

Supporting Information

Design and Discovery of 6-[(3S,4S)-4-methyl-1-(pyrimidin-2-ylmethyl)pyrrolidin-3-yl]-1-(tetrahydro-2H-pyran-4-yl)-15-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (PF-04447943) a Selective Brain Penetrant PDE9 Inhibitor for the Treatment of Cognitive Disorders

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Crystal Structure Experimental Conditions:

Human PDE9 catalytic domain expression, purification and soaking with compound **20** (PDB 4E90) 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.

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 $MgCl_2$. 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 $MgCl_2$ and 2.8mM $CaCl_2$. 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).

#	PDE9-Mean (nM)	pKi	STD error	PDE1c- Mean (nM)	pKi	STD error	n
3	0.99	-9.00	0.13	14	-7.96	0.09	4
8	4.9	-8.31	0.12	>1000	<-6.0		4
9	152	-6.82	0.12	5601	-5.36	0.07	4
10	1.7	-8.77	0.22	431	-6.45	0.21	4
11	89	-7.05	0.11	3421	-5.60	0.10	4
12	4.6	-8.34	0.15	889	-5.73	0.15	4
13	1.2	-8.92	0.13	639	-6.21	0.07	4
14	0.94	-9.03	0.16	165	-7.05	0.12	4

15	3.4	-8.47	0.13	466	-6.45	0.11	4
16	6.3	-8.20	0.17	1858	-6.08	0.13	4
17	3.8	-8.42	0.19	979	-6.38	0.16	4
18	12	-7.94	0.15	1603	-5.81	0.12	4
19	4.6	-8.34	0.12	1872	-6.03	0.12	4
20	8.3	-8.08	0.09	1394	-5.90	0.10	4

Table 2. PDE Selectivity data for compound **20**.

PDE1c	1394 nM
PDE2	>10000 nM
PDE3A	>10000 nM
PDE4C	>10000 nM
PDE5	>10000 nM
PDE6	>10000 nM
PDE7B	>10000 nM
PDE8B	>10000 nM
PDE10	>10000 nM
PDE11	>10000 nM

Pharmacokinetic Experimentals. All animals were housed and procedures conducted in accordance with IACUC guidelines. Compound **20** was tested in rats and dogs to assess pharmacokinetic (PK) parameters

including maximum concentration (C_{max}), area under the plasma concentration-time curve (AUC), clearance (CL), steady state volume of distribution (V_{ss}), half life (t_{1/2}), and bioavailability (F).

Jugular vein-cannulated male Sprague-Dawley rats were obtained and housed at least two days prior to treatment. Rats were administered **20** as either an intravenous (IV) bolus or oral (PO) gavage dose at 1 mg/kg. Male and female dogs were housed in metabolism cages during the conduct of the studies and were administered **20** as an intravenous bolus dose at 0.3 mg/kg and oral dose at 1 mg/kg. Both rats and dogs were fasted overnight, allowed unrestricted access to water prior to and throughout the study, and fed four hours post-dose. For all PK studies, the IV formulation was prepared in 5/95 DMSO/saline whereas the PO formulation was prepared in 0.5% methylcellulose. Pharmacokinetic parameters were determined from individual animal data using non-compartmental analysis in Watson Bioanalytical LIMS (version 7.2.0.03). Concentrations below the limit of quantification (BLQ) were recorded as ignored for use in calculations.

In the IV and PO PK studies, blood samples [0.4 mL – rats, 1 mL – dog] were harvested from the jugular vein immediately before dosing and at selected intervals post-dose. Whole blood samples were placed in Microtainer and Vacutainer tubes containing heparin for rat and dog, respectively, and stored on ice until centrifuged at 2040 x g for 5 min in an Eppendorf microfuge 5415C. The plasma was transferred to 0.5-mL polypropylene tubes and stored frozen at –80°C until analysis.

Neuropharmacokinetics Experimentals. Male Sprague-Dawley rats and FVB/N mice were administered compound **20** as a subcutaneous bolus at 1 mg/kg followed by euthanasia by carbon dioxide asphyxiation at a single time point for rats (0.5 h post-dose) and multiple time points for mice (0.5 and 2 h post-dose). Whole blood was collected via cardiac puncture and placed into Vacutainer and Microtainer tubes containing heparin for rats and mice, respectively, and stored on ice until centrifuged for the preparation of plasma. Cerebrospinal fluid (CSF) was collected from the rats only by puncture of the cistern magna using a 23-gauge needle fitted with polyethylene tubing (PE50) and a 1 mL syringe. Whole brains were collected by decapitation, rinsed in phosphate-buffered saline, and weighed. Both CSF and whole brains were immediately frozen on dry ice upon collection. All samples were stored frozen at –80°C until analysis.

