Supporting Information

Abbreviations Used

AE, adverse effect; Cl, clearance; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; %F, percent bioavailability; Gln, glutamine; HTS, high throughput screening; Ile, isoleucine; Leu, leucine; mg/kg, milligrams per kilogram; nM, nanomolar; NOR, novel object recognition; OR, object retrieval; PDB, protein data bank; PDE, phosphodiesterase; Phe, phenylalanine; PPB, plasma protein binding; Tyr, tyrosine; $t_{1/2}$, half-life; Vdss, volume of distribution

EXPERIMENTAL SECTION

General Chemistry Information: ^{1}H NMR spectra were recorded on a Varian Inova 400 (400 MHz) spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with tetramethylsilane as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), integration. Low resolution mass spectra were recorded on a Bruker Daltonics 3T Fourier transform ion cyclotron resonance mass spectrometer (FT/ICR) with electrospray ionization. Analytical thin layer chromatography was performed on EM Reagent 0.25 mm silica gel 60-F plates. Automated flash chromatography was performed on an ISCO Combiflash Sg 100c with Biotage Flash 40 cartridges. Preparative reverse phase chromatography was performed using a Gilson 215 liquid handler and a YMC CombiPrep Pro C18 column (50 mm x 20 mm i.d.) with a linear gradient over 20 minutes (95:5 to 5:95 water/acetonitrile, containing 0.1% TFA) with collection triggered by UV detection at 220 or 254 nm. Compound purity was determined to be >95% by analytical HPLC analysis on an Agilent 1090 HPLC with binary pump and diode array detector with area quantification performed at 214 nM (Method 1: Zorbax RX-C18, 75 mm x 4.6 mm, 3.5 μM, 98% A/2% B to 100% B over 5.5 min then 100% B to 6.0 min (A = 0.1% H_3P0_4/water v/v; B = acetonitrile). Method 2: Luna C8(2), 75 mm x 4.6 mm, 3 μM 98% A/2% B to 100% B over 5.5 min then 100B to 6.0 min (A = 0.1% H_3PO_4/water v/v; B = acetonitrile). Solvents for extraction and chromatography were HPLC grade. Unless otherwise noted all reactions were conducted in oven (80C) or flame-dried glassware with magnetic stirring under an inert atmosphere of dry nitrogen. High resolution mass spectra (HRMS) were recorded using a Waters Synapt G1 Quadrapole-Time of Flight (Q-TOF) high definition mass spectrometer (HDMS).

Chemistry

(S)-3,6-dimethyl-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**3**).

To a stirred solution of 1-(4-(trifluoromethyl)phenyl)ethanone (2.0 g, 10.63 mmol) in THF (5.32 ml) and heptane (15.95 ml) was added tert-butyl hydrazinecarboxylate (1.545 g, 11.69 mmol) and the resulting mixture was heated to reflux for 12 hours followed by concentration under vacuum giving a white solid that was used without purification. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.89 (d, *J* = 8.2 Hz, 2H), 7.78 (s, 1H), 7.61 (d, *J* = 8.3 Hz, 2H), 2.21 (s, 3H), 1.56 (s, 9H).

To a stirred solution of tert-butyl 2-(1-(4-(trifluoromethyl)phenyl)ethylidene)-hydrazinecarboxylate (3.21 g, 10.62 mmol) in MeOH (11.80 ml) and AcOH (11.80 ml) was

added sodium cyanoborohydride (0.667 g, 10.62 mmol) as a solid and the resulting mixture was stirred 16 hours at ambient temperature. All volatiles were removed under vacuum and the residue diluted with saturated aqueous NaHCO3 and extracted three times with DCM. The combined organics were washed with brine, dried over MgSO4, filtered and evaporated to give a white solid that was used without purification. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.59 (d, *J* = 8.1 Hz, 2H), 7.47 (d, *J* = 8.1 Hz, 2H), 5.95 (s, 1H), 4.23 (d, *J* = 34.3 Hz, 2H), 1.43 (s, 9H), 1.32 (d, *J* = 6.6 Hz, 3H).

To a stirred solution of tert-butyl 2-(1-(4-(trifluoromethyl)phenyl)ethyl)-hydrazinecarboxylate (3.23 g, 10.61 mmol) in dioxane (5 ml) was added 4 M HCl in dioxane (26.5 ml, 106 mmol) and the resulting mixture was stirred at ambient temperature for 5 h. All volatiles were removed under vacuum giving the HCl salt as a yellow solid. MS m/z: $[M + H]^+$ Calcd for $C_9H_{11}F_3N_2$ 205.10; Found 205.3.

To a stirred solution of 2-(1-ethoxyethylidene)malononitrile (1.425 g, 10.47 mmol) in EtOH (25 ml) at 0 °C was added solid (1-(4-(trifluoromethyl)phenyl)ethyl)hydrazine dihydrochloride (2.9 g, 10.47 mmol). The resulting solution was allowed to stir for 15 minutes, after which sodium methoxide (25% in MeOH) (5.03 ml, 21.98 mmol) was added in one portion. The reaction mixture was stirred for 1 hr, then allowed to warm to ambient temperature and stirred an additional hour. The mixture was then heated to 60 °C for 6 hours. The mixture was cooled to ambient temperature and evaporated to remove all volatiles. The residue was diluted with saturated aqueous sodium bicarbonate solution and ethyl acetate. The layers were separated and the aqueous phase extracted with ethyl acetate two times. The combined organic layers were washed with brine and dried over $MgSO_4$. The suspension was then filtered, evaporated and the residue purified by silica chromatography (0-50% EtOAc in hexanes gradient) to afford the product (1.2 g, 4.08 mmol, 39.0 % yield) as a yellow solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.62 (d, *J* = 8.2 Hz, 2H), 7.31 (d, *J* = 8.1 Hz, 2H), 5.23 (d, *J* = 7.0 Hz, 1H), 4.12 (d, *J* = 7.2 Hz, 1H), 3.98 (s, 2H), 2.30 (s, 3H), 2.04 (s, 1H), 1.88 (d, *J* = 7.0 Hz, 3H). MS m/z: $[M + H]^+$ Calcd for $C_{14}H_{13}F_3N_4$ 295.12; Found 295.1.

