Bifunctional curcumin analogue for two-photon imaging and inhibiting crosslinking of amyloid beta in Alzheimer's disease

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Supplemental Materials

1. Experimental section

Reagents used for the synthesis were purchased from Aldrich and used without further purification. Column chromatography was performed on silica gel (SiliCycle Inc., 60 Å, 40-63 mm) slurry packed into glass columns. Synthetic Aβ peptides (1-40/42) were purchased from rPeptide (Bogart, GA, 30622). ^{1}H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz respectively, and reported in ppm downfield from tetramethylsilane. Fluorescence measurements were carried out using an F-4500 Fluorescence Spectrophotometer (Hitachi). Mass spectra were obtained at Harvard University, Department of Chemistry Instrumentation Facility. Transgenic female APP/PS1 mice were purchased from Jackson Laboratory. All animal experiments were approved by the Institutional Animal Use and Care Committee at Massachusetts General Hospital.

Synthesis of CRANAD-28 and -44: The syntheses were performed following a modified procedure that was based on our previous reports [1]. **CRANAD-28:** 2,2-Difluro-1,3-dioxaboryl-pentadione (40 mg, 0.25 mmol) was dissolved in acetonitrile (4.0 mL),

followed by the additions of acetic acid (0.1 mL), tetrahydroisoquinoline (20 μL), and N-phenyl-3-methyl-1H-pyrazole-carboxaldehyde (93 mg, 0.5 mmol). The resulted solution was stirred at 60 °C for 4 hours. An orange precipitate was formed during the reaction, which was collected by filtrating the reaction mixture and by washing twice with a mixture solvent of ethyl acetate/hexanes (1:5). 90 mg, yield: 74.4%. m.p. > 250°C; ^{1}H NMR ($CDCl_3$) δ(ppm) 2.46 (s, 6H), 5.95 (s, 1H), 6.50 (d, 2H, J = 16Hz), 7.44 (m, 6H), 7.54 (m, 4H), 7.97 (d, 2H, J = 16Hz), 7.99 (s, 2H); ^{13}C NMR ($CDCl_3$) δ(ppm) 11.1, 101.2, 117.6, 117.9, 125.0, 128.6, 129.3, 137.2, 138.7, 139.2, 141.6, 179.0; ^{19}F NMR ($CDCl_3$) δ(ppm) 141.2, 141.3; ESI-MS (M^+) m/z = 484.2; HRMS (ESI-TOF) ($M+H^+$) m/z = 485.1948 (calculated: 485.1955, relative Error = 2.4ppm).

CRANAD-44: Yield: 68.6%. m.p. > 250°C; ^{1}H NMR ($CDCl_3$) δ(ppm) 2.39 (s, 6H), 6.40 (s, 1H), 6.68 (d, 2H, J = 16Hz), 7.83 (d, 2H, J = 16Hz), 8.01 (s, 2H); ^{13}C NMR ($CDCl_3$) δ(ppm) 10.2, 100.2, 116.0, 117.3, 138.2, 138.7, 140.5, 179.0; ^{19}F NMR ($CDCl_3$) δ(ppm) 141.2, 141.3; ESI-MS ($M+H^+$) m/z = 333.10; HRMS (ESI-TOF) ($M+H^+$) m/z = 333.1326 (calculated: 333.1329, relative Error = 1.7ppm).

Quantum yield: The measurements were performed by the procedure described in ref. [2].

Aβ species preparation: Aβ40 aggregates for *in vitro* studies were generated by slow stirring of Aβ40 in PBS buffer for 3 days at room temperature. Aβ40/42 monomers were prepared and characterized by following our previously reported procedures [1].

Fluorescence spectral testing: The tests of CRANAD-28 and -44 with Aβ42 monomers, Aβ40 monomers, and Aβ40 aggregates were performed following our previously reported procedure [1].

Binding constant (Kd) measurement: To PBS solution (1.0 mL) of CRANAD-28 (25 nM), various amounts of Aβs (aggregates, oligomers, dimers, and monomers respectively) were added to final Aβs concentrations of 25.0, 50.0, 75.0, 100.0, 125.0, and 150 nM, and their fluorescence intensities at 580 nm were recorded (Ex: 498 nm). Kd calculation was based on the fluorescence decrease ($\Delta FI = F_0 - F_{(C(A\beta))}$), where F_0 is the fluorescence intensity of CRANAD-28 without Aβs, and $F_{(C(A\beta))}$ is the fluorescence intensity of CRANAD-28 with a tested concentration of Aβs. The Kd binding curve was

generated using Prism 5.0 software with nonlinear one-site binding regression (SI Fig.1b).