Male and female Beagle dogs were administered compound **20** by oral gavage at 1 mg/kg followed by collection of both blood and CSF samples in a time course manner. Whole blood was collected from the jugular vein into Vacutainer tubes containing heparin and stored on ice until centrifugation for plasma preparation. The CSF was collected from the cistern magna via an IVAP port and frozen immediately on dry ice following collection. All samples were stored frozen at –80°C until analysis.

Analysis of Compound 16 in Biological Matrices (plasma, brain, CSF, buffer). Concentrations of **20** in various matrices (plasma, brain, CSF) obtained from preclinical species were determined via LC-MS/MS.

Brains were homogenized in 4 volumes of water and homogenates were processed and analyzed in a manner similar to other matrices. Aliquots of samples (50 μ L volume of standard, control, or sample) were treated with the addition 20 μ L of internal standard and 0.7 mL methyl tert-butyl ether (MTBE), shaken vigorously, and then centrifuged ($1640 \times g$) for 5 minutes. The resultant supernatant was transferred and evaporated under the flow of nitrogen at 37° Celsius. The samples were reconstituted in 50/50 methanol/water and directly injected onto the LC-MS/MS system.

The reverse-phase HPLC system consisted of a Phenomenex Luna C18(2) 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 and 0.1% isopropanol 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 (95:5 A:B) that was followed by a 1.3 minute linear gradient to a mobile phase solvent ratio of 20:80 (A:B). The intermediate condition was held for 0.2 minute followed by an immediate return to the starting conditions. The starting conditions were then maintained for a one-minute period of re-equilibration. Retention times for **16** and the internal standard were approximately 1.1 and 1.4 minutes, respectively. 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 Ultima 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 transition of 396.3 \rightarrow 203.2 for compound **20**.

From a ratio of the peak area responses of **20** relative to the internal standard, a standard curve was constructed using a linear least squares regression with a 1/X² weighting. The lower (BLQ) and upper (ALQ) limits of quantification were 0.6 and 1000 or 1500 ng/mL, respectively. Quality controls were prepared in the low, medium, and high regions of the dynamic range of the standard curve and were within 20% of nominal concentrations.

Plasma Protein Binding. The binding of compound **20** to plasma proteins and non-specific binding to brain tissue was determined by the 96-well equilibrium dialysis technique using cellulose membranes (HTdialysis, Gales Ferry, CT) with a molecular weight cutoff of 12-14 kDa. Rat brain tissue was diluted with two volumes of phosphate buffered saline and homogenized via probe sonication. For protein binding, plasma was fortified with compound **20** at a concentration of 100 and 1000 ng/mL and 1/3 brain homogenate was fortified at a concentration of 1000 ng/g. The fortified matrix samples (150 μ L; donor) were subjected to equilibrium dialysis (n = 3) against an equal volume of phosphate buffered saline (pH 7.4; receiver) at 37 ° Celsius with gentle shaking in a 95/5 O₂/CO₂ incubator for approximately 6 h.

Following equilibration, an aliquot of the plasma, brain, and buffer samples were removed from each equilibration cell and stored frozen at -80°C until analysis via LC-MS/MS (methods described above). The fraction unbound of compound **20** in plasma was determined by the following equation:

$$f_u = \left[\frac{C_{receiver}}{C_{donor}} \right]$$

Where C_{receiver} and C_{donor} are the concentrations measured in the buffer and plasma samples, respectively. The unbound fraction in undiluted brain was calculated via the below equation where D and $f_{u,\text{meas}}$ represent the fold dilution of brain tissue and the associated free fraction determined as the ratio of concentrations in buffer versus diluted brain tissue.

$$\text{Undiluted } f_u = \left[\frac{1/D}{((1/f_{u,\text{meas}}) - 1) + 1/D} \right]$$

Microsomal Stability Studies. The in vitro stability of compound **20** in liver microsomes from human, rat, and dog was determined using the well-stirred model described previously by Obach et al 1997. Compound **20** was examined in duplicate following incubation with microsomes (substrate concentration equal to 0.1 μM ; cytochrome P450 concentration equal to 0.25 μM) in 0.1 M potassium phosphate buffer containing 3.3 mM magnesium chloride (pH 7.4) at 37° Celsius. The reaction mixture was pre-warmed at 37° Celsius for 5 min before addition of NADPH (1.0 mM). Aliquots (50 μl) of the reaction mixture at $t = 0, 2, 5, 10, 15$ and 30 min were added to acetonitrile (50 μl) containing internal standard to quench the reaction. The samples were centrifuged at 1640 x g for 5 min and the resultant supernatant was subjected to LC-MS/MS analysis as described previously. For control experiments, NADPH was omitted from these incubations. The microsomal half-life ($t_{1/2}$) obtained as a slope of substrate depletion was scaled to calculate the intrinsic clearance.