Supporting Information

for

PF-06827443 displays robust allosteric agonist and positive allosteric modulator activity in high receptor reserve and native systems.

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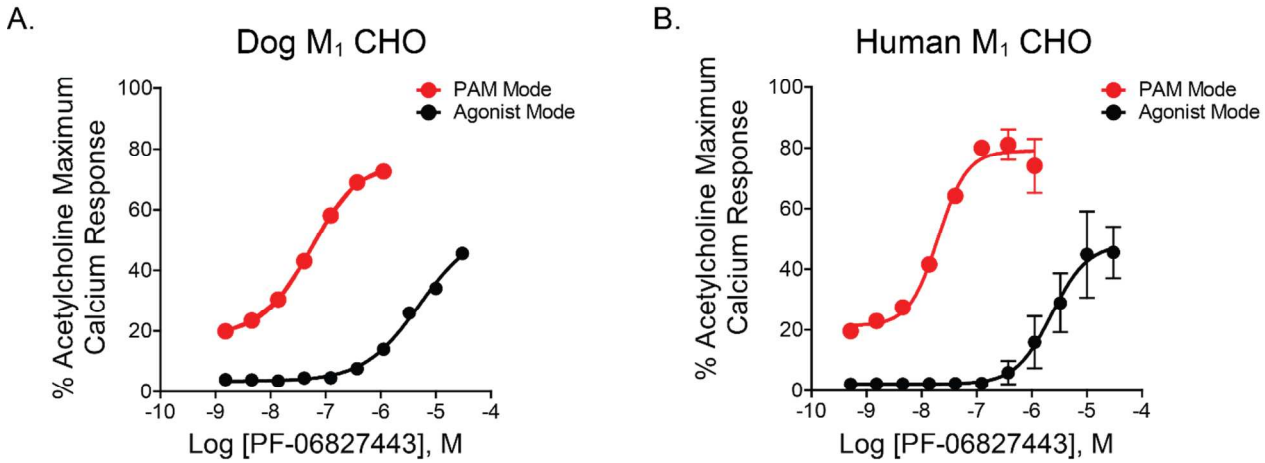
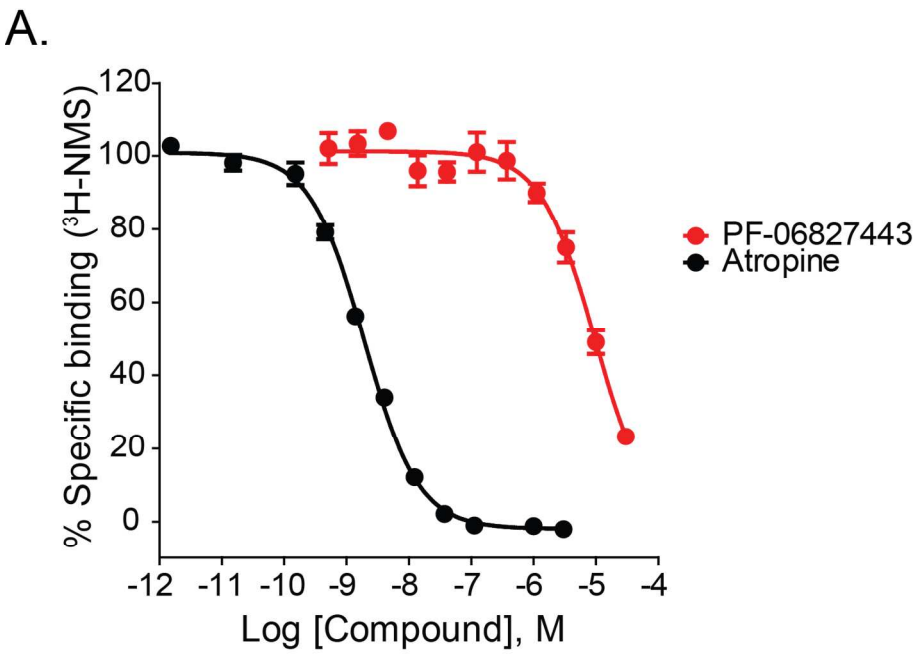
Supplemental Figure 1



Supplemental Figure 1 PF-06827443 displays intrinsic agonist activity in M_1-expressing CHO cells, independent of species. (**A**) PF-06827443 concentration-response curves (CRC) of calcium mobilization assay in CHO cells stably expressing the dog M_1 receptor in the absence of ACh (Agonist Mode; Black) and the presence of an EC_{20} of ACh (PAM Mode; Red). (**B**) Under similar conditions, concentration response curves were generated for PF-06827443 in CHO cells stably expressing the human M_1 receptor. Data represent mean ± S.E.M. from 3 independent experiments performed in triplicate.

