Synthesis and Structure-Activity Relationships of 5,6,7-substituted Pyrazolopyrimidines: Discovery of a novel TSPO PET Ligand for Cancer Imaging

Supporting Information

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1. General information.

All commercially available reagents were used without further purification. Microwave reactions were carried out with a Biotage **Initiator™ Sixty microwave system** (Uppsala, Sweden)**. Reaction residues were purified using a CombiFlash** purification system (TELEDYNE ISCO) with silica cartridges. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker 400 MHz spectrometer in the Vanderbilt Small Molecule NMR Facility. Chemical shifts are

reported in ppm using the residual of chloroform as the internal standard (7.26 ppm for ^{1}H and 77.160 ppm for ^{13}C, respectively). The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet. All CH/CHN elemental analyses were performed by Atlantic Microlab, INC (Norcross, GA, USA). High-resolution mass spectra were acquired with a Waters Synapt hybrid quadrupole/oa-QTof mass spectrometer equipped with a dual channel ES-CI source. All compounds used for biological assays were purified by HPLC and were ≥ 95% purity based on analytical LCMS monitored at 254 nm.

2. Synthetic and analytic data for the compounds.

*3-Cyano-***N,N***-diethyl-4-(4-methoxyphenyl)-4-oxobutanamide (3)*

To a solution of 3-(4-methoxyphenyl)-3-oxopropanenitrile (1.0 g, 5.71 mmol, **1**) and 2-chloro-*N,N*-diethylacetamide (0.854 g, 5.71 mmol, **2**) in 80% EtOH (40 mL) were added NaOH (0.22 g, 5.71 mmol) and KI (2.84 g, 17.1 mmol). The mixture was microwaved at 80 °C for 40 min. The reaction was monitored by TLC (hexane/ethyl acetate = 50/50, v/v). When completed, the mixture was filtered and extracted with CH_2Cl_2. The residue was concentrated and purified by flash chromatography on silica gel (hexane/ethyl acetate = 50/50, v/v) to afford **3** (1.14 g, 70%) as a brown oil. [Found: C, 62.06; H, 6.59; N, 8.56. $C_{16}H_{20}N_2NaO_3$ requires C, 61.73; H, 6.48; N, 9.00%]. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.05 (d, 2H, *J* = 8.8 Hz), 6.85 (d, 2H, *J* = 8.8 Hz), 5.00 (dd, 1H, *J* = 9.2 Hz, 4.5 Hz), 3.89 (s, 3H), 3.39 (m, 4H), 3.33 (m, 1H), 2.86 (m, 1H), 1.28 (t, 3H, *J* = 7.2 Hz), 1.09 (t, 3H, *J* = 7.2 Hz). ^{13}C-NMR ($CDCl_3$, 400 MHz) δ 187.7, 167.1, 164.5, 131.4, 127.1, 117.5, 114.2, 55.5, 41.9, 40.6, 33.6, 32.5, 14.05, 12.9. HRMS calcd for $C_{16}H_{20}N_2O_3Na$ *m/z* = 311.1372 $(M + Na)^+$, found 311.1381.

2-(3-amino-5-(4-methoxyphenyl)-1H-pyrazol-4-yl)*-N,N-*diethylacetamide (4)

To a solution of **3** (1.14 g, 3.95 mmol) in EtOH (18 mL) were added hydrazine (0.126 g, 3.95 mmol) and AcOH (0.13 mL). The mixture was irradiated at 90 °C for 40 min in a Biotage Initiator. The reaction was monitored with TLC (CH_2Cl_2/MeOH = 90/10, v/v). When completed, the residue was washed with saturated aqueous Na_2CO_3 (50 mL) and extracted with CH_2Cl_2 (50 mL). The organic layer was collected and concentrated *in vacuo*. Purification of the residue with flash chromatography on silica gel (CH_2Cl_2/MeOH = 100/0-90/10, v/v) afforded **4** (0.5 g, 42%) as yellow crystals. [Found: C, 58.60; H, 6.59; N, 16.90. $C_{16}H_{22}N_4NaO_2$ requires C, 59.06, H, 6.82, N, 17.22%]. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.32 (d, 2H, *J* = 8.8 Hz), 6.97 (d, 2H, *J* = 8.8 Hz), 3.85 (s, 3H), 3.51 (s, 2H), 3.32 (m, 2H), 3.06 (m, 2H), 1.08 (t, 3H, *J* = 7.2 Hz), 0.93 (t, 3H, *J* = 7.2 Hz). ^{13}C-NMR ($CDCl_3$, 400 MHz) δ 170.2, 159.8, 129.0, 126.6, 122.8, 114.3, 55.3, 42.3, 40.4, 28.5, 14.0, 13.0. HRMS calcd for $C_{16}H_{23}N_2O_2$ *m/z* = 303.1821 (MH^+), found 303.1814.

