Supporting Information

Discovery of VU6028418: A Highly Selective and Orally Bioavailable M_4 Muscarinic Acetylcholine Receptor Antagonist

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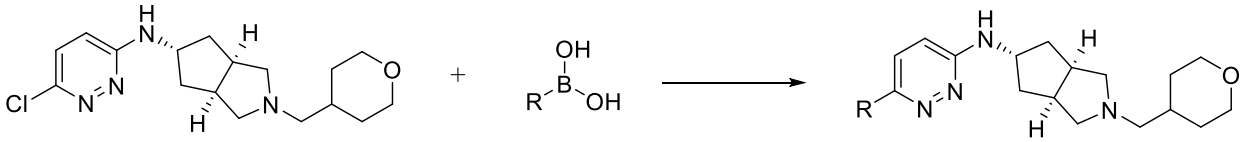
General Methods

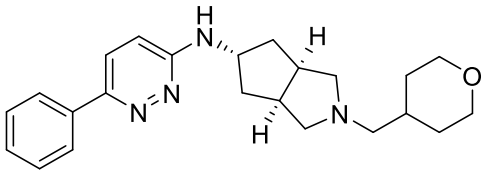
All reactions were carried out employing standard chemical techniques under inert atmosphere. Solvents used for extraction, washing, and chromatography were HPLC grade. All reagents were purchased from commercial sources and were used without further purification. Low resolution mass spectra were obtained on an Agilent 6120 or 6150 with UV detection at 215 nm and 254 nm along with ELSD detection and electrospray ionization, with all final compounds showing >95% purity and a parent mass ion consistent with the desired structure. All NMR spectra were recorded on a 400 MHz Brüker AV-400 instrument. ^{1}H chemical shifts are reported as δ values in ppm relative to the residual solvent peak ($CDCl_3$ = 7.26, MeOD = 3.34). Data are reported as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, dd = doublet of doublets, ddd = doublet of doublet of doublets, td = triplet of doublets, dt = doublet of triplets, m = multiplet), coupling constant, and integration. ^{13}C chemical shifts are reported as δ values in ppm relative to the residual solvent peak ($CDCl_3$ = 77.16, MeOD = 49.86). Automated flash column chromatography was performed on a Biotage Isolera 1 and a Teledyne ISCO Combi-Flash system. Microwave synthesis was performed in a Biotage Initiator microwave synthesis reactor. RP-HPLC purification of final compounds was performed on a Gilson preparative LC system. High resolution mass spectra were obtained on an Agilent 6540 UHD Q-TOF with ESI source. Melting points were recorded on an OptiMelt automated melting point system by Stanford Research Systems.

General Procedure A



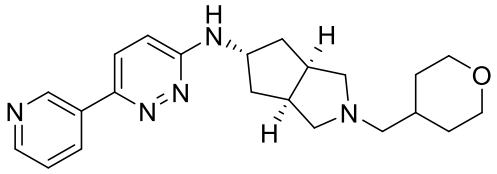
(3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (1 eq), boronic acid (1.5 eq), potassium carbonate (3 eq), and BrettPhos-Pd-G3 (0.1 eq) were sealed in a vial and placed under an inert atmosphere. 5:1 1,4-dioxane/H_2O solution (degassed under vacuum) was added via syringe and the reaction mixture was stirred at 100 °C for 2 h or until complete by LCMS. The reaction mixture was cooled, diluted with H_2O and extracted in DCM. Solvents were concentrated and the crude residue was purified by RP-HPLC. Fractions containing product were basified with sat. $NaHCO_3$ solution (if purified under acidic conditions), and extracted with DCM. Combined organic extracts were filtered through a phase separator and concentrated to give the final products.

(3aR,5s,6aS)-N-(6-phenylpyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (8a)



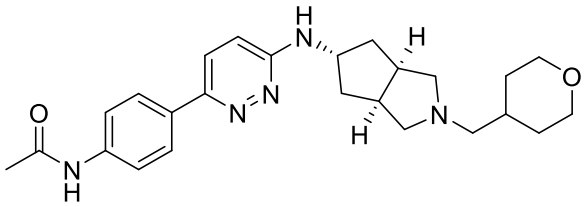
Followed General Procedure A with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine **7**[1] (40 mg, 0.12 mmol), phenylboronic acid (21.7 mg, 0.18 mmol), potassium carbonate (50 mg, 0.36 mmol), and BrettPhos-Pd-G3 (10.8 mg, 0.01 mmol) to give the title compound as a white solid after purification by RP-HPLC (5-40% MeCN in 0.1% TFA solution over 10 min) (4.4 mg, 10%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 – 7.92 (m, 2H), 7.60 (d, *J* = 9.3 Hz, 1H), 7.50 – 7.41 (m, 2H), 7.41 – 7.34 (m, 1H), 6.70 (d, *J* = 9.3 Hz, 1H), 4.77 (d, *J* = 7.3 Hz, 1H), 4.45 – 4.34 (m, 1H), 3.97 (dd, *J* = 11.3, 4.0 Hz, 2H), 3.39 (td, *J* = 11.8, 1.9 Hz, 2H), 2.77 – 2.70 (m, 2H), 2.63 – 2.54 (m, 2H), 2.33 (dd, *J* = 9.2, 3.3 Hz, 2H), 2.26 (d, *J* = 6.7 Hz, 2H), 2.01 – 1.93 (m, 2H), 1.76 – 1.64 (m, 5H), 1.34 – 1.23 (m, 2H). ^{13}C NMR (101 MHz, CDCl3) δ 158.4, 151.4, 137.2, 128.9, 128.7, 126.0, 125.6, 113.3, 68.1, 62.3, 62.2, 53.3, 40.3, 39.8, 34.4, 32.0. HRMS (TOF, ES+), $C_{23}H_{31}N_4O$ $[M+H]^+$ calc. mass 379.2492, found 379.2487. m.p.= 195 – 198 °C.

(3aR,5s,6aS)-N-(6-(pyridin-3-yl)pyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (8b)



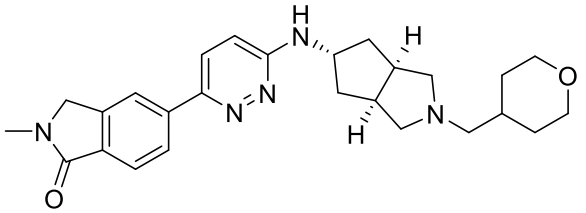
Followed General Procedure A with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (40 mg, 0.12 mmol), pyridine-3-boronic acid pinacol ester (36.5 mg, 0.18 mmol), potassium carbonate (50 mg, 0.36 mmol), and BrettPhos-Pd-G3 (10.8 mg, 0.01 mmol) to give the title compound as a white solid after purification by RP-HPLC (2-38% MeCN in 0.1% TFA solution over 9 min) (12.7 mg, 28%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.11 (d, *J* = 2.3 Hz, 1H), 8.61 (dd, *J* = 4.8, 1.7 Hz, 1H), 8.34 (dt, *J* = 8.0, 2.0 Hz, 1H), 7.61 (d, *J* = 9.3 Hz, 1H), 7.38 (dd, *J* = 8.0, 4.7 Hz, 1H), 6.72 (d, *J* = 9.3 Hz, 1H), 5.01 (d, *J* = 7.2 Hz, 1H), 4.45 – 4.35 (m, 1H), 3.95 (dd, *J* = 11.5, 3.8 Hz, 2H), 3.38 (td, *J* = 11.8, 1.9 Hz, 2H), 2.76 – 2.68 (m, 2H), 2.61 – 2.53 (m, 2H), 2.33 (dd, *J* = 9.2, 3.3 Hz, 2H), 2.25 (d, *J* = 6.7 Hz, 2H), 2.00 – 1.93 (m, 2H), 1.77 – 1.64 (m, 5H), 1.34 – 1.20 (m, 2H).^{13}C NMR (101 MHz, $CDCl_3$) δ 158.7, 149.7, 148.8, 147.2, 133.3, 132.9, 125.3, 123.8, 113.3, 68.1, 62.3, 62.1, 53.3, 40.3, 39.8, 34.4, 31.9. HRMS (TOF, ES+), $C_{22}H_{30}N_5O$ $[M+H]^+$ calc. mass 380.2445, found 380.2444. m.p.= 146 – 151 °C.

