Supporting Information for:

Discovery of VU0467485: An M_4 PAM Evaluated as a Preclinical Candidate for the Treatment of Schizophrenia

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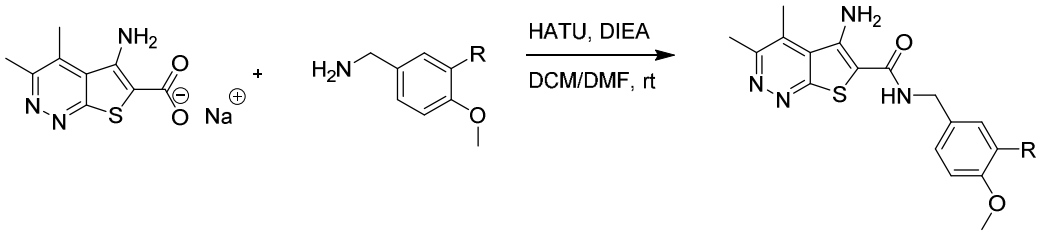
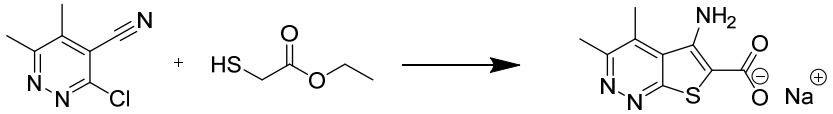
<u>Experimental Section</u>

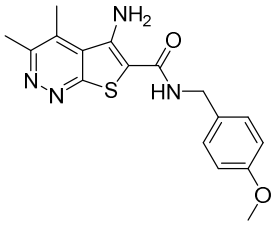
All reactions were carried out employing standard chemical techniques under inert atmosphere. Solvents used for extraction, washing, and chromatography were HPLC grade. Unless otherwise noted, all reagents were purchased from Aldrich Chemical Co. and were used without further purification. Analytical thin layer chromatography was performed on 250 μm silica gel plates from Sorbent Technologies. Analytical HPLC was performed on an Agilent 1200 LCMS 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 (MeOD = 3.31, $CDCl_3$ = 7.26). Data are reported as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet), coupling constant (Hz), and integration. ^{13}C chemical shifts are reported as δ values in ppm relative to the residual solvent peak (MeOD = 49.0, $CDCl_3$ = 77.16). Low resolution mass spectra were obtained on an Agilent 1200 LCMS with electrospray ionization. High resolution mass spectra were recorded on a Waters QToF-API-US plus Acquity system with electrospray ionization. Automated flash column chromatography was performed on a Teledyne ISCO Combiflash Rf system. Preparative purification of library compounds was performed on a Gilson 215 preparative LC system.



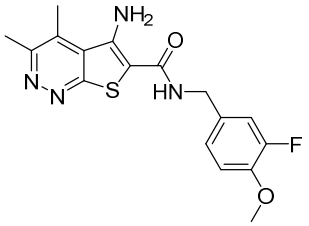
General Procedure for Library Synthesis. Compounds **6** were synthesized in parallel according to the following procedure. Sodium carboxylate **5** (0.34 mmol) was dissolved in DMF and *N,N*-diisopropylethylamine (0.5 mmol) added. Then, the appropriate benzyl amine was added at rt and allowed to stir for 2 hours. Next, the reactions were partitioned into $CHCl_2$ and H_2O and then passed through disposable phase-separator columns (Biotage Isolute). The organics were concentrated on a heated air-drying block and then analyzed by LCMS. Purification by mass-directed Preparative HPLC afforded desired products **6** as solids and lightly colored (55-97%) with >98% purity by ELSD and 214 nm/254 nm UV analysis.



5-amino-3,4-dimethylthieno[2,3-c]pyridazine-6-carboxylate (5): This compound was synthesized as previously described: *ACS Chem. Neurosci*. **2014**, *5*, 920-941. LCMS: Rt=0.320 min, M+H=224.0; >98% @ 215 and 254 nm; ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.92 (br s, 2H), 2.73 (s, 3H), 2.71 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.8, 160.9, 154.9, 148.0, 133.5, 127.0, 20.2, 15.1.



5-amino-*N*-(4-methoxybenzyl)-3,4-dimethylthieno[2,3-*c*]pyridazine-6-carboxamide (6a, VU0464090): A solution of 5-amino-3,4-dimethylthieno[2,3-*c*]pyridazine-6-carboxylate **5** (1.0 eq), HATU (1.0 eq), and *N,N*- diisopropylethylamine (3.0 eq) in DMF (0.4M) at rt was stirred for 5 min. To the reaction mixture was added (4-methoxyphenyl)methanamine (1.1 eq). After LCMS confirmed desired product, the reaction mixture was diluted with 1N NaOH (aq). After 1h of vigorous stirring, the precipitate was filtered and dried in the 50 °C oven overnight. The product was collected (94% yield). LCMS: Rt=0.913 min, M+H=342.8; >98% @ 215 and 254 nm; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.63 (t, *J* = 5.6 Hz, 1H), 7.26 (d, *J* = 8.4 Hz, 2H), 6.90 (d, *J* = 8.3 Hz, 2H), 4.37 (d, J = 5.9 Hz, 2H), 3.73 (s, 3H), 2.73 (s, 3H), 2.72 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.0, 160.2, 158.7, 154.8, 146.6, 133.7, 132.0, 129.2, 128.0, 114.2, 103.7, 55.5, 42.4, 20.0, 15.1; HRMS (ESI): calc'd for $C_{17}H_{18}N_4O_2S$ [M]; 342.1150. Found: 342.1152.