N,N-*diethyl-2-(2-(4-methoxyphenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (5a, DPA-713)*

To a solution of **4** (0.05 g, 0.165 mmol) in EtOH (8.0 mL) was added 2,4-pentanedione (0.0165 g, 0.165 mmol). The mixture was irradiated with a Biotage Initiator at 180 °C for 45 min. The reaction was monitored by TLC (CH_2Cl_2/MeOH = 90/10, v/v). Following the reaction, the residue was concentrated *in vacuo* and purified with flash chromatography (CH_2Cl_2/MeOH = 100/0-90/10, v/v) to afford **5** (0.055 g, 91%) as brown crystals. [Found: C, 68.61; H, 6.98; N, 15.11. $C_{21}H_{26}N_4O_2$ requires C, 68.83; H, 7.15; N, 15.29%] ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.78 (d, 2H, *J* = 8.8 Hz), 6.99 (d, 2H, *J* = 8.8 Hz), 6.50 (s, 1H), 3.92 (s, 2H), 3.85 (s, 3H), 3.50 (m, 2H), 3.41 (m, 2H), 2.74 (s, 3H), 2.54 (s, 3H), 1.20 (t, 3H, *J* = 7.2 Hz), 1.12 (t, 3H, *J* = 7.2 Hz).

^{13}C-NMR ($CDCl_3$, 400 MHz) δ 170.0, 159.7, 157.3, 154.9, 147.6, 144.6, 129.9, 126.3, 113.8, 108.0, 100.7, 55.2, 42.2, 40.5, 28.1, 24.6, 16.8, 14.3, 13.0. HRMS calcd for $C_{21}H_{26}N_4O_2Na$ *m/z* = 389.1953 $(M + Na)^+$, found 389.1940.

2-(5,7-diethyl-2-(4-methoxyphenyl)pyrazolo[1,5-*a*]pyrimidin-3-yl)-*N,N*-diethylacetamide (5b)

This compound was prepared from **4** with 3,5-heptadione using the same method as **5a**. [Found: C, 65.35; H, 7.30; N, 12.22. $C_{23}H_{30}N_4O_2$ requires C, 70.02; H, 7.66; N, 14.20%] ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.68 (d, 2H, *J* = 8.8 Hz), 7.00 (d, 2H, *J* = 8.8 Hz), 6.61 (s, 1H), 4.01 (s, 2H), 3.86 (s, 3H), 3.50 (m, 2H), 3.42 (m, 2H), 3.26 (m, 2H), 2.94 (m, 2H), 1.47 (t, 3H, *J* = 7.48 Hz), 1.37 (t, 3H, *J* = 7.6 Hz), 1.21 (t, 3H, *J* = 7.2 Hz), 1,13 (t, 3H, *J* = 7.08). ^{13}C-NMR ($CDCl_3$, 400 MHz) δ 170.3, 159.6, 156.9, 154.0, 145.9, 141.7, 129.9, 126.5, 119.9, 113.9, 100.3, 55.2, 42.2, 40.5, 28.0, 23.3, 21.3, 14.3, 14.1, 13.0, 12.9. HRMS calcd for $C_{23}H_{30}N_4O_2$, *m/z* = 385.2447 (MH^+), found 385.2436.

2-(5,7-diisopropyl-2-(4-methoxyphenyl)pyrazolo[1,5-*a*]pyrimidin-3-yl)-*N,N*-diethylacetamide (5c)

This compound was prepared from **4** with 2,6-dimethyl-3,5-heptadione using the same method as **5a**. ^{1}H-NMR ($CDCl_3$, 400 MHz) ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.89 (d, 2H, *J* = 8.8 Hz), 7.00 (d, 2H, *J* = 8.8 Hz), 6.50 (s, 1H), 3.93 (s, 2H), 3.89 (m, 1H), 3.86 (s, 3H), 3.62 (m, 2H), 3.41(m, 2H), 3.04 (m, 1H), 1.44 (d, 6H, *J* = 6.8 Hz), 1.32 (d, 6H, *J* = 6.8 Hz), 1.22 (t, 3H, *J* = 7.2 Hz), 1.11 (t, 3H, *J* = 7.2 Hz). ^{13}C-NMR ($CDCl_3$, 400 MHz) δ 170.6, 165.9, 159.7, 154.7, 154.1, 147.5, 126.5, 113.8, 101.7, 100.7, 55.2, 42.5, 40.7, 36.4, 28.1, 28.0, 20.0, 14.4, 13.1. HRMS calcd for $C_{25}H_{34}N_4O_2$, *m/z* = 423.2760 (MH^+), found 423.2762.

N,N-diethyl-2-(2-(4-methoxyphenyl)-5,6,7-trimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (5d)

This compound was prepared with **4** and 3-methyl-2,4-pentanedione using the same method as **5a**. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.56 (d, 2H, J = 7.2 Hz), 7.01 (d, 2H, J = 7.2 Hz), 4.08 (s, 2H), 3.87 (s, 3H), 3.44 (m, 4H), 2.96 (s,3H), 2.78 (s, 3H), 2.39 (s, 3H), 1.33 (t, 3H, J = 7.2 Hz), 1.16 (t, 3H, J = 7.1 Hz). HRMS calcd for $C_{22}H_{28}N_4O_2$, m/z = 381.2291 (MH^+), found 381.2295.

N,N-diethyl-2-(6-ethyl-2-(4-methoxyphenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (5e)

This compound was prepared with **4** and 3-ethyl-2,4-pentanedione using the same method as **5a**. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.56 (d, 2H, J = 8.8 Hz), 7.01 (d, 2H, J = 8.8 Hz), 4.07 (s, 2H), 3.87 (s, 3H), 3.44 (m, 4H), 2.95 (s, 3H), 2.82 (m, 2H), 2.81 (s, 3H), 1.25 (m, 6H), 1.15 (t, 3H, 7.1 Hz). HRMS calcd for $C_{23}H_{30}N_4O_2$, m/z = 395.2447 (MH^+), found 395.2448.