N-(4-(6-(((3aR,5s,6aS)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-yl)amino)pyridazin-3-yl)phenyl)acetamide (8c)



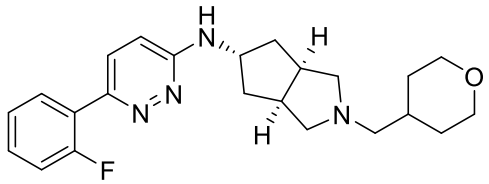
Followed General Procedure A with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (20 mg, 0.06 mmol), 4-acetylaminophenyl boronic acid (16 mg, 0.09 mmol), potassium carbonate (25 mg, 0.18 mmol), and BrettPhos-Pd-G3 (5.4 mg, 0.006 mmol) to give the title compound as a white solid after purification by RP-HPLC (1-30% MeCN in 0.1% TFA solution over 9 min) (7.3 mg, 28%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (d, *J* = 8.6 Hz, 2H), 7.80 (s, 1H), 7.61 (d, *J* = 8.6 Hz, 2H), 7.55 (d, *J* = 9.3 Hz, 1H), 6.72 (d, *J* = 9.4 Hz, 1H), 4.88 (d, *J* = 7.3 Hz, 1H), 4.48 – 4.38 (m, 1H), 3.96 (dd, *J* = 11.4, 3.3 Hz, 2H), 3.38 (td, *J* = 11.8, 1.9 Hz, 2H), 2.83 – 2.73 (m, 4H), 2.39 – 2.30 (m, 4H), 2.19 (s, 3H), 1.98 (dd, *J* = 12.9, 5.8 Hz, 2H), 1.79 – 1.66 (m, 5H), 1.35 – 1.23 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.7, 158.1, 151.0, 138.7, 132.8, 126.6, 125.4, 120.1, 114.0, 68.0, 62.1, 61.9, 53.0, 40.1, 39.3, 34.1, 31.9, 24.8. HRMS (TOF, ES+), $C_{25}H_{34}N_5O_2$ $[M+H]^+$ calc. mass 436.2707, found 436.2706. m.p.= 229 – 234 °C.

2-methyl-5-(6-(((3aR,5s,6aS)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-yl)amino)pyridazin-3-yl)isoindolin-1-one (8d)



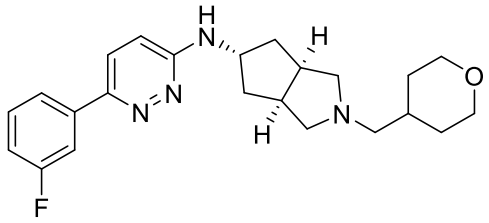
Followed General Procedure A with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (40 mg, 0.12 mmol), 2-methyl-1-isoindolinone-5 boronic acid (34.0 mg, 0.18 mmol), potassium carbonate (50 mg, 0.36 mmol), and BrettPhos-Pd-G3 (10.8 mg, 0.01 mmol) to give the title compound as a yellow solid after purification by RP-HPLC (2-38% MeCN in 0.1% TFA solution over 9 min) (9.6 mg, 18%). ^{1}H NMR (400 MHz, MeOD) δ 8.10 (s, 1H), 8.03 (dd, *J* = 8.0, 1.5 Hz, 1H), 7.81 (dd, *J* = 10.2, 8.3 Hz, 2H), 6.94 (d, *J* = 9.4 Hz, 1H), 4.56 (s, 2H), 4.55 – 4.47 (m, 1H), 3.94 (dd, *J* = 11.3, 3.4 Hz 2H), 3.43 (td, *J* = 11.9, 1.9 Hz, 2H), 3.22 (s, 3H), 2.84 – 2.74 (m, 4H), 2.32 (d, *J* = 6.8 Hz, 2H), 2.28 – 2.22 (m, 2H), 2.00 – 1.92 (m, 2H), 1.80 – 1.70 (m, 5H), 1.33 – 1.21 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 169.9, 159.1, 150.6, 143.3, 141.2, 132.9, 126.8, 126.3, 123.8, 120.9, 116.8, 68.3, 62.9, 62.3, 52.8, 52.7, 40.9, 38.7, 34.7, 32.3, 29.1, HRMS (TOF, ES+), $C_{26}H_{34}N_5O_2$ $[M+H]^+$ calc. mass 448.2707, found 448.2703. m.p.= 166 – 174 °C.

(3aR,5s,6aS)-N-(6-(2-fluorophenyl)pyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (8e)



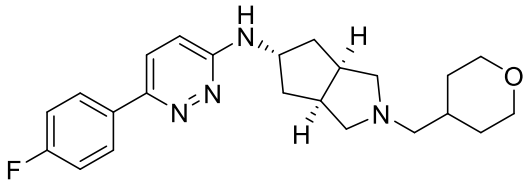
Followed General Procedure A with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (40 mg, 0.12 mmol), 2-fluorophenyl boronic acid (24.9 mg, 0.18 mmol), potassium carbonate (50 mg, 0.36 mmol), and BrettPhos-Pd-G3 (10.8 mg, 0.01 mmol) to give the title compound as a white solid after purification by RP-HPLC (2-38% MeCN in 0.1% TFA solution over 9 min) (21.9 mg, 47%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (t, *J* = 8.0 Hz, 1H), 7.65 (dd, *J* = 9.3, 1.8 Hz, 1H), 7.37 – 7.29 (m, 1H), 7.26 – 7.20 (m, 1H), 7.12 (dd, *J* = 11.6, 8.1 Hz, 1H), 6.69 (d, *J* = 9.3 Hz, 1H), 5.07 (d, *J* = 7.2 Hz, 1H), 4.45 – 4.35 (m, 1H), 3.95 (dd, *J* = 11.5, 4.4 Hz, 2H), 3.37 (td, *J* = 11.6 Hz, 1.6 Hz, 2H), 2.81 – 2.68 (m, 4H), 2.38 – 2.28 (m, 4H), 2.00 – 1.94 (m, 2H), 1.76 – 1.63 (m, 5H), 1.34 – 1.21 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.3 (d, *J* = 248.5 Hz), 158.2 (d, *J* = 5.1 Hz), 148.0 (d, *J* = 1.7 Hz), 130.3 – 130.2 (m, 2C), 128.9 (d, *J* = 9.4 Hz), 125.2 (d, *J* = 11.5 Hz), 124.7 (d, *J* = 3.3 Hz), 116.1 (d, *J* = 22.6 Hz), 112.7, 67.9, 62.0, 61.9, 52.9 40.1, 39.3, 34.2, 31.8.HRMS (TOF, ES+), $C_{23}H_{30}FN_4O$ $[M+H]^+$ calc. mass 397.2398, found 397.2393. m.p.= 154 – 158 °C.

(3aR,5s,6aS)-N-(6-(3-fluorophenyl)pyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (8f)



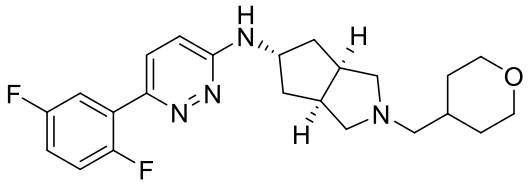
Followed General Procedure A with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (40 mg, 0.12 mmol), 3-fluorophenyl boronic acid (24.9 mg, 0.18 mmol), potassium carbonate (50 mg, 0.36 mmol), and BrettPhos-Pd-G3 (10.8 mg, 0.01 mmol) to give the title compound as a white solid after purification by RP-HPLC (2-38% MeCN in 0.1% TFA solution over 9 min) (10.2 mg, 22%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 – 7.69 (m, 2H), 7.58 (d, *J* = 9.3 Hz, 1H), 7.44 – 7.38 (m, 1H), 7.11 – 7.04 (m, 1H), 6.70 (d, *J* = 9.3 Hz, 1H), 4.82 (d, *J* = 7.3 Hz, 1H), 4.46 – 4.36 (m, 1H), 3.97 (dd, *J* = 11.3, 4.7, 2H), 3.39 (td, *J* = 11.9, 1.9 Hz, 2H), 2.82 – 2.70 (m, 2H), 2.68 – 2.54 (m, 2H), 2.39 – 2.31 (m, 2H), 2.32 – 2.24 (m, 2H), 2.03 – 1.94 (m, 2H), 1.79 – 1.67 (m, 5H), 1.37 – 1.22 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.5 (d, *J* = 246.7 Hz), 158.5, 150.2 (d, *J* = 2.2 Hz), 139.4 (d, *J* = 7.9 Hz), 130.4 (d, *J* = 8.4 Hz), 125.5, 121.4 (d, *J* = 3.0 Hz), 115.5 (d, *J* = 21.4 Hz), 113.3, 112.9 (d, *J* = 22.9 Hz), 68.1, 62.3, 62.1, 53.3, 40.3, 39.7, 34.4, 32.0. HRMS (TOF, ES+), $C_{23}H_{30}FN_4O$ $[M+H]^+$ calc. mass 397.2398, found 397.2393. m.p.= 196 – 199 °C.