5-amino-*N*-(3-fluoro-4-methoxybenzyl)-3,4-dimethylthieno[2,3-*c*]pyridazine-6-carboxamide (6c, VU0467485): A solution of 5-amino-3,4-dimethylthieno[2,3-*c*]pyridazine-6-carboxylate **5** (1.0 eq), HATU (1.2 eq), and *N,N*-diisopropylethylamine (3.0 eq) in DCM:DMF (7:1; 0.2M) at rt was stirred for 5 min. To the reaction mixture was added (4-methoxyphenyl)methanamine (1.1 eq). After LCMS confirms desired product, the reaction mixture was diluted with 2N NaOH (aq) and extracted with 1:1 [3:1 ($CHCl_3$/IPA)/ DCM] (3x). The combined organic extractions were washed with water (3x), dried ($MgSO_4$), and concentrated *in vacuo*. The solid residue was sonicated in IPA for 5 min and filtered. The solid was washed with cold IPA and dried. The product was collected as a brown solid (66% yield). LCMS: Rt=0.935 min, M+H=360.8; >98% @ 215 and 254 nm; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.64 (t, *J* = 5.8 Hz, 1H), 7.27-7.01 (m, 5H), 4.36 (d, J = 5.8 Hz, 2H), 3.81 (s, 3H), 2.73 (s, 3H), 2.71 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.9, 160.2, 154.3, 151.7 (d, *J*(C,F) = 242.0 Hz), 146.6, 146.4 (d, *J*(C,F) = 10.6 Hz), 135.5, 132.9 (d, *J*(C,F) = 5.6 Hz), 129.0, 124.0 (d, *J*(C,F) = 3.3 Hz), 115.5 (d, *J*(C,F) = 18.1 Hz), 114.2 (d, *J*(C,F) = 1.8 Hz), 104.5, 56.5, 42.1, 19.3, 15.2; HRMS (ESI): calc'd for $C_{17}H_{17}FN_4O_2S$ [M]; 360.1056. Found: 360.1057.

In Vitro Pharmacology Methods

Calcium Mobilization Assays. Compound-evoked increases in intracellular calcium were measured using Chinese hamster ovary (CHO) cells stably expressing rat, human, or cynomolgus monkey muscarinic receptors (M_1−M_5; M_2 and M_4 cells were cotransfected with G_{qi5}). Cells were plated in 384-well, black-walled, clear-bottomed plates in 20 μL of assay medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed fetal bovine serum, 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid (HEPES), and 1 mM sodium pyruvate) at a density of 15000 cells/well and grown overnight at 37 °C/5% CO_2. The next day, medium was removed, and the cells were incubated with 20 μL/well of 1 μM Fluo-4AM (Invitrogen, Carlsbad, CA) prepared as a 2.3 mM stock in dimethyl sulfoxide (DMSO), mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127, and diluted in calcium assay buffer (Hank's balanced salt solution [HBSS], Invitrogen, Carlsbad, CA) supplemented with 20 mM HEPES and 2.5 mM probenecid, pH 7.4, for 50 min at 37 °C. Dye was removed and replaced with 20 μL/well of assay buffer. For PAM potency curves, M_4 compounds were diluted in calcium assay buffer and added to the cells followed by the addition of an EC_{20} concentration of ACh 140 s later and then an EC_{80} concentration of ACh 120 s later. For fold shift experiments, multiple fixed concentrations (50 nM to 30 μM) of M_4 compound or vehicle was added followed by the addition of a concentration−response curve of ACh 140 s later. Calcium flux was measured over time as an increase in fluorescence using a functional drug screening system 6000 (FDSS 6000, Hamamatsu, Japan). The change in relative fluorescence over basal was calculated before 936 dx. normalization to the maximal response to ACh. As described previously, shifts of ACh concentration−response curves by the M_4 modulators were globally fitted to an operational model of allosterism.21 Data (means ± SEM, n = 3) were analyzed using GraphPad Prism V.5.04 (GraphPad Software, San Diego, CA). Previously described in detail in: *ACS Chem. Neurosci*. **2014**, *5*, 920-941.