2-(6-acetyl-2-(4-methoxyphenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)-N,N-diethylacetamide (5f)

This compound was prepared with **4** and triacetylmethane using the same method as **5a**. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.67 (d, 2H, J = 8.8 Hz), 7.00 (d, 2H, J = 8.8 Hz), 3.98 (s, 2H), 3.87 (s, 3H), 3.49 (m, 2H), 3.43 (m, 2H), 2.78 (s, 3H), 2.60 (s, 3H), 2.55 (s, 3H), 1.23 (t, 3H, J = 7.16), 1.13 (t, 3H, 7.1 Hz). HRMS calcd for $C_{23}H_{28}N_4O_2$, m/z = 409.2240 (MH^+), found 409.2241.

2-(6-chloro-2-(4-methoxyphenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)-N,N-diethylacetamide (5g)

This compound was prepared with **4** and 3-chloro-2,4-pentanedione *via* the same method as **5a**. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.72 (d, 2H, *J* = 8.8 Hz), 7.00 (d, 2H, *J* = 8.8 Hz), 3.94 (s, 2H), 3.87 (s, 3H), 3.49 (m, 2H), 3.43 (m, 2H), 2.91 (s, 3H), 2.66 (s, 3H), 1.23 (t, 3H, *J* = 7.16), 1.13 (t, 3H, 7.1 Hz). HRMS calcd for $C_{21}H_{25}ClN_4O_2$, *m/z* = 401.1744 (MH^+), found 401.1747.

N,N-*diethyl-2-(2-(4-(2-fluoroethoxy)phenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (6a, DPA-714)*

To a solution of **5** (55 mg, 0.15 mmol) in aqueous HBr (4 mL) was added HTPB (8.0 mg, 0.015 mmol). The reaction mixture was irradiated with a Biotage Initiator at 110 °C for 40 min. The reaction was monitored by TLC (CH_2Cl_2/MeOH = 90/10, v/v). When completed, the mixture was washed with saturated aqueous $NaHCO_3$ (50 mL) and extracted with CH_2Cl_2 (50 mL x 2). The organic layer was collected and concentrated *in vacuo*. The crude product was then added to a suspension of NaH (7.4 mg, 0.30 mmol) in dry THF (4.0 mL). The reaction mixture was stirred for 10 min at 0 °C and then allowed to warm to room temperature before addition of a solution of 2-fluoroethyl-4-methylbenzenesulfonate (100 mg, 0.46 mmol) in dry THF (2.0 mL). The mixture was irradiated at 120 °C for 30 min with a Biotage Initiator. The reaction was monitored with TLC (CH_2Cl_2/MeOH = 95/5, v/v). When complete, the reaction residue was partitioned between aqueous 1.0 M HCl (50 mL) and CH_2Cl_2 (50 mL). The organic layer was collected and concentrated *in vacuo*. Final purification of the residue using a Gilson HPLC afforded **6** (40 mg, 64%) as white crystals. [Found: C, 66.56; H, 6.94. $C_{22}H_{27}FN_4O_2$ requires C, 66.31; H, 6.83%]. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.68 (d, 2H, *J* = 8.8 Hz), 7.02 (d, 2H, *J* = 8.8 Hz), 6.58 (s, 1H), 4.85 (t, 1H, 4.2Hz), 4.73 (t, 1H, 4.2Hz) 4.30 (t, 1H, 4.4Hz), 4.23 (t, 1H, 4.4Hz), 3.98 (s, 2H), 3.47 (m, 2H), 3.42 (m, 2H), 2.79 (s, 3H), 2.62 (s, 3H), 1.21 (t, 3H, *J* = 7.2 Hz), 1.12 (t, 3H, *J* =

7.2 Hz). ^{13}C-NMR ($CDCl_3$, 400 MHz) δ 170.1, 158.9, 157.8, 155.8, 147.04, 145.70, 130.0, 126.0, 114.7, 108.1, 100.2, 82.64, 80.95, 67.2, 67.0, 42.31, 40.91, 27.9, 23.3, 17.0, 14.0, 12.8. HRMS calcd for $C_{22}H_{27}FN_4O_2Na$ *m/z* = 421.2016 $(M + Na)^+$, found 421.2006.

2-(5,7-diethyl-2-(4-(2-fluoroethoxy)phenyl)pyrazolo[1,5-*a*]pyrimidin-3-yl)-*N,N*-diethylacetamide (6b)

This compound was prepared with **5b** *via* the same method as **6a**. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.80 (d, 2H, *J* = 8.8 Hz), 7.02 (d, 2H, *J* = 8.8 Hz), 6.54 (s, 1H), 4.85 (t, 1H, 4.2 Hz), 4.73 (t, 1H, *J* = 4.3 Hz) 4.31 (t, 1H, *J* = 4.2 Hz), 4.23 (t, 1H, *J* = 4.2 Hz), 3.95 (s, 2H), 3.54 (m, 2H), 3.41 (m, 2H), 3.21 (m, 2H), 2.86 (m, 2H), 1.45 (t, 3H, *J* = 7.2 Hz), 1.34 (t, 3H, *J* = 7.2 Hz), 1.20 (t, 3H, *J* = 7.2 Hz), 1.11 (t, 3H, *J* = 7.2 Hz). ^{13}C-NMR ($CDCl_3$, 400 MHz) δ 170.40, 162.40, 158.60, 154.91, 150.36, 146.97, 130.05, 126.76, 114.61, 104.80, 100.55, 82.69, 80.99, 67.17, 66.96, 42.40, 40.74, 31.01, 27.98, 23.30, 14.22, 12.94, 12.84, 10.21. HRMS calcd for $C_{24}H_{31}FN_4O_2$ *m/z* = 427.2509 (MH^+), found 427.2500.