(3aR,5s,6aS)-N-(6-(4-fluorophenyl)pyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (8g)



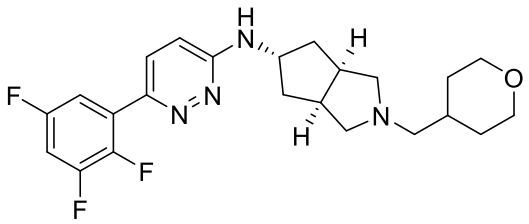
Followed General Procedure A with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (40 mg, 0.12 mmol), 4-fluorophenyl boronic acid (24.9 mg, 0.18 mmol), potassium carbonate (50 mg, 0.36 mmol), and BrettPhos-Pd-G3 (10.8 mg, 0.01 mmol) to give the title compound as a white solid after purification by RP-HPLC (2-38% MeCN in 0.1% TFA solution over 9 min) (5.3 mg, 11%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 – 7.90 (m, 2H), 7.55 (d, *J* = 9.3 Hz, 1H), 7.17 – 7.10 (m, 2H), 6.70 (d, *J* = 9.3 Hz, 1H), 4.78 (d, *J* = 7.3 Hz, 1H), 4.45 – 4.36 (m, 1H), 3.97 (dd, *J* = 11.4, 4.1 Hz, 2H), 3.39 (td, *J* = 11.8, 1.9 Hz, 2H), 2.80 – 2.69 (m, 2H), 2.67 – 2.57 (m, 2H), 2.34 (dd, *J* = 9.4, 3.4 Hz, 2H), 2.28 (d, *J* = 6.8 Hz, 2H), 2.02 – 1.94 (m, 2H), 1.76 – 1.65 (m, 5H), 1.35 – 1.22 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.4 (d, *J* = 248.4 Hz), 158.3, 150.6, 133.3 (d, *J* = 3.2 Hz), 127.8 (d, *J* = 8.3 Hz), 125.4, 115.9 (d, *J* = 21.5 Hz), 113.6, 68.1, 62.3, 62.1, 53.3, 40.3, 39.7, 34.4, 32.0. HRMS (TOF, ES+), $C_{23}H_{30}FN_4O$ $[M+H]^+$ calc. mass 397.2398, found 397.2394. m.p.= 170 – 173 °C.

(3aR,5s,6aS)-N-(6-(2,5-difluorophenyl)pyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (8h)



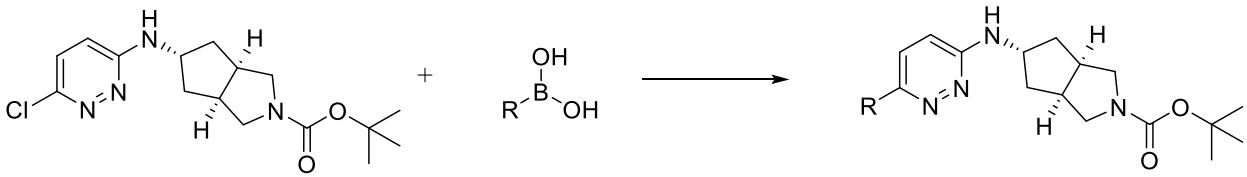
Followed General Procedure A with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (50 mg, 0.15 mmol), 2,5-difluorophenyl boronic acid (35.2 mg, 0.22 mmol), potassium carbonate (62 mg, 0.45 mmol), and BrettPhos-Pd-G3 (13.5 mg, 0.015 mmol) to give the title compound as a white solid after purification by RP-HPLC (5-40% MeCN in 0.1% TFA solution over 9 min) (17.8 mg, 29%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 – 7.82 (m, 1H), 7.71 – 7.67 (m, 1H), 7.12 – 6.98 (m, 2H), 6.68 (d, *J* = 9.4 Hz, 1H), 5.11 – 4.99 (m, 1H), 4.43 – 4.34 (m, 1H), 3.96 (dd, *J* = 11.3, 4.3 Hz, 2H), 3.38 (td, *J* = 11.7, 1.6 Hz, 2H), 2.81 – 2.73 (m, 2H), 2.67 – 2.59 (m, 2H), 2.38 – 2.32 (m, 2H), 2.28 (d, *J* = 6.7 Hz, 2H), 2.01 – 1.93 (m, 2H), 1.78 – 1.66 (m, 5H), 1.36 – 1.19 (m, 2H).^{13}C NMR (101 MHz, $CDCl_3$) δ 159.2 (dd, *J* = 242.0, 2.1 Hz), 158.5, 156.4 (dd, *J* = 244.1, 2.3 Hz), 146.9, 128.7 (d, *J* = 10.9 Hz), 126.6 (dd, *J* = 13.2, 8.2 Hz), 117.3 (dd, *J* = 25.0, 8.5 Hz), 116.6 (dd, *J* = 26.7, 9.5 Hz), 116.3 (dd, *J* = 25.6, 3.4), 112.3, 68.0, 62.2, 62.1, 53.2, 40.3, 39.7, 34.3, 31.9. HRMS (TOF, ES+), $C_{23}H_{29}F_2N_4O$ $[M+H]^+$ calc. mass 415.2304, found 415.2302. m.p.= 182 – 184 °C.

(3aR,5s,6aS)-2-((tetrahydro-2H-pyran-4-yl)methyl)-N-(6-(2,3,5-trifluorophenyl)pyridazin-3-yl)octahydrocyclopenta[c]pyrrol-5-amine (8i)



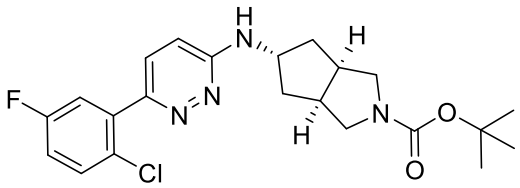
Followed General Procedure A with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (40 mg, 0.12 mmol), 2,3,5-trifluorophenyl boronic acid (27.2 mg, 0.15 mmol), potassium carbonate (50 mg, 0.36 mmol), and BrettPhos-Pd-G3 (10.8 mg, 0.01 mmol) to give the title compound as a white solid after purification by RP-HPLC (10-40% MeCN in 0.1% TFA solution over 9 min) (5.4 mg, 11%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (dd, *J* = 9.4, 2.1 Hz, 1H), 7.66 – 7.62 (m, 1H), 6.97 – 6.90 (m, 1H), 6.71 (d, *J* = 9.4 Hz, 1H), 5.17 (d, *J* = 7.2 Hz, 1H), 4.47 – 4.37 (m, 1H), 4.96 (dd, *J* = 11.4, 3.9 Hz, 2H), 3.38 (td, *J* = 11.8, 1.9 Hz, 2H), 2.85 – 2.77 (m, 2H), 2.77 – 2.67 (m, 2H), 2.43 – 2.36 (m, 2H), 2.34 (d, *J* = 6.0 Hz, 2H), 2.04 – 1.95 (m, 2H), 1.80 – 1.67 (m, 5H), 1.36 – 1.23 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.6, 157.9 (ddd, *J* = 244.3, 10.9, 3.0 Hz), 150.9 (ddd, *J* = 249.9, 16.9, 13.1 Hz), 145.3 (ddd, *J* = 245.8, 13.8, 3.9 Hz), 146.2 – 146.0 (m, 1C), 128.6 (d, *J* = 10.6 Hz), 127.8 (t, *J* = 9.9 Hz), 112.5, 111.0 – 110.6 (m, 1C), 105.4 (dd, *J* = 26.7, 21.0 Hz), 68.0, 62.1, 62.0, 53.1, 40.2, 39.4, 34.2, 31.9. HRMS (TOF, ES+), $C_{23}H_{28}F_3N_4O$ $[M+H]^+$ calc. mass 433.2210, found 433.2207. m.p.= 201 – 203 °C.

General Procedure B



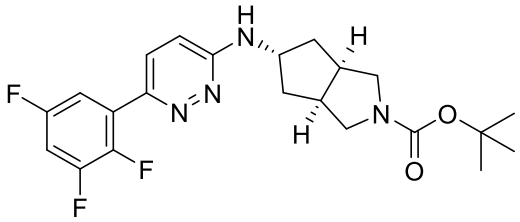
(3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (1 eq), boronic acid (1.5 eq), potassium carbonate (3 eq), and BrettPhos-Pd-G3 (0.1 eq) were sealed in a vial and placed under an inert atmosphere. 5:1 1,4-dioxane/H_2O solution (degassed) was added via syringe and the reaction stirred at 100 °C for 2 h. The reaction was cooled, diluted with H_2O and extracted in DCM. Solvents were concentrated and the crude residue was purified by column chromatography (EtOAc in hexanes) to yield the title compound.

tert-butyl (3aR,5s,6aS)-5-((6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (9a)



Followed General Procedure B with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (500 mg, 1.5 mmol), 2-chloro-5-fluorophenyl boronic acid (309 mg, 1.77 mmol), potassium carbonate (621 mg, 4.4 mmol), and BrettPhos-Pd-G3 (134 mg, 0.15 mmol) to give the title compound as a white solid after purification by column chromatography (3-80% EtOAc in hexanes) (422 mg, 66%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (d, *J* = 9.3 Hz, 1H), 7.47 (dd, *J* = 9.1, 3.1 Hz, 1H), 7.40 (dd, *J* = 8.8, 5.0 Hz, 1H), 7.07 – 7.01 (m, 1H), 6.69 (d, *J* = 9.3 Hz, 1H), 5.33 (d, *J* = 6.7 Hz, 1H), 4.43 – 4.34 (m, 1H), 3.62 – 3.52 (m, 2H), 3.21 (d, *J* = 11.3 Hz, 2H), 2.90 – 2.80 (m, 2H), 2.09 – 2.00 (m, 2H), 1.94 – 1.85 (m, 2H), 1.46 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.5, (d, *J* = 247.2 Hz), 158.2, 154.7, 150.3, 138.3 (d, *J* = 8.1 Hz), 131.5 (d, *J* = 8.5 Hz), 129.3, 127.1 (d, *J* = 3.2 Hz), 118.4 (d, *J* = 24.1 Hz), 116.8 (d, *J* = 22.6 Hz), 111.8, 79.5, 53.2, 52.3, 41.9 – 40.3 (m, 2C), 39.37, 29.81, 28.63. HRMS (TOF, ES+), $C_{22}H_{27}ClFN_4O_2$ $[M+H]^+$ calc. mass 433.1801, found 433.1797. m.p.= 182 – 185 °C.

