Supplemental Data

Development of a selective and high affinity radioligand, 
\[{}^3\text{H}\]VU6013720, for the M₄ muscarinic receptor

Aidong Qi, Haley E. Kling, Natasha Billard, Alice L. Rodriguez, Li Peng, Jonathan W. Dickerson, Julie L. Engers, Aaron M. Bender, Mark S. Moehle, Craig W. Lindsley, Jerri M. Rook, and Colleen M. Niswender

Supplemental Figures

Supplemental Figure 1. VU6013719 fully competes for binding of \([{}^3\text{H}]\)VU6013720. Increasing concentrations of VU6013719 were added with \([{}^3\text{H}]\)VU6013720 to rat M₄-expressing cells. Data are the Mean ± SD from a representative assay of three independent experiments performed in triplicate.
Supplemental Figure 2. [³H]VU6013720 and [³H]NMS label a similar number of sites in M₄-expressing cells. Increasing concentrations of [³H]VU6013720 (white) or [³H]NMS (black) were added to membranes from rat M₄ cells and specific binding was assessed. Bₘₐₓ values were 4500 ± 700 with [³H]NMS and 4100 ± 500 fmol/mg with [³H]VU6013720, and pKₐ values were 10 ± 0.1 with [³H]NMS and 9.6 ± 0.3 with [³H]VU6013720, respectively. Data represent Mean ± SD performed three times in triplicate; data shown are representative of a single experiment.
Supplemental Figure 3. $[^3]H$VU6013720 specifically binds to the human and mouse $M_4$ receptors. Specific binding of $[^3]H$VU6013720 to human (A) and mouse (B) $M_4$ receptors was evaluated using saturation binding assays, as described in Methods and Figure 3. $[^3]H$VU6013720 binds to human and mouse $M_4$ receptors with high affinity and in a saturable manner (refer to Table 2 for affinities). Data are the Mean ± SD from four experiments performed in quadruplicate (human) and 1 assay (mouse) performed in triplicate. Atropine was used as the nonspecific binding control.
**Synthesis of VU6013719.**

\[
\text{tert-butyl (3aR,5s,6aS)-5-((6-chloropyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (3) was prepared in a similar manner as Intermediate 3 from (Moehle, Bender et al. 2021). tert-butyl (3aR,5s,6aS)-5-aminohexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (1, 5.0 g, 22.1 mmol, 1 eq) and 3,6-dichloropyridazine (2, 9.87 g, 66.3 mmol, 3 eq) were combined in tert-butanol (40 mL) and DIPEA (11.5 mL, 66.3 mmol, 3 eq) was added. The resulting solution was heated to 150 °C under microwave irradiation for 2 h, after which time the reaction mixture was concentrated under reduced pressure, and crude residue was purified by normal phase...}
\]
column chromatography on silica gel (3-100% EtOAc in hexanes) to give the title compound as a tan solid (4.82 g, 64%). ¹H-NMR (400 MHz, MeOD) δ 7.27 (d, J = 9.4 Hz, 1H), 6.87 (d, J = 9.4 Hz, 1H), 4.41 (p, J = 6.3 Hz, 1H), 3.55 (dd, J = 11.1, 8.0 Hz, 2H), 3.19 (dd, J = 11.4, 3.8 Hz, 2H), 2.90 – 2.80 (m, 2H), 1.90 – 1.92 (m, 2H), 1.89 – 1.81 (m, 2H), 1.46 (s, 9H). ¹³C-NMR (101 MHz, MeOD) δ 159.3, 156.3, 146.8, 130.3, 120.5, 80.8, 53.7, 53.2 (signal broadening is observed), 42.3 (signal broadening is observed), 39.5, 28.8; ES-MS [M+H-t-butyl]⁺ = 283.2.