In Vivo Pharmacology Methods

Locomotor Activity Studies in Rats. Open field activity was tested using a SmartFrame Open Field System (Kinder Scientific, San Diego, CA) with a 16 × 16 array of infrared photobeams located 2.5 cm above the floor of the chamber as previously described.9,18 To establish the dose−response relationship for amphetamine on locomotor activity, rats were habituated for 60 min in the open field and then injected with vehicle (sterile water SC) or dose of amphetamine (0.75 mg/kg, SC), and locomotor activity was recorded for an additional 60 min. To determine the effects of VU0467485 (**6c**) on reversing amphetamine-induced hyperlocomotion, rats were habituated in the open field for 30 min, followed by administration of vehicle (10% (v/v) Tween 80 in sterile water) or a dose of VU0467485 (**6c**) (1−10 mg/kg PO). After an additional 30 min, vehicle (sterile water) or amphetamine (0.75 mg/kg SC) was injected, and locomotor activity was recorded for another 60 min. The time course of drug-induced changes in ambulation is expressed as mean number of beam breaks per 5 min bin over the 120 min session. Total locomotor activity was calculated as the total number of beam breaks from the time of amphetamine administration [t = 60 min] to the end of the experiment [t = 120 min]. Total activity and

time course data (means ± SEM) were analyzed by one-way and two-way ANOVA, respectively, and post hoc comparisons were made by Dunnett's test using GraphPad Prism (La Jolla, CA).

In Vitro DMPK Methods

PPB, BHB, Hepatic Microsomal CL_{int}, and CYP_{450} Inhibition Assays. Plasma protein binding (PPB), brain homogenate binding (BHB), hepatic microsomal intrinsic clearance (CL_{int}), and CYP_{450} inhibition assays were performed as described previously [J. Med. Chem. 56: 5208-5212; Drug. Metab. Dispos. 41: 1703-1714; ACS Chem. Neurosci. 7: 1192-1200].

CYP_{450} Induction Assay. Evaluation of the CYP induction potential of compound **6c** (VU0467485) was performed using an activity-based assay design with CYP-specific probe substrates and a 5-day induction protocol in human hepatocytes. A starter kit for the assessment of CYP induction in cryoplateable human hepatocytes was purchased from Celsis In Vitro Technologies (IVT), now Bioreclamation IVT (Baltimore, MD), that included plating and induction media, assay buffer, and cryostored hepatocytes from a single female donor. All other reagents and materials were commercially available. On day 1, cells were rapidly thawed and viability determined by trypan blue exclusion. Cells were plated in 96-well format at a concentration of 5 x 10^4 cells/well, incubated for 4 hr (37 ^oC, 5 % CO_2), with subsequent media change and overnight incubation. On days 3 and 4, CYP-specific inducers (positive controls) were added to the cells over a pre-determined concentration range (n =3) as follows: omeprazole, (CYP 1A2), rifampicin, (CYP 3A4), and an internal compound, (CYP 2B6). Test compound was added in similar fashion over a range of concentrations (0.3 – 100 μM). On day 5, changes in CYP activity indicative of induction were assessed by incubating CYP-specific probe substrates (phenacetin, CYP 1A2; midazolam, CYP 3A4; bupropion, CYP 2B6) for 1 hr, followed by termination with ice-cold acetonitrile. The plates were then centrifuged at 3000 rcf for 15 min and the resultant supernatant was removed and diluted 1 x 4 in water for LC-MS/MS analysis on an AB Sciex API-4000 triple-quadrupole instrument. Mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each CYP-specific metabolite (acetaminophen, CYP 1A2; 1-hydroxymidazolam, CYP 3A4; hydroxybupropion, CYP 2B6). Control- and compound-dependent changes in metabolite formation were quantitated against a 12-point standard curve and induction potential was reported both as an E_{max} (fold-induction) and EC_{50} value (μM).

Metabolite ID. Metabolite identification experiments were conducted using hepatic S9 fractions from multiple species (rat [Sprague Dawley], dog [beagle], nonhuman primate [cynomolgus]; male, pooled; BD Biosciences, San Jose, CA) and human hepatic microsomes (mixed gender, pooled, BD Biosciences, San Jose, CA) according to the following procedure. Test compound (**6c**) was incubated (25 μM) in a solution of potassium phosphate-buffer (0.1 M, pH 7.4) containing hepatic S9 (5 mg/mL) or hepatic microsomes (1 mg/mL) and relevant cofactors (NADPH, 2 mM in all incubations; UDGPA and PAPs, 2 mM in S9 incubations) at 37 ºC in a borosilicate glass vessels under ambient atmosphere for approximately 1 hour. Protein was then precipitated by addition of cold acetonitrile (2X volumes) and centrifugation (3000 rcf) for 10 minutes. Resulting supernatants were dried under a stream of nitrogen gas before reconstitution in an 85:15 (v/v) mixture of aqueous ammonium formate (10 mM, pH 4.1) and

acetonitrile. Samples were then analyzed via LC-UV/MS^n essentially as described previously [Drug. Metab. Dispos. 41: 1703-1714].

Reactive Metabolism/Bioactivation Assay. Potential for metabolic bioactivation of compound **6c** was investigated using human hepatic microsomes via a procedure identical to that used for metabolite identification experiments, except the incubations were additionally fortified with glutathione (3 mM), and LC-UV/MS^n analysis was performed to identify potential conjugates.