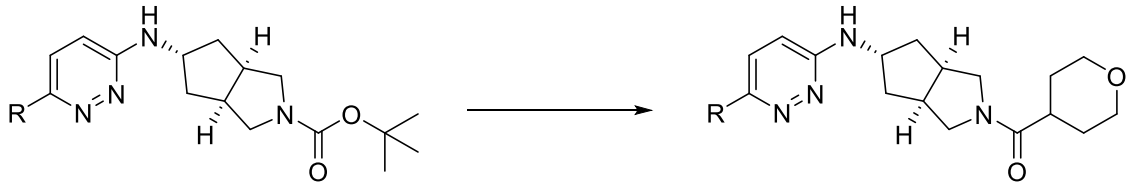
tert-butyl (3aR,5s,6aS)-5-((6-(2,3,5-trifluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (9b)



Followed General Procedure B with (3aR,5s,6aS)-N-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (80 mg, 0.24 mmol), 2,3,5-trifluorophenyl boronic acid (49.8 mg, 0.28 mmol), potassium carbonate (99 mg, 0.71 mmol), and BrettPhos-Pd-G3 (21.4 mg, 0.024 mmol) to give the title compound as a white solid after purification by column chromatography (3-80% EtOAc in hexanes) (55.7 mg, 54%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (dd, *J* = 9.4, 1.8 Hz,2 1H), 7.66 – 7.61 (m, 1H), 6.97 – 6.89 (m, 1H), 6.70 (d, *J* = 9.4 Hz, 1H), 5.51 (d, *J* = 6.8 Hz, 1H), 4.45 – 4.36 (m, 1H), 3.62 – 3.51 (m, 2H), 3.21 (d, *J* = 11.1 Hz, 2H), 2.91 – 2.81 (m, 2H), 2.10 – 2.00 (m, 2H), 1.95 – 1.86 (m, 2H), 1.46 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.9, (ddd, *J* = 245.6, 11.7, 2.9 Hz), 158.4, 154.7, 150.9 (ddd, *J* = 249.7, 15.1 13.0, Hz), 145.3, (ddd, *J* = 247.6, 13.6, 4.3 Hz), 146.2 – 146.1 (m, 1C), 128.6 (d, *J* = 10.6 Hz), 127.8 (t, *J* = 9.9 Hz), 112.4, 111.0 – 110.6 (m, 1C), 105.4 (dd, *J* = 27.9, 20.9 Hz), 79.5,

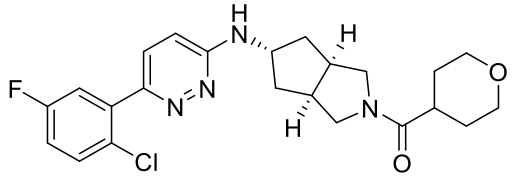
53.1, 52.3, 41.9 – 40.2 (m, 2C), 39.3, 28.3. HRMS (TOF, ES+), $C_{22}H_{26}F_3N_4O_2$ $[M+H]^+$ calc. mass 435.2002, found 435.2001. m.p.= 180 – 183 °C.

General Procedure C



To a solution of Boc-protected intermediate compound in 1,4 dioxane was added 4M HCl solution in dioxane. After 1.5 h or completion by LCMS, the reaction was concentrated to yield the HCl salt. The resulting HCl salt (1 eq) was suspended in DMF and DIPEA (3 eq) was added, followed by the addition of tetrahydro-2H-pyran-4-carboxylic acid (1.2 eq) and HATU (1.5 eq) After stirring at r.t. for 1 h or until completion by LCMS, the reaction mixture was purified directly by RP-HPLC. Fractions containing product were basified with sat. $NaHCO_3$ solution (if purified under acidic conditions), and extracted with DCM. Combined organic extracts were filtered through a phase separator and concentrated to give the final products.

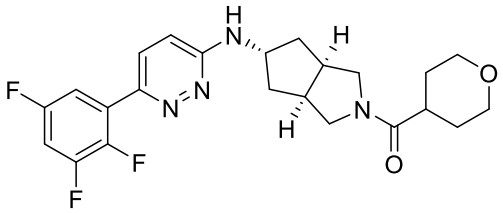
((3aR,5s,6aS)-5-((6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrol-2(1H)-yl)(tetrahydro-2H-pyran-4-yl)methanone (10a)



Followed General Procedure C: tert-butyl (3aR,5s,6aS)-5-((6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (420 mg, 0.97 mmol), in 1,4 dioxane (5 mL) was added 4M HCl solution in dioxanes (5 mL). After 1.5 h the reaction was concentrated to yield the HCl salt as a white solid (360 mg, 100%). The resulting HCl salt (360 mg, 0.97 mmol) was suspended in DMF (5 mL) and DIPEA (0.51 mL, 2.9 mmol) followed with the addition of tetrahydro-2H-pyran-4-carboxylic acid (150 mg, 0.12 mmol) and HATU (560 mg, 1.5 mmol). After 1 h at r.t., the crude was purified by RP-HPLC (30-70% MeCN in 0.1% TFA solution over 20 min) to yield the title compound as a white solid (380 mg, 88% over 2 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (d, *J* = 9.3 Hz, 1H), 7.45 (dd, *J* = 9.2, 3.1 Hz, 1H), 7.40 (dd, *J* = 8.7, 5.1 Hz, 1H), 7.07 – 7.01 (m, 1H), 6.69 (d, *J* = 9.3 Hz, 1H), 5.46 (d, *J* = 6.6 Hz, 1H), 4.48 – 4.39 (m, 1H), 4.06 – 3.98 (m, 2H), 3.77 – 3.69 (m, 2H), 3.48 – 3.34 (m, 4H), 3.05 – 2.94 (m, 1H), 2.92 –

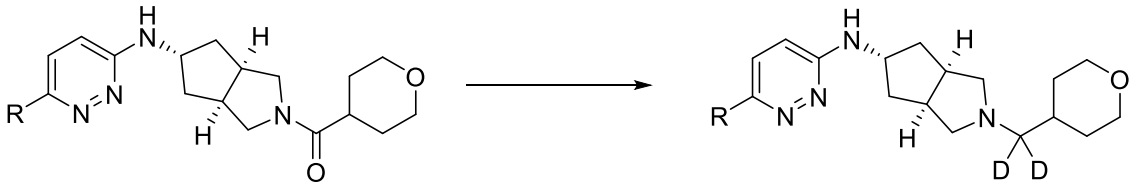
2.82 (m, 1H), 2.59 (tt, *J* = 11.4, 3.8 Hz, 1H), 2.13 – 1.83 (m, 6H), 1.68 – 1.56 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.1, 161.5, (d, *J* = 247.3 Hz), 158.0, 150.4 (d, *J* = 1.6 Hz), 138.3 (d, *J* = 11.3 Hz), 131.5 (d, *J* = 8.2 Hz,), 129.3, 127.1 (d, *J* = 3.4 Hz), 118.3 (d, *J* = 24.0 Hz), 116.9 (d, *J* = 22.6 Hz) 112.2, 67.4, 67.4, 53.0, 52.4, 52.0, 42.1, 39.9, 39.8, 39.3, 39.2, 28.8, 28.6. HRMS (TOF, ES+), $C_{23}H_{27}ClFN_4O_2$ $[M+H]^+$ calc. mass 445.1801, found 445.1799. *A 1:1 ratio of amide rotamers is observed in ^{13}C spectra*. m.p.= 188 – 195 °C.

