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Supporting information

Synthesis of radioiodinated probes to evaluate the biodistribution of a potent TRPC3 inhibitor

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Materials and methods

Chemicals were of analytic grade and used as received. $Na^{125}I$ was purchased from MP Biomedical LLC. ^1H NMR spectra were measured using a Varian UNITY 400 (400 MHz) or a Varian Mercury 300 (300 MHz) or a Bruker ascend 600 (600 MHz) spectrometer. Chemical shifts are reported as ppm (in DMSO-d_6 or $CDCl_3$). Mass spectra (MS) were recorded on a Waters LCT Premier XE system. HRMS were recorded using an Applied Biosystems Mariner System 5299 spectrometer. All melting points were determined using a Yanaco melting point apparatus (Yanagimoto Ind. Co., Kyoto, Japan) and are uncorrected. Infrared (IR) spectra were recorded in potassium bromide pellets using a Shimadzu-FT-IR 8400. HPLC analyses were performed using a Shimadzu LC-6AD equipped with a Shimadzu SPD-10A UV detector, a Shimadzu LC-6A equipped with a Shimadzu SPD-6A UV detector and a EG&G 40101A radioactivity detector, or a GL Science GL-7540 equipped with a Perkin Elmer FSA 505TR radioactivity detector.

Chemistry

(2-Bromo-4-nitrophenyl)hydrazine (2)

To a solution of 2-bromo-1-fluoro-4-nitrobenzene (325 mg, 1.5 mmol) in EtOH (10 mL), hydrazine hydrate was slowly added (75 mg, 1.5 mmol) over 15 min at 85°C. The solution was stirred for 1.5 h at 85°C and then allowed to cool to room temperature. The resulting precipitate was collected by filtration and **2** (301 mg, 89% yield) was obtained as a yellow solid, mp 138°C–139°C. IR (KBr, cm^{-1}) ν: 3354, 3320, 1349. ^1H NMR (400 MHz, DMSO-d_6) δ 8.22 (s, 1H, 3-H), 8.09 (d, 1H, J = 8.6 Hz, 5-H), 7.94 (brs, 1H, N<u>H</u>NH_2), 7.22 (d, 1H, J = 8.6 Hz, 6-H), 4.60 (brs, 2H, NHN<u>H_2</u>).

Ethyl 1-(2-bromo-4-nitrophenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (3)

To a solution of **2** (299 mg, 1.3 mmol) in EtOH (12 mL), sulfuric acid was added (0.04 mL). The solution was refluxed for 4 h and then allowed to cool to room temperature. The resulting precipitate was collected by filtration and **3** (297 mg, 56% yield) was obtained as a milky white solid, mp 113°C–114°C. IR (KBr, cm^{-1}) ν: 3122, 1717. ^1H NMR (400 MHz, $CDCl_3$) δ 8.61 (s, 1H, 3-H), 8.34 (d, 1H, J = 7.2 Hz, 5'-H), 8.23 (s, 1H, 3'-H), 7.62 (d, 1H, J = 8.6 Hz, 6'-H), 4.40 (q, 2H, J = 7.2 Hz, $CO_2C\underline{H_2}CH_3$), 1.40 (t, 3H, J = 7.2 Hz, $CO_2CH_2C\underline{H_3}$). MS (FAB) m/z: 410 [M + H^+] Anal. Calcd. for $C_{13}H_9BrF_3N_3O_4$ = 406.9729: C, 38.26; H, 2.22; N, 10.30%. Found: C, 38.29; H, 2.16; N, 10.36%.

Ethyl 1-(4-amino-2-bromophenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (4)

To a solution of **3** (2.89 g, 7.1 mmol) in EtOAc (260 mL), saturated ammonium chloride solution (44 mL) and zinc dust (6.0 g) were added, and the mixture was stirred for 4 h at 0°C. Saturated sodium hydrogen carbonate solution (80 mL) was added to the solution and the zinc dust was removed by filtration. The filtrate was diluted with EtOAc. The organic layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layer were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification by column chromatography on silica gel ($CHCl_3$:EtOAc = 9:1 eluent) yielded **4** (2.27 g, 85% yield) as a white solid, mp 93°C –94°C. IR (KBr, cm^{-1}) ν: 3381, 3476, 1713. ^1H NMR (400 MHz, $CDCl_3$) δ 8.13 (s, 1H,