Supplemental Figure 2



Supplemental Figure 2: PF-06827443 displaces [^{3}H]-NMS at rat M1 receptors. (A) Inhibition of orthosteric radioligand binding with [^{3}H]-NMS by PF-06827443 and atropine control. Data are plotted as a percentage of specific [^{3}H]-NMS binding. Data represent the mean ± SEM from three separate experiments performed in triplicate.

Supplemental Table 1: Receptor densities determined from saturation binding assay[a]

Rat M_1-TREx CHO	Bmax (fmol/mg) ± SEM
5 ng TET	48 ± 4
15 ng TET	139 ± 8
25 ng TET	1066 ± 27
1000 ng TET	5859 ± 188
rM1-CHO	1305 ± 208
hM1-CHO	1479 ± 129

[a] Data represents values determined from displacement of varying concentration of [^{3}H]-NMS ranging from 3nM to 0.003 nM) using the cell membranes isolated from rat M1-TREx CHO that induced with different TET amount compared to the CHO cells stably expressing rM1 and hM1 receptors. Values represent the mean ± SEM of two experiments performed in triplicate.

Supplemental Table 2: Comparison of Acetylcholine concentration to elicit EC_{20} response in each TET condition in rat M_1 TREx-CHO and rat M_1-CHO. There is a leftward shift of ACh potency wth increasing amount of TET. Incubation with 25 ng TET shows comparable ACh potency to one in rat M_1-CHO.[a]

Condition	ACh concentration (nM) (± SEM)	EC_{20} % response
5 ng TET	45.3 ± 1.3	16 ± 2
15 ng TET	19.3 ± 1.3	16 ± 1
25 ng TET	1.4 ± 0.2	15 ± 1
1 ug TET	0.2 ± 0.0	13 ± 1
rM1-CHO	1.2 ± 0.0	14 ± 1

[a] Data values determined from ACh concentration necessary to elicit a ~EC_{20} % response in the various cell lines. Values represent the mean ± SEM of three independent experiments performed in triplicate.

Supplemental Methods

Cell line and calcium mobilization assay: To determine the functional activity of M_1 PAMs at dog M_1, the dog M_1 full-length open reading frame (ORF) was amplified from the dog hippocampus cDNAs (Zyagen, San Diego, CA). The ORF was then subcloned into the EcoR I and Xho I sites of pcDNA3.1 (+) vector (Life Technologies, Carlsbad, CA). Sequencing of the plasmid confirmed the presence of dog M_1 ORF (XM_540897). CHO cells were transfected with dog M_1 expression plasmid using Fugene 6 (Promega, Madison, WI), and next day, the transfected cells were incubated with the selection medium containing 1 mg/ml G418 for 2 weeks. The resulting polyclones were used for the calcium mobilization assay described below. Similarly, rat M_1 ORF was subcloned into pcDNA5/TO TET-inducible expression vector (Life Technologies, Carlsbad, CA). The expression plasmid was transfected into TREx-CHO cells (Life Technologies, Carlsbad, CA). After 2 weeks of hygromycin-selection, the polyclones were used for generating monoclones. For calcium mobilization assay, all of the CHO cells constitutively expressing dog, human and rat M1 receptors (M_1-CHO) were plated in black-walled, clear-bottomed 384 well plates (Greiner Bio-One, Monroe, NC) the day before assay. Tetracycline-inducible rat M_1-TREx-CHO cells were plated in the presence of varying amounts of TET (5, 15, 25, 1000 ng/mL). The next day, cells were washed with assay buffer (Hank's balanced salt solution, 20 mM HEPES, 4.16 mM sodium bicarbonate, and 2.5 mM probenecid) and immediately incubated with 20 µL of 1.15 µM Fluo-4-acetomethoxyester (Fluo-4 AM) dye solution prepared in assay buffer for 45 min at 37 °C. M_1 PAMs were serial diluted (1:3) in DMSO for 10-point concentration−response curves (CRC), and further diluted in assay buffer at starting final concentration 30µM using Echo liquid handler (Labcyte, Sunnyvale, CA). To determine the ACh concentration for EC_{20} response, ACh CRC was performed in each TET-condition in rat M_1-TREx-CHO, and rat M_1-CHO cells. After removing dye, cells were washed with assay buffer. Immediately, calcium flux was measured using the Functional Drug Screening System (FDSS7000, Hamamatsu, Japan). The

serially diluted compounds or DMSO vehicle were added to cells for 2.5 min and then an EC_{20} concentration of acetylcholine (ACh) was added and incubated for 2 min. EC_{max} concentration was also added to cells that were incubated with DMSO vehicle to ensure the EC_{20} calcium response. To determine the potency and efficacy of the agonist and PAM, data were analyzed to generate a concentration-response curve using a four-point logistical equation in GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