To a solution of 1 N aqueous sodium hydroxide (4.67 ml, 4.67 mmol) at 0 °C was added 35% aqueous hydrogen peroxide solution (1.860 ml, 21.24 mmol) followed by 5-amino-3-methyl-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1H-pyrazole-4-carbonitrile (1.25 g, 4.25 mmol) as a solution in MeOH (28.3 ml) over 2 minutes. The resulting mixture was stirred 8 hours while gradually warming to ambient temperature. The reaction was quenched with a saturated aqueous sodium bisulfite solution, and then evaporated to remove all volatiles. The residue was diluted with water and extracted with ethyl acetate (4x). The combined organic layer was washed with water, brine, dried over $MgSO_4$, filtered and evaporated to dryness to afford a yellow solid. This material was used without further purification. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.60 (d, *J* = 8.2 Hz, 2H), 7.31 (d, *J* = 8.1 Hz, 2H), 5.39 (s, 2H), 5.24 (d, *J* = 7.1 Hz, 1H), 5.12 (s, 2H), 2.43 (s, 3H), 1.89 (d, *J* = 7.0 Hz, 3H). MS m/z: $[M + H]^+$ Calcd for $C_{14}H_{15}F_3N_4O$ 313.13; Found 313.2.

To a solution of 5-amino-3-methyl-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1H-pyrazole-4-carboxamide (100 mg, 0.320 mmol) in dioxane (1.6 ml) was added acetyl chloride (27.6 mg, 0.352 mmol) and the resulting mixture was heated to 100 °C for 90 minutes. The mixture was cooled and concentrated under vacuum. The residue was purified by silica chromatography (0-5% MeOH in DCM gradient) to afford the racemic product. The enantiopure title compound was obtained by chiral SFC chromatography (OJ, 10% MeOH (0.2% DEA/CO_2)). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 11.72 (br s, 1 H), 7.56 (d, *J* = 8.0 Hz, 2 H), 7.48 (d, *J* = 8.0 Hz, 2 H), 6.04

(q, *J* = 7.1 Hz, 1 H), 2.58 (s, 3H), 2.51 (s, 3H), 1.95 (d, *J* = 7.1 Hz, 3 H). HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{16}H_{16}F_3N_4O$ 337.1271; Found 337.1257.

(S)-3-(hydroxymethyl)-6-methyl-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**4**).

To a stirred solution of 2-(2-(benzyloxy)-1-methoxyethylidene)malononitrile (556 mg, 2.44 mmol) in EtOH (6 ml) at 0 °C was added solid (1-(4-(trifluoromethyl)phenyl)ethyl)-hydrazine dihydrochloride (675 mg, 2.44 mmol). The resulting solution was allowed to stir for 15 minutes, after which sodium methoxide (25% in MeOH) (1.11 g, 5.12 mmol) was added in one portion. The reaction mixture was stirred and allowed to warm to ambient temperature for 1.5 hours. The mixture was then evaporated to remove all volatiles. The residue was diluted with saturated aqueous sodium bicarbonate solution and ethyl acetate. The layers were separated and the aqueous phase extracted with ethyl acetate two times. The combined organic layers were washed with brine and dried over MgSO4. The suspension was then filtered, evaporated and the residue purified by silica chromatography (0-5% MeOH in DCM gradient) to afford the product (810 mg, 2.02 mmol, 83% yield) as a solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.62 (d, *J* = 8.0 Hz, 2H), 7.42 (d, *J* = 7.2 Hz, 2H), 7.36 (t, *J* = 7.9 Hz, 2H), 7.30 (d, *J* = 8.1 Hz, 3H), 5.26 (d, *J* = 7.1 Hz, 1H), 4.65 (s, 2H), 4.54 (s, 2H), 3.98 (s, 2H), 1.89 (d, *J* = 7.0 Hz, 3H). MS m/z: $[M + H]^+$ Calcd for $C_{21}H_{19}F_3N_4O$ 401.16; Found 401.3.

To a solution of 1 N aqueous sodium hydroxide (2.20 ml, 2.20 mmol) at 0 °C was added 35% aqueous hydrogen peroxide solution (0.875 ml, 9.99 mmol) followed by 5-amino-3-((benzyloxy)methyl)-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1H-pyrazole-4-carbonitrile (0.80 g, 2.00 mmol) as a solution in MeOH (13.3 ml) over 2 minutes. The resulting mixture was stirred 16 hours while gradually warming to ambient temperature. The reaction was quenched with a saturated aqueous sodium bisulfite solution, and then evaporated to remove all volatiles. The residue was purified by silica chromatography (0-5% MeOH in DCM gradient) to afford the product (641 mg, 1.53 mmol, 77% yield) as a solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.61 (d, *J* = 8.2 Hz, 2H), 7.39 – 7.29 (m, 7H), 5.27 (d, *J* = 6.9 Hz, 1H), 5.14 (s, 2H), 4.71 (s, 2H), 4.56 (s, 2H), 1.90 (d, *J* = 7.0 Hz, 3H). MS m/z: $[M + H]^+$ Calcd for $C_{21}H_{21}F_3N_4O_2$ 419.17; Found 419.4.