CYP_{450} Reaction Phenotyping and Mapping. In order to determine the extent of involvement of various CYPs to the metabolism of **6c** and to estimate the relative contributions of these CYPs to the formation of its specific metabolites, *in vitro* incubations were carried out using commercially available human EasyCYP bactosomes (Cypex, available through Xenotech, Lenexa, KS). EasyCYP bactosomes express human CYPs and reductase (with or without cytochrome b_5) at standardized CYP concentrations and total protein concentrations. Briefly, the test compound was pre-incubated for 5 min (final concentration 10-25 μM) in 100 mM potassium phosphate buffer (pH 7.4) containing 125 pmol of CYP enzyme at 37 ^oC, prior to initiation of the reaction with NADPH (1 mM). Control bactosomes that do not express any CYP enzymes are also simultaneously incubated with the test compound and either buffer or NADPH was then added after the 5 min pre-incubation time. The reactions were allowed to proceed for 1 hr, followed by termination with ice-cold acetonitrile (2X volumes) and centrifugation at 3000 rcf, 4 ^oC, for 10 min. The resulting supernatant was removed and transferred to a separate tube and dried under nitrogen gas prior to reconstitution for LC-UV/MS^n analysis essentially as described previously [Drug. Metab. Dispos. 41: 1703-1714]. Resulting data were then used to map individual CYPs to the formation of each metabolite and, in conjunction with the *in vitro* CL_{int} data (from human hepatic microsomes), the fractional (%) contribution of each CYP to the total CYP-mediated metabolism of **6c** was determined via an intersystem extrapolation factor (ISEF)-based approach [Drug Metab. Dispos. 39: 373-382].

Caco-2 and MDCK-MDR1 Permeability/Efflux Assays. Monodirectional (A→B) permeability of compound **6c** was determined in Caco-2 cells, and bidirectional permeability was determined in MDCK-MDR1 (human P-gp) cells via Absorption Systems (Exton, PA) using their standard transwell assay procedures and a 5 μM concentration of test compound.

In Vivo DMPK Methods

Animal Care and Use. All animal procedures were approved by the respective Institutional Animal Care and Use Committees and conducted in accordance with the National Institutes of health *Principles of Laboratory Animal Care* and/or the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

Rat Brain Distribution. Distribution of compound **6c** from systemic plasma to brain tissue was determined 1.5 hours following a single oral administration (1, 3, or 10 mg/kg; vehicle: 10% [v/v] tween80 in water, 10 mL/kg dose volume) of the mono-HCl salt to male, Sprague Dawley rats (n = 7-8 per dose level; fasted). Sample preparation and LC-MS/MS bioanalysis was performed as described previously [J. Med. Chem. 56: 5208-5212; Drug. Metab. Dispos. 41: 1703-1714; ACS Chem. Neurosci. 7: 1192-1200]. The observed total brain:plasma distribution partition coefficients (K_p) were similar

across each of the dose groups, and thus K_p values from all animals/groups were averaged (arithmetic mean). The mean unbound brain:unbound plasma distribution partition coefficient ($K_{p,uu}$) was calculated by division of the mean K_p by [rat fu_{plasma} / rat fu_{brain}] derived from the in vitro PPB and BHB assays.

Dog Brain Distribution. Distribution of compound **6c** from systemic plasma to brain tissue was determined 2.0 hours following a single oral administration (3 mg/kg; vehicle: 30% [w/v] hydroxy-propyl-β-cyclodextrin in water, 5 mL/kg dose volume; fasted) of the mono-HCl salt to a female, Mongrel dog (n = 1), which was previously/independently scheduled for euthanasia. Sample preparation and LC-MS/MS bioanalysis was performed essentially as described previously [J. Med. Chem. 56: 5208-5212; Drug. Metab. Dispos. 41: 1703-1714; ACS Chem. Neurosci. 7: 1192-1200], except the brain sample was subjected to manual sectioning and homogenization by a sonication probe. $K_{p,uu}$ was calculated by division of the K_p by [dog fu_{plasma} / dog fu_{brain}] derived from in vitro PPB and BHB data.

Rat Pharmacokinetics. The plasma pharmacokinetics of compound **6c** was determined in male, Sprague Dawley rat following a single IV administration (1 mg/kg; vehicle: 10% EtOH 60% PEG400 30% saline [v/v/v], 1 mL/kg dose volume; n = 1) of the freebase, and separately, following a single PO administration (3 mg/kg; vehicle: 0.1% [v/v] Tween80 0.5% methylcellulose in water, 10 mL/kg dose volume; n = 2; fasted) of the mono-HCl salt using procedures for sample collection, sample preparation, and LC-MS/MS bioanalysis described previously [J. Med. Chem. 56: 5208-5212; Drug. Metab. Dispos. 41: 1703-1714; ACS Chem. Neurosci. 7: 1192-1200]. Raw time-concentration data from individual animals were subjected to noncompartmental analysis (NCA) via Phoenix® WinNonlin® (v.5.3; Certara USA Inc., Princeton, NJ), and in the case of the oral study, resulting PK parameters from each animal were averaged.

