Supplementary Material (ESI) for Medicinal Chemistry Communications

Supporting information for

Phenyldiazenyl benzothiazole derivatives as probes for *in vivo* imaging of neurofibrillary tangles in Alzheimer's Disease brains

Kenji Matsumura,[a] Masahiro Ono,[a,*] Shun Hayashi,[a] Hiroyuki Kimura,[a] Yoko Okamoto,[b] Masafumi Ihara,[b] Ryosuke Takahashi,[b] Hiroshi Mori,[c] Hideo Saji,[a,*]

[a]Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan. [b]Graduate School of Medicine, Kyoto University, [c]Department of Neuroscience, Osaka City University Medical School, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585.

*To whom correspondence should be addressed: Phone: +81-75-753-4608, Fax: +81-75-753-4568. E-mail: ono@pharm.kyoto-u.ac.jp for M. Ono. Phone: +81-75-753-4556, Fax: +81-75-753-4568. E-mail: hsaji@pharm.kyoto-u.ac.jp for H. Saji.

Experimental

All reagents were commercial products and used without further purification unless indicated otherwise. ^{1}H NMR spectra were recorded on a JEOL JNM-LM400 with TMS as an internal standard. Coupling constans are reported in Hertz. Multiplicity was defined as singlet (s), doulet (d), triplet (t), and multiplet (m). Mass spectra were obtained on a SHIMADZU LCMS-2010 EV. HPLC was performed with a Shimadzu system (a LC-20AT pump with a SPD-20A UV detector, $\lambda = 254$ nm) using a Cosmosil C 18 column (Nacalai Tesque, 5C18-AR-Ⅱ, 4.6×150 mm) and acetonitrile/water (8/2) as the mobile phase at a flow rate of 1.0 mL/min. All key compounds were proven by this method to show >99% purity.

Chemistry

(E)-4-((6-Bromobenzo[d]thiazol-2-yl)diazenyl)aniline (1). A suspension of 2-amino-6-bromobenzothiazole (1.14 g, 5.0 mmol) in 50% H_2SO_4 (30 mL) was stirred at 0^oC for 30 min. $NaNO_2$ (345 mg, 5.0 mmol) was added drop-wise addition (30 min at 0^oC). After 1 h, a solution of aniline (465 μL, 5.0 mmol) in a mixed solvent (30 mL,

H_2O/conc.HCl = 9/1) was added at 0°C. The solution was stirred at 0°C for 1 h. The mixture was extracted with $CHCl_3$ (200 mL). The organic layer was dried over $MgSO_4$ and filtered. Evaporation of the solvent afforded a residue, which was purified by silica gel chromatography (hexane/ethyl acetate = 2/1) to give 400 mg of **1** (24.1%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.70 (s, 2H), 6.76 (d, J = 9.2 Hz, 2H), 7.65 (d, J = 10.8 Hz, 1H), 7.77 (d, J = 8.8 Hz, 2H), 7.91 (d, J = 8.8 Hz, 1H), 8.30 (s, 1H), MS (APCI) m/z 333 [MH^+].

(E)-4-((6-Bromobenzo[d]thiazol-2-yl)diazenyl)-N-methylaniline (2). The same reaction described above to prepare **1** was used, and 389 mg of **2** was obtained in a yield of 22.4% from 2-amino-6-bromobenzothiazole and *N*-methylaniline. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.89 (s, 3H), 6.77 (d, J = 9.6 Hz, 2H), 7.64 (d, J = 10.8 Hz, 1H), 7.77 (s, 1H), 7.83 (d, J = 9.6 Hz, 2H), 7.90 (d, J = 8.8 Hz, 1H), 8.29 (s, 1H). MS (APCI) m/z 347 [MH^+].

(E)-4-((6-Bromobenzo[d]thiazol-2-yl)diazenyl)-N,N-dimethylaniline (3). The reaction

described above to prepare **1** was used, and 500 mg of **3** was obtained in a yield of 27.8% from 2-amino-6-bromobenzothiazole and *N,N*-dimethylaniline. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.17 (s, 6H), 6.76 (d, *J* = 8.4 Hz, 2H), 7.56 (d, *J* = 8.8 Hz, 1H), 7.90 (d, *J* = 8.8 Hz, 2H), 7.97-8.00 (m, 3H). MS (APCI) m/z 361 [MH^+].