To a solution of 5-amino-3-((benzyloxy)methyl)-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1H-pyrazole-4-carboxamide (357 mg, 0.853 mmol) in dioxane (4.3 ml) was added acetyl chloride (100 mg, 1.28 mmol) and the resulting mixture was heated to 100 °C for 4 hours. Temperature was reduced to 75 °C and the mixture was then stirred 16 hours. The mixture was cooled and concentrated under vacuum. The residue was purified by silica chromatography (0-10% MeOH in DCM gradient) to afford the product (326 mg, 0.738mmol, 86% yield) as a brown oil. MS m/z: $[M + H]^+$ Calcd for $C_{23}H_{23}F_3N_4O_2$ 443.17; Found 443.4.

To a solution of 3-((benzyloxy)methyl)-6-methyl-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (326 mg, 0.738 mmol) in EtOH (10 ml) was added 10% palladium on activated carbon (200 mg, 0.187 mmol) and the resulting mixture was pressurized to 50 psi under H_2 for 16 hours. The mixture was filtered through Celite and the pad rinsed with MeOH. Combined filtrates were concentrated under vacuum and purified by preparative HPLC (5-95% ACN in water (0.1% TFA)) to afford the racemic product. The enantiopure title compound was obtained by chiral SFC chromatography (OJ, 10% MeOH (0.2% DEA/CO_2)). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 12.18 (s, 1H), 7.57 (d, *J* = 8.1 Hz, 2H), 7.50

(d, *J* = 8.1 Hz, 2H), 6.07 (q, *J* = 7.0 Hz, 1H), 4.90 (d, *J* = 6.2 Hz, 2H), 4.73 (t, *J* = 6.6 Hz, 1H), 2.56 (s, 3H), 1.95 (d, *J* = 7.1 Hz, 3H). HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{16}H_{16}F_3N_4O_2$ 353.1225; Found 353.1202.

(R)-3-(hydroxymethyl)-6-methyl-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**5**) ^{1}H NMR (500 MHz, Chloroform-*d*) δ 12.18 (s, 1H), 7.57 (d, *J* = 8.1 Hz, 2H), 7.50 (d, *J* = 8.1 Hz, 2H), 6.07 (q, *J* = 7.0 Hz, 1H), 4.90 (d, *J* = 6.2 Hz, 2H), 4.73 (t, *J* = 6.6 Hz, 1H), 2.56 (s, 3H), 1.95 (d, *J* = 7.1 Hz, 3H). HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{16}H_{15}F_3N_4O_2$ 353.1225; Found 353.1228.

(S)-1-(2,2-dimethyl-1-(4-(trifluoromethyl)phenyl)propyl)-3-(hydroxymethyl)-6-methyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**6**) The procedure of Example **4** was followed using (2,2-dimethyl-1-(4-(trifluoromethyl)phenyl)propyl)hydrazine. ^{1}H NMR (400 MHz, CD_3OD) δ 7.84 (d, *J* = 12 Hz, 2H), 7.67 (d, *J* = 8.8 Hz, 2H), 5.84 (s, 1H), 4.91 (s, 2H), 2.48 (s, 3H), 1.06 (s, 9H). HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{19}H_{21}F_3N_4O_2$ 395.1695; Found 395.1715.

(S)-1-(1-(3-fluoro-4-(trifluoromethyl)phenyl)ethyl)-3-(hydroxymethyl)-6-methyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**7**) The procedure of Example **4** was followed using (1-(3-fluoro-4-(trifluoromethyl)phenyl)ethyl)hydrazine. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 10.82 (s, 1H), 7.56 (t, *J* = 7.9 Hz, 1H), 7.23 (d, *J* = 10.3 Hz, 2H), 6.08 – 5.96 (m, 1H), 4.90 (d, *J* = 6.7 Hz, 2H), 4.63 (t, *J* = 6.8 Hz, 1H), 2.55 (s, 3H), 1.95 (d, *J* = 7.1 Hz, 3H). HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{16}H_{14}F_4N_4O_2$ 371.1131; Found 371.1137.

(S)-6-(3,4-dimethoxybenzyl)-3-(hydroxymethyl)-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**8**). The procedure of Example **4** was followed using 2-(3,4-dimethoxyphenyl)acetyl chloride. ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.57 (d, *J* = 8.2 Hz, 2H), 7.48 (d, *J* = 8.2 Hz, 2H), 6.93 (d, *J* = 1.4 Hz, 1H), 6.90 – 6.81 (m, 2H), 6.10 (d, *J* = 7.2 Hz, 1H), 4.82 (s, 2H), 3.92 (s, 2H), 3.80 (s, 3H), 3.72 (s, 3H), 1.95 (d, *J* = 7.2 Hz, 3H). HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{24}H_{23}F_3N_4O_4$ 489.1749; Found 489.1758.

(S)-6-hydroxy-3-(hydroxymethyl)-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**9**).

To a solution of 5-amino-3-((benzyloxy)methyl)-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1H-pyrazole-4-carboxamide (100 mg, 0.239 mmol) in dioxane (1.2 ml) at 0 °C was added triphosgene (36 mg, 0.119 mmol) followed by dropwise addition of TEA (33 µl, 0.239 mmol). The resulting mixture was heated to 100 °C for 2.5 hours. The mixture was cooled and concentrated under vacuum. The residue was purified by silica chromatography (0-5% MeOH in DCM gradient) to afford the product (57 mg, 0.128 mmol, 54% yield). MS m/z: $[M + H]^+$ Calcd for $C_{22}H_{19}F_3N_4O_3$ 445.15; Found 445.3.