Dog Pharmacokinetics. The plasma pharmacokinetics of compound **6c** was determined in male, Beagle dog (n = 2) following a single IV administration (1 mg/kg; vehicle: 10% EtOH 60% PEG400 30% saline [v/v/v], 1 mL/kg dose volume), and separately, following a single PO administration (3 mg/kg; vehicle: 0.1% [v/v] Tween80 0.5% [v/v] in water, 5 mL/kg dose volume; fasted) of the mono-HCl salt via a crossover design (i.e., animals were dosed IV and then PO following a washout period). The in-life phases, sample preparation, LC-MS/MS bioanalysis, and subsequent NCA PK analysis of individual animal raw time-concentration data were performed via Frontage Laboratories (Exton, PA) in accordance with their established Standard Operating Procedures (SOPs) and IACUC protocols.

Nonhuman Primate Pharmacokinetics. The plasma pharmacokinetics of compound **6c** was determined in male, cynomolgus monkey (n = 1) following a single IV administration (0.2 mg/kg, vehicle: 10% EtOH 50% PEG400 40% saline [v/v/v], 1 mL/kg dose volume) of the free base. The in-life phase, sample preparation, LC-MS/MS bioanalysis, and subsequent NCA PK analysis of individual animal raw time-concentration data were performed via Frontage Laboratories (Exton, PA) in accordance with their established Standard Operating Procedures (SOPs) and IACUC protocols.

Ancillary Pharmacology, Physiochemical Properties, and Mutagenicity

Mini-Ames Test. The mutagenic potential of **6c** was investigated by a mini-Ames test (TA98/TA100; +/- human hepatic S9) performed internally by AstraZeneca (AZ) using standard assay procedures.

Ancillary Pharmacology and Physiochemical Properties. The ancillary pharmacology and physiochemical properties of VU0467485/AZ13713945 (**6c**) were evaluated internally at AZ and via Pharmaron (Beijing, China) and Cerep (Celle L'Evescault, France) using standard assay procedures, and the results of these experiments are presented below.

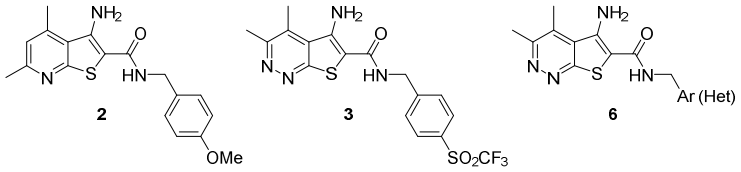
Test Name	Result Type	Result
hERG Hu CHO IF Ephs CR	Mean IC50 (μM)	>33.3
hERG Hu CHO IF Ephs SP	Mean % Inhib (at 10 μM)	4.512
Na1.5 Hu CHO IF Ephs CR	Mean IC50 (μM)	>33.3
IKs Hu CHO IF Ephs CR	Mean IC50 (μM)	>33.3
Ito Kv4.3 Hu CHO IF Ephs CR	Mean IC50 (μM)	>33.3
D2 Hu Bind FW CR	Mean IC50 (μM)	>100
a1B Hu Bind FW CR	Mean IC50 (μM)	>100
a2C Hu Bind FW CR	Mean IC50 (μM)	>100
EtA Hu Bind FW CR	Mean IC50 (μM)	>100
GABAa1b2g2 Hu Bind FW CR	Mean IC50 (μM)	>100
H1 Hu Bind FW CR	Mean IC50 (μM)	>100
H2 Hu Bind FW CR	Mean IC50 (μM)	>30
GR HU Cyto Bind FW CR	Mean IC50 (μM)	>100
DOP2 Hu Bind FW CR	Mean IC50 (μM)	>100
b1 Hu HEK cAMP TRF CR	Mean IC50 (μM)	>100
D1 Hu CHO cAMP TRF CR	Mean IC50 (μM)	>100
Ang2 AT1 Hu HEK [Ca2+] Ag CR	Mean EC50 (μM)	>100
Ang2 AT1 Hu [Ca2+] Antag CR	Mean IC50 (μM)	>30
DAT hu Bind FW CR	Mean IC50 (μM)	>100
D3 Hu Bind FW CR	Mean IC50 (μM)	>100
A1 Hu CHO Imped CDS Ag CR	Mean EC50 (μM)	>30
A1 Hu CHO Imped CDS Antag CR	Mean IC50 (μM)	>30
a1A Hu CHO [Ca2+] Ag CR	Mean EC50 (μM)	>30
a1A Hu CHO [Ca2+] Antag CR	Mean IC50 (μM)	>30
a2A Hu CHO Imped CDS Ag CR	Mean EC50 (μM)	>30
b1 Hu HEK cAMP TRF Ag CR	Mean EC50 (μM)	>100
5HT2B Hu Bind FW CR	Mean IC50 (μM)	>100
A1 Hu Bind FW CR	Mean IC50 (μM)	>100
a1A Hu Bind FW CR	Mean IC50 (μM)	>100
CB1 Hu Bind FW CR	Mean IC50 (μM)	>100
ACHE HU Enz Photometry CR	Mean IC50 (μM)	>100
A2A Hu Bind FW CR	Mean IC50 (μM)	>100
PDE3? Hu Enz TRF CR	Mean IC50 (μM)	>100
PDE4D Hu Enz TRF CR	Mean IC50 (μM)	>100
5-HT1A Hu Bind FW CR	Mean IC50 (μM)	>100
5-HT1D Rat Bind FW CR	Mean IC50 (μM)	>100