3-H), 7.13 (d, 1H, *J* = 8.4 Hz, 6'-H), 6.95 (s, 1H, 3'-H), 6.64 (d, 1H, *J* = 8.4 Hz, 5'-H), 4.37 (q, 2H, *J* = 7.2 Hz, $CO_2C\underline{H_2}CH_3$), 1.38 (t, 3H, *J* = 7.2 Hz, $CO_2CH_2C\underline{H_3}$). MS (ESI) m/z: 400 [M + Na^+] Anal. Calcd. for $C_{13}H_{11}BrF_3N_3O_2$ = 376.9987: C, 41.29; H, 2.93; N, 11.11%. Found: C, 41.53; H, 2.98; N, 11.05%.

Ethyl 1-(4-amino-2-(tributylstannyl)phenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (5)

To a solution of **4** (1.13 g, 3.0 mmol) in toluene (5.0 mL), bis(tri-n-butyltin) (3.0 mL, 6.0 mmol) and tetrakis(triphenylphosphine)palladium (173 mg, 0.015 mmol) were added, and the solution was refluxed for 2 h. The reaction mixture was quenched with saturated potassium fluoride solution and filtered through Celite. The filtrate was extracted with EtOAc and the combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification by column chromatography on silica gel ($CHCl_3$:EtOAc = 9:1 eluent) yielded **5** (964 mg, 55% yield) as a reddish-brown oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (s, 1H, 3-H), 6.99 (d, 1H, *J* = 8.4 Hz, 6'-H), 6.81 (s, 1H, 3'-H), 6.62 (d, 1H, *J* = 8.6 Hz, 5'-H), 4.37 (q, 2H, *J* = 7.2 Hz, $CO_2C\underline{H_2}CH_3$), 1.43–1.35 (m, 9H, $CO_2CH_2C\underline{H_3}$, $Sn(CH_2C\underline{H_2}CH_2CH_3)_3$), 1.25 (m, 6H, $Sn(CH_2C\underline{H_2}CH_2CH_3)_3$) 0.85 (t, 6H, *J* = 7.6 Hz, $Sn(CH_2CH_2C\underline{H_2}CH_3)_3$), 0.77 (t, 9H, *J* = 8.6 Hz, $Sn(CH_2CH_2CH_2C\underline{H_3})_3$). MS (ESI) m/z: 612 [M + Na^+], HRMS (ESI) Calcd. for $C_{25}H_{38}F_3N_3O_2Sn$ [M + Na^+]: 612.1836, found 612.1840.

Ethyl 1-(4-amino-2-iodophenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (6)

To a solution of **5** (59 mg, 0.10 mmol) in CH_2Cl_2 (3.4 mL), 0.1 M iodine solution (29 mg, 0.24 mmol) was added, and the solution was stirred at room temperature. After

stirring for 30 min, the mixture was quenched with sodium sulfite solution, and the resulting mixture was diluted with water. The mixture was extracted with CH_2Cl_2 and the combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification by column chromatography on silica gel (hexane:EtOAc = 5:1 eluent) yielded **6** (37 mg, 87% yield) as a white solid, mp 149°C–150°C. IR (KBr, cm^{-1}) ν: 3349, 3364, 1728. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (s, 1H, 3-H), 7.19 (d, 1H, J = 8.6 Hz, 6'-H), 7.09 (s, 1H, 3'-H), 6.67 (d, 1H, J = 8.6 Hz, 5'-H), 4.37 (q, 2H, J = 7.2 Hz, $CO_2C\underline{H_2}CH_3$), 1.44 (t, 3H, J = 7.2 Hz, $CO_2CH_2C\underline{H_3}$). MS (ESI) m/z: 426 [M + H^+]. HRMS (ESI) Calcd. for $C_{13}H_{11}F_3IN_3O_2$ [M + H^+]: 425.9927, found 425.9938. Anal. Calcd for $C_{13}H_{11}F_3IN_3O_2$ = 424.9848: C, 36.73; H, 2.61; N, 9.88%. Found: C, 36.77; H, 2.60; N, 9.88%.