Histological staining of brain slice: A 30-micron brain slice from an 18-month old APP/PS1 mouse was fixed with 4% formalin for 5 min, and washed with distilled water twice for 5 min. Then the slice was incubated with CRANAD-28 solution (1.0 μM in 20% ethanol and 80% dd water) for 15 minutes, and then washed with 20% ethanol followed by washing with dd water. After drying, the slice was covered with VectaShield mounting media. Thioflavin S and CRANAD-44 staining were followed using a similar procedure. Florescence images were observed using Nikon Eclipse 50i microscope.

Thinning skull surgery: An APP/PS1 mouse (9 months old) was anesthetized with Ketamine/xylazine (70 mg/kg) and a thin-skull imaging window was surgically prepared as described in ref. [3].

Two-Photon imaging: CRANAD-28 (2.0 mg/kg in a fresh solution containing 15% cremorphor, 15% DMSO and 70% PBS) was injected intravenously at time 0 min by a bolus injection during image acquisition. Two-photon fluorescence excitation was accomplished with a 900-nm laser (Prairie Ultima). Imaging was performed using a two-photon microscope (Prairie Technologies) equipped with a 20x water immersion objective (N.A. 1.0, Zeiss). Images were collected for 15 seconds per frame 512x512 μm matrix for 60 min. Texas-red dextran (70,000 MW) (50 μL) was injected in the tail vein at 60 min after CRANAD-28 injection, and images outlining the blood vessels were obtained. In BBB penetration study, a wild type mouse (C57BL6) was imaged for 5 min to obtain pre-injection background. CRANAD-28 (2.0 mg/kg in a fresh solution containing 15% cremorphor, 15% DMSO and 70% PBS) was then injected in the tail vein, and images were collected for 20 seconds per frame 512x512 μm matrix for 30 min. Images were analyzed with ImageJ software using 2 ROIs for each data group.

Gel electrophoresis and Western blotting: Samples were separated on 4−20% gradient Tris-glycine mini gels (Invitrogen). For FAM-Aβ42 gels, the images were acquired using IVIS®Spectrum (Perkin Elmer) with excitation = 465 nm, and emission = 520 nm. For native Aβ42 gels, the gel was transferred onto a nitrocellulose membrane in a cooled transferring buffer and the membrane was blocked at room temperature for 2 hours. After blocking, the membrane was incubated in a solution of 6E10 anti-Aβ primary antibody

(1:2000 dilution, Covance, Dedham, MA) at 4°C overnight. After washing with TBS buffer, the membrane was incubated with the secondary antibody for 2 hours at room temperature. Western Breeze Chemiluminescent kit (Invitrogen) was used to visualize the bands. The images were acquired using IVIS®Spectrum under a bioluminescence imaging setting. SeeBlue®plus2 (Invitrogen)(4-250KD) was used as a molecular weight marker.

All the samples used for SDS-PAGE gel and Western blot were prepared using the same procedure as described below. A 5 μL HFIP (hexafluoroisopropanol) solution (25 μM) of native Aβ42 or FAM-Aβ42 was added to a 1.5 mL eppendorf tube (Protein LoBind). After evaporating the organic solvent under vacuum, a 5 μL DMSO or DMSO solution of CRANAD-28 (25 μM) was added to the tube, followed by the addition of 15 μL of Vitamin C solution in PBS (33.3 μM) and 5 μL of copper sulfate solution in PBS (12.5 μM). The resulting mixture was incubated at 37°C for 4 hours, and was then subjected to gel electrophoresis. For natural crosslinking, no Vc and Copper were added. The resulting mixture was incubated at 37°C for 24 hours.