N,N*-diethyl-2-(2-(4-(2-fluoroethoxy)phenyl)-5,7-diisopropylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (6c)

This compound was prepared with **5c** *via* the same method as **6a**. ^{1}H-NMR ($CDCl_3$, 400 MHz δ 7.91 (d, 2H, *J* = 8.8 Hz), 7.02 (d, 2H, *J* = 8.8 Hz), 6.50 (s, 1H), 4.85 (t, 1H, *J* = 4.3 Hz), 4.73 (t, 1H, *J* = 4.3 Hz), 4.31 (t, 1H, *J* = 4.2 Hz), 4.23 (t, 1H, *J* = 4.2 Hz), 3.93 (s, 2H), 3.88 (m, 1H), 3.63 (m, 2H), 3.41 (m, 2H), 3.05 (m, 1H), 1.44 (d, 6H, *J* = 5.6 Hz), 1.33 (d, 6H, *J* = 5.6 Hz), 1.23 (t, 3H, *J* = 7.1 Hz), 1.11 (t, 3H, *J* = 7.1 Hz). HRMS calcd for $C_{26}H_{35}FN_4O_2$ *m/z* = 455.2822 (MH^+), found 455.2815.

N,N-*diethyl-2-(2-(4-(2-fluoroethoxy)phenyl)-5,6,7-trimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (6d)*

This compound was prepared with **5e** *via* the same method as **6a**. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.66 (d, 2H, *J* = 8.8 Hz), 7.02 (d, 2H, *J* = 8.8 Hz), 4.85 (t, 1H, *J* = 4.2 Hz), 4.73 (t, 1H, *J* = 4.3 Hz), 4.31 (t, 1H, *J* = 4.2 Hz), 4.23 (t, 1H, *J* = 4.1 Hz), 4.00 (s, 2H), 3.44 (m, 4H), 2.87 (s, 3H), 2.67 (s, 3H), 2.34 (s, 3H), 1.20 (s, 3H), 1.12 (t, 3H, *J* = 7.2 Hz). HRMS calcd for $C_{23}H_{29}FN_4O_2$ *m/z* = 413.2353 (MH^+), found 413.2349.

N,N-*diethyl-2-(6-ethyl-2-(4-(2-fluoroethoxy)phenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (6e)*

This compound was prepared with **5f** *via* the same method as **6a**. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.78 (d, 2H, *J* = 8.8 Hz), 7.01 (d, 2H, *J* = 8.8 Hz), 4.84 (t, 1H, *J* = 4.2 Hz), 4.72 (t, 1H, *J* = 4.2 Hz), 4.30 (t, 1H, *J* = 4.2 Hz), 4.23 (t, 1H, *J* = 4.2 Hz), 3.92 (s, 2H), 3.51 (m, 2H), 3.41 (m, 2H), 2.79 (s, 3H), 2.73 (m, 2H), 2.60 (s, 3H), 1.20 (m, 6H), 1.12 (d, 3H, *J* = 8.5 Hz). HRMS calcd for $C_{24}H_{31}FN_4O_2$ *m/z* = 427.2509 (MH^+), found 427.2496.

2-(6-acetyl-2-(4-(2-fluoroethoxy)phenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)-*N,N*-diethylacetamide (6f)

This compound was prepared with **5g** *via* the same method as **6a**. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.77 (d, 2H, *J* = 8.8 Hz), 7.02 (d, 2H, *J* = 8.8 Hz), 4.85 (t, 1H, *J* = 4.2 Hz), 4.73 (t, 1H, *J* = 4.2 Hz), 4.31 (t, 1H, *J* = 4.2 Hz), 4.24 (t, 1H, *J* = 4.2 Hz), 3.92 (s, 2H), 3.52 (m, 2H), 3.43 (m, 2H), 2.76 (s, 3H), 2.59 (s, 3H), 2.53 (s, 3H), 1.25 (t, 3H, *J* = 7.2 Hz), 1.13 (t, 3H, *J* = 7.2 Hz). HRMS calcd for $C_{24}H_{29}FN_4O_3$ *m/z* = 441.2302 (MH^+), found 441.2295.

2-(6-chloro-2-(4-(2-fluoroethoxy)phenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)-*N,N*-diethylacetamide (6g)

This compound was prepared with **5h** *via* the same method as **6a**. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.79 (d, 2H, J = 8.8 Hz), 7.02 (d, 2H, J = 8.8 Hz), 4.84 (t, 1H, J = 4.2 Hz), 4.72 (t, 1H, J = 4.2 Hz), 4.30 (t, 1H, J = 4.2 Hz), 4.23 (t, 1H, J = 4.1 Hz), 3.90 (s, 2H), 3.51 (m, 2H), 3.42 (m, 2H), 2.91 (s, 3H), 2.65 (s, 3H), 1.23 (t, 3H, J = 7.2 Hz), 1.12 (t, 3H, J = 7.2 Hz). HRMS calcd for $C_{22}H_{26}ClFN_4O_2$ m/z = 433.1807(MH^+), found 433.1790.