(E)-4-((6-(Tributylstannyl)benzo[d]thiazol-2-yl)diazenyl)aniline (4). A mixture of **1** (89.7 mg, 0.26 mmol), bis(tributyltin) (260 μL), and $(Ph_3P)_4Pd$ (127 mg, 0.11 mmol) in a mixed solvent (15 mL, dioxane/Et_3N = 2/1) was stirred under reflux for 12 h. The solvent was removed, and the residue was purified by silica gel chromatography (hexane/ethyl acetate = 2/1) to give 40 mg of **4** (28.4%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88-1.57 (m, 27H), 4.41 (s, 2H), 6.74 (d, *J* = 8.8 Hz, 2H), 7.57 (d, *J* = 8.0 Hz 1H), 7.93-7.96 (m, 3H), 8.05 (d, *J* = 8.0 Hz, 1H). MS (APCI) m/z 545 [MH^+].

(E)-N-Methyl-4-((6-(tributylstannyl)benzo[d]thiazol-2-yl)diazenyl)aniline (5). The reaction described above to prepare **4** was used, and 49 mg of **5** was obtained in a yield of 23.7% from **2**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.84-1.56 (m, 27H), 2.88 (s, 3H),

6.77 (d, *J* = 9.2 Hz, 2H), 7.54 (d, *J* = 7.6 Hz, 1H), 7.64 (s, 1H), 7.83 (d, *J* = 8.8 Hz, 2H) 7.94 (d, *J* = 8.0 Hz, 1H), 8.06 (s, 1H). MS (APCI) m/z 559 [MH^+].

(E)-N,N-Dimethyl-4-((6-(tributylstannyl)benzo[d]thiazol-2-yl)diazenyl)aniline (6). The reaction described above to prepare **4** was used, and 100 mg of **6** was obtained in a 67.3% yield from **3**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88-1.68 (m, 27H), 3.18 (s, 6H), 6.77 (d, *J* = 9.2 Hz, 2H), 7.54 (d, *J* = 7.6 Hz, 1H), 7.92 (s, 1H), 7.99-8.03 (m, 3H). MS (APCI) m/z 573 [MH^+].

(E)-4-((6-Iodobenzo[d]thiazol-2-yl)diazenyl)aniline (7). To a solution of **4** (23.7 mg, 0.043 mmol) in chloroform (10 mL) was added a solution of iodine in chloroform (1 mL, 0.25 M) at room temperature. The mixture was stirred at room temperature for 20 min, and a saturated $NaHSO_3$ solution (15 mL) was added. The organic layer was separated, dried over $MgSO_4$, and filtered. The solvent was removed, and the residue was subjected to silica gel chromatography (hexane/ethyl acetate = 2/1) to give 10.0 mg of **7** (60.2%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.70 (s, 2H), 6.76 (d, *J* = 9.2 Hz, 2H), 7.65

(d, J = 10.8 Hz, 1H), 7.77 (d, J = 8.8 Hz, 2H), 7.91 (d, J = 8.8 Hz, 1H), 8.30 (s, 1H). MS (APCI) m/z 381 [MH^+].

(E)-4-((6-Iodobenzo[d]thiazol-2-yl)diazenyl)-N-methylaniline (8). The reaction described above to prepare **7** was used, and 15 mg of **8** was obtained in a yield of 70.2% from **5**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.01 (s, 3H), 6.75 (d, J = 8.8 Hz, 2H), 7.76-7.80 (m, 3H), 7.97 (d, J = 9.2 Hz, 2H), 8.17 (s, 1H). MS (APCI) m/z 395 [MH^+].

(E)-4-((6-Iodobenzo[d]thiazol-2-yl)diazenyl)-N,N-dimethylaniline (9). The reaction described above to prepare **7** was used, and 24.5 mg of **9** was obtained in a yield of 70.2% from **6**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.18 (s, 6H), 6.77 (d, J = 9.2 Hz, 2H), 7.75-7.78 (m, 3H), 8.00 (d, J = 9.2 Hz, 2H), 8.17 (s, 1H). MS (APCI) m/z 409 [MH^+].