Extracellular field electrophysiology: 6-10 week old male C57BL6/J mice (Jackson Laboratories) were anesthetized using a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively, intraperitoneal injection), transcardially perfused with ice-cold cutting solution (in mM: 230 sucrose, 2.5 KCl, 8 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 10 D-glucose, 26 $NaHCO_3$), then the brains were removed and submerged in ice-cold cutting solution. Coronal slices containing the prelimbic prefrontal cortex were cut at 400 µm and were transferred to a holding chamber containing NMDG-HEPES recovery solution (in mM: 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 D-glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 $MgSO_4$, 0.5 $CaCl_2$, 12 N-acetyl-L-cysteine, pH 7.3, <310 mOsm) for 8-10 minutes at 32 ºC. Slices were then transferred to a room temperature holding chamber for at least 1.5 hours containing ACSF (in mM: 126 NaCl, 1.25 NaH_2PO_4, 2.5 KCl, 10 D-glucose, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$) supplemented with 600 µM sodium ascorbate for slice viability. All buffers were continuously bubbled at room temperature with 95% O_2/5% CO_2. Subsequently, slices were transferred to a 30-32 ºC submersion recording chamber (Warner Instruments) and perfused with ACSF at a rate of 2 mL/min. Recording pipettes were constructed from thin-walled borosilicate capillary glass tubing (I.D. 1.17 mm, O.D. 1.50 mm; Warner Instruments, Hamden, CT), pulled with a horizontal pipette puller (P-97 Sutter Instrument Co., Novata, CA) to a resistance of 1-3MΩ when filled with ACSF. Field excitatory postsynaptic potentials (fEPSPs) were recorded from layer V of the prelimbic cortex and evoked electrically by a concentric bipolar stimulating electrode (200 µs duration, 0.05 Hz; inter-pulse interval of

50 ms) in the superficial layers II/III. Layer II/III was visualized using a Olympus BX50WI upright microscope (Olympus, Lake Success, NY) microscope according to landmarks illustrated in the Allen mouse brain atlas and the recording electrode was laterally placed approximately 200µm away from layer II/III in layer V so that the recording and stimulating electrodes were along a line perpendicular to the midline. Signals were amplified by a Multiclamp 700B, were filtered at 0.5kHz and sampled at a rate of 20,000 kHz with a Digidata 1322A, and data was collected and analyzed using pClamp 9.2, Clampex 10.6.2, and Clampfit 10.2 software (Molecular Devices) running on a Dell PC (Round Rock, TX). PF-06827443 was prepared in DMSO (<0.1% final) and subsequently diluted into ACSF to the appropriate concentration and applied to the bath for 20 minutes using a peristaltic pump perfusion system.

Radioligand binding assay: Competition binding assays were performed using [^{3}H]-N-methylscopolamine ([^{3}H-]NMS, Perkin Elmer. Boston, MA) as previously described (Add Rook et al 2017). Briefly, compounds were serial diluted 1:3 in DMSO for an 11 point CRC, then further diluted for a final top concentration of 30 µM in binding buffer (20 mM HEPES, 10 mM MgCl2, and 100 mM NaCl, pH 7.4). Membranes from rat M_1-CHO cells (10 µg) were incubated with the serially diluted compounds in the presence of a K_d concentration of [^{3}H-]NMS, 0.088 nM, at room temperature for 3 hr with constant shaking. Nonspecific binding was determined in the presence of 10 µM atropine. Similarly, saturation binding was performed using varying concentration of [^{3}H-]NMS, 3nM to 0.003 nM. Binding was terminated by rapid filtration through GF/B Unifilter plates (PerkinElmer) using a Brandel 96-well plate Harvester (Brandel Inc., Gaithersburg, MD), followed by three washes with ice-cold harvesting buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl). Plates were air-dried overnight, 50 µl of Microscint20 added to the plate, and radioactivity was counted using a TopCount Scintillation Counter (PerkinElmer Life and Analytical Sciences).