2-(4-(3-(2-(diethylamino)-2-oxoethyl)-5,7-diethylpyrazolo[1,5-*a*]pyrimidin-2-yl)phenoxy)ethyl 4-methylbenzenesulfonate (7)

To a solution of **5** (260 mg, 0.66 mmol) in aqueous HBr (20 mL) was added hydroxyl-terminated polybutadiene (HTPB) (35.5 mg, 0.07 mmol). The reaction mixture was irradiated with microwaves at 110 °C for 40 min. The reaction was monitored by TLC (CH_2Cl_2/MeOH = 90/10, v/v). When complete, the mixture was washed with saturated aqueous $NaHCO_3$ (100 mL) and extracted with CH_2Cl_2 (50 mL x 3). The organic layer was collected and concentrated in *vacuo*. The crude product was then added to a suspension of NaH (17 mg, 0.71 mmol) in dry THF (10.0 mL). The reaction mixture was stirred for 10 min at 0 °C and then allowed to warm to room temperature before addition of a solution of 2-fluoroethyl-4-methylbenzenesulfonate (366 mg, 0.99 mmol) in dry THF (5.0 mL). The mixture was irradiated at 120 °C for 30 min with a Biotage Initiator. The reaction was monitored with TLC (CH_2Cl_2/MeOH = 95/5, v/v). When complete, the reaction residue was partitioned between aqueous 1.0 M HCl (50 mL) and CH_2Cl_2 (50 mL). The organic layer was collected, dried over $MgSO_4$ and concentrated *in vacuo*. Final purification of the residue on a CombiFlash (TELEDYNE ISO) afforded **7** (170 mg, 45%) as

yellow crystals. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.83 (d, 4H, 8.5 Hz), 7.36 (d, 2H, *J* = 8.0 Hz), 6.86 (d, 2H, *J* = 8.8 Hz), 6.50 (s, 1H), 4.40 (t, 2H, *J* = 5.0 Hz), 4.19 (t, 2H, 4.6 Hz), 3.91 (s, 2H), 3.57 (m, 2H), 3.41 (m, 2H), 3.19 (m, 2H), 2.83 (m, 2H), 2.45 (s, 3H), 1.44 (t, 3H, *J* = 7.5 Hz), 1.34 (t, 3H, 7.6 Hz), 1.22 (t, 3H, 7.1 Hz), 1.11 (t, 3H, 7.1 Hz). ^{13}C-NMR ($CDCl_3$, 400 MHz) δ 170.27, 162.20, 158.05, 154.54, 149.62, 147.52, 144.91, 132.81, 129.99, 129.81, 127.96, 127.19, 114.47, 104.87, 100.77, 68.04, 65.34, 42.38, 40.60, 31.33, 27.99, 23.22, 21.59, 14.34, 13.04, 12.76, 10.25.

3. Measurement of Log $P_{7.5}$

The lipophilicity of each library entrant was examined by determination of the log $P_{7.5}$ value using a HPLC method previously described.[28] Samples were analyzed using a C18 Dynamax column (Varian, 250 x 4.6 mm) and a mobile phase of MeOH and phosphate buffer (85/15, v/v, pH = 7.5) with a flow rate of 1.0 mL/min. The lipophilicity of each ligand was estimated by comparison of retention time to that of standards having known log P values. The standards used were catechol, aniline, benzene, bromobenzene, ethyl benzene, trimethylbenzene, and hexachlorobenzene, each dissolved in an appropriate solvent. All sample injections were done in triplicate and the results averaged to provide the final values. Relative retention times, RRT (to catechol), were calculated, and a calibration curve of log P *versus* log RRT was generated. The calibration equations were polynomial with an r^2 of 0.994 or greater.

4. X-Ray Crystallography

A crystal of **6b** was mounted on a glass fiber and maintained at 100 K during data collection. Data collection was done using an Agilent Technologies Xcalibur PX2 Ultra with a sealed-tube

copper X-ray source and an ONYX CCD area detector. Data reduction was done using Chrysalis software (Agilent Technologies). The data were phased using SIR2011.

5. Radiosynthesis

^{18}F-6b and the radioligand precursor (**7**) were prepared according to our published methods.[22] In brief, using a commercial apparatus (TRACERlab FX F-N; GE Healthcare), we dried aqueous ^{18}F-fluoride ion (~3 Ci/111 GBq) by iterative cycles of addition and evaporation of acetonitrile, followed by complexation with K^+-$K_{2.2.2}$/K_2CO_3. The complex was then reacted with 2-(4-(3-(2-(diethylamino)-2-oxoethyl)-5,7-diethylpyrazolo[1,5-*a*]pyrimidin-2-yl)phenoxy)ethyl 4-methylbenzenesulfonate (**7**) (4.0 mg) at 99 °C for 20 min in anhydrous dimethyl sulfoxide (0.6 mL). **^{18}F-6b** was purified using reversed-phase high-performance liquid chromatography (C18, Dynamax 250 x 21.4 mm; Varian), eluting at 6.0 mL/min with 10 mM NaH_2PO_4 buffer (pH 6.7) and ethanol (47.5/52.5, v/v). **^{18}F-6b** was collected directly into 140 mL of water (deionized), passed through a C-18 Sep-Pak (Waters) followed by sequential elution with 200 proof ethanol (1.0 mL) and saline (9.0 mL) into a sterile vial. Typical specific activities were 4203 Ci/mmol (156 TBq/mmol) or greater.