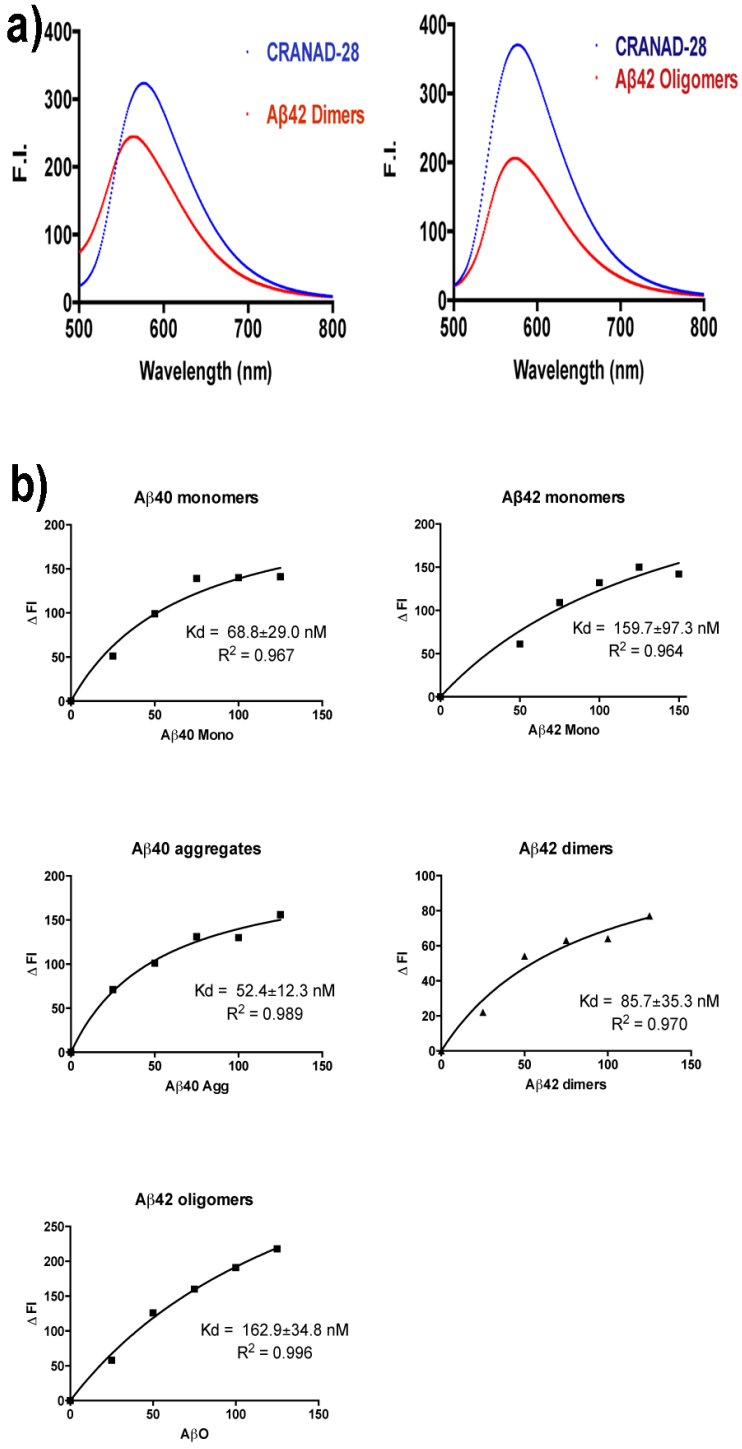
Reference:

[1] a) C. Ran, X. Xu, S. B. Raymond, B. J. Ferrara, K. Neal, B. J. Bacskai, Z. Medarova, A. Moore, *J Am Chem Soc* **2009**, *131*, 15257-15261; b) C. Ran, Zhao, W., Moir, RD, Moore, A. , *PLoS One* **2011**, *6*, e19362.

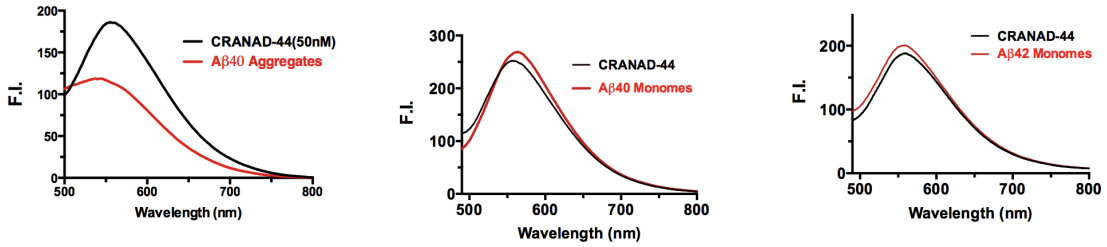
[2] S. Fery-Forgues, Lavabre, D., *J. Chem. Edu.* **1999**, *76*, 1260-1264.