To a solution of 3-((benzyloxy)methyl)-6-hydroxy-1-(1-(4-(trifluoromethyl)phenyl)-ethyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (56 mg, 0.126 mmol) in EtOH (1.2 ml) was added 10% palladium on activated carbon (26.8 mg, 0.374 mmol) and the resulting mixture was stirred under a hydrogen balloon for 2 hours. The mixture was filtered through Celite and the

pad rinsed with MeOH. Combined filtrates were concentrated under vacuum and purified by preparative HPLC (10-95% ACN in water (0.1% TFA)) to afford the racemic product. The enantiopure title compound was obtained by chiral SFC chromatography (OJ, 10% MeOH (0.2% DEA/CO_2)). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.52 (d, *J* = 8.1 Hz, 2H), 7.38 (d, *J* = 8.0 Hz, 2H), 5.79 (q, *J* = 6.9 Hz, 1H), 5.30 (s, 1H), 4.78 (s, 2H), 1.88 (d, *J* = 7.0 Hz, 3H). HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{15}H_{14}F_3N_4O_3$ 355.1013; Found 355.0991.

(S)-3-(fluoromethyl)-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1H-pyrazolo[3,4-d]pyrimidine-4,6-diol (**10**).

To a solution of *(S)-6-hydroxy-3-(hydroxymethyl)-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one* (**9**) (50 mg, 0.141 mmol) in DCM (706 µl) at 0 °C was added DAST (187 µl, 1.41 mmol) and the resulting mixture was warmed to ambient temperature and stirred overnight. Added saturated aqueous sodium bicarbonate solution and extracted the mixture three times with DCM. The combined organic layer was washed with brine, dried over MgSO4, filtered, and concentrated under vacuum. The residue was purified by preparative HPLC (10-95% ACN in water (0.1% TFA)) to afford the product as a solid. ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.66 (d, *J* = 8.2 Hz, 2H), 7.48 (d, *J* = 8.0 Hz, 2H), 5.86 – 5.70 (m, 1H), 5.64 – 5.51 (m, 1H), 5.50 – 5.41 (m, 1H), 1.93 (d, *J* = 6.9 Hz, 3H). HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{15}H_{13}F_4N_4O_2$ 357.0975; Found 357.0980.

(S)-6-hydroxy-3-(2,2,2-trifluoroethyl)-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**11**)

To a mixture of malonitrile (0.451 g, 6.83 mmol) in THF (13.65 ml) at ambient temperature was added NaH (0.546 g, 13.65 mmol) followed by dropwise addition of 3,3,3-trifluoropropionyl chloride (1 g, 6.83 mmol). The resulting mixture 1.5 hours. 1N HCl was added to quench, and the mixture was extracted with EtOAc three times, and the combined organic layer was dried over MgSO4, filtered, and concentrated under vacuum. This material was used without further purification. To a mixture of 2-(3,3,3-trifluoro-1-hydroxypropylidene)malononitrile (1.2g, 6.81 mmol) in trimethyl orthoformate (11.36, 6.81 mmol) was added p-toluenesulfonic acid (0.587 g, 3.41 mmol) and the resulting mixture was heated to reflux overnight. The mixture was evaporated to remove volatiles, and the residue purified by silica chromatography (25% EtOAc in hexanes) to afford the product (0.584 g, 3.07 mmol, 45%). ^{1}H NMR (300 MHz, CDCl3) δ 4.43 (2, 3 H), 3.45 (q, *J* = 9.3 Hz, 2H), MS m/z: $[M + H]^+$ Calcd for $C_7H_5F_3N_2O$ 191.05; Found 191.0.

(*S*)-6-hydroxy-3-(2,2,2-trifluoroethyl)-1-(1-(4-(trifluoromethyl)phenyl)ethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one was synthesized from 2-(3,3,3-trifluoro-1-methoxypropylidene)malononitrile using procedures similar to those shown for compounds **3** and **9**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.19 (s, 1H), 10.98 (s, 1H), 7.73 (d, *J* = 8.2 Hz, 2H), 7.50 (d, *J* = 8.1 Hz, 2H), 5.88 (q, 1H), 3.82 (q, *J* = 11.9 Hz, 2H), 1.81 (d, *J* = 6.8 Hz, 3H). HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{16}H_{13}F_6N_4O_2$ 407.0943; Found 407.0943.