Ethyl 1-(2-(tributylstannyl)-4-(2,3,3-trichloroacrylamido)phenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (7)

A mixture of thionyl chloride (9 mL, 124 mmol) and trichloroacrylic acid (515 mg, 2.96 mmol) was stirred at 75°C for 16.5 h. After the solution was concentrated *in vacuo*, the resulting mixture was diluted with CH_2Cl_2. A solution of **5** (572 mg, 1.0 mmol) in CH_2Cl_2 and *N*,*N*-diisopropylethylamine (0.5 mL, 2.91 mmol) were added to the mixture and the solution was stirred at 0°C for 2.5 h. After water was added to the reaction mixture, the mixture was extracted with CH_2Cl_2, and the combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification by column chromatography on silica gel (hexane:EtOAc = 8:1 eluent) yielded **7** (381 mg, 53% yield) as a reddish-brown oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (s, 1H, 3-H), 7.75 (d, 1H, J = 8.6 Hz, 5'-H), 7.61 (s, 1H, 3'-H), 7.22 (d, 1H, J = 8.6 Hz, 6'-H), 4.38 (q, 2H, J

= 7.2 Hz), 1.39 (t, 3H, *J* = 7.2 Hz, $CO_2CH_2C\underline{H}_3$), 1.22–1.35 (m, 12H, $Sn(C\underline{H}_2C\underline{H}_2CH_2CH_3)_3$), 0.92 (t, 6H, *J* = 7.2 Hz, $Sn(CH_2CH_2C\underline{H}_2CH_3)_3$), 0.85 (9H, t, *J* = 7.2 Hz, $Sn(CH_2CH_2CH_2C\underline{H}_3)_3$). MS (ESI) m/z: 768 [M + Na^+]. HRMS (ESI) calcd. for $C_{28}H_{37}Cl_3F_3N_3O_3Sn$ [M + Na^+] 768.0752, found 768.0733.

Ethyl 1-(2-iodo-4-(2,3,3-trichloroacrylamido)phenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (I-Pyr3)

A mixture of thionyl chloride (3 mL, 41 mmol) and trichloroacrylic acid (164 mg, 0.94 mmol) was stirred at room temperature for 15 min, and then slowly warmed to 75°C. After the solution was stirred for 6.5 h, thionyl chloride (3 mL, 41 mmol) was added and the solution was stirred for 30 min. After the solution was concentrated *in vacuo*, the resulting mixture was diluted with CH_2Cl_2. Added to the mixture was a solution of **6** (132 mg, 0.31 mmol) in CH_2Cl_2 and *N*,*N*-diisopropylethylamine (0.1 mL, 0.31 mmol), and the solution was stirred at 0°C for 1 h. After water was added to the reaction mixture, the mixture was extracted with CH_2Cl_2, and the combined organic layer was dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification by column chromatography on silica gel (hexane/EtOAc 3/1) gave I-Pyr3 (139 mg, 60% yield) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.26 (1H, s, NH), 8.18 (s, 1H, 3'-H), 8.15 (s, 1H, 3-H), 7.72 (d, 1H, *J* = 8.6 Hz, 5'-H), 7.34 (q, 1H, *J* = 8.6 Hz, 6'-H), 4.39 (q, 2H, *J* = 7.2 Hz, $CO_2C\underline{H}_2CH_3$), 1.39 (t, 3H, *J* = 7.2 Hz, $CO_2CH_2C\underline{H}_3$). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 160.7, 157.9, 143.0, 139.0, 138.9, 130.2, 129.4, 128.5, 122.8, 120.0, 119.7, 117.9, 116.5, 96.7, 61.4, 14.1. MS (ESI) m/z: 581 [M + H^+] HRMS (ESI) Calcd. for $C_{16}H_{10}Cl_3F_3IN_3O_3$ [M + H^+]: 581.8864, found 581.8868. Anal. Calcd for

$C_{16}H_{10}Cl_3F_3IN_3O_3$ = 580.8785: C, 32.99; H, 1.73; N, 7.21%. Found: C, 33.03; H, 1.74; N, 7.17%.

1-(2-Iodo-4-(2,3,3-trichloroacrylamido)phenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylic acid (I-Pyr8)

To a solution of I-Pyr3 (78 mg, 0.10 mmol) in EtOH (5 mL), 1M potassium hydroxide solution (0.1 mL) was added at 0°C. The resulting solution was stirred at room temperature. The reaction mixture was neutralized with HCl and extracted with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification by column chromatography on silica gel (EtOAc eluent) yielded **12** (13 mg, 18% yield) as a yellow solid, mp 199°C–200°C. 1H NMR (300 MHz, DMSO-d_6) δ 11.37 (s, 1H, $CO_2\underline{H}$), 8.30 (s, 1H, 3-H), 8.28 (s, 1H 3'-H), 7.71 (d, 1H, J = 8.7 Hz, 5'-H), 7.63 (q, 1H, J = 8.7 Hz, 6'-H). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 161.7, 159.0, 142.8, 140.0, 138.1, 129.1, 128.9, 128.2, 123.8, 122.9, 119.9, 117.9, 116.9, 98.0. MS (ESI) m/z: 553 [M − H^+] HRMS (ESI) Calcd. for $C_{14}H_6Cl_3F_3IN_3O_3$ [M − H^+]: 551.8394, found 551.8403.