(tetrahydro-2H-pyran-4-yl)((3aR,5s,6aS)-5-((6-(2,3,5-trifluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrol-2(1H)-yl)methanone (10b)



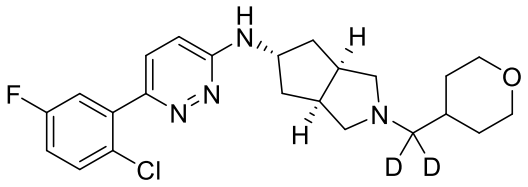
Followed General Procedure C: To tert-butyl (3aR,5s,6aS)-5-((6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (15 mg, 0.035 mmol), in MeOH (0.5 mL) was added 4M HCl solution in dioxanes (0.5 mL). After 30 min, the reaction was concentrated to yield the HCl salt as a white solid (12.8 mg, 100%). The resulting HCl salt (12.8 mg, 0.035 mmol) was suspended in DMF (1 mL) and DIPEA (30 µL, 0.17 mmol) followed with the addition of tetrahydro-2H-pyran-4-carboxylic acid (6.7 mg, 0.052 mmol) and HATU (19.7 mg, 0.052 mmol). After 1 h at r.t., the crude was purified by RP-HPLC (30-70% MeCN in 0.05% NH_4OH solution in over 20 min) to yield the title compound as a white solid (12.1 mg, 78% over 2 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (dd, *J* = 9.4, 2.1 Hz, 1H), 7.68 – 7.62 (m, 1H) 6.99 – 6.91 (m, 1H), 6.71 (d, *J* = 9.4 Hz, 1H), 5.34 (d, *J* = 6.6 Hz, 1H), 4.52 – 4.43 (m, 1H), 4.07 – 3.99 (m, 2H), 3.79 – 3.71 (m, 2H), 3.49 – 3.36 (m, 4H), 3.05 – 2.95 (m, 1H), 2.94 – 2.84 (m, 1H), 2.60 (tt, *J* = 11.4, 3.8 Hz, 1H), 2.15 – 2.03 (m, 2H), 2.00 – 1.85 (m, 4H), 1.68 – 1.58 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.1, 159.1 (ddd, *J* = 244.7, 10.8, 2.7 Hz), 158.0, 150.9 (ddd, *J* = 250.5, 15.2, 13.0 Hz), 145.4 (ddd, *J* = 246.8, 14.1, 3.9 Hz), 146.5 – 146.3 (m, 1C), 128.7 (d, *J* = 10.6 Hz), 127.6 (t, *J* = 9.9 Hz), 113.0, 111.0 – 110.6 (m, 1C), 105.6 (dd, *J* = 27.2, 21.1 Hz), 67.4, 67.4 53.0, 52.4, 52.0, 42.2, 39.9, 39.8, 39.3, 39.2, 28.8, 28.7. HRMS (TOF, ES+), $C_{23}H_{26}F_3N_4O_2$ $[M+H]^+$ calc. mass 447.2002, found 447.1999. *A 1:1 ratio of amide rotamers is observed in ^{13}C spectra.* m.p.= 200 – 207 °C.

General Procedure D



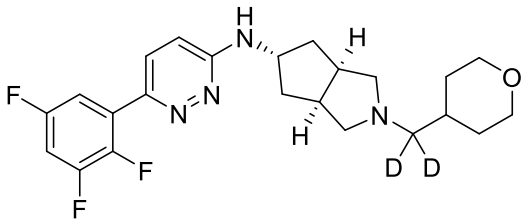
Amide intermediate (1 eq) was suspended in THF and cooled to 0 °C. Lithium aluminum deuteride (3 eq) was added and the reaction mixture was stirred at 0 °C for 30 min or until completion by LCMS. The mixture was quenched with H_2O, and 1N NaOH aq. solution, and stirred for an additional 30 min. Solids were removed by filtration and solvents were concentrated. Crude residue was purified by RP-HPLC. Fractions containing product were basified with sat. $NaHCO_3$ solution (if purified under acidic conditions), and extracted with DCM. Combined organic extracts were filtered through a phase separator and concentrated to give the final products.

(3aR,5s,6aS)-N-(6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl-d2)octahydrocyclopenta[c]pyrrol-5-amine (11a)



Followed General Procedure D with ((3aR,5s,6aS)-5-((6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrol-2(1H)-yl)(tetrahydro-2H-pyran-4-yl)methanone (33 mg, 0.074 mmol) in THF (1 mL) to give the title compound as a white solid after purification by RP-HPLC (5-35% MeCN in 0.1% TFA solution over 10 min) (8.1 mg, 25%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, *J* = 9.3 Hz, 1H), 7.48 (dd, *J* = 9.2, 3.1 Hz, 1H), 7.41 (dd, *J* = 8.8, 5.1 Hz, 1H), 7.07 – 7.01(m, 1H), 6.68 (d, *J* = 9.3 Hz, 1H), 4.97 (d, *J* = 7.2 Hz, 1H), 4.41 – 4.31 (m, 1H), 3.96 (dd, *J* = 11.3, 3.9 Hz, 2H), 3.38 (td, *J* = 11.8, 1.9 Hz, 2H), 2.78 – 2.70 (m, 2H), 2.62 – 2.52 (m, 2H), 2.34 (dd, *J* = 9.3, 3.1 Hz, 2H), 2.01 – 1.93 (m, 2H), 1.81 – 1.62 (m, 5H), 1.34 – 1.22 (m, 2H).^{13}C NMR (101 MHz, $CDCl_3$) δ 161.6, (d, *J* = 247 Hz), 158.5 (d, *J* = 5.5 Hz), 150.2 (d, *J* = 1.8 Hz), 138.5 (d, *J* = 8.2 Hz), 131.4 (d, *J* = 8.6 Hz), 129.2, 127.1 (d, *J* = 3.4 Hz), 118.4 (d, *J* = 24.0 Hz), 116.8 (d, *J* = 22.8 Hz), 111.5, 68.1, 62.1, 53.4, 53.3, 40.3, 39.8, 34.2, 31.9. HRMS (TOF, ES+), $C_{23}H_{27}D_2ClFN_4O$ $[M+H]^+$ calc. mass 433.2134, found 433.2131. m.p.= 198 – 204 °C.

(3aR,5s,6aS)-2-((tetrahydro-2H-pyran-4-yl)methyl-d2)-N-(6-(2,3,5-trifluorophenyl)pyridazin-3-yl)octahydrocyclopenta[c]pyrrol-5-amine (11b)



Followed General Procedure D with (tetrahydro-2H-pyran-4-yl)((3aR,5s,6aS)-5-((6-(2,3,5-trifluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrol-2(1H)-yl)methanone (37 mg, 0.083 mmol) in THF (1 mL) to give the title compound as a white solid after purification by RP-HPLC (5-35% MeCN in 0.1% TFA solution over 5 min) (5 mg, 14%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (dd, *J* = 9.6, 2.1 Hz, 1H),7.67 – 7.63 (m, 1H), 6.98 – 6.90 (m, 1H), 6.71 (d, *J* = 9.4 Hz, 1H), 5.06 (d, *J* = 6.8 Hz, 1H), 4.50 – 4.39 (m, 1H), 3.97 (dd, *J* = 11.7, 4.8, 2H), 3.39 (td, *J* = 11.8, 1.9 Hz, 2H), 2.95 – 2.75 (m, 4H), 2.46 – 2.34 (m, 2H), 2.06 – 1.97 (m, 2H), 1.80 – 1.69 (m, 5H), 1.37 – 1.23 (m, 2H).^{13}C NMR (101 MHz, $CDCl_3$) δ 158.5, 158.0 (ddd, *J* = 246.0, 11.0, 2.8 Hz), 150.9 (ddd, *J* = 249.9, 15.4, 12.8 Hz), 146.2, 145.3, (ddd, *J* = 246.8, 14.4, 4.0 Hz), 128.6 (d, *J* = 10.6 Hz), 127.8 (t, *J* = 9.7 Hz), 112.6, 111.1 – 110.7 (m, 1C), 105.4 (dd, *J* = 28.6, 21.1 Hz), 67.9, 62.4, 61.8, 53.0, 40.1, 39.3, 33.9, 31.8. HRMS (TOF, ES+), $C_{23}H_{26}D_2F_3N_4O$ $[M+H]^+$ calc. mass 435.2335, found 435.2331. m.p.= 199 – 202 °C.

Drug Metabolism and Pharmacokinetics

Intrinsic Clearance

Determination of test compounds' *in vitro* intrinsic clearance (CL_{int}) and prediction of hepatic clearance (CL_{hep}) was performed via an in-house assay using human and/or rat hepatic microsomes with a substrate depletion approach according to a previously described methodology [1-4].

CYP_{450} Inhibition

Initial determination of test compounds' potency for reversible inhibition of four major human CYP_{450}s (1A2, 2C9, 2D6, 3A4) was performed via an in-house assay using human hepatic microsomes incubated with a 'cocktail' of isoform-specific probe substrates according to a previously described methodology [1-4].

More definitive determination of VU6028418's potential for CYP_{450} inhibition, including assessment of reversible vs. time-dependent (+/- NADPH) inhibition potencies of a broader set of enzymes (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) was performed via contract by Q2 Solutions (Indianapolis, IN) using human hepatic microsomes with isoform-specific probe substrates according to their established standard assay methodologies.

CYP_{450} Induction

Determination of VU6028418's human CYP_{450} (1A2, 2B6, 3A4) induction potential was performed via contract by Q2 Solutions (Indianapolis, IN) using cryopreserved hepatocytes from three donors and RT-PCR measurement of CYP_{450} mRNA according to their established standard assay methodologies.