The radiotracer preparation was visually inspected for clarity, absence of color, and particulates. Specific activity of the radiotracer was calculated from 33 individual radiolabeling processes based on HPLC chromatography. The specific radioactivity was determined as follows: a known amount of radioactive product was injected and the area of the UV absorbance peak corresponding to the radiolabelled product was determined (integration value) from the HPLC chromatogram. This value was then compared with a standard curve, generated from

nonradioactive **6b**, relating mass to UV absorbance. The quotient of activity injected and mass gave the specific activity value.

6. In vitro TSPO-Binding Assay

C6 cells were collected and washed with PBS buffer three times, and then frozen and thawed three times in lysis buffer (5.0 mM HEPES, 0.21 M D-mannitol, 0.07 M sucrose, 2.0 mM benzamidine, 2.0 mM toluenesulfonyl fluoride, 4.0 mM $MgCl_2$, pH 7.4) to produce C6 cell lysate (0.5 mg/mL). The obtained C6 cell lysate (0.3 mL) was then incubated with ^{3}H-PK 11195 (final concentration 0.6 nM) (Perkin Elmer, Waltham, MA, USA) and TSPO ligands (**Table 1**) (10^{-5} to 10^{-12} M) in a total volume of 1.0 mL for 2 h at 25 °C. The reaction was terminated by rapid filtration through a Brandel harvester (Gaithersburg, MD, USA) and collection onto a filter presoaked with 0.3% polyethyleneimine. Filters were then punched out into scintillation vials and bound radioactivity measured on a Beckman LS 6000 Scintillation Counter (Brea, California, USA). Binding affinity (*K*i) was calculated using Prism GraphPad (La Jolla, CA, USA) using 5 nM as the *K*d for PK 111 95 and a radioligand concentration of 0.6 nM.

7. *In vitro* CBR-Binding Assay

Male Wistar rats were decapitated and the cerebral cortex collected and dissected. The cerebral cortex was homogenized in 20 volumes of ice-cold PBS buffer (pH 7.4) with a Brinkmann microhomogenizer (Kinematica AG, Luzern, SW). The homogenate was collected at a protein concentration of 5.0 mg/mL and stored at -80 °C. The crude cerebral cortex preparation (0.3 mL) was incubated with ^{3}H-flunitrazepam (final concentration 0.6 nM) (Perkin Elmer) and **6b** (10^{-5} to 10^{-7} M) in a total volume of 1.0 mL for 2 h at 25 °C. The reaction was terminated by rapid

filtration through a Brandel harvester and collection onto a filter presoaked with 0.3% polyethyleneimine. Filters were then punched out into scintillation vials and bound radioactivity measured on a Beckman LS 6000 Scintillation Counter.

8. Rat Model

All studies involving animals were conducted in compliance with federal and institutional guidelines. Two weeks before imaging, healthy male Wistar rats were stereotactically inculcated in the right hemisphere with 1.0 x 10^5 C6 glioma cells (American Type Tissue Collection). Prior to imaging, all rats were affixed with venous and arterial catheters.

9. MRI Imaging

MRI was used to localize C6 tumors. Rats were secured prone in a radiofrequency coil (38-mm inner diameter) and placed in a 4.7-T horizontal bore imaging system (Varian Inc., Palo Alto, CA, USA). A constant body temperature of 37 °C was maintained using heated airflow. An initial multislice gradient-echo imaging sequence (repetition time, 150 ms; echo time, 3.5 ms; matrix, 128 x 128; field of view, 40 x 40 mm^2; slice thickness, 2 mm) was used to acquire 7 slices in each imaging plane (axial, coronal, sagittal) for proper positioning of subsequent scans. A multislice T_2-weighted fast spin-echo scan with 8 echoes and 8.0-ms echo spacing (effective echo time, 32 ms) was then collected with a repetition time of 2,000 ms; field of view of 32 x 32 mm^2; matrix of 128 $\times$ 128; 16 acquisitions; and 8 coronal slices of 2-mm thickness.

10. PET/CT Imaging

PET/CT was performed within 24 hours of MRI in rats with confirmed tumors. Tumor-bearing rats were administered **^{18}F-6b** *via* jugular catheter while in a microPET Focus 220 scanner (Siemens, Munich, DE). Data were collected in list-mode format for 90 minutes, followed by a CT scan (microCAT II; Siemens) for attenuation correction. For reconstruction, the dynamic PET acquisition was divided into twelve 10-s frames for the first two minutes, three 60-second frames for the following three minutes, and seventeen 300-second frames for the duration of the scan. The raw data within each frame were then binned into three-dimensional sinograms, with a span of three and ring difference of 47. The sonograms were reconstructed into tomographic images (128 x 128 x 95) with voxel sizes of 0.095 x 0.095 x 0.08 cm^3, after scatter and attenuation corrections were applied, using a two-dimensional ordered-subsets expectation-maximization algorithm with 16 subsets and four iterations. Attenuation correction was accomplished by generating an attenuation map from the CT images. The CT image was first coregistered with the small animal PET image, segmented, and then projected into sonogram space with a span of 47 and ring difference of 23. Time-activity curves were generated by manually drawing 3-dimensional volumes of interest over tumor and contralateral brain using ASIPro (Siemens).