Ethyl 1-(4-(2,3,3-trichloroacrylamido)phenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (Pyr3)[18]

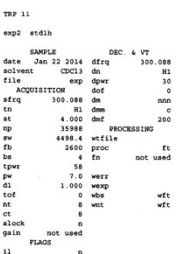
To a solution of ethyl 1-(4-aminophenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (23.3 mg, 0.08 mmol) in dry DMF (2 mL), 2,3,3-trichloroacrylic acid (32 mg, 0.18 mmol), BOP reagent (127 mg, 0.29 mmol), and *N*,*N*-diisopropylethylamine (4 mg, 0.03 mmol) were added. The reaction mixture was refluxed under an argon atmosphere for 6 h at room temperature. After the solution was concentrated *in vacuo*,

the residue was diluted with EtOAc, washed with 10% citric acid solution and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification by column chromatography on silica gel (hexane:EtOAC = 6:1 eluent) yielded Pyr3 (15 mg, 42% yield) as a white solid, mp 148°C–150°C. IR (KBr, cm^{-1}) ν: 3246, 1744, 1659. ^1H NMR (400 MHz, $CDCl_3$) δ 8.06 (s, 1H, 3-H), 7.73 (d, 4H, J = 8.6 Hz, 5'-H), 7.44 (d, 4H, J = 8.6 Hz, 6'-H), 4.38 (q, 2H, J = 7.2 Hz, $CO_2C\underline{H_2}CH_3$), 1.39 (t, 3H, J = 7.2 Hz, $CO_2CH_2C\underline{H_3}$). MS (ESI) m/z: 479 [M + Na^+].

1-(4-(2,3,3-Trichloroacrylamido)phenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylic acid (Pyr8)

To a solution of Pyr3 (48 mg, 0.10 mmol) in EtOH (5 mL), 1 M potassium hydroxide solution (0.1 mL) was added at 0°C, and the solution was stirred at room temperature. The reaction mixture was neutralized with HCl and extracted with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification by column chromatography on silica gel (EtOAc eluent) yielded Pyr8 (45 mg, 100% yield) as a white solid. IR (KBr, cm^{-1}) ν: 3448, 3286, 1667, 1258. ^1H NMR (400 MHz, $CDCl_3$) δ 8.18 (s, 1H, 3-H), 7.74 (d, 4H, J = 8.8 Hz, 5'-H), 7.45 (q, 4H, J = 8.8 Hz, 6'-H). MS (ES) m/z: 451 [M + Na^+].

Spectral data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tpwr	58		
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d1	1.000	wexp	
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Fig. S1 ^{1}H NMR of I-Pyr3 in $CDCl_3$