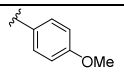
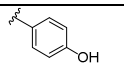
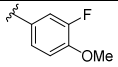
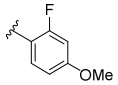
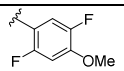
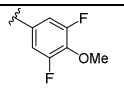
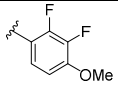
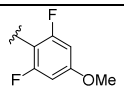
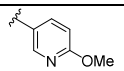
GABA? Rat Bind FW CR	Mean IC50 (μM)	>100
5-HT2C Hu Bind FW CR	Mean IC50 (μM)	>100
5HT3 Hu Bind FW CR	Mean IC50 (μM)	>100
5HT4 Hu Bind FW CR	Mean IC50 (μM)	>100
D3 Hu CHO cAMP TRF Ag CR	Mean EC50 (μM)	>100
D3 Hu CHO cAMP TRF Antag CR	Mean IC50 (μM)	>100
MR2 Hu CHO cAMP RF Antag CR	Mean IC50 (μM)	>100
SST4 Hu CHO cAMP TRF Ag CR	Mean EC50 (μM)	>100
SST4 Hu CHO cAMP TRF Antag CR	Mean IC50 (μM)	>100
D2 Hu HEK Imped CDS Ag CR	Mean EC50 (μM)	>30
SST4 Hu Bind FW CR	Mean IC50 (μM)	>100
D2 Hu HEK Imped CDS Antag CR	Mean IC50 (μM)	>30
H2 Hu CHO cAMP TRF Antag CR	Mean IC50 (μM)	>100
OPRk1 Rat CHO cAMP TRF Ag CR	Mean EC50 (μM)	>100
OPRk1 Rat CHO cAMP TRF Antag CR	Mean IC50 (μM)	>100
M1 Hu CHO [Ca2+] Ag CR	Mean EC50 (μM)	34.8
M1 Hu CHO [Ca2+] Antag CR	Mean IC50 (μM)	>30
H1 Hu HEK [Ca2+] Ag CR	Mean EC50 (μM)	>100
H1 Hu HEK [Ca2+] Antag CR	Mean IC50 (μM)	>30
H2 Hu CHO cAMP TRF Ag CR	Mean EC50 (μM)	>100
5HT1B Ham CHO Imped Antag CR	Mean IC50 (μM)	>30
CB1 Hu CHO cAMP TRF Ag CR	Mean EC50 (μM)	>100
a2A Hu Bind FW CR	Mean IC50 (μM)	>100
CB1 Hu CHO cAMP TRF Antag CR	Mean IC50 (μM)	>100
b1 Hu Bind FW CR	Mean IC50 (μM)	>100
D1 Hu Bind FW CR	Mean IC50 (μM)	>100
5HT2B Hu CHO IP1 TRF Antag CR	Mean IC50 (μM)	>100
D1 Hu CHO cAMP TRF Ag CR	Mean EC50 (μM)	>100
M2 Hu CHO cAMP TRF Ag CR	Mean EC50 (μM)	>100
EtA Hu SKN [Ca2+] Antag CR	Mean IC50 (μM)	>30
M2 Hu Bind FW CR	Mean IC50 (μM)	>100
OPRm1 Hu Bind FW CR	Mean IC50 (μM)	>100
NAchA1 Hu Bind FW CR	Mean IC50 (μM)	>100
NAchA4 Hu Bind FW CR	Mean IC50 (μM)	>100
NET Hu Bind FW CR	Mean IC50 (μM)	>100
5HT1B Ham CHO Imped Ag CR	Mean EC50 (μM)	>30
EtA Hu [Ca2+] Ag CR	Mean EC50 (μM)	99.8
a2C Hu CHO cAMP TRF Ag CR	Mean EC50 (μM)	>100
a2C Hu CHO cAMP TRF Antag CR	Mean IC50 (μM)	>100
OPRk1 Rat Bind FW CR	Mean IC50 (μM)	>100
a1B Hu CHO cAMP TRF Ag CR	Mean EC50 (μM)	>100
M1 Hu Bind FW CR	Mean IC50 (μM)	>100
M5 Hu Bind FW CR	Mean IC50 (μM)	>100

