau aggregates and $K_d = 45.4$ nM and $B_{\text{max}} = 38.9$ pmol/nmol $A\beta_{1-42}$ protein for $A\beta_{1-42}$ aggregates). This result showed that FPPDB had almost equal affinity for tau and $A\beta_{1-42}$

aggregates, which was reflected the K_i values of FPPDB for both aggregates in the competitive inhibition assays using ThS.

$[^{18}\text{F}]$ FPPDB was investigated for its affinity for NFTs in vitro by autoradiography in human AD brain sections (Figure 4).

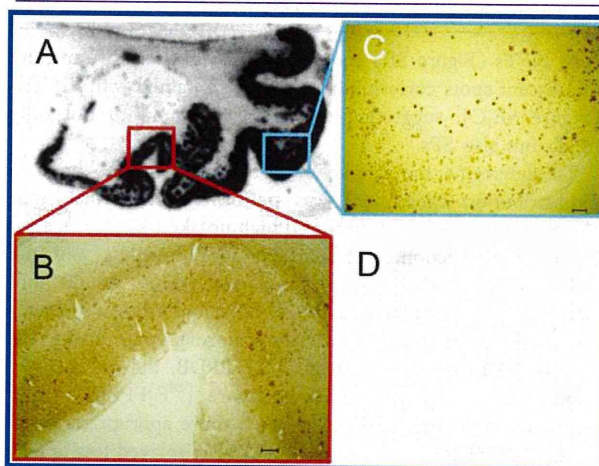


Figure 4. Autoradiogram of $[^{18}\text{F}]$ FPPDB (A) and immunohistochemical staining with antibodies against hyperphosphorylated tau (B) and $A\beta_{1-42}$ (C) in brain sections from the same patient. Autoradiogram of a control brain (D). Bars indicate 200 μm .

Autoradiographic images of $[^{18}\text{F}]$ FPPDB showed the accumulation of radioactivity in the AD brain sections with little nonspecific binding in white matter (Figure 4A). The accumulation corresponded with the results of immunohistochemical staining with both the anti phosphorylated tau antibody (AT8) and the anti $A\beta_{1-42}$ antibody (BC05) (Figure 4B,C, respectively). Conversely, $[^{18}\text{F}]$ FPPDB showed almost no accumulation in normal human brain sections (Figure 4D). These results suggested that $[^{18}\text{F}]$ FPPDB had enough affinity to label NFTs in AD brain sections, although its affinity for tau aggregates was lower than that of iodinated PDB derivatives. However, the results also indicated that $[^{18}\text{F}]$ FPPDB did not possess enough selectivity for NFTs to show high contrast between NFTs and SPs in an autoradiographic study, similarly to the iodinated PDB derivatives reported previously.

To further confirm the affinity of FPPDB for NFTs and SPs in the AD brain, fluorescent staining was carried out using brain sections from the same AD patient (Figure 5). Numerous fluorescent spots were detected in the entorhinal cortex of AD brain sections (Figure 5A,C) as reflected by the high affinity for recombinant tau and $A\beta_{1-42}$ aggregates in both competitive inhibition and saturation binding assays in vitro. ThS stained

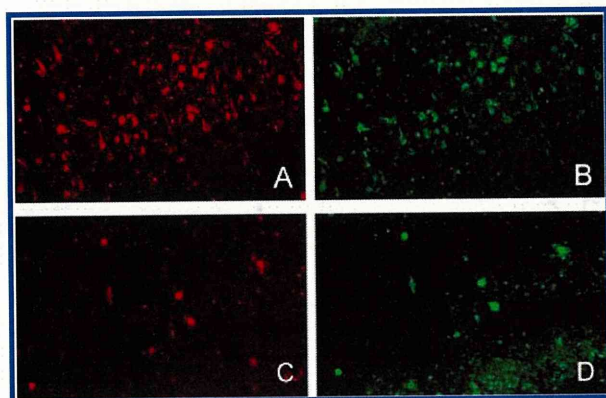


Figure 5. Fluorescent staining with FPPDB (A and C) and ThS (B and D) in the entorhinal cortex of AD brain sections. Many NFTs were clearly stained with FPPDB (A) and ThS (B). SPs were also stained with FPPDB (C) and ThS (D).

both NFTs (Figure 5B) and SPs (Figure 5D) clearly, and the fluorescent spots corresponded to those obtained with FPPDB (Figure 5A,C), suggesting that FPPDB bound to not only NFTs but also SPs.