[3] D. F. Marker, M. E. Tremblay, S. M. Lu, A. K. Majewska, H. A. Gelbard, *JoVE* **2010**, *43,* pii: 2059.

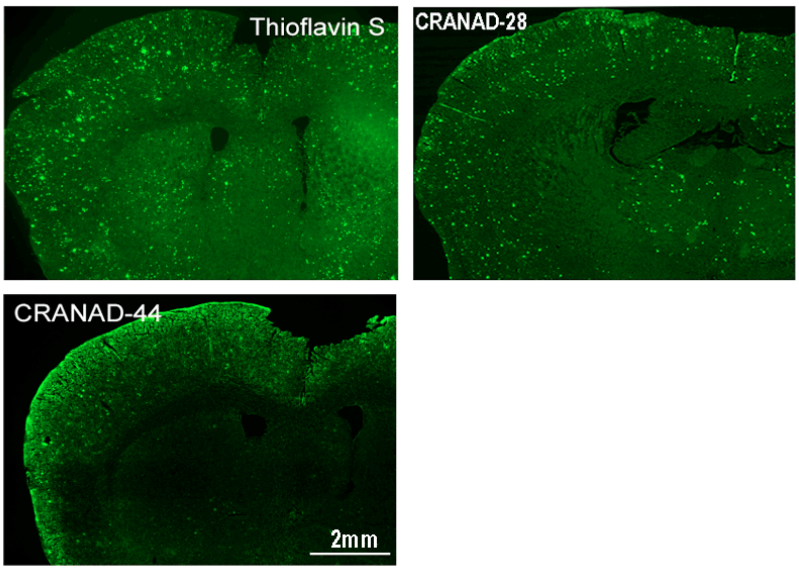
2. Supplemental Figures



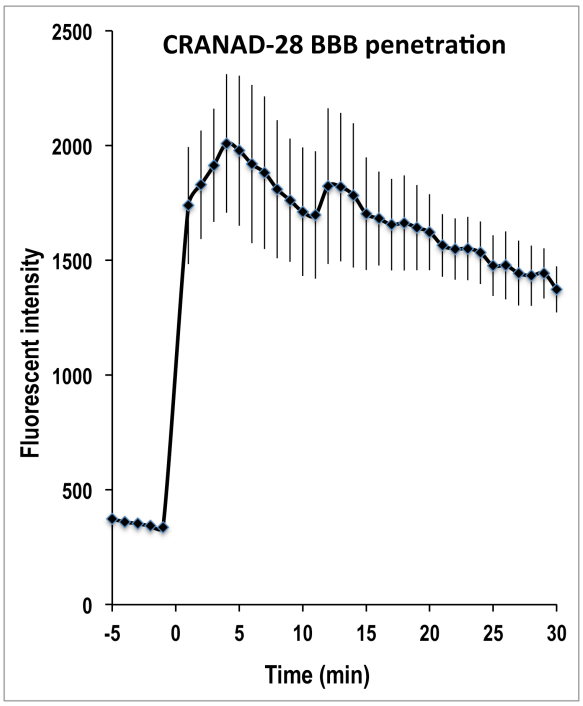
SI Fig.1 (a) Fluorescence properties changes upon interaction of CRANAD-28 with Aβ42 dimers and Aβ42 oligomers. (b) Kd measurement of CRANAD-28 with various Aβs.



SI Fig.2 Fluorescence properties changes upon interaction of CRANAD-44 with Aβ40 aggregates, Aβ40 monomers and Aβ42 monomers. No significant changes were observed for CRANAD-44 with Aβ40/42 monomers.



SI Fig.3 Histological staining of consecutive brain slices from an 18-month old APP/PS1 AD mouse. CRANAD-28 showed excellent staining capability, which was similar to Thioflavin S, a standard staining agent for Aβ plaques. However, compared to Thioflavin S and CRANAD-28, a lesser number of plaques were observed with CRANAD-44.



SI Fig.4 BBB penetration of CRANAD-28 observed by two-photon imaging in a wild type mouse. The intensities of two ROIs nearby blood vessels were measured and averaged.