11. HPLC Radiometabolite Analysis

Arterial blood (200 μL) was collected at 2, 12, 30, 60, and 90 min following injection of 1.5 mCi of ^{18}F-6b. After centrifugation, the plasma was extracted with acetonitrile/water (340 μL, 7/1, v/v). The mixture was then centrifuged and the supernatant used for reversed-phase HPLC analysis with 0.1 M aqueous ammonium acetate (NH_4OAc) (pH 10) and acetonitrile (30/70, v/v)

at 1.0 mL/min on a C18 Dynamax 250 x 4.6 mm column (Varian). Radiochromatographic data were recorded and collected using a radioisotope detector (Bioscan, Washington, DC, USA).

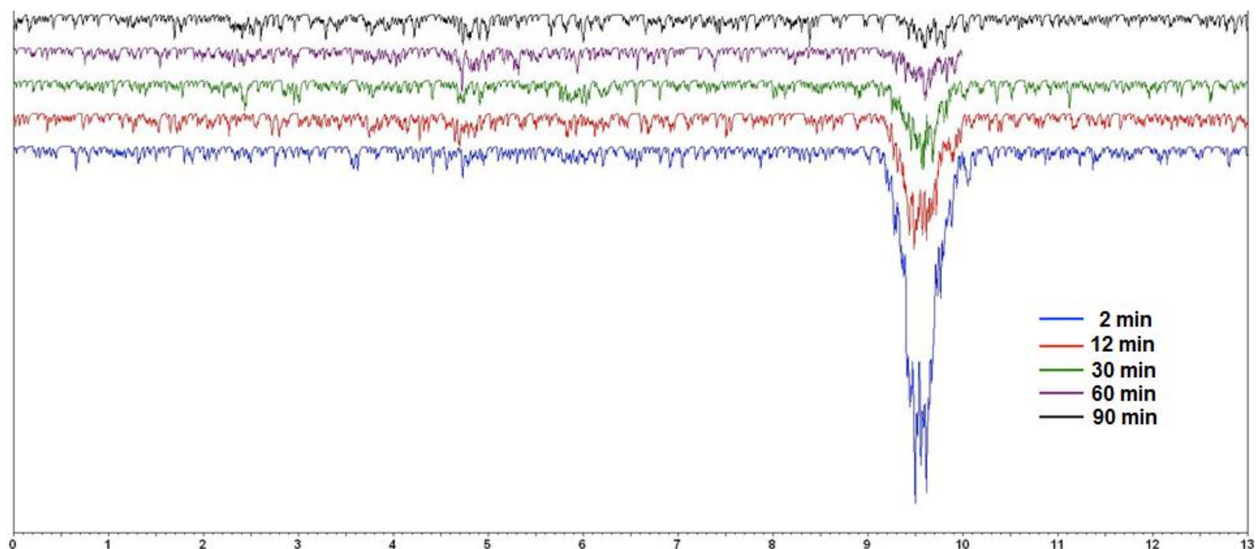
12. Histology

Whole brains were harvested and fixed in 4% formalin for 48 hours, followed by paraffin embedding for immunohistochemistry. Tissue sections of 5.0-μm thickness were taken and stained with TSPO-specific rabbit polyclonal antibody, a gift from Professor Vassilios Papadopoulos of McGill University. Immunoreactivity was assessed using a horseradish peroxidase detection kit (Dako, Glostrup, DK). Hematoxylin and eosin staining was used to evaluate cell density and tumor localization.

