

Supporting Information

Knowledge Driven Prospective Design and Chemical Enablement as Strategies for Rapid Lead Optimization and Identification of a Selective Brain Penetrant PDE9A Inhibitor

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Crystal Structure Experimental Conditions:

Human PDE9 catalytic domain expression, purification and soaking with compounds **1** (PDB 3JS1) and compound **6** (PDB 3JSW) was carried out as described previously described (1).

¹Liu, S; Mansour, M. N.; Dillman, K. S.; Perez, J. R.; Danley, D. E.; Aeed, P. A.; Simons, S. P.; Lemotte, P. K.; Menniti, F. S. Structural Basis for the Catalytic Mechanism of Human Phosphodiesterase 9. *Proc. Natl. Acad. Sci. USA* **2008**, *105*(36), 13309-14.

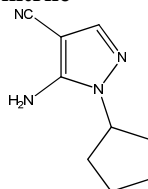
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Chemistry Experimental

Experimentals

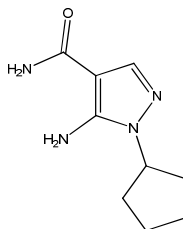
All reagents and solvents were used as purchased from commercial sources. Reactions were carried out under a blanket of nitrogen. Silica gel chromatography was done using the appropriate size Biotage pre-packed silica filled cartridges. Mass Spectral data was collected on a Micromass ADM Atmospheric Pressure Chemical Ionization instrument (LRMS APCI). NMR spectra were generated on a Varian 400 MHz instrument. Chemical shifts were recorded in ppm relative to tetramethylsilane (TMS) with multiplicities given as s (singlet), bs (broad singlet), d (doublet), t (triplet), dt (double of triplets) m (multiplet). All target compounds were analyzed using ultra high performance liquid chromatography/UV/evaporative light scattering detection coupled to time-of-flight mass spectrometry (UHPLC/UV/ELSD/TOFMS). Unless otherwise noted, all tested compounds were found to be >95% pure by this testing method. UHPLC/MS Analysis. The UHPLC was performed on a Waters ACQUITY UHPLC system (Waters, Milford, MA), which was equipped with a binary solvent delivery manager, column manager, and sample manager coupled to ELSD and UV detectors (Waters, Milford, MA). Detection was performed on a Waters LCT premier XE mass spectrometry (Waters, Milford, MA). The instrument was fitted with an Acquity BEH (bridged ethane hybrid) C18 column (30 mm × 2.1 mm, 1.7 μ m particle size, Waters, Milford, MA) operated at 60 °C.

5-amino-1-cyclopentyl-1H-pyrazole-4-carbonitrile



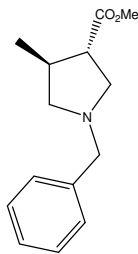
To a solution of cyclopentylhydrazine hydrogen chloride (10g, 73.2 mmol), in ethanol (50 mL) was added 2-(methoxymethylene)malononitrile (8.9g, 1 eq.) and sodium methoxide (2.5g, 1.5 eq). The reaction mixture was heated at reflux for 18h and concentrated. The reaction mixture was partitioned between brine and ethyl acetate. The organic layer was separated, dried with magnesium sulfate, filtered and concentrated. The title compound was used as is in the next step (7.0g, 54%). ¹H NMR (400 MHz, D₂O) δ : 8.06(s, 1H), 4.4(m, 1H), 1.91-1.97(m, 2H), 1.73-1.81 (m, 4H), 1.58-1.70 (m, 2H); MS: (M⁺H m/z = 177).