PDE2 Biochemical Assay and Counterscreening Protocols

The activity of the compounds as PDE2 inhibitors were determined using a fluorescence polarization (FP) methodology (Huang, W., et al., J. Biomol. Screen, 2002, 7: 215). In a typical experiment the PDE2 inhibitory activity of the compounds was determined in accordance with the following experimental method. Rhesus PDE2A3 was amplified from rhesus macaque brain cDNA (Biochain Institute, Hayward, CA) using primers based on human PDE2A sequence (accession NM_002599.3) where the forward primer containing a Kozak consensus was 5'-gccaccatggggcaggcatgtggc-3' and the reverse primer was 5'- tcactcagcatcaaggctgca-3'. Amplification with Easy-A High-Fidelity PCR cloning enzyme (Stratagene, La Jolla, CA) was 95 ºC for 2 minutes followed by thirty three cycles of 95°C for 40 seconds, 52ºC for 30 seconds, and 72ºC for 2 minutes 48 seconds. Final extension was 72ºC for 7 minutes. The PCR product was TA cloned into pcDNA3.3-TOPO (Invitrogen, Carlsbad, CA) according to standard protocol. A consensus sequence was developed from multiple clones and then deposited into GenBank (EU812167). AD293 cells (Stratagene, La Jolla, CA) with 70-80% confluency were transiently transfected with rhesus PDE2A3 / pcDNA3.3-TOPO using Lipofectamine 2000 according to manufacturer specifications (Invitrogen, Carlsbad, CA). Cells were harvested 48 hours post-transfection and lysed by sonication (setting 3, 10 X 5 sec pulses) in a buffer containing 20 mM HEPES pH 7.4, 1 mM EDTA and Complete Protease Inhibitor Cocktail Tablets (Roche, Indianapolis, IN). Lysate was collected by centrifugation at 75,000 x g for 20 minutes at 4˚C and supernatant utilized for evaluation of PDE2 activity. The fluorescence polarization assay for cyclic nucleotide phosphodiesterases was performed using an IMAP® FP kit supplied by Molecular Devices, Sunnyvale, CA (product # R8139). IMAP® technology has been applied previously to examine the effects of phosphodiesterase inhibitors (Huang, W., et al., J. Biomol Screen, 2002, 7: 215). Assays were performed at room temperature in 384-well microtiter plates with an incubation volume of 20.2 µL. Solutions of test compounds were prepared in DMSO and serially diluted with DMSO to yield 8 µL of each of 10 solutions differing by 3-fold in concentration, at 32 serial dilutions per plate. 100% inhibition was determined using a known PDE2 inhibitor, Bay 60-7550 (Ki-~0.2nM) at 1 µM concentration for 100% inhibition. Bay 60-7550 was obtained from Axxora via Fisher Scientific (cat# ALX-270-421-M025 / cat# NC9314773 A Labcyte Echo 555 (Labcyte, Sunnyvale, CA) is used to dispense 200 nL from each well of the titration plate to the 384 well assay plate. Ten microliters of a solution of enzyme (1/2000 final dilution from aliquots; sufficient to produce 20% substrate conversion) was added to the assay plate. Next 10 uL of a separate solution of the substrate FAM-labeled cAMP (50 nM final concentration product # R7506 from Molecular Devices) and the activator cGMP (1 uM final concentration), prepared in the assay buffer (10 mM Tris HCl, pH 7.2, 10 mM $MgCl_2$, 0.05% NaN_3 0.01% Tween-20, and 1 mM DTT) was added to the assay plate and shaken to mix. The reaction is allowed to proceed at room temperature for 60 minutes. A binding solution is then made from the kit components, comprised of 80% Solution A, 20% Solution B and binding reagent at a volume of 1/600 the total binding solution. The enzymatic reaction is stopped by addition of 60 µL of the binding solution to each well of the assay plates and the plates are sealed and shaken for 30 seconds. The plate was incubated at room temperature for at least one hour prior to determining the fluorescence polarization (FP). The parallel and perpendicular fluorescence of each well of the plate was measured using a Tecan Genios Pro plate reader (Tecan, Switzerland) or Perkin Elmer EnVision™ plate reader (Waltham, MA). Fluorescence polarization (mP) was calculated from the parallel (S) and

perpendicular (P) fluorescence of each sample well and the analogous values for the median control well, containing only substrate (So and Po), using the following equation:

Polarization (mP) = 1000*(S/So-P/Po)/(S/So+P/Po).

Dose-inhibition profiles for each compound were characterized by fitting the mP data to a four-parameter equation given below. The apparent inhibition constant (K_I), the maximum inhibition at the low plateau relative to "100% Inhibition Control" (Imax; e.g. 1=> same as this control), the minimum inhibition at the high plateau relative to the "0% Inhibition Control" (Imin, e.g. 0=> same as the no drug control) and the Hill slope (nH) are determined by a non-linear least squares fitting of the mP values as a function of dose of the compound using an in-house software based on the procedures described by Mosser et al., JALA, 2003, 8: 54-63, using the following equation:

$$mP = \frac{(0\%mP - 100\%mP)(\mathrm{Imax} - \mathrm{Imin})}{1 + \left[\dfrac{[\mathrm{Drug}]}{(10^{-\mathrm{pK_I}}(1 + \dfrac{[Substrate]}{\mathrm{K}_M})} \right]^{\mathrm{nH}}} + 100\%mP + (0\%mP - 100\%mP)(1 - \mathrm{Imax})$$

The median signal of the "0% inhibition controls" (0%mP) and the median signal of the "100% inhibition controls" (100%mP) are constants determined from the controls located in columns 1-2 and 23-24 of each assay plate. An apparent (K_M) for FAM-labeled cAMP of ~10 uM was used.

Selectivity for PDE2, as compared to other PDE families, was assessed using the IMAP® technology. Human PDE10A2 enzyme was prepared from cytosolic fractions of transiently transfected HEK cells. All other PDE's were GST Tag human enzyme expressed in insect cells and were obtained from BPS Bioscience (San Diego, CA): PDE1A (Cat# 60010), human PDE2A1(Cat# 60020), PDE3A (Cat# 60030), PDE4A1A (Cat# 60040), PDE5A1 (Cat# 60050), PDE6C (Cat# 60060), PDE7A (Cat# 60070), PDE8A1 (Cat# 60080), PDE9A2 (Cat# 60090), PDE11A4 (Cat# 60110).