Iododestannylation reaction

The radioiodinated forms of compounds **7**, **8**, and **9** were prepared from the corresponding tributyltin derivatives by iododestannylation. Briefly, to initiate the

reaction, 100 μL of H_2O_2 (3%) was added to a mixture of a tributyltin derivative (150 μg/150 μL EtOH), [^{125}I]NaI (0.1–0.2 mCi, specific activity 81.4 TBq/mmol), and 100 μL of 1 N HCl in a sealed vial. The reaction was allowed to proceed at room temperature for 10 min and terminated by addition of $NaHSO_3$. After neutralization with sodium biocarbonate and extraction with ethyl acetate, the extract was dried by passing through an anhydrous Na_2SO_4 column and then blown dry with a stream of nitrogen gas. The radioiodinated ligand was purified by HPLC on a Cosmosil C18 column with an isocratic solvent of acetonitrile/H_2O (8/2) at a flow rate of 1.0 mL/min.

Saturation Assay with ThS using recombinant tau and A$\beta_{1–42}$ aggregates

The 441-aa isoform of human tau was expressed from a cDNA clone in *Escherichia coli* and purified as described previously.[1] Tau aggregates were prepared by incubating tau protein (1 mg/mL in MES buffer, pH 6.8) at 37^{o}C for 8 days with gentle and constant shaking in the presence of 0.1 mg/mL heparin. A solid form of A$\beta_{1–42}$ was purchased from Peptide Institute (Osaka, Japan). Aggregation was achieved by gently dissolving the peptide (0.25 mg/mL) in PBS solution (pH 7.4). The solutions were

incubated at 37 ^{o}C for 42 h with gentle and constant shaking. The binding experiments were carried out in Protein LoBind Tubes (Eppendorf). A mixture of tau aggregates (final conc., 0.2 μM) or $A\beta_{1\text{-}42}$ aggregates (final conc., 2.2 μM) were incubated at room temperature for 30 min in the presence of ThS (final conc., 0.2-10 μM), dispensed to MULTI WELL PLATE (0.4 mL × 96 wells flatbottom, SUMITOMO BAKELITE CO., LTD, Japan), and subjected to fluorescence spectroscopy (λ_{ex} = 440 nm; λ_{em} = 510 nm for tau aggregates and λ_{ex} = 440 nm; λ_{em} = 480 nm for $A\beta_{1-42}$ aggregates). The fluorescence intensity (λ_{ex} = 440 nm; λ_{em} = 510 nm for tau aggregates and λ_{ex} = 440 nm; λ_{em} = 480 nm for $A\beta_{1-42}$ aggregates) was plotted and K_d values of ThS for recombinant tau and $A\beta_{1-42}$ aggregates were calculated from saturation curves using GraphPad Prism software (Graph Pad software, San Diego, CA). The results are shown in Figure S1.

Inhibition assay with PDB derivatives using recombinant tau and $A\beta_{1\text{-}42}$ aggregates

The binding experiments were carried out in Protein LoBind Tubes (Eppendorf). ThS was used as a competitive ligand. Tau aggregates (final conc., 0.2 μM) or $A\beta_{1\text{-}42}$ aggregates (final conc., 2.2 μM) were incubated at room temperature for 30 min in the

presence of ThS (final conc., 1.5 μM) and PDB derivatives and 4-*N*-methylamino-4'-hydroxystilbene (SB-13)[2] (0.16 nM-12.5 μM), then the mixture was dispensed to MULTI WELLPLATE (0.4 mL × 96 wells flatbottom, SUMITOMO BAKELITE CO., LTD, Japan) and subjected to fluorescence spectroscopy (λ_{ex} = 440 nm; λ_{em} = 510 nm for tau aggregates and λ_{ex} = 440 nm; λ_{em} = 480 nm for $A\beta_{1-42}$ aggregates). The fluorescent intensity was plotted and values for the half maximal inhibitory concentration (IC_{50}) were calculated from displacement curves of three independent experiments using GraphPad Prism, and those for the inhibition constant (K_i) were calculated using the Cheng-Prusoff equation[3]: $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is the concentration of ThS used in the assays, and K_d is the dissociation constant of ThS. The K_d values of ThS for tau and Aβ aggregates, determined according to the method described above, were 0.26 and 1.0 μM, respectively.