5-amino-1-cyclopentyl-1H-pyrazole-4-carboxamide



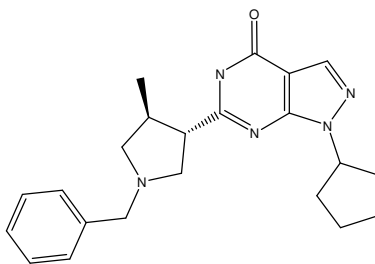
To 5-amino-1-cyclopentyl-1H-pyrazole-4-carbonitrile (45g) cooled at 0 °C was slowly added concentrated H₂SO₄ (261 mL). The mixture was stirred until the starting material was completely dissolved. The reaction mixture was poured onto ice and made basic with a saturated aqueous ammonia solution. The precipitate was separated by filtration and recrystallize from methanol to give the title compound (46 g, 50%). ¹H NMR (400 MHz, DMSO-d₆) δ: 7.95 (s, 1H), 7.22 (bs, 1H), 6.75 (bs, 1H), 5.38 (bs, 2H), 4.38 (m, 1H), 1.95 (m, 2H), 1.80 (m, 2H), 1.71 (m, 2H), 1.58 (m, 2H); MS: (M⁺H m/z = 195).

(3,4-trans)-methyl 1-benzyl-4-methylpyrrolidine-3-carboxylate



To a solution of (E)-methyl but-2-enoate (1.6 g, 0.014 mol) was added toluene (30 mL), N-(methoxymethyl)(phenyl)-N-((trimethylsilyl)methyl)methanamine (3.7g, 1.2 eq) and trifluoroacetic acid (1.5g, 1 eq). The reaction mixture was heated at 50 °C for 18h. The reaction mixture was concentrated, quenched with saturated sodium bicarbonate, extracted with methylene chloride, dried with magnesium sulfate, filtered and concentrated. Purification via MPLC chromatography eluting with 20-30% ethyl acetate/hexanes provided the title compound (1.5g, 44%). 400 MHz ¹H NMR (CDCl₃) δ 7.33-7.20 (m, 5H), 4.15-4.08 (m, 1H), 3.66-3.53 (m, 2H), 2.87-2.74 (m, 2H), 2.53-2.44 (m, 2H), 2.23-2.19 (m, 1H), 1.23 (t, *J* = 7.1 Hz, 3H), 1.11 (d, *J* = 6.6 Hz, 3H); MS: (M⁺H m/z = 234)..

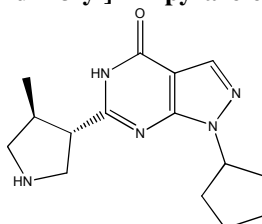
(7) 6-[(3,4-trans)-1-benzyl-4-methylpyrrolidin-3-yl]-1-cyclopentyl-15-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one



To 5-amino-1-cyclopentyl-1H-pyrazole-4-carboxamide (1.0g, 5.15mmol) and (3,4-trans)-methyl 1-benzyl-4-methylpyrrolidine-3-carboxylate (1.44g, 1.2 eq) was added a solution of potassium t-Butoxide in (1M) in THF (25mL, 5 eq.) The reaction mixture was heated at reflux for 16h and poured into saturated sodium bicarbonate. The aqueous layer was extracted with ethyl acetate, dried with magnesium sulfate, filtered and concentrated. MPLC biotage chromatography eluting with 1-4% methanol/methylene chloride with 0.5% saturated ammonium hydroxide provided the title compound as the racemate (1.67g, 86%) It was

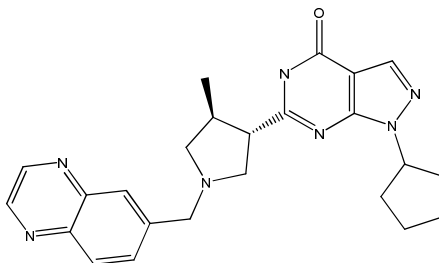
separated by the Chiral Separations Lab: Preparative conditions: Proportions: 75/25; Mobile Phase: CO₂/MeOH; Modifier: None; Column : Chiralcel OD, Dimensions : 4.6mm x 25cm ; Flow Rate: 1 mL/min, Mobile phase: 90:10 Heptane: EtOH; Retention Times: 6.81 min. Enantiomer 1; 8.12 min. Enantiomer 2; 400 MHz ¹H NMR (CD₃OD) δ 8.00 (s, 1H), 7.38-7.23 (m, 5H), 5.14-5.10 (m, 1H), 3.80-3.57 (m, 2H), 3.34 (t, *J* = 8.3 Hz, 1H), 2.97 (d, *J* = 9.9 Hz, 1H), 2.80-2.78 (m, 1H), 2.53-2.49 (m, 1H), 2.41-2.38 (m, 1H), 2.10-1.89 (m, 7H), 1.70-1.66 (m, 2H), 1.17 (d, *J* = 6.6 Hz, 3H). MS: (M⁺H m/z = 378.1).