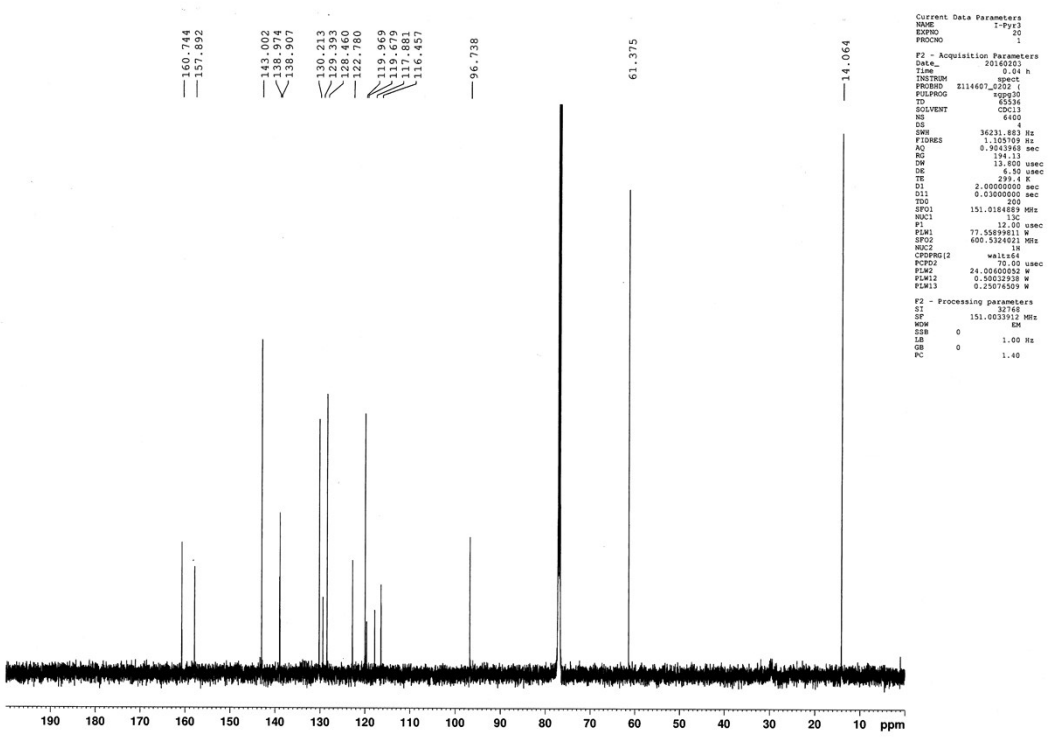

Fig. S2 ^{13}C NMR of I-Pyr3 in $CDCl_3$

Fig. S3 ^{1}H NMR of I-Pyr8 in DMSO-d_6

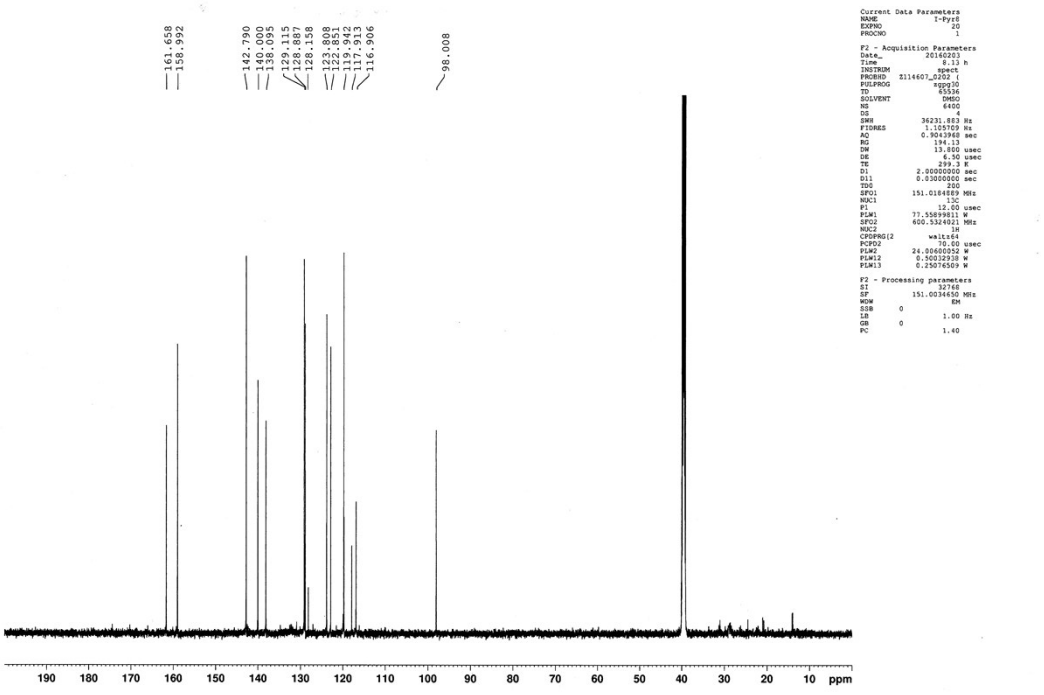

Fig. S4 ^{13}C NMR of I-Pyr8 in DMSO-d_6

Measurement of Intracellular Ca^{2+} Concentration

HEK293 cells were transfected with TRPC3-pCIneo with or without pEGFP-F vector using fugene 6. Twenty-four hour after transfection, the cells were trypsinized and plated in a 24-well plate containing coverslips at 2 × 10^4 cells/well. The cells were loaded with 1 μM Fura-2 AM at 37°C for 40 min. Images of the Fura-2 fluorescence of GFP-positive cells were recorded in Hepes-buffured saline (10 mM Hepes (pH 7.4), 140 mM NaCl, 1.13 mM $MgCl_2$, 4.7 mM KCl, 2 mM $CaCl_2$, and 10 mM glucose) and analyzed with a video image analysis system (Aqua Cosmos, Hamamatsu Photonics). Results are represented as the mean ± SEM (n = 45–92). GFP-positive cells were measured in each experiment and all experiments were repeated at least twice.