CYP_{450} Metabolic Phenotype

Determination of VU6028418's human CYP_{450} metabolic phenotype (i.e., relative contribution of metabolism by 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, and 3A5 to CL_{hep}) was performed via contract by Q2 Solutions (Indianapolis, IN) using recombinant enzymes and human hepatic microsomes with a relative activity factor (RAF) approach to prediction of fraction metabolized ($f_{m,CYP}$) values for each enzyme in accordance with their established standard assay methodologies.

Metabolite Identification

Qualitative *in vitro* profiling with tentative identification of VU6028418's metabolites was performed via contract by Q2 Solutions (Indianapolis, IN) using a four hour incubation of parent (10 µM) in cryopreserved hepatocytes from multiple species (pooled male Sprague-Dawley rat, male beagle dog, male cynomolgus monkey, and mixed gender human) according to their established standard assay methodologies.

Results from these experiments revealed a total of 16 unique metabolites (8 primary; 8 secondary/tertiary) generated in one or more species. The proposed metabolite structures and their relative abundance (by %UV and %MS total peak areas) are shown in the following tables and figure:

Table S1.

: Percent UV Total Peak Areas of VU6028418 and Metabolites in Each Species

Peak ID	Tentative Metabolite Identification	% Total Peak Area			
		Rat	Dog	Monkey	Human
VU6028418	Parent (P)	80.69	88.57	87.11	86.16
M1	P + O	0.99	ND	ND	ND
M2	P + O	11.88	3.33	ND	ND
M4	P + N-acetylglucosamine	ND	ND	2.22	ND
M6	P + glucuronide	ND	ND	ND	1.79
M7	P + glucuronide	ND	ND	ND	0.89
M8	P + O + O	6.44	8.10	8.44	11.16
M14	N-dealkylation	ND	ND	2.22	ND

ND = Not detected

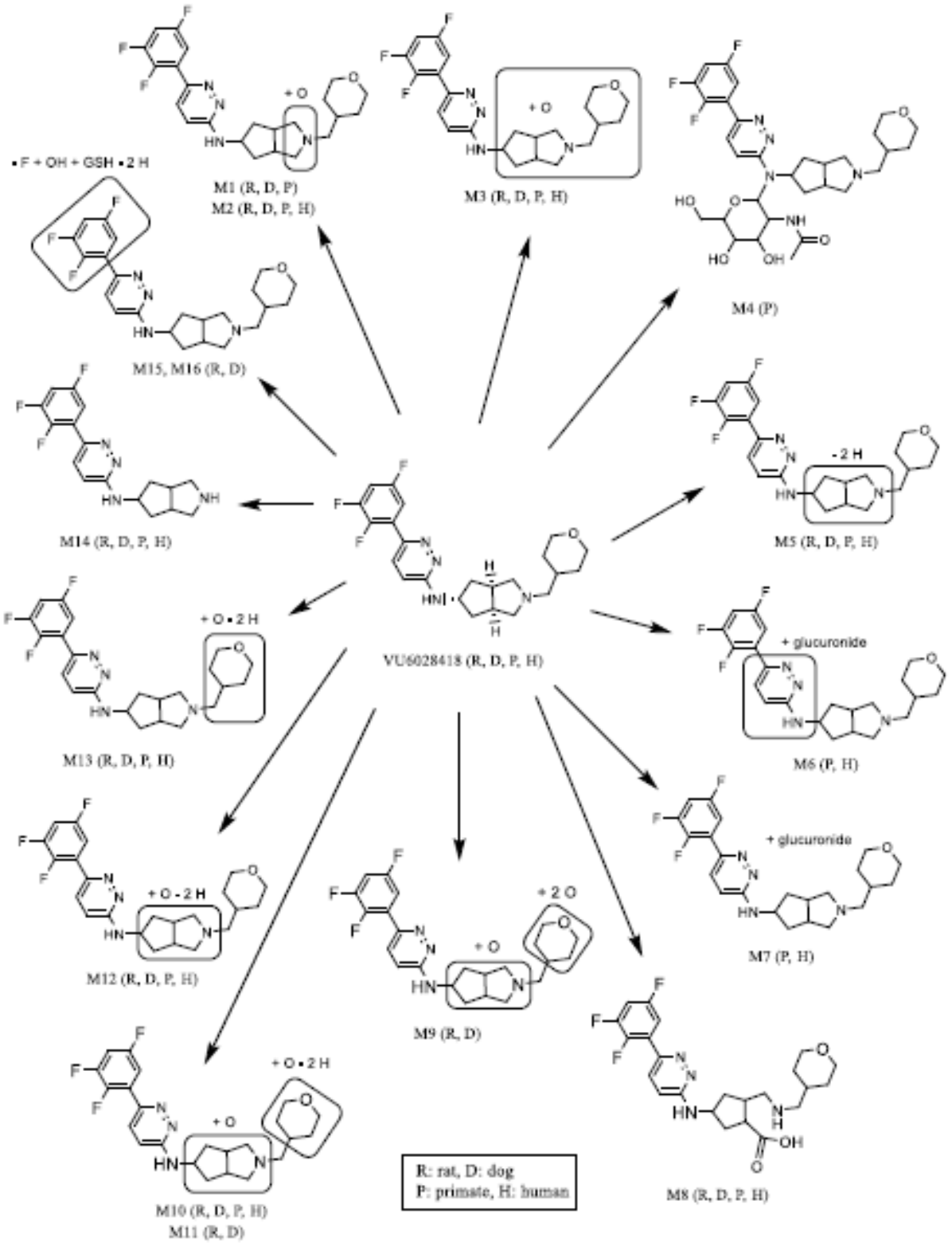
Table S2.

Percent MS Total Peak Areas of VU6028418 and Metabolites in Each Species

Peak ID	Tentative Metabolite Identification	% Total Peak Area			
		Rat	Dog	Monkey	Human
VU6028418	Parent (P)	82.62	90.12	88.35	93.06
M1	P + O	0.79	0.06	0.01	ND
M2	P + O	11.80	3.23	0.02	0.01
M3	P + O	0.42	1.04	0.50	0.82
M4	P + N-acetylglucosamine	ND	ND	3.31	ND
M5	P – 2 H	1.32	0.37	2.42	0.39
M6	P + glucuronide	ND	ND	0.03	0.25
M7	P + glucuronide	ND	ND	0.09	0.19
M8	P + O + O	1.74	3.27	3.77	4.49
M9	P + O + O + O	0.62	0.37	ND	ND
M10	P + O + O – 2 H	0.17	0.01	0.17	< 0.01
M11	P + O + O – 2 H	0.09	0.25	ND	ND
M12	P + O – 2 H	0.12	0.01	0.10	0.02
M13	P + O – 2 H	0.10	0.62	0.44	0.57
M14	N-dealkylation	0.07	0.11	0.80	0.20
M15	P – F + OH + GSH – 2 H	0.04	0.29	ND	ND
M16	P – F + OH + GSH – 2 H	0.10	0.26	ND	ND

ND = Not detected

Figure S1.


+ O
M1 (R, D, P)
M2 (R, D, P, H)
M3 (R, D, P, H)
M4 (P)
- F + OH + GSH - 2 H
M15, M16 (R, D)
M14 (R, D, P, H)
- 2 H
M5 (R, D, P, H)
VU6028418 (R, D, P, H)
+ O - 2 H
M13 (R, D, P, H)
+ glucuronide
M6 (P, H)
+ O - 2 H
M12 (R, D, P, H)
+ glucuronide
M7 (P, H)
+ 2 O
+ O
M9 (R, D)
+ O - 2 H
+ O
M10 (R, D, P, H)
M11 (R, D)
R: rat, D: dog
P: primate, H: human
M8 (R, D, P, H)

P-gp Efflux

Initial determination of test compounds' potential for efflux by human P-gp at a single concentration (5 µM; in duplicate) was performed via contract by Absorption Systems (Exton, PA) using a bidirectional permeability assay with MDCK-MDR1 cells in accordance with their established standard assay methodology.

Definitive assessment of VU6028418 efflux by human P-gp at a single concentration (5 µM; in triplicate) was performed via contract by Solvo Biotechnology (Szeged, Hungary) using a bidirectional permeability assay with LLC-PK1-MDR1 cells in accordance with their established standard assay methodology.

Rat IV/PO Pharmacokinetics

Determination of *in vivo* pharmacokinetics (PK) of VU6028418 following single IV (1 mg/kg, solution dose in 10% EtOH 40% PEG400 50% saline vehicle, 1 mL/kg) or PO (3 mg/kg, suspension dose in 0.1% Tween80 0.5% MC in water, 10 mL/kg) administration to male, Sprague-Dawley rat (*n* = 2, serially-sampled) was performed in-house using in-life phase, bioanalytical, and PK analysis methodologies essentially as previously described [1-4].