1-cyclopentyl-6-[(3S,4S)-4-methylpyrrolidin-3-yl]-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one



A solution of 6-[(3S,4S)-1-benzyl-4-methylpyrrolidin-3-yl]-1-cyclopentyl-15-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (9.25g) in ethanol 100 mL was added to a parr bottle. 2 mL of concentrated HCl followed by 3g of palladium hydroxide was added. The reaction mixture was placed on a hydrogenator under 45 psi of H₂ gas for 4h. The reaction mixture was filtered through celite and concentrated to provide the title compound as an HCl salt (57%). 400 MHz ¹H NMR (CDCl₃) δ 8.00 (s, 1H), 5.14-5.10 (m, 1H), 4.89-3.84 (m, 1H), 3.72-3.67 (m, 1H), 3.38-3.31 (m, 1H), 3.03-2.98 (m, 1H), 2.85-2.81 (m, 1H), 2.08-1.85 (m, 7H), 1.69-1.61 (m, 2H), 1.19-1.10 (m, 3H). MS: (M⁺H m/z = 288.2).

(19)-1-cyclopentyl-6-[(3S,4S)-4-methyl-1-(quinoxalin-6-ylmethyl)pyrrolidin-3-yl]-15-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one

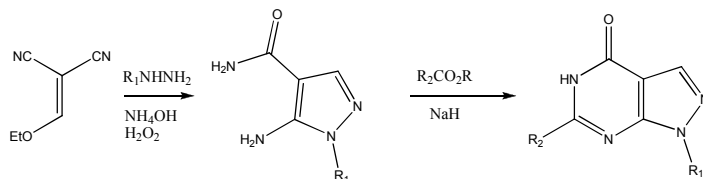


To a solution of 1-cyclopentyl-6-[(3,4-trans)-4-methylpyrrolidin-3-yl]-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (70 mg, 2.4mmol) in 1,2-dichloroethane (12 mL) was added acetic acid (0.3g 2 eq.), quinoxaline-6-carbaldehyde (0.5g, 1.5 eq.) and sodium triacetoxy borohydride (1.0g, 2eq). The reaction mixture was heated at 40 °C for 4h, poured into saturated sodium bicarbonate, extracted with methylene chloride, dried with magnesium sulfate, filtered and concentrated. Purification via Biotage MPLC eluting with 1-4% methanol/methylene chloride/0.5% ammonium hydroxide provided the title compound (301mg, 68%). 400 MHz ¹H NMR (CDCl₃) δ 8.82 (s, 2H), 8.14 (d, *J* = 8.3 Hz, 1H), 8.07-7.93 (m, 3H), 5.15-5.08 (m, 1H), 4.00-3.87 (m, 2H), 3.37 (t, *J* = 8.7 Hz, 1H), 3.04 (d, *J* = 9.5 Hz, 1H), 2.85-2.84 (m, 1H), 2.66-2.62 (m,

1H), 2.46-2.42 (m, 1H), 2.11-1.92 (m, 7H), 1.71-1.63 (m, 2H), 1.20 (d, $J = 7.05$ Hz, 3H). MS: (M^+H m/z = 430.1).

Combinatorial Experimental Conditions:

General Parallel Procedure for Compounds 5-7.