Preparation of [^{125}I]I-Pyr3

To a solution of tributyltin derivative **7** (100 μg, 0.13 μmol) in methanol (10 μL), a solution of chloramine-T (300 μg, 1.31 μmol) in methanol (40 μL), acetic acid (10 μL), and a solution of sodium [^{125}I]iodide (1.2 MBq) were added, and adjusted to pH 5.0 with 0.1 M NaOH. The reaction mixture was incubated for 2 h at 35°C and a solution of 10 mg/mL $Na_2S_2O_5$ (20 μL) was added to quench the reaction. The product was purified using HPLC on a 4.6 × 250 mm COSMOSIL 5C18-AR Ⅱ column eluted with acetonitrile and water containing 0.1% TFA (65:35) at a flow rate of 0.9 mL/min.

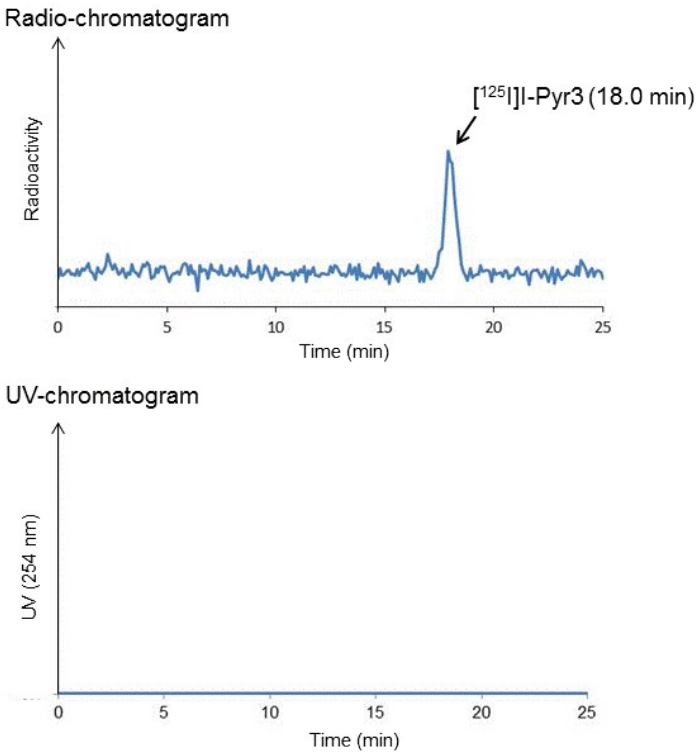

Fig. S5 The radio- and UV-chromatograms of [^{125}I]I-Pyr3. The retention time of [^{125}I]I-Pyr3 (18.0 min) was measured with a radioactivity detector.

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Preparation of [^{125}I]I-Pyr8

To a solution of [^{125}I]I-Pyr3 (500 μL, 0.52 MBq) in methanol/H_2O (3/1), a solution of 1M NaOH (50 μL) was added. The reaction mixture was incubated for 30 min at 50°C. The product was purified using HPLC on a 4.6 × 250 mm COSMOSIL 5C18-AR Ⅱ column eluted with acetonitrile and water containing 0.1% TFA (65:35) at a flow rate of 0.9 mL/min.