Assays for PDE 1 through 11 were performed in parallel at room temperature in 384-well microtiter plates with an incubation volume of 20.2 µL. Solutions of test compounds were prepared in DMSO and serially diluted with DMSO to yield 30 µL of each of ten solutions differing by 3-fold in concentration, at 32 serial dilutions per plate. 100% inhibition was determined by adding buffer in place of the enzyme and 0% inhibition is determined by using DMSO (1% final concentrations). A Labcyte POD 810 (Labcyte, Sunnyvale, CA) was used to dispense 200 nL from each well of the titration plate to make eleven copies of the assay plate for each titration, one copy for each PDE enzyme. A solution of each enzyme (dilution from aliquots, sufficient to produce 20% substrate conversion) and a separate solution of FAM-labeled cAMP or FAM-labeled cGMP from Molecular Devices (Sunnyvale, CA, product # R7506 or cGMP#R7508), at a final concentration of 50 nM were made in the assay buffer (10 mM Tris HCl, pH 7.2, 10 mM $MgCl_2$, 0.05% NaN_3 0.01% Tween-20, and 1 mM DTT). Note that the substrate for PDE2 is 50 nM FAM cAMP containing 1000 nM of cGMP. The enzyme and the substrate were then added to the assay plates in two consecutive additions of 10 µL and then shaken to mix. The reaction was allowed to proceed at room temperature for 60 minutes. A

binding solution was then made from the kit components, comprised of 80% Solution A, 20% Solution B and binding reagent at a volume of 1/600 the total binding solution. The enzymatic reaction was stopped by addition of 60 µL of the binding solution to each well of the assay plate. The plates were sealed and shaken for 10 seconds. The plates were incubated at room temperature for one hour, then the parallel and perpendicular fluorescence was measured using a Tecan Genios Pro plate reader (Tecan, Switzerland). The apparent inhibition constants for the compounds against all 11 PDE's was determined from the parallel and perpendicular fluorescent readings as described for PDE10 FP assay using the following apparent K_M values for each enzyme and substrate combination: PDE1A (FAM cGMP) 70 nM, human PDE2A1 (FAM cAMP) 10,000 nM, PDE3A (FAM cAMP) 50 nM, PDE4A1A (FAM cAMP) 1500 nM, PDE5A1 (FAM cGMP) 400 nM, PDE6C (FAM cGMP) 700 nM, PDE7A (FAM cAMP) 150 nM, PDE8A1 (FAM cAMP) 50 nM, PDE9A2 (FAM cGMP) 60 nM, PDE10A2 (FAM cAMP) 150nM, PDE11A4 (FAM cAMP) 1000 nM.

In vivo studies materials and methods

All experimental protocols described in this study were approved by the Merck and Co., Inc. Institutional Animal Care and Use Committee and conducted in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). In addition, the self-ordered spatial search (SOSS) protocol was approved by the Maccine Institutional Animal Care and Use Committee and conducted in accordance with the National Advisory Committee for Laboratory Animal Research (NACLAR). All efforts were made to minimize animal suffering, to reduce the number of animals used and to use alternatives to in vivo methods where possible. Specific details of species, strain, and weight are given within the methods for each section. Rats, rhesus, and cynomolgus macaques were used in these studies in order to determine whether the effects of PQCA are similar across species. We believe that examining the effects of PQCA in multiple species increases our confidence that the compound might have effects in additional species, such as humans. Temperature and relative humidity were maintained at 21–24 °C and 50–55 %, respectively. Food and water were available ad libitum unless otherwise noted. PQCA was prepared as previously described (Kuduk et al. 2011a).

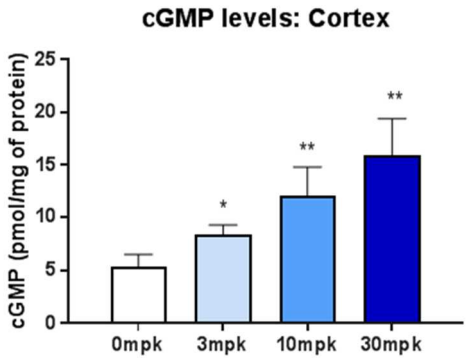
In vitro cGMP measurement

The cortex was dissected, frozen on dry ice, and stored at −80 °C until analysis. Cortical brain tissues were weighed out and diluted 1:10 by weight to 0.01g/mL in 0.1M HCl. Brain tissues were homogenized on ice using a probe sonicator (Thermofisher) for 30 seconds. Samples were then centrifuged at 6,000g at 4°C for 10 minutes (Thermofisher). The supernatant was retained and moved to centrifuge tube with filter (0.22uM nylon polypropylene tube) (Corning, 8169) and centrifuged at 13,000g at 4°C for 10 minutes. Total protein was determined using a modified Lowry method (BioRad DC Protein Assay, BioRad Laboratories).

cGMP measurement was measured in the cytosolic fraction of cortical tissue using the direct cGMP ELISA kit (Enzo, ADI-901-014) according to manufacturing instructions Briefly, the cGMP standard was prepared using a cGMP stock solution in 0.1M HCl. 50ul of neutralizing reagent was added per well followed by 100ul of standards and samples. Next, 50ul of blue conjugate and 50ul of yellow conjugate were added per well. Plate was incubated on a plate shaker at 500 rpm at room temperature (RT) for 2hr. Next, the plate was washed 3x with 400ul

of 1X Wash Buffer. 200uL of pNpp Substrate was added per well followed by incubation of plate at RT 1hr without shaking. Finally, 50uL of stop solution was added per well and plate was read immediately at 405nm on Spectramax (Molecular Devices). Data analysis was performed using microsoft excel and graphprism to generate standard curve, extrapolate values, and normalize to total protein (pmol/mg total protein).

Figure 6: Effects of compound **4** on cortex cGMP levels in rat brain.