A solution of 2-(ethoxymethylene)malononitrile (5 mmol) in EtOH (10 mL) was prepared in an 40 mL vial equipped with a septum cap. A solution of the hydrazine (5 mmol) in EtOH (10 mL) was added. If the hydrazine was the HCl salt, 10 mmol of triethylamine was added to the solution. The reaction mixture was shaken overnight at room temperature. NH_4OH (28% aqueous, 5mL) and H_2O_2 (30% aqueous, 1000 μ L) were added and the mixture was shaken at room temperature for two days. A saturated $Na_2S_2O_3$ aqueous solution (7 mL) and n-BuOH (7 mL) were added. The mixture was vortexed and centrifuged, the top layer was transferred to new vials and evaporated to dryness. EtOH (10 mL) and n-BuOH (10 mL) were added. The reaction mixture was shaken at 60°C for 1 hour, filtered and evaporated to dryness for the next step.

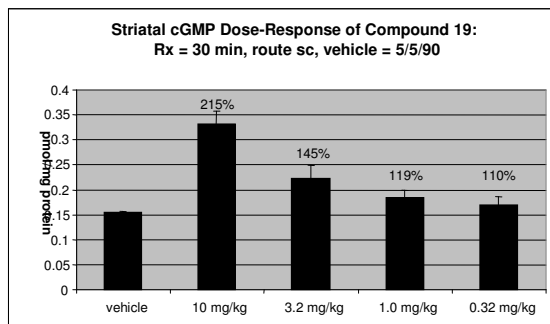
The amino-pyrazole-carboxamide obtained were split into 150 μ mol scale reaction. To amino-pyrazole-carboxamide in dioxane (500 μ L of a 0.3M solution) was added a solution of the ester in dioxane (1000 μ L of a 0.5M solution) and a suspension of NaH in dioxane (95%, 1000 μ L of a 1.5 M suspension) was added. The reaction mixture was shaken for 4 hours at room temperature. EtOH was slowly added (150 μ L) then EtOH (500 μ L) and n-BuOH (150 μ L) added. The reaction mixture was shaken at 80°C for 2 hours, cooled to room temperature, vortexed and centrifuged. The top layer was transferred to collection vials. The solvent was removed by Genevac. DMSO (1500 μ L) was added and the products were purified by HPLC.

General Parallel Procedure for the preparation of Compound 8-18

The aldehydes (0.07 mmol, 1.4 eq.) were weighed into vials. To the aldehydes was added 1-cyclopentyl-6-[(3S4S)-4-methylpyrrolidin-3-yl]-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (14.4 mg, 0.0445 mmol) as a 0.5 ml solution in dichloro ethane. To the reaction was added an acetic acid solution in dichloroethane (2 eq.). Sodium triacetoxy borohydride (21 mg, 0.1 mmol, 2 eq.) was added and the reactions shaken for 20 hours. The reaction was partitioned between 1.5 ml of 1 N sodium hydroxide and 2.5 ml of ethyl acetate. The layers were separated and the organic layer loaded onto a SCX SPE (6 ml, 1g, silicycle) column. The resin was washed with 5 ml of methanol. The compounds were eluted from the resin with 7.5 ml of N triethylamine in methanol. The solvent was evaporated and purified via HPLC to provide the library compounds in >95% purity.

Biology Experimental

Striatal cGMP levels following a dose-dependant subcutaneous administration of **19**.



The effect of **19** on striatal cGMP levels in mice. Multiple doses of PF-4181366 (**19**) or vehicle (40 % β -cyclodextrin) were administered subcutaneously to male CD-1 mice. After thirty minutes, animals were sacrificed by focused microwave irradiation, after which striatal tissue was microdissected and cGMP levels determined as previously described. Bars are means and lines are S.E.M.s (n = 4 animals/group).²

² Schmidt, C. J.; Chapin, D. S.; Cianfroga, J.; Corman, M. L.; Hajos, M.; Harms, J. F.; Hoffman, W. E.; Lebel, L. A.; McCarthy, S. A.; Nelson, F. R.; Proulx-LaFrance, C.; Majchrzak, A. D.; Ramirez, A. D.; Schmidt, K.; Seymour, P. A.; Siuciak, J. A.; Tingley III, F. d.' Williams, r. D.; Verhoest, P. R.; Menniti, F. S. Preclinical Characterization of Selective Phosphodiesterase 10A Inhibitors: A New Therapeutic Approach to the Treatment of Schizophrenia. *J. Pharm. Exp. Ther.* **2008**, 325 (2), 681-690.