To determine the uptake of [^{18}F]FPPDB in the brain, a biodistribution experiment was performed in normal mice (Table 1). [^{18}F]FPPDB displayed high uptake (4.28% ID/g) at 2 min postinjection, sufficient for PET imaging, and the radioactivity in the brain cleared with time. At 60 min postinjection, the uptake was 2.53% ID/g, indicating a relatively fast washout from the brain. Since normal brain tissue has neither NFTs nor SPs to trap [^{18}F]FPPDB, the radioactivity should wash out quite rapidly. Therefore, [^{18}F]FPPDB's rapid clearance from normal brain makes it more appropriate as an imaging agent for AD brain. [^{125}I]PDB-3 displayed less uptake into and a slower washout from the brain (0.94 and 2.89% ID/g at 2 and 60 min postinjection, respectively) than [^{18}F]FPPDB.¹⁴ The improved pharmacokinetics of [^{18}F]FPPDB were achieved by replacing iodine with a fluoro-pegylated group at position 6. The log *P* value of [^{18}F]FPPDB and [^{125}I]PDB-3 was 2.05 and 3.84, respectively, suggesting that [^{18}F]FPPDB is less lipophilic than [^{125}I]PDB-3. Although lipophilicity is just one of the factors influencing the uptake of a compound into the brain, it may explain in the favorable pharmacokinetics of [^{18}F]FPPDB. However, as compared with several $A\beta$ imaging

probes under clinical study such as [^{18}F]BAY94-9172⁷ and [^{18}F]AV-45,⁸ [^{18}F]FPPDB's uptake into and washout from the brain were not particularly satisfactory. The replacement of the dimethylamino group of [^{18}F]FPPDB with a less lipophilic group may lead to the development of more promising probes for diagnosing AD. Because defluorination, as reflected by the uptake of [^{18}F]FPPDB into bone, was low (1.82–1.92% ID/g), interference with the imaging is expected to be relatively minor. [^{18}F]FPPDB was cleared from plasma by not only the hepatobiliary system (20.2% ID/g in the liver at 2 min postinjection) but the renal system (13.9% ID/g in the kidney at 2 min postinjection). The hepatobiliary excretion to the intestines was also rather fast, and radioactivity was observed to accumulate within the intestine at later time points (22.9% ID/g at 60 min postinjection).

In conclusion, we designed, synthesized, and evaluated a PDB derivative, [^{18}F]FPPDB, as a novel PET imaging agent for diagnosing AD. In binding experiments in vitro, the derivative displayed high affinity for both tau and $A\beta_{1-42}$ aggregates. NFTs and SPs were stained in experiments using autoradiography and fluorescent staining with AD brain sections, reflecting the results of the in vitro assays. Although FPPDB had lower selectivity for tau aggregates than PDB-3 in vitro, in biodistribution experiments using normal mice, [^{18}F]FPPDB had improved pharmacokinetics as compared with [^{125}I]PDB derivatives. Replacement of iodine with fluorine in the PDB scaffold was highly effective in improving the radioactive pharmacokinetics of [^{18}F]FPPDB in the brain. However, it resulted in a decrease in selective binding for tau aggregates. Further structural optimization based on the PDB scaffold, such as changing the position substituted in the fluoro-pegylated group or replacing the dimethylamino group with different groups to improve the affinity for tau aggregates may lead to the development of more useful probes for the in vivo imaging of NFTs in AD brains.

■ ASSOCIATED CONTENT

5 Supporting Information

Full experimental procedures and characterization data for all new compounds described in this study. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Table 1. Biodistribution of Radioactivity after Injection of [^{18}F]FPPDB in Normal Mice^a

tissue	time after injection (min)			
	2	10	30	60
blood	3.04 (0.40)	1.83 (0.82)	2.19 (0.75)	2.68 (0.63)
liver	20.2 (1.19)	19.3 (1.00)	13.1 (1.45)	9.32 (1.51)
kidney	13.9 (1.50)	7.67 (3.42)	5.08 (3.67)	4.00 (3.75)
intestine	2.94 (0.38)	4.99 (0.95)	14.4 (1.44)	22.9 (1.38)
spleen	3.47 (0.80)	3.41 (0.80)	2.71 (0.80)	2.14 (0.76)
pancreas	5.41 (0.69)	4.01 (1.03)	2.87 (0.63)	2.18 (0.67)
heart	7.45 (0.84)	4.14 (1.87)	3.05 (1.64)	2.62 (1.45)
lung	13.5 (3.51)	4.37 (4.60)	3.36 (3.70)	3.59 (3.18)
stomach ^b	1.11 (0.07)	1.42 (0.35)	2.69 (0.44)	3.37 (1.26)
brain	4.28 (0.45)	4.26 (0.37)	3.47 (0.54)	2.53 (0.54)
bone	1.88 (0.47)	1.82 (0.50)	1.82 (0.49)	1.92 (0.33)

^aEach value represents the mean (SD) for five animals. ^bExpressed as % injected dose per organ.