Rat PO Dose-Escalation Pharmacokinetics

Determination of *in vivo* PK of VU6028418 following single, escalating PO administrations (1, 3, 10, 30, 100, and 300 mg/kg, in 0.1% Tween80 0.5% MC in water, 10 mL/kg) to male, Sprague-Dawley rat (*n* = 3, serially sampled) was performed via contract by Frontage Laboratories (Exton, PA) in accordance with their established standard in-life phase, bioanalytical, and PK analysis methodologies. The results from this study are shown in the following tables.

Table S3.

Pharmacokinetic Profile of VU6028418 following Single Oral Doses of 1, 3, 10, 30, 100 and 300 mg/kg to Male Sprague-Dawley Rats

Compound	Dose Group	Animal	Nominal PO Dosage (mg/kg)	Actual PO Dosage (mg/kg)	No pts used for $t_{1/2}$	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{Inf} (h*ng/mL)	AUC Extr (%)	MRT_{Inf} (h)	AUC/D (h*kg*ng/mL/mg)
VU6028418	Group 1	Rat 1	1.0	1.01	4	19.8	2.00	34.0	700	756	7	24.3	751
		Rat 2	1.0	0.99	3	12.5	6.00	68.6	805	859	6	16.0	870
		Rat 3	1.0	1.01	4	13.7	4.00	38.5	1030	1060	3	21.7	1040
					N	3	3	3	3	3	3	3	3
					Mean	15.3	4.00	47.0	845	892	5	20.7	887
					SD	3.91	2.00	18.8	169	155	2	4.25	145
					%CV	25.6	50.0	40.0	20.0	17.4	40.0	20.5	16.3

Last time point for AUC_{last}: 72 h for Rats 1 and Rat 3; 48 h for Rat 2

T_{last} for all animals was greater than 3-fold the estimated t1/2. Extrapolated PK parameters are reported.

Compound	Dose Group	Animal	Nominal PO Dosage (mg/kg)	Actual PO Dosage (mg/kg)	No pts used for $t_{1/2}$	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{Inf} (h*ng/mL)	AUC Extr (%)	MRT_{Inf} (h)	AUC/D (h*kg*ng/mL/mg)
VU6028418	Group 2	Rat 4	3.0	3.02	3	20.2	6.00	79.1	2950	3240	9	31.4	1080
		Rat 5	3.0	2.98	3	16.2	0.50	74.5	1870	2000	6	27.9	670
		Rat 6	3.0	3.00	3	19.1	0.50	112	3160	3420	8	27.7	1140
					N	3	3	3	3	3	3	3	3
					Mean	18.5	2.33	88.5	2660	2890	8	29.0	963
					SD	2.07	3.18	20.5	692	773	2	2.08	256
					%CV	11.2	136.5	23.2	26.0	26.7	25.0	7.2	26.6

Last time point for AUC_{last}: 72 h for all animals

T_{last} for all animals was greater than 3-fold the estimated t1/2. Extrapolated PK parameters are reported.

Compound	Dose Group	Animal	Nominal PO Dosage (mg/kg)	Actual PO Dosage (mg/kg)	No pts used for $t_{1/2}$	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{Inf} (h*ng/mL)	AUC Extr (%)	MRT_{Inf} (h)	AUC/D (h*kg*ng/mL/mg)
VU6028418	Group 3	Rat 7	10.0	9.87	3	32.1	0.50	125	6820	8860	23	50.8	897
		Rat 8	10.0	10.16	3	30.9	0.25	232	6780	8370	19	42.8	823
		Rat 9	10.0	10.00	3	20.9	2.00	302	8210	9000	9	28.3	900
					N	3	3	3	3	3	3	3	3
					Mean	28.0	0.92	220	7270	8740	20	40.6	873
					SD	6.15	0.95	89.1	814	331	7	11.4	43.6
					%CV	22.0	103.2	40.5	11.2	3.8	35.0	28.1	5.0

Last time point for AUC_{last}: 72 h for all animals

T_{last} for Rats 7 and 8 was less than 3-fold but greater than twice the estimated t1/2. Extrapolated PK parameters are reported.

T_{last} for Rat 9 was greater than 3-fold the estimated t1/2. Extrapolated PK parameters are reported.

Compound	Dose Group	Animal	Nominal PO Dosage (mg/kg)	Actual PO Dosage (mg/kg)	No pts used for $t_{1/2}$	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{Inf} (h*ng/mL)	AUC Extr (%)	MRT_{Inf} (h)	AUC/D (h*kg*ng/mL/mg)
VU6028418	Group 4	Rat 10	30.0	29.62	NR	NR	4.00	369	17400	NR	NR	NR	NR
		Rat 11	30.0	30.00	4	16.1	0.25	738	19100	20000	4	22.2	666
		Rat 12	30.0	30.37	4	28.8	4.00	833	27200	33000	18	40.8	1090
					N	2	3	3	3	2	2	2	2
					Mean	22.5	2.75	647	21200	26500	10	31.5	878
					SD		2.17	245	5240	9190			
					%CV		78.9	37.9	24.7	34.7			

Last time point for AUC_{last}: 72 h for all animals

NR: Not reported; extrapolated PK parameters were not reported for Rat 10 since t_{last} was less than twice the estimated t1/2 and AUC extrapolated was greater than 25% .

T_{last} for Rat 11 was greater than 3-fold the estimated t1/2. Extrapolated PK parameters are reported.

T_{last} for Rat 12 was less than 3-fold but greater than twice the estimated t1/2. Extrapolated PK parameters are reported.

Compound	Dose Group	Animal	Nominal PO Dosage (mg/kg)	Actual PO Dosage (mg/kg)	No pts used for $t_{1/2}$	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{Inf} (h*ng/mL)	AUC Extr (%)	MRT_{Inf} (h)	AUC/D (h*kg*ng/mL/mg)
VU6028418	Group 5	Rat 13	100	100.00	NR	NR	8.00	637	31000	NR	NR	NR	NR
		Rat 14	100	100.86	NR	NR	1.00	1900	77800	NR	NR	NR	NR
		Rat 15	100	101.54	NC	NC	4.00	1240	66100	NR	NR	NR	NR
					N		3	3	3				
					Mean		4.33	1260	58300				
					SD		3.51	632	24400				
					%CV		81.1	50.2	41.9				

Last time point for AUC_{last}: 72 h for all animals

NR: Not reported; extrapolated PK parameters were not reported for Rats 13 and 14 since t_{last} was less than twice the estimated t1/2 and AUC extrapolated was greater than 25% .

NC: Not calculated; extrapolated PK parameters were not calculated for Rat 15 since there was insufficient data to define the elimination phase.

Compound	Dose Group	Animal	Nominal PO Dosage (mg/kg)	Actual PO Dosage (mg/kg)	No pts used for $t_{1/2}$	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{Inf} (h*ng/mL)	AUC Extr (%)	MRT_{Inf} (h)	AUC/D (h*kg*ng/mL/mg)
VU6028418	Group 6	Rat 16	300	298.25	NC	NC	72.0	1850	90400	NC	NC	NC	NC
		Rat 17	300	304.62	NC	NC	4.00	1860	122000	NC	NC	NC	NC
		Rat 18	300	301.78	NC	NC	72.0	1760	89500	NC	NC	NC	NC
					N		3	3	3				
					Mean		49.3	1820	101000				
					SD		39.3	55.1	18500				
					%CV		79.7	3.0	18.3				

Last time point for AUC_{last}: 72 h for all animals

NC: Not calculated; extrapolated PK parameters were not calculated since there was insufficient data to define the elimination phase.

Dog IV/PO Pharmacokinetics

Determination of *in vivo* pharmacokinetics (PK) of VU6028418 following single IV (1 mg/kg, solution dose in 10% EtOH 70% PEG400 20% saline vehicle, 1 mL/kg) or PO (3 mg/kg, suspension dose in 0.1% Tween80 0.5% MC in water, 10 mL/kg) administration to male, beagle dog (*n* = 3, serially-sampled) was performed via contract by Frontage Laboratories (Exton, PA) using their established standard in-life phase, bioanalytical, and PK analysis methodologies.

Rat Brain and CSF Distribution

Determination of VU6028418's brain and CSF distribution, on a total and unbound concentration basis, following a single PO administration (3 m/kg, suspension dose in 0.1% Tween80 0.5% MC in water, 10 mL/kg) to male, Sprague-Dawley rat (*n* = 3, non-serially sampled from 0.25 to 72 hr) was performed in-house essentially in accordance with previously described methodologies [3].

Calculation of the total brain to total plasma, unbound brain to unbound plasma, and total CSF to unbound plasma distribution partition coefficients (K_p, $K_{p,uu}$, $K_{p,u}$, respectively) was performed using mean AUCs from each matrix, and where relevant, values for fraction unbound in rat plasma or brain ($f_{u,plasma}$, $f_{u,brain}$, respectively) determined *in vitro* from plasma protein binding or brain homogenate binding assays performed in-house in accordance with previously described methodologies [1-4].