tert-butyl (3aR,5s,6aS)-5-((6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (5). tert-butyl (3aR,5s,6aS)-5-((6-chloropyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (3, 102 mg, 0.3 mmol, 1.0 eq), 2-chloro-5-fluorophenylboronic acid (4, 68.0 mg, 0.39 mmol, 1.3 eq), potassium carbonate (126 mg, 0.9 mmol, 3.0 eq) and BrettPhos-Pd-G3 (27.2 mg, 0.03 mmol, 0.1 eq) were combined in a flask, and 1,4-dioxane (2.4 mL) and water (0.6 mL) were added. The resulting mixture was evacuated and purged with nitrogen (3x). The resulting mixture was stirred under an inert atmosphere at 100 °C. After 2 h, the reaction mixture was diluted with EtOAc, filtered through a pad of Celite which was rinsed thoroughly with EtOAc, and the filtrate was concentrated under reduced pressure. The crude residue was purified using normal phase column chromatography on silica gel (0-100% EtOAc in hexanes) to provide the title compound as a pale-yellow powder (84 mg, 64%). ¹H NMR (400 MHz, DMSO) δ 7.61 (dd, J = 8.9, 5.2 Hz, 1H), 7.54 (d, J = 9.3 Hz, 1H), 7.44 (dd, J = 9.3, 3.1 Hz, 1H), 7.33 (ddd, J = 8.8, 8.1, 3.2 Hz, 1H), 7.17 (d, J = 6.7 Hz, 1H), 6.86 (d, J = 9.3 Hz, 1H), 4.45 (h, J = 6.6 Hz, 1H), 3.49 (dd, J = 11.1, 8.2 Hz, 2H), 3.30 (s, 2H),
(3aR,5s,6aS)-N-(6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)-2-(cyclohexylmethyl)octahydrocyclopenta[c]pyrrol-5-amine (VU6013719). tert-butyl (3aR,5s,6aS)-5-((6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (5, 83.6 mg, 0.19 mmol, 1.0 eq) was dissolved in 1,4-dioxane (1.9 mL). A solution of HCl in 1,4-dioxane (4M, 0.72 mL, 2.96 mmol, 15 eq) was added dropwise. After 2 h at r.t., the reaction mixture was concentrated under reduced pressure to give the HCl salt, which was dried under vacuum and carried forward without additional purification (71 mg). The HCl salt was suspended in DCE (2.0 mL) and THF (0.5 mL) and cyclohexanecarbaldehyde (64.7 mg, 0.58 mmol, 3.0 eq) was added. The resulting mixture was stirred at r.t. for 15 min and sodium triacetoxyborohydride (122 mg, 0.58 mmol, 3.0 eq.) was added. After 1 h at 50 °C, sat. NaHCO₃ solution was added to the reaction mixture, and the aqueous layer was extracted with DCM (3x). The combined organic extracts were washed with brine, dried over Na₂SO₄, filtered and concentrated. Purification using normal phase chromatography on silica gel (0-10% MeOH in DCM) provided the title compound (56 mg, 68% over 2 steps); ¹H NMR (400 MHz, DMSO) δ 7.61 (dd, J = 8.9, 5.2 Hz, 1H), 7.53 (d, J = 9.3 Hz,
1H), 7.44 (dd, J = 9.3, 3.1 Hz, 1H), 7.32 (ddd, J = 8.8, 8.1, 3.2 Hz, 1H), 7.04 (d, J = 6.9 Hz, 1H), 6.83 (d, J = 9.3 Hz, 1H), 4.49 – 4.41 (m, 1H), 2.67 – 2.61 (m, 1H), 2.50 – 2.46 (m, 2H), 2.31 – 2.23 (m, 2H), 2.15 (d, J = 7.2 Hz, 2H), 1.86 – 1.75 (m, 4H), 1.70 – 1.57 (m, 5H), 1.46 – 1.35 (m, 1H), 1.28 – 1.08 (m, 3H), 0.89 – 0.79 (m, 2H); 13C NMR (101 MHz, DMSO) δ 160.8 (d, J = 246.4 Hz), 157.9, 148.9 (d, J = 2.0 Hz), 138.9 (d, J = 8.1 Hz), 131.6 (d, J = 9.1 Hz), 128.3, 126.6 (d, J = 2.0 Hz), 117.8 (d, J = 24.2 Hz), 116.8 (d, J = 22.2 Hz), 113.4, 62.4, 61.9, 52.0, 36.4, 31.4, 26.4, 25.6; ES-MS [M+H]+ = 429.1.