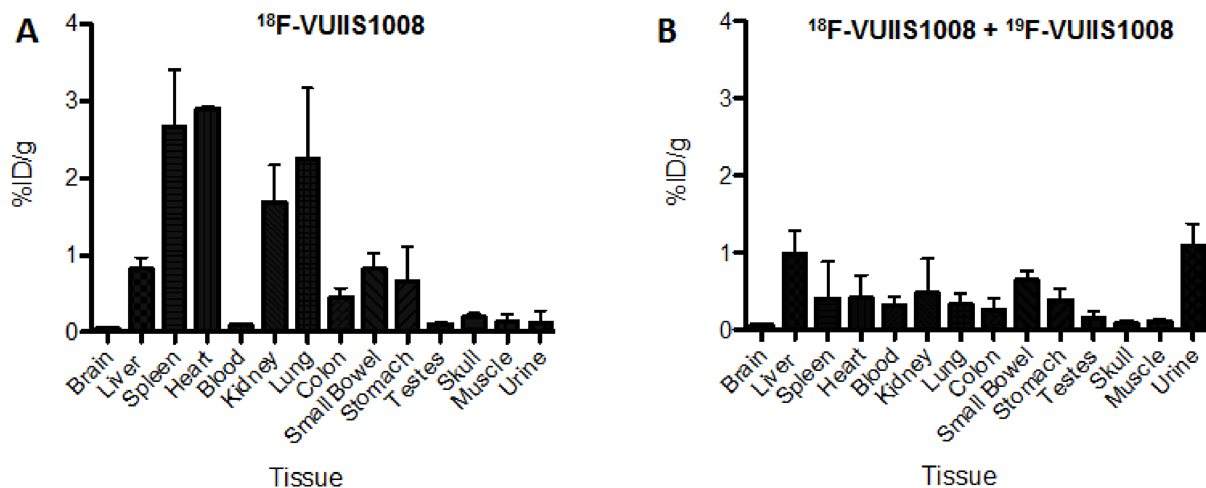
13. Biodistribution

Fourteen different tissue samples were collected for the biodistribution study 60 minutes of 1.0 – 1.5 mCi of ^{18}F-6b injection. Tissues included brain, liver, spleen, heart, blood, kidney, lung, colon, small bowel, stomach, testes, skull, muscle, and urine. For rats with displacement, **6b** (10 mg/kg) was injected 30 minutes after **^{18}F-6b** injection. Radioactivity of the samples was determined with a NaI well counter (Capintec, Ramsey, NJ, USA). %ID/g value of each sample was calculated according to the weight and radioactivity of the samples.

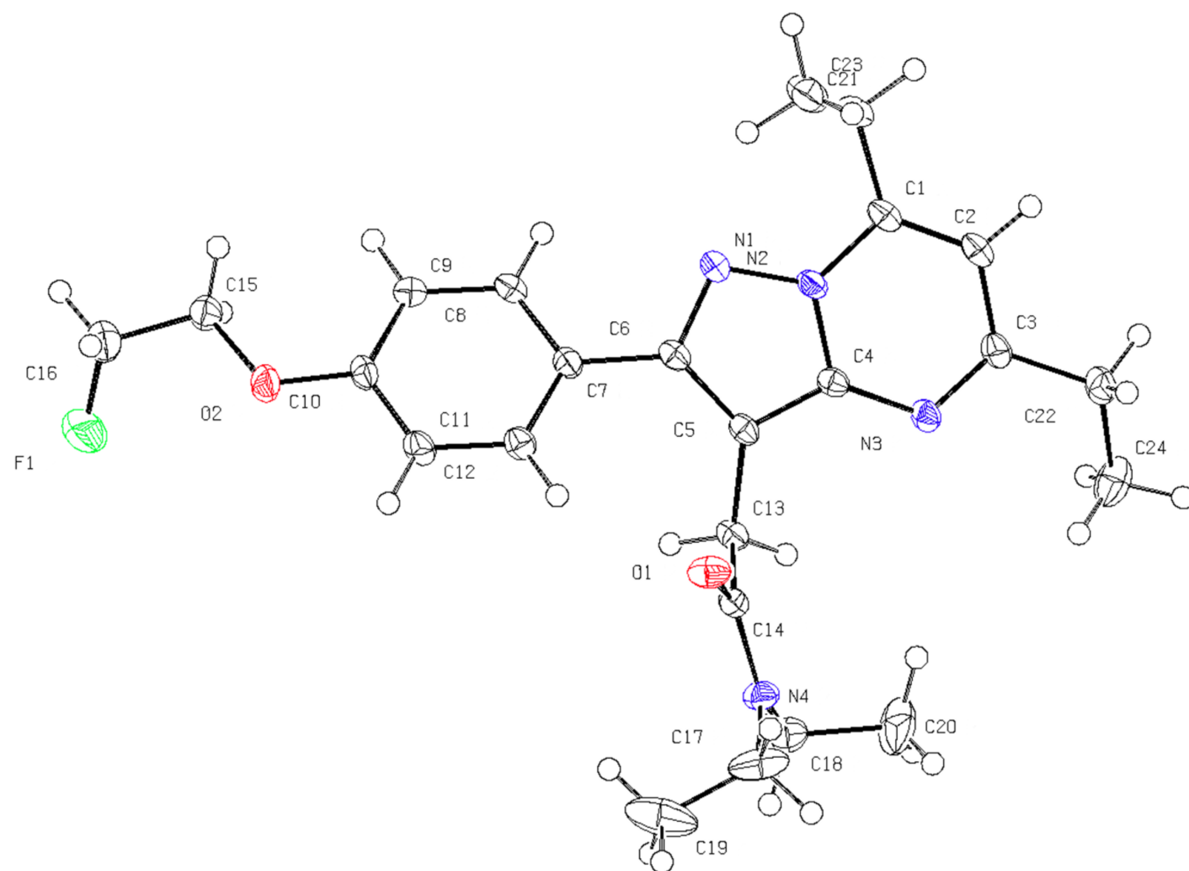
14. Supplemental Figures:



Supplemental Figure 1. HPLC radiometabolite analysis of plasma extract following administration of ^{18}**F-6b**. Post-administration times denoted by color. Inset: Integrated area of the corresponds to ^{18}**F-6b** parent peak over time.



Supplemental Figure 2. Biodistribution of ^{18}**F-6b** in normal rat tissues, 90 minutes post tracer administration. (A) ^{18}**F-6b** uptake without cold analog (6b) challenge. (B) ^{18}**F-6b** uptake with cold analog (**6b**) challenge administered 30 minutes following ^{18}**F-6b** administration.



Supplemental Figure 3. Crystal Structure for **6b**.