Molecular Pharmacology

Calcium Mobilization Assays. Compound-evoked decreases to an EC_{80} concentration of ACh in intracellular calcium were measured using Chinese hamster ovary (CHO) cells stably expressing rat or human muscarinic receptors (M_1–M_5; M_2 and M_4 cells were co-transfected with G_{qi5}). Cell culture reagents were purchased from Gibco-ThermoFisher Scientific (Waltham, MA) unless otherwise noted. Cells (15,000 cells/20 µL/well) were plated in Greiner black wall / clear bottom 384 well plates in F12 medium containing 10% FBS, 20 mM *2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid* (HEPES), and 1X Antibiotic/Antimycotic. The cells were grown overnight at 37 °C in the presence of 5% CO_2. The next day, the medium was removed and replaced with 20 µL of 1 µM Fluo-4, AM (Invitrogen, Carlsbad, CA) prepared as a 2.3 mM stock in dimethyl sulfoxide (DMSO) and mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127 (Invitrogen, Carlsbad, CA) and diluted in Assay Buffer (Hank's Balanced Salt Solution (HBSS), 20 mM HEPES, 4.16 mM sodium bicarbonate (Sigma-Aldrich, St. Louis, MO) and 2.5 mM Probenecid (Sigma-Aldrich, St. Louis, MO)) for 50 minutes at room temperature. Dye was removed and replaced with 20 µL of Assay Buffer. Compounds were serially diluted 1:3 into 10 point concentration response curves in DMSO using the AGILENT Bravo Liquid Handler (Atlantic Lab Equipment, Santa Clara, CA), transferred to daughter plates using an Echo acoustic plate reformatter (Labcyte, Sunnyvale, CA) and diluted in Assay Buffer to a 2X final concentration. Ca^{2+} flux was measured using a Functional Drug Screening System 6000 or 7000 (FDSS6000/7000, Hamamatsu, Japan). After establishment of a fluorescence baseline for 2-3 seconds (2-3 images at 1 Hz; excitation, 480 ± 20 nm; emission, 540 ± 30 nm), 20 µL of test compound was added to the cells, and the response was measured. 140 seconds later, 10 µL (5X) of an EC_{20} concentration of ACh (Sigma-Aldrich, St. Louis, MO) was added to the cells, and the response of the cells was measured. Approximately 125 seconds later, an EC_{80} concentration of ACh was added. Calcium fluorescence was recorded as fold over basal fluorescence and raw data were normalized to the maximal response to agonist. Potency (IC_{50}) and maximum response (% ACh Max) for compounds was determined using a four-parameter logistical equation using GraphPad Prism (La Jolla, CA) or the Dotmatics software platform:

$$y = bottom + \frac{top - bottom}{1 + 10^{(LogEC50 - A)Hillslope}}$$

where *A* is the molar concentration of the compound; *bottom* and *top* denote the lower and upper plateaus of the concentration-response curve; HillSlope is the Hill coefficient that describes the steepness of the curve; and EC_{50} is the molar concentration of compound required to generate a response halfway between the *top* and *bottom*.

Radioligand Binding Assays

Membranes were made from CHO cells stably expressing the human M4 receptor (co-expressing Gqi5). Radioligand competition binding assays were performed as previously described.[1] In brief, M4 antagonists were serially diluted into assay buffer and added to each well of a 96-well plate, along with 10 µg/well cell membrane and approximately 80 pM [^{3}H]-NMS (PerKinElmer, Boston, MA). Following a 3-hour incubation period on shaker at room temperature, the membrane-bound ligand was separated from free ligand by filtration through 96-well glass fiber filter plates (Unifilter-96, GF/B; PerkinElmer, Boston, MA). Forty microliters of scintillation fluid was added to each well, and the membrane-bound radioactivity was determined by scintillation counting (TopCount; PerkinElmer Life and Analytical Sciences, Boston, MA). Nonspecific binding was determined using 10 µM atropine. Data were analyzed with GraphPad Prism 7.0.

Ancillary Pharmacology

Table S4.

Cardiac Ion Channel Panel – Charles River Laboratories

Ion Channel	Mean % Inhibition	Standard Deviation	Standard Error	n
hCav1.2	24.5	2.6	1.5	3
hCav3.2	41.7	10.9	6.3	3
hHCN2	3.0	1.4	0.8	3
hERG	96.0	1.8	0.8	5
hKv1.3	24.2	9.6	5.5	3
hKv1.5	-2.9	5.8	3.4	3
hKvLQT1/hminK	5.6	7.3	3.3	5
hNav1.5 (Tonic)	19.0	6.2	3.1	4
hNav1.5 (Phasic)	21.7	6.1	3.0	4

The *in vitro* effects of VU6028418 were evaluated at room temperature using the QPatch HT® (Sophion Bioscience A/S, Denmark), an automatic parallel patch clamp system. VU6028418 was exposed to each ion channel at 10 µM and tested in at least three cells (n ≥ 3). The duration of exposure to each test article concentration was a minimum of three (3) minutes.

Table S5.

Lead Profiling Screen – Eurofins Panlabs

Assay Name	Species	% inh at 10 µM
Adenosine A2A	hum	2
Adenosine A3	hum	4
Adrenergic α1A	hum	11
Adrenergic α1B	hum	3
Adrenergic α1D	hum	15
Adrenergic α2A	hum	-1
Adrenergic β1	hum	11
Adrenergic β2	hum	1
Androgen (Testosterone)	hum	4
Bradykinin B1	hum	10
Bradykinin B2	hum	4
Calcium Channel L-Type, Benzothiazepine	hum	1
Calcium Channel L-Type, Dihydropyridine	rat	50
Calcium Channel N-Type	rat	23
Cannabinoid CB1	rat	-2
Dopamine D1	hum	3
Dopamine D2S	hum	30
Dopamine D3	hum	14
Dopamine D4.4	hum	15
Endothelin ETA	hum	4
Endothelin ETB	hum	-5
Epidermal Growth Factor (EGF)	hum	7
Estrogen ERα	hum	-1
GABAA, Flunitrazepam, Central	hum	0
GABAA, Muscimol, Central	rat	-7
GABAB1A	rat	15
Glucocorticoid	hum	2
Glutamate, Kainate	hum	5
Glutamate, NMDA, Agonism	rat	-6

Glutamate, NMDA, Glycine	rat	5
Glutamate, NMDA, Phencyclidine	rat	5
Histamine H1	hum	13
Histamine H2	hum	-2
Histamine H3	hum	47
Imidazoline I2, Central	rat	-8
Interleukin IL-1 R1	hum	3
Leukotriene, Cysteinyl CysLT1	hum	6
Melatonin MT1	hum	0
Muscarinic M1	hum	22
Muscarinic M2	hum	61
Muscarinic M3	hum	42
Neuropeptide Y Y1	hum	-6
Neuropeptide Y Y2	hum	-1
Nicotinic Acetylcholine α1, Bungarotoxin	hum	10
Nicotinic Acetylcholine α3β4	hum	43
Opiate δ1 (OP1, DOP)	hum	4
Opiate κ (OP2, KOP)	hum	14
Opiate μ (OP3, MOP)	hum	21
Phorbol Ester	mouse	2
Platelet Activating Factor (PAF)	hum	-1
Potassium Channel [KATP]	ham	-4
Potassium Channel hERG	hum	64
Prostanoid EP4	hum	5
Purinergic P2X	rat	-4
Purinergic P2Y, Non-Selective	rat	12
Rolipram	rat	3
Serotonin (5-Hydroxytryptamine) 5-HT1A	hum	-3
Serotonin (5-Hydroxytryptamine) 5-HT2B	hum	9
Serotonin (5-Hydroxytryptamine) 5-HT3	hum	12
Sigma σ1	hum	101
Sodium Channel, Site 2	rat	34
Tachykinin NK1	hum	17
Thyroid Hormone	rat	14
Transporter, Dopamine (DAT)	hum	29
Transporter, GABA	rat	-5
Transporter, Norepinephrine (NET)	hum	13
Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	hum	5

Haloperidol-Induced Catalepsy

All rodent PK experiments were conducted in accordance with the National Institute of Health regulations of animal care covered in Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985) and were approved by the Institutional Animal Care and Use Committee.

Adult male Sprague-Dawley rats (weighing 290-326 grams) were used for the haloperidol-induced catalepsy studies. The rats were injected with 1.5 mg/kg of haloperidol (i.p.; 1 ml/kg) and then returned to the home cage. The rats were then administered VU6028418 (8i) (0.3-3 mg/kg p.o.; 10 ml/kg) or vehicle (2% Tween 80/0.5% methylcellulose) thirty minutes later. Cataleptic behavior was determined one hour later by placing the forelimbs on a bar raised 6 cm above the table and recording the amount of time it takes for the rat to withdraw the forelimbs. Data are expressed as mean latency to withdraw + SEM or percent inhibition of catalepsy + SEM. The data for these dose-response studies were analyzed by a one-way ANOVA. If there was a main effect of dose, then each dose group was compared with vehicle treated control animals using GraphPad Prism (version 4.03, GraphPad, La Jolla, CA).[5]

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