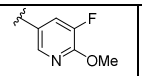
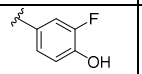
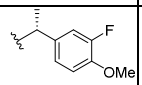
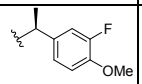
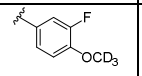
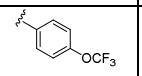
MR2 Hu Bind FW CR	Mean IC50 (µM)	>100
NK1 Hu Bind FW CR	Mean IC50 (µM)	20.3
a1B Hu CHO cAMP TRF Antag CR	Mean IC50 (µM)	>100
M2 Hu CHO cAMP TRF Antag CR	Mean IC50 (µM)	>100
OPRM1 Hu CHO cAMP TRF Ag CR	Mean EC50 (µM)	27.5
OPRM1 Hu CHO cAMP TRF Antag CR	Mean IC50 (µM)	>100
5HT2B Hu CHO IP1 TRF Ag CR	Mean EC50 (µM)	>100
5-HT1D Rat CHO Imped Antag CR	Mean IC50 (µM)	>30
5HT4 Hu CHO cAMP TRF Ag CR	Mean EC50 (µM)	>100
GABAA Rat Bind FW CR	Mean IC50 (µM)	1.20
ARG1 Hu Enz Photometry CR	Mean IC50 (µM)	>100
5HT4 Hu CHO cAMP TRF Antag CR	Mean IC50 (µM)	>100
5-HT7 Hu CHO cAMP TRF Ag CR	Mean EC50 (µM)	>100
5-HT7 Hu CHO cAMP TRF Antag CR	Mean IC50 (µM)	>100
BK2 Hu CHO [Ca2+] Ag CR	Mean EC50 (µM)	>100
BK2 Hu CHO [Ca2+] Antag CR	Mean IC50 (µM)	>30
b2 Hu CHO cAMP TRF Ag CR	Mean EC50 (µM)	>100
b2 Hu CHO cAMP TRF Antag CR	Mean IC50 (µM)	>100
NAchA7 Hu Bind FW CR	Mean IC50 (µM)	>100
NMDA Rat Bind FW CR	Mean IC50 (µM)	>100
Sigma1 Hu Bind FW CR	Mean IC50 (µM)	18.0
5-HT1A Hu HEK Imped CDS Ag CR	Mean EC50 (µM)	>30
5-HT1D Rat CHO Imped Ag CR	Mean EC50 (µM)	>30
CatS Hu Enz Fluorimetry CR	Mean IC50 (µM)	>30
PPARgamma Hu Bind FW CR	Mean IC50 (µM)	>100
5-HT2C Hu HEK IP1 TRF Ag CR	Mean EC50 (µM)	>100
5-HT1A Hu HEK Imped Antag CR	Mean IC50 (µM)	>30
ROCK2 Hu Enz TRF CR	Mean IC50 (µM)	>100
5HT7 Hu Bind FW CR	Mean IC50 (µM)	>100
TSPO Rat Bind CR	Mean IC50 (µM)	>100
GABAB Hu Bind FW CR	Mean IC50 (µM)	>100
Ghre Hu Binding CR	Mean IC50 (µM)	11.8
COX1 Hu Enz TRF CR	Mean IC50 (µM)	>100
cKIT Hu Enz TRF CR	Mean IC50 (µM)	>100
GSK3b Hu Enz TRF CR	Mean IC50 (µM)	>100
PDE6 Bov Enz CR	Mean IC50 (µM)	>100
PDK1 Hu Enz TRF CR	Mean IC50 (µM)	>100
ROCK1 Hu Enz TRF CR	Mean IC50 (µM)	>100
MAO-B Hu Enz Lumin CR	Mean IC50 (µM)	>100
MMP2 Hu Enz Fluorimetry CR	Mean IC50 (µM)	>100
PDE10A1 Hu Enz TRF CR	Mean IC50 (µM)	>100
ALK4 Hu Enz TRF CR	Mean IC50 (µM)	>100
FGFR1 Hu Enz FRET CR	Mean IC50 (µM)	>100