Fluorescent staining of AD brain sections

The experiments with animals were conducted in accordance with our institutional

guidelines and approved by the Kyoto University. Postmortem brain tissue from an autopsy-confirmed case of AD (a 93-year-old, female) was obtained from the Graduate School of Medicine, Kyoto University. Six-micrometer-thick serial sections of paraffin-embedded blocks were used for staining. Paraffin sections were subjected to two 15-min incubations in xylene, two 1-min incubations in 100% EtOH, one 1-min incubation in 90% EtOH, one 1-min incubation in 80% EtOH, and one 1-min incubation in 70% EtOH to completely deparaffinize them, followed by two 2.5-min washes in water. The brain tissue was incubated with a 50% ethanol solution (300 μM) of **9** for 30 min. Finally, the sections were washed in ethanol for 30 sec. Fluorescent observation was performed with the Keyence system (excitation filter, 540-580 nm; emission filter, 600-660 nm; DM filter; 595 nm). After being immersed in EtOH overnight, the same sections were stained with ThS, a pathological dye used for staining NFTs in the brain, and observed with the Keyence system (excitation filter, 450-490 nm; emission filter, 510-560 nm; DM filter; 495 nm). This experiment was performed under a fluorescence microscope (BIOREVO BZ-9000, Keyence Corp., Osaka, Japan).

Immunohistochemical staining of NFT in human AD brain sections

The experiments with animals were conducted in accordance with our institutional guidelines and approved by the Kyoto University. Postmortem brain tissue from an autopsy-confirmed case of AD (a 93-year-old, female) was obtained from the Graduate School of Medicine, Kyoto University. Six-micrometer-thick serial sections of paraffin-embedded blocks were used for staining. The sections were subjected to two 15-min incubations in xylene, two 1-min incubations in 100% EtOH, one 1-min incubation in 90% EtOH, one 1-min incubation in 80% EtOH, and one 1-min incubation in 70% EtOH to completely deparaffinize them, followed by two 2.5-min washes in water. They were then autoclaved for 15 min. in 0.01 M citric acid buffer (pH6.0) to activate the antigen. After two 5-min incubations in PBS-Tween20, the sections were incubated at room temperature with anti phosphorylated tau (AT8) or $A\beta_{1-42}$ primary antibody overnight. After three two-min incubations in PBS-Tween20, they were incubated with biotinylated goat anti-mouse IgG at room temperature for 1 h. After three 5-min. incubations in PBS-Tween, the sections were incubated with Streptavidin-Peroxidase complex at room temperature for 30 min. After three 2-min

incubations in PBS-Tween, they were incubated with DAB as a chromogen for 30 min. After being washed with water, the sections were observed under a microscope (BIOREVO BZ-9000, Keyence Corp., Osaka, Japan).

***In vitro* autoradiography**

Postmortem brain tissues from an autopsy-confirmed case of AD (a 93-year-old, female) and a control (a 73-year-old, female) were obtained from the Graduate School of Medicine, Kyoto University and BioChain Institute Inc., respectively. The presence and location of NFTs in the sections were confirmed with immunohistochemical staining using AT8 anti-phosphoylated tau antibody. The sections were incubated with [^{125}I]**9** (92.5 kBq/100 μL) for 1 h at room temperature. They were then dipped in 50% EtOH for 2 h and washed with water for 30 s. After drying, the ^{125}I-labeled sections were exposed to a BAS imaging plate (Fuji Film, Tokyo, Japan) for 2 h. Autoradiographic images were obtained using a BAS5000 scanner system (Fuji Film).

***In vivo* biodistribution in normal mice.**

A saline solution (100 μL) of [^{125}I]**7**, **8**, and **9** (8.51-11.1 kBq) containing ethanol (10 μL) was injected intravenously directly into the tail of ddY mice (5-week-old, 26-28 g). The mice were sacrificed at various time points post-injection. The organs of interest were removed and weighed, and the radioactivity was measured with an automatic gamma counter (Perkin Elmer, Wizard 1470).