Enzyme Data Experimentals (PDE9A)

PDE9 and PDE1c enzyme assay protocol

PDE9A and PDE1C were generated from full-length recombinant human clones transfected into Sf9 cells. The enzymes were extracted from cell pellets in lysis buffer (20 mM Tris, 2 mM benzamidine, 1.0 mM Na₂EDTA, 0.25 M sucrose, 100 uM PMSF, pH 7.5 at room temperature) and stored frozen at -80 °C. PDE activity was measured using a plate-based Scintillation Proximity Assay (SPA) modified from an Amersham Biosciences protocol (TRKQ7090). For competitive enzyme inhibition assays, concentration of a substrate at or below the K_m of the enzyme was utilized. The concentration of enzyme was adjusted to convert less than 10% of available substrate to end product during the assay.

Test compounds were initially dissolved in DMSO and diluted such that the final DMSO assay concentration was 3%. The drugs were added to a 384-well plate, followed by substrate. For the PDE9A assay, 50nM [3H]-cGMP was used as substrate and diluted in an assay buffer containing 50 mM Tris and

1.3 mM MgCl₂. PDE9A enzyme (in assay buffer) was added to the plate to initiate the reaction and incubation was allowed to proceed for 30 minutes at room temperature. The reaction was stopped with the addition of 10uM of a potent PDE9A inhibitor, followed by 0.2 mg/well PDE SPA beads (Amersham Biosciences; RPNQ0150).

For the PDE1C assay, 20nM [3H]-cAMP was used as substrate and diluted in an assay buffer containing 50 mM Tris, 1.3 mM MgCl₂ and 2.8mM CaCl₂. PDE1C enzyme was diluted in assay buffer containing calmodulin (approximately 100 units per well, final in assay) and added to the plate to initiate the reaction. Incubation was terminated after 30 minutes at room temperature with the addition of 0.2 mg/well PDE SPA beads (Amersham Biosciences; RPNQ0150).

Table 1. PDE9A and PDE1C IC₅₀ values, the standard error of the mean and the number of determinations (N).

Compound	PDE9A pIC ₅₀ ± SEM, M (N)	PDE9A IC ₅₀ , nM	PDE1C pIC ₅₀ ± SEM, M (N)	PDE1C IC ₅₀ , nM
2	8.68 ± 0.14 (N=3)	2.1	8.67 ± 0.06 (N=16)	2.1
6	6.81 ± 0.25 (N=3)	150	5.83 ± 0.13 (N=3)	1500
7	7.18 ± 0.23 (N=3)	66	5.67 ± 0.09 (N=3)	2100
8	7.20 ± 0.30 (N=3)	63	6.01 ± 0.18 (N=3)	970
9	7.42 ± 0.20 (N=3)	40	6.25 ± 0.12 (N=3)	563
10	6.71 ± 0.28	200	5.88 ± 0.15	1300

	(N=3)		(N=3)	
11	7.54 ± 0.37 (N=3)	29	6.57 ± 0.31 (N=3)	270
12	7.80 ± 0.21 (N=3)	16	6.71 ± 0.18 (N=3)	190
13	7.96 ± 0.27 (N=3)	11	6.39 ± 0.03 (N=3)	400
14	7.83 ± 0.40 (N=3)	15	6.68 ± 0.28 (N=3)	210
15	7.95 ± 0.34 (N=3)	11	6.71 ± 0.13 (N=3)	200
16	7.97 ± 0.24 (N=3)	11	6.96 ± 0.16 (N=3)	110
17	7.91 ± 0.27 (N=3)	12	6.66 ± 0.10 (N=3)	220
18	8.72 ± 0.30 (N=3)	1.9	7.14 ± 0.16 (N=3)	73
19	8.75 ± 0.24 (N=3)	1.8	7.30 ± 0.19 (N=3)	50

Table 2. PDE Selectivity data for compound **19**.