a2A Hu CHO Imped Antag CR	Mean IC50 (µM)	>30
A2A Rat PC12 cAMP TRF Ag CR	Mean EC50 (µM)	>100
A2A Rat PC12 cAMP TRF Antag CR	Mean IC50 (µM)	>100
DOP2 Rat PC12 CDS Ag CR	Mean EC50 (µM)	>30
DOP2 Rat PC12 CDS Antag CR	Mean IC50 (µM)	>30
MR2 Hu CHO cAMP TRF Ag CR	Mean EC50 (µM)	>100
Na+/K+? ATPase Pig Photomet CR	Mean IC50 (µM)	>100
EGFR kinase Hu Enz TRF CR	Mean IC50 (µM)	>100
src Hu Enz TRF CR	Mean IC50 (µM)	>100
MAP3K7 Hu Enz TRF CR	Mean IC50 (µM)	>100
TRKA Hu Enz TRF CR	Mean IC50 (µM)	>100
eNOS Hu HUVEC Enz CR	Mean IC50 (µM)	>100
NK1 Hu U-373 MG [Ca2+] Ag CR	Mean EC50 (µM)	84.5
TXA2 sy Hu Platelet Enz EIA CR	Mean IC50 (µM)	84.5
5-HT2C Hu HEK IP1 TRF Antag CR	Mean IC50 (µM)	>100
AT Gpig Bind FW CR	Mean IC50 (µM)	1.51
BK2 Hu Bind FW CR	Mean IC50 (µM)	>100
ECE1 Hu Enz Fluorimetry CR	Mean IC50 (µM)	>100
b2 Hu Bind FW CR	Mean IC50 (µM)	>100
KDR Hu Enz TRF CR	Mean IC50 (µM)	>100
SET Hu Bind FW CR	Mean IC50 (µM)	>100
INSR Hu Enz TRF CR	Mean IC50 (µM)	>100
COX2 Hu Enz TRF CR	Mean IC50 (µM)	>30
RARa Hu Binding SPA CR	Mean IC50 (µM)	>100
Ghre Hu CHO [Ca2+] Ag CR	Mean EC50 (µM)	>30
AurKA Hu Enz TRF CR	Mean Ki (µM)	>100
GABAB hu CHO Imped CDS Ag CR	Mean EC50 (µM)	>30
GABAB hu CHO Imped CDS Antag CR	Mean IC50 (µM)	>30
CaV-L Rat extract Bind CR	Mean IC50 (µM) Mean Ki (µM)	66.8 33.4
CaV-L Rat extract Bind CR	Mean IC50 (µM) Mean Ki (µM)	30.1 23.4
THP1 Fluo tox	Mean IC50 (µM)	>250

Structures, Activities, and *In Vitro* Intrinsic Clearance of 2, 3, and Analogues 6.



Entry	Ar (Het) for Analogues 6	hM_4 EC_{50} (nM)[a] [% ACh Max ±SEM]	hM_4 pEC_{50} (±SEM)	rM_4 EC_{50} (nM)[a] [% ACh Max ±SEM]	rM_4 pEC_{50} (±SEM)	human CL_{int},[b] (mL/min/kg)	rat CL_{int},[b] (mL/min/kg)
2	-	631 [81.4±0.9]	6.20±0.01	257 [69.0±5.0]	6.59±0.07	60.8	206
3	-	627 [54.9±3.3]	6.20±0.06	17.7 [68.1±1.6]	7.75±0.06	19.8	47.7
6a		130 [83.7±3.5]	6.89±0.06	59.7 [78.1±1.7]	7.22±0.06	35.9	80.5
6b		96.7 [69.8±2.2]	7.01±0.08	70.8 [70.3±10.9]	7.15±0.11	17.7	24.7
6c		78.8 [80.6±0.7]	7.10±0.01	26.6 [68.7±3.4]	7.57±0.05	26.1	73.4
6d		90.6 [75.5±2.4]	7.04±0.14	24.5 [80.0 (n=2)]	7.61 (n=2)	87.7	223
6e		41.4 [68.5±1.4]	7.38±0.05	28.8 [91.4 (n=2)]	7.54 (n=2)	42.8	162
6f		84.1 [75.3±1.1]	7.07±0.03	17.4 [91.0 (n=2)]	7.76 (n=2)	86.9	131
6g		43.4 [71.2±3.6]	7.36±0.02	17.8 [92.6±2.9]	7.75±0.15	46.9	217
6h		100 [87.1±1.9]	6.99±0.06	29.5 [97.0 (n=1)]	7.53 (n=1)	36.3	134
6i		239 [76.9±2.8]	6.62±0.11	107.2 [86.1±7.6]	6.97±0.08	16.8	33.2

6j		142 [80.8±1.5]	6.85±0.08	25.1 [77.9 (n=1)]	7.60 (n=1)	28.9	77.4
6k		59.1 [83.1±2.9]	7.22±0.07	33.1 [80.8 (n=2)]	7.48 (n=2)	58.2	52.7
6l		>10	>5.0	n.d	n.d	n.d.	n.d.
6m		508 [62.4±2.5]	6.29±0.09	n.d	n.d	n.d.	n.d.
6n		55.7 [87.1±0.8]	7.25±0.04	n.d	n.d	49.2	173
6o		213[b] [60.9]	6.72[b]	29.5 [68.4 (n=1)]	7.53 (n=1)	61.1	92.6

[a]Calcium mobilization assays with human or rat $M_{4/Gqi5}$-CHO cells performed in the presence of an EC_{20} fixed concentration of acetylcholine; values represent means from three (*n*=3) independent experiments performed in triplicate, unless otherwise indicated.

[b]Loss-of-parent vs. time (i.e., $t_{1/2}$ method; 1 μM test compound concentration) in human or rat hepatic microsomes with incorporation of species-specific physiological scaling factors; values represent results from one to three (*n*=1-3) determinations performed in triplicate.