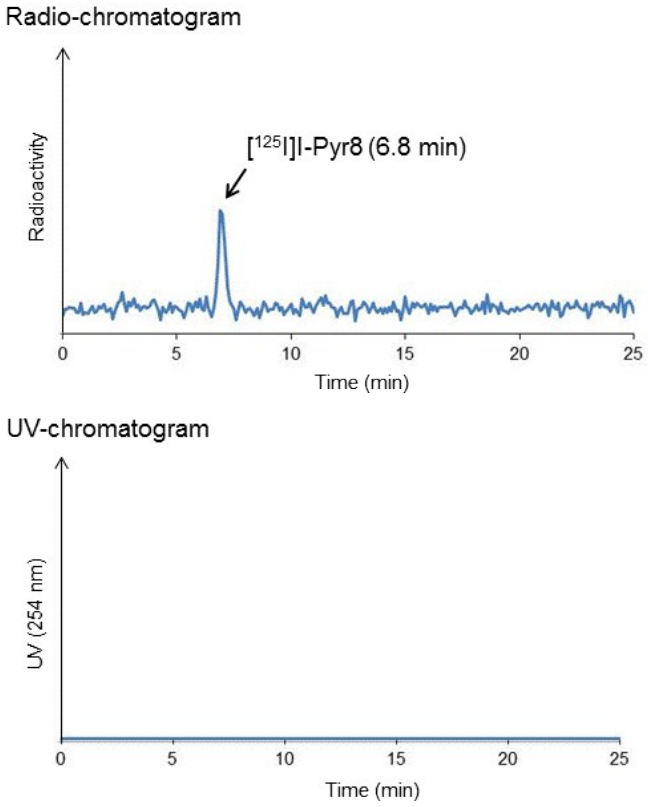

Fig. S6 The radio- and UV-chromatograms of [^{125}I]I-Pyr8. The retention time of [^{125}I]I-Pyr8(6.8 min) was measured with a radioactivity detector.

Stability study

The stability of [^{125}I]I-Pyr3 in PB and mouse plasma was determined by incubating 44 kBq purified [^{125}I]I-Pyr3 in solutions containing 20 mM PB (pH 7.4) or 250 μL mouse plasma at 37°C for 0, 1, 3, 6, and 24 h. Plasma proteins were precipitated by adding 500 μL methanol after centrifugation at 1000 ×*g* for 5 min at 4°C. The radiochemical purity was analyzed by TLC (hexane:EtOAc = 2:1 eluent).

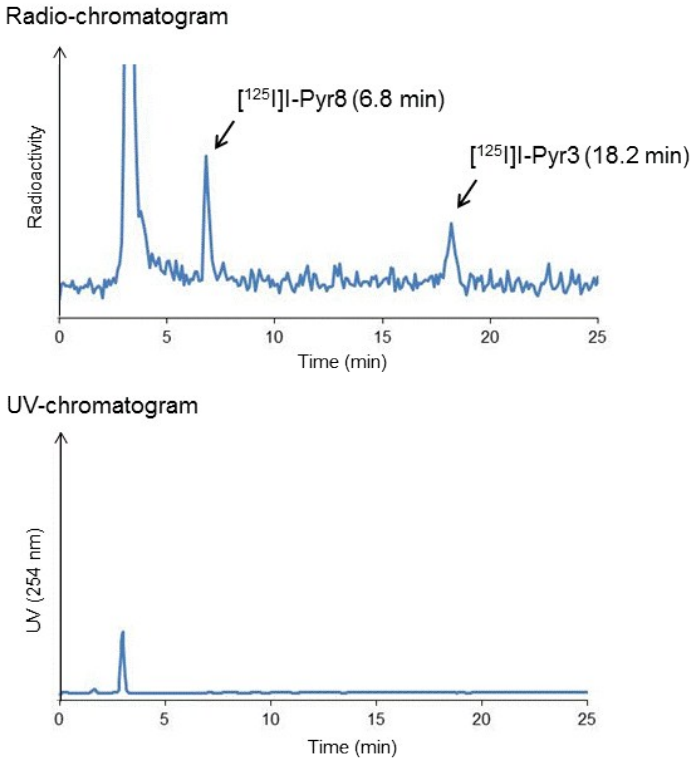

Fig. S7 Metabolite profile of [^{125}I]I-Pyr3 after incubation for 30 min in mouse plasma.

In vivo biodistribution in normal mice

In vivo biodistribution studies were approved by the Kobe Pharmaceutical University Committee on Animal Research and Ethics and performed in six-week-old male ddy mice. A solution of radioiodinated ligands (18.5 kBq, 100 μL) in PB (pH 7.4) was injected directly into the tail vein. The mice were sacrificed at various time points (10 min, 30 min, 1 h, 3 h, and 24 h) post-injection. The organs of interest were removed and weighed. The radioactivity of the organs was counted with an automatic γ-counter (Perkin Elmer 2480 WIZARD^2). The percentage dose per gram of organ and the percentage dose per organ were calculated.