References

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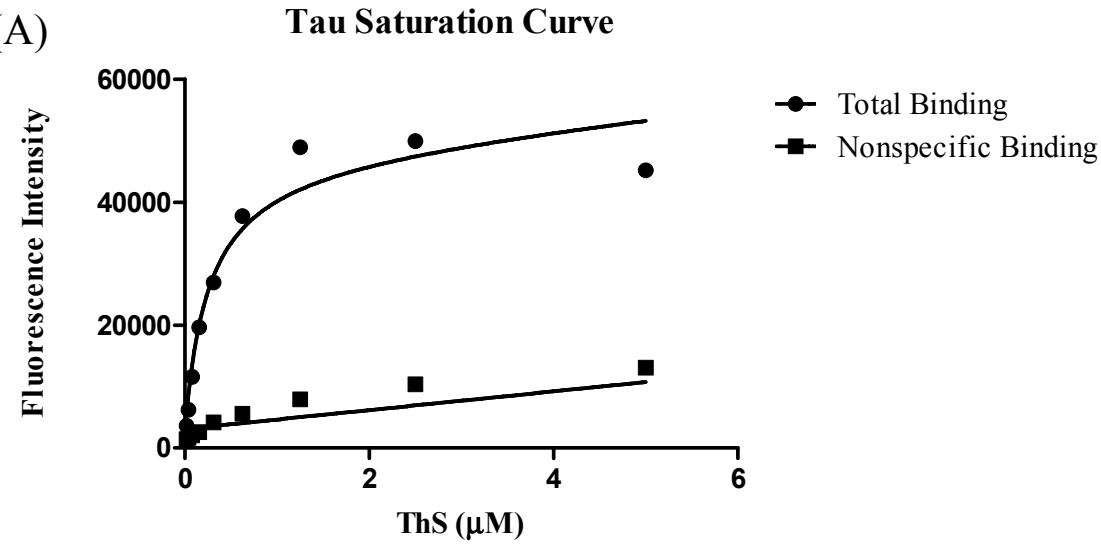
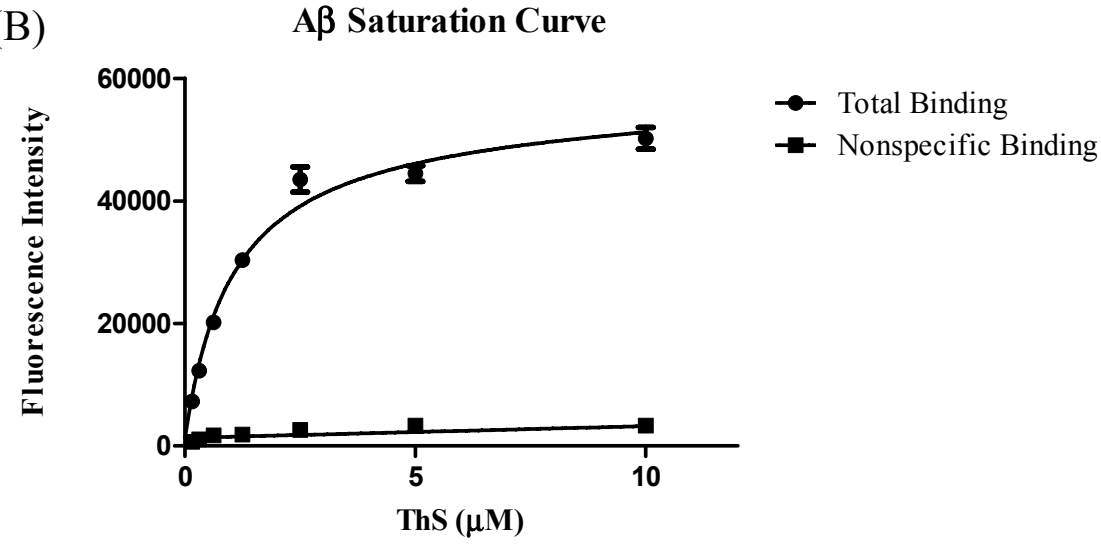
(A)



K_d = 0.26 μM

(B)



K_d = 1.0 μM

Figure S1. Saturation curves of thoflavin-S for tau (A) and $A\beta_{1\text{-}42}$ (B) aggregates.