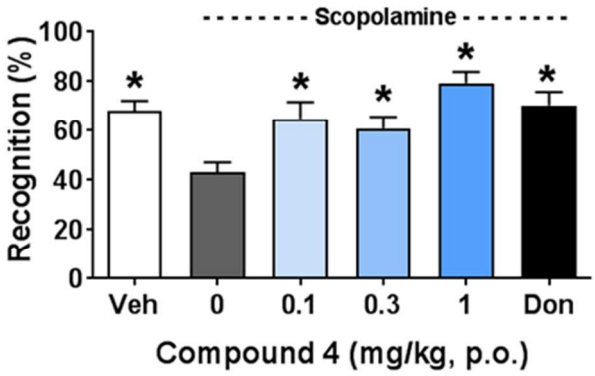


n = 7 – 8 per group
One way ANOVA, multiple comparison across groups using Dunnett's Test compared to vehicle group.
* = p value < 0.05
** = p value < 0.01

Novel object recognition

Male Wistar Hannover rats (Charles Rivers Laboratory) weighing 200–250 g were housed 2/cage under reverse 12 h light:dark conditions (lights off 06:00) with food and water available ad libitum. One hour prior to testing, animals were brought to the testing room and habituated. Testing was performed under dim light conditions. On the day of testing each rat was first habituated to the test arena for 3 min without any objects present. The test arena consisted of a vinyl, opaque cylinder approximately 80 cm in diameter with walls 40 cm tall. Activity of the animals was video recorded and scored using visual tracking software (Cleversys). Following habituation to the test arena, animals were returned to their home cage for 5 min before being placed back in the arena for 3 min with two identical objects (T1). Objects used were custom fabricated geometric shapes (cone and sphere) similar in overall size (approximately 8 cm in height × 8 cm in diameter). Exploration was recorded when the animal's nose was pointed in the direction of the object and was <2 cm from the object. One hour later each rat was again placed in the testing arena for 3 min and exposed to one identical object and one novel object (T2). The amount of time animals explored the novel object relative to the familiar object was the primary measure. In addition, time spent exploring the objects during T1 was also recorded and analyzed. Prior to T1, animals were given scopolamine (Sigma Aldrich, 1 mg/kg; i.p.) or vehicle (saline) and donepezil (Sequoia Research Products, 1.8 mg/kg; i.p.), PQCA (3, 10, or 30 mg/kg; i.p.) or vehicle (5 % beta cyclodextrin). All injections were given 30 min prior to T1.

Compound **4** in scopolamine-impared Novel Object Recognition in adult rats.



Dose (mg/kg)	Plasma concentration (µM)	Unbound plasma drug levels (µM)
0.1	0.174	0.025
0.3	0.717	0.103
1.0	0.949	0.137

n=8-16 per group
One way ANOVA followed by Dunnett's Multiple Comparison Test * = $p<0.05$ compared to scopolamine (0) group.

Object retrieval detour task

Six single-housed male rhesus macaques (*Macaca mulatta*), 4–6 years old, 6.5–9.5 kg, participated as subjects in this experiment. Subjects were maintained on a 12 h light:dark cycle (lights on at 06:30) with room temperatures maintained at 22 ± 2 °C. Testing was performed in each subject's home cage, between 12:00 and 15:00 h. Subjects were fed their full daily regimen of food (Purina High Protein Monkey Diet no. 5045) after testing. Water was available ad libitum. In addition to their chow, monkeys were given a variety of fresh fruits and vegetables daily. The object retrieval detour task requires subjects to retrieve food objects (diced apple squares, 1 × 1 in.) from a clear acrylic box with a single open plane. Sessions consisted of a fixed arrangement of eight "easy" and nine "difficult" trials. For easy trials the reward was positioned either (1) inside the box, with the open plane (and reward) directly in the line of sight and reach of the subject, (2) slightly protruding from the box with the open plane to the left or right of the subject, or (3) just inside the box with the open plane either to the left or right of the subject. Performance on easy trials was measured to detect potential adverse events under drug conditions (e.g., motor, motivational, or visuospatial impairments). For difficult trials, the reward was placed deep inside the box opposite the open plane. Performance on difficult, but not easy trials, are disrupted by scopolamine and prefrontal cortex lesions and is thought to require greater attention, planning, and impulse control, relative to easy trials. Figure 1 provides an illustration of the "easy" and "difficult" trial types. Trials were scored

according to the subject's initial attempt to retrieve the reward and were scored as correct if subjects successfully reached into the open plane of the box to retrieve the reward. Reaches were scored as incorrect if the subject contacted one of the solid planes of the box on the initial attempt to retrieve the reward. Subjects were not punished for incorrect reaches and all subjects eventually retrieved all rewards. A newly cleaned box was presented for every trial, to eliminate visual cues from previous handling. Prior to each trial, a barrier was placed in front of the acrylic box to prevent the subject from observing the baiting process or the position of the reward prior to the commencement of each trial.