PDE1c	55 nM
PDE2	>1000 nM
PDE3A	>1000 nM
PDE4C	>1000 nM
PDE5	349 nM
PDE6	127 nM
PDE7B	>1000 nM
PDE8B	>1000 nM
PDE10	>1000 nM
PDE11	>1000 nM

Pharmacokinetic Experimentals

All animals were housed and studies conducted in accordance with IACUC guidelines. Male Long-Evans rats were obtained from Charles River Breeding Laboratories and housed for two days prior to treatment. The rats were not fasted overnight and allowed unrestricted access to water throughout the study.

Rats were subcutaneously (SC) administered **19** at a dose of 10.0mg/kg. The 10-mg/kg formulation was prepared as a 5.0-mg/mL solution in 5:5:90 DMSO:emulphor:saline and delivered at 2 mL/kg in the mid-back region. Blood, CSF and brain samples were harvested from each animal immediately before dosing and at 0.5, 1, 2 and 4 hours post-dose. At each sampling point, the rats (n=3) were anesthetized with carbon dioxide and whole blood was collected via cardiac puncture into vacutainer tubes (anti-coagulant: sodium heparin). Blood samples were stored on ice until centrifuged at 2060 x g for 5 min in a Beckman Allegra 6R centrifuge. The plasma was transferred to 0.5-mL polypropylene tubes and stored at –20°C until analysis. CSF was collected by puncture of the cisterna magna via a 23-gauge needle fitted with polyethylene tubing (PE50) and a 1-mL syringe. The CSF was dispensed into 0.5-mL polypropylene tubes and stored at –20°C until analysis. Whole brains were collected via decapitation and

thoroughly rinsed with saline. Each individual whole brain was then blotted dry, placed in tared polypropylene vials for weighing and stored on dry ice until transferred to a -20°C freezer.

Plasma samples (50 µL of standard, control or sample) were transferred into 1.2-mL Marsh tubes in a 96-well plate and add 100 µL acetonitrile spiked with the internal standard (0.2 µg/mL of an internal standard) to each well. The resultant supernatant was transferred to a clean 96-well block for analysis and evaporated to dryness under a stream of nitrogen at 37°C. The samples were reconstituted in 50/50 methanol/water (80 uL) for LC-MS/MS analysis.

The reverse-phase HPLC system consisted of a Phenomenex Primesphere C18-HC column (2.0 x 30 mm, 5-µ particle size) and a discontinuous elution gradient. The mobile phase solvents were: solvent A = 0.1% formic acid in 20 mM ammonium acetate and solvent B = acetonitrile. During the first half-minute of the chromatographic run, the mobile phase solvents were held at a constant ratio (85:15 A:B) that was followed by a 1.3-minute linear gradient to a mobile phase solvent ratio of 20:80 (A:B). This condition was held for 0.2 minutes followed by an immediate return to the starting conditions which was maintained for an additional one-minute period of re-equilibration. A flow rate of 0.6 mL/min was employed in this study and the effluent was monitored by mass spectrometric detection on a Micromass Quattro Premier instrument fitted with an electrospray ionization Z-spray interface that was operated in a positive ion mode. Drug and internal standard were analyzed in an MRM mode using the transitions 430.4→143.2 and 378.4→191.1 for **19** and internal standard, respectively.

From a ratio of the peak area responses of either **19** relative to the internal standard, a standard curve was constructed using a linear least squares regression with either a 1/X or 1/X² weighting for the plasma, CSF and brain homogenate analysis. The lower (LLOQ) and upper (ULOQ) limits of quantification were 0.6 and 1000 ng/mL, respectively. Quality controls were prepared in the low (0.7-7.0 ng/mL), medium (70 ng/mL) and high (700 ng/mL) regions of the dynamic range of the standard curve and at least 75% of the QC samples were within 20% of nominal concentrations.