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Notes

The authors declare no competing financial interest.

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BODIPY-Based Molecular Probe for Imaging of Cerebral β -Amyloid Plaques

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Supporting Information

ABSTRACT: We designed and synthesized a BODIPY-based probe (BAP-1) for the imaging of β -amyloid plaques in the brain. In binding experiments *in vitro*, BAP-1 showed excellent affinity for synthetic $A\beta$ aggregates. β -Amyloid plaques in Tg2576 mouse brain were clearly visualized with BAP-1. In addition, the labeling of β -amyloid plaques was demonstrated *in vivo* in Tg2576 mice. These results suggest BAP-1 to be a useful fluorescent probe for the optical imaging of cerebral β -amyloid plaques in patients with Alzheimer's disease.

KEYWORDS: Alzheimer's disease, β -amyloid plaque, BODIPY, optical imaging



The formation of β -amyloid ($A\beta$) plaques is a critical neurodegenerative change in Alzheimer's disease (AD).^{1,2} Since the imaging of $A\beta$ plaques *in vivo* may enable the pre-symptomatic diagnosis of AD, several imaging technologies including positron emission tomography (PET),^{3–21} single photon emission computed tomography (SPECT),^{22–24} magnetic resonance imaging,^{25–28} and optical imaging^{29–33} have been applied for this purpose. In particular, several PET probes have shown the feasibility of imaging $A\beta$ plaques in AD brains. PET imaging is an established clinical modality that provides good sensitivity deep in tissue. However, it is limited by a time-consuming data acquisition process, exposure to radioactivity, the need for expensive equipment and highly skilled personnel, and a relatively poor spatial resolution.

Conversely, optical imaging with fluorescent probes is a relatively new modality that offers real-time, nonradioactive, and, depending on the technique, high-resolution imaging,³⁴ leading to a rapid, inexpensive, and nonradioactive drug screening system for AD. However, there have been fewer reports regarding the development of fluorescent probes than PET probes despite their significance, although AOI-987,²⁹ NIAD-4,³⁰ CRANAD-2,³² ANCA-11,³⁵ and BMAOI³⁶ have been reported for the imaging of $A\beta$ plaques.

Compounds containing a boron dipyrromethane (BODIPY) scaffold have widespread applications as dyes, fluorescent probes in biological systems, and materials for incorporation into electroluminescent devices.^{37–40} Their broad utility is due to their high thermal and photochemical stability, chemical robustness, and tunable fluorescence properties. We have previously reported a dual SPECT/fluorescent probe based on the BODIPY scaffold, for the imaging of $A\beta$ plaques *in vivo*.⁴¹ Despite good affinity for synthetic $A\beta(1–42)$ aggregates and the clear labeling of $A\beta$ plaques in sections of the mouse brain, the BODIPY-based probe was not suitable for imaging *in vivo* due to its poor uptake into the brain. Two other papers have

reported BODIPY-based probes targeting $A\beta$ plaques.^{42,43} However, these derivatives have not been applied to imaging *in vivo* perhaps due to their low brain uptake and short excitation/emission wavelength, though they showed high affinity for $A\beta$ plaques *in vitro*. The findings of these previous studies suggested that additional structural changes may modify the properties of BODIPY derivatives to improve their suitability for imaging *in vivo*.

Many $A\beta$ -imaging probes for PET applied in clinical trials possess a dimethylamino styryl group as a consensus structure as reported previously.^{3,22,44–46} Then, in the present study, we designed and synthesized a new BODIPY-based $A\beta$ probe (BAP-1) with a dimethylamino styryl group which plays an important role in binding to $A\beta$ aggregates. BAP-1 belongs to a class of dyes that are collectively called molecular rotors, where the dimethyl-aniline is the donor, and the BODIPY unit is the acceptor.⁴⁷ This motif is typical of $A\beta$ -imaging probes. Here, we report the *in vitro* and *in vivo* evaluation of BAP-1 as a new probe for the optical imaging of cerebral $A\beta$ plaques.

RESULTS AND DISCUSSION

The target BODIPY derivative (BAP-1) was synthesized as shown in Scheme 1. Although the synthetic route for this compound has been reported,³⁷ we made some modifications. The key step in the formation of the BODIPY backbone (**3**) was accomplished by the condensation of pyrrole 2-carboxyaldehyde (**1**) and 2,4-dimethylpyrrole (**2**) at low temperature, followed by the addition of $\text{BF}_3 \cdot \text{OEt}_2$ in a yield of 36.1%. Compound **4** (BAP-1) was successfully prepared by the condensation of **3** and 4-dimethylaminobenzaldehyde in the presence of piperidine and acetic acid (50.6% yield).

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