Open image in new window

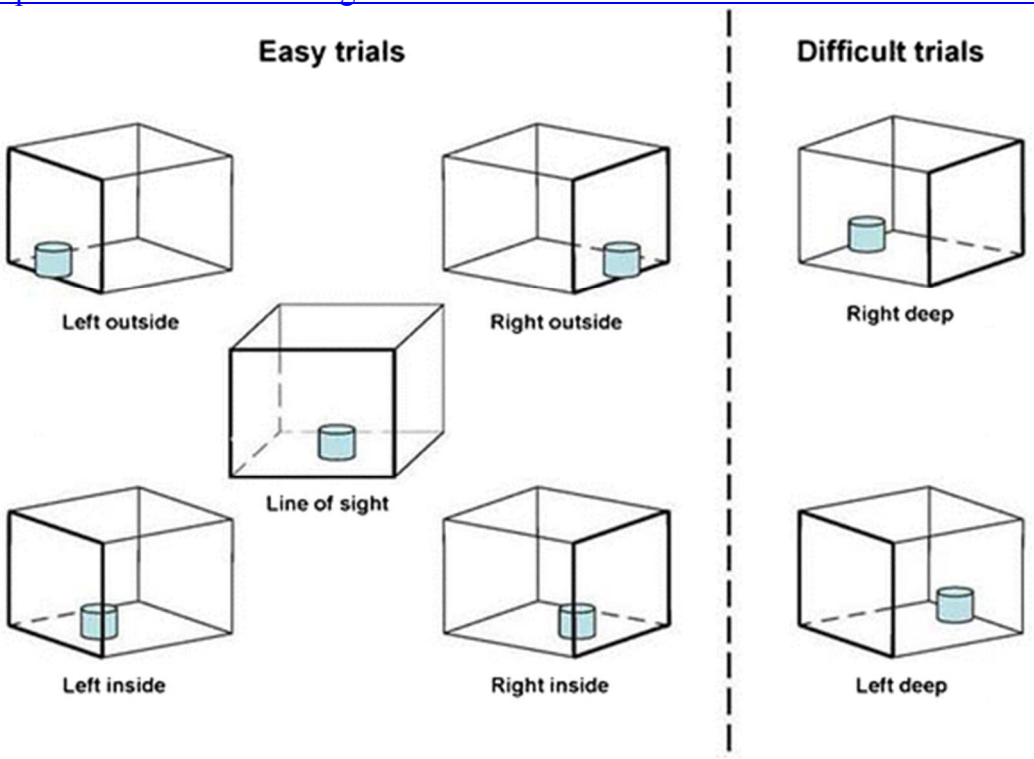


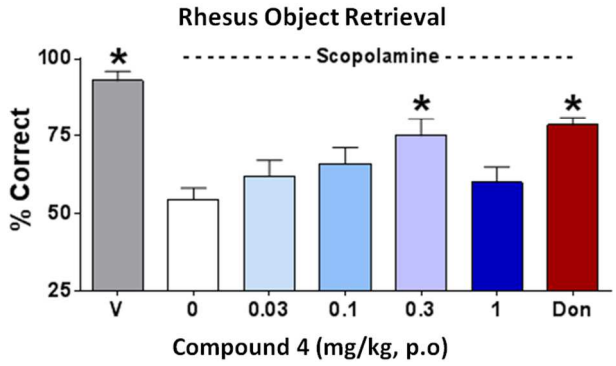
Fig. 1

Diagram of the box position (*bold plane* is open) and food reward in the OR tack. The *left segment* illustrates five variations of easy trials; the *right segment* illustrates the two variations of difficult trials. All illustrations represent the trial as viewed by the monkey

Subjects were tested two times weekly, with at least 3 days between test sessions. Subjects were first tested under vehicle-only conditions (IM saline in the case of scopolamine and p.o. 20 % vitamin E TPGS for PQCA) until their performance stabilized. Performance was considered stable when the SEM of the last three sessions was ≤10 on both trial types. Next, the subjects were characterized on scopolamine to demonstrate sufficient impairment compared to the vehicle baseline. Due to individual differences in sensitivity to scopolamine, each subject's "best dose" (defined as the dose that produces a >20 % deficit on difficult trials and does not significantly impact easy trial performance) was identified and subsequently replicated 2–3 times to ensure reliability. Once vehicle and scopolamine baseline performance stability was successfully attained, PQCA characterization was initiated. Utilizing a Latin-square study

design, scopolamine (or vehicle) and PQCA (or vehicle) were administered 30 min and 4 h prior to testing, respectively.

Compound **4** in scopolamine-impaired Rhesus Object Retrieval in adult monkeys



Dose (mg/kg)	Plasma concentration (µM)	Unbound plasma drug levels (µM)
0.03	0.061	0.006
0.1	0.270	0.027
0.3	0.598	0.060
1	2.03	0.200

n=10
One way ANOVA followed by Dunnett's Multiple Comparison Test * = $p<0.05$ compared to scopolamine (0) group.

Pharmacokinetic studies

The concentration of PQCA in plasma from satellite rats was sampled 30 min after oral administration and in plasma from satellite rhesus monkey and from cynomolgus monkeys following SOSS performance, 4 and 4.25 h after oral administration. Mice were sampled as described above. PQCA levels were quantified using a Transcend LX2 Multiplexed UPLC system coupled with a SCIEX API4000 Q-Trap mass spectrometer (Applied Biosystems, Ontario, Canada). Protein was precipitated from plasma, then, supernatants were separated on an Ascentis Express C18 column (50 mm × 3.0 mm × 2.7 µ) operated at 60 ° C. The flow rate was 0.750 ml/min and a gradient mobile phase consisting of solvent A (water with 0.1 % formic acid) and solvent B (acetonitrile with 0.1 % formic acid) was used during the separation. The concentration of PQCA in the samples were determined using MultiQuant 1.2 based on standard curve and quality control samples

Statistics

Novel object recognition and total object exploration during T1 and T2 were analyzed with a one-way analysis of variance followed by Fisher's LSD post hoc tests to examine group

differences. For the object retrieval detour task and self-ordered spatial search, a one-way repeated-measures analysis of variance was used to determine main effects of treatment, and within-subjects *t* tests were performed to compare differences versus the vehicle–scopolamine group. Laser Doppler blood flow data were analyzed via two-way analysis of variance (time and group as within and between subject measures, respectively).