National Institute of Mental Health Psychoactive Drug Screening Program

(NIMH PDSP)

ASSAY PROTOCOL BOOK

Version III

March 2018

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This is an updated version as of March 2018 of the previous PDSP assay protocol book (2012), and contains detailed descriptions of experimental procedures, data analysis, and representative figures for radioligand binding and functional assays performed by the NIMH-PDSP.

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Section 1: Radioligand binding assays

1.1. Drug plate preparation for radioligand binding assays: Hamilton ASM® and STAR®

The NIMH PDSP uses an **Integrated Sample Storage, Retrieval & Liquid Handling Robotic System** (see SOW) to prepare assay plates for the research staff to perform experiments. Briefly, the system consists of a refrigerated storage module that stores the PDSP samples and reference compounds; a retrieval module that selects, thaws, uncaps and delivers the compounds; and a multi-station liquid handling platform to transfer the samples and standards to make assay plates. The system is controlled by four PC computers utilizing Hamilton's Microlab STAR Venus One and Sample Manager software suites. Considerable training is required to operate the system. Advanced training is required to program and trouble-shoot the system.

A set of steps to instruct the machines to create a plate is called a "method." Each method has hundreds of lines of instructions. Below is an overview of the steps required to create assay plates for primary and secondary assay plates. This is an overview of the liquid handling and major plate movement steps. Robotic movements for tip set-up, tip movements, tip recycling, plate staging, etc. are not included.

A. Primary Plate Preparation:

1. A "work list" is created by a senior staff member utilizing our PDSP Database.
2. The work list is downloaded onto the ASM computer.
3. The "run control" program is opened on ASM server computer.
4. The assay method corresponding to the assay to be run is selected; the ASM server and storage module, de-capper and robotic arms perform self-checks.
5. The user is prompted to select the work list to be run.
6. The "run control" program verifies the work list, prompts the user to select start and thaw time variables or defaults.
7. Sample retrieval begins in ASM storage module, which sends messages to appropriate liquid handling station.

    B. The ASM storage module retrieves samples using the 2-dimensional bar code on each tube

C. Samples are collected in an empty rack, inside the ASM Storage module.
D. When all samples are collected, the rack is sent to the ASM server via an automated trolley and the server's internal single-grip arm.
E. Samples are thawed with forced hot air according to protocol, with an option to enter thaw time.
F. The rack is then delivered via the ASM Server single-grip arm and hand-off arm to the Capper/Decapper
G. Sample tubes are uncapped and sent to the appropriate STAR deck

8. The user prepares the STAR liquid handler deck with proper plates, buffers and pipette tips. (Steps 8-14 are performed in parallel to step 7.)
9. The STAR computer run control program (this is a separate instance from the ASM run control program) prompts the user for tip-counting verification; user also confirms that the protocol and plate count are correct.
10. STAR platform begins plating procedure.
11. Assay plates are distributed to the STAR deck (1 per final plate); the plate barcode is recorded and used throughout the remainder of the process to verify the integrity of sample tracking.
12. 97.5 microliters of buffer are added to rows A and E of each assay plate.
13. DMSO (vehicle control) is added to wells A1 and E1 of each assay plate.
14. STAR platform pauses with user input, and waits for drug delivery from the ASM (step 7f above).
15. Samples are mixed after step 7G is completed and the sample rack is delivered to the STAR deck.
16. 2.5 microliters of control reference compound are aspirated from the sample tube and distributed into the assay plate in wells A12 and E12 using the eight single-channel pipetters.
17. 2.5 microliters of each sample is aspirated from sample tubes and distributed into the assay plate in wells A2 through A11 and E2 through E11 using the eight single-channel pipetters.
18. Row A is then mixed and aliquoted from A to B and A to C and A to D with a single-row selection of pipette tips by the 96-well head, each well then has 25 microliters of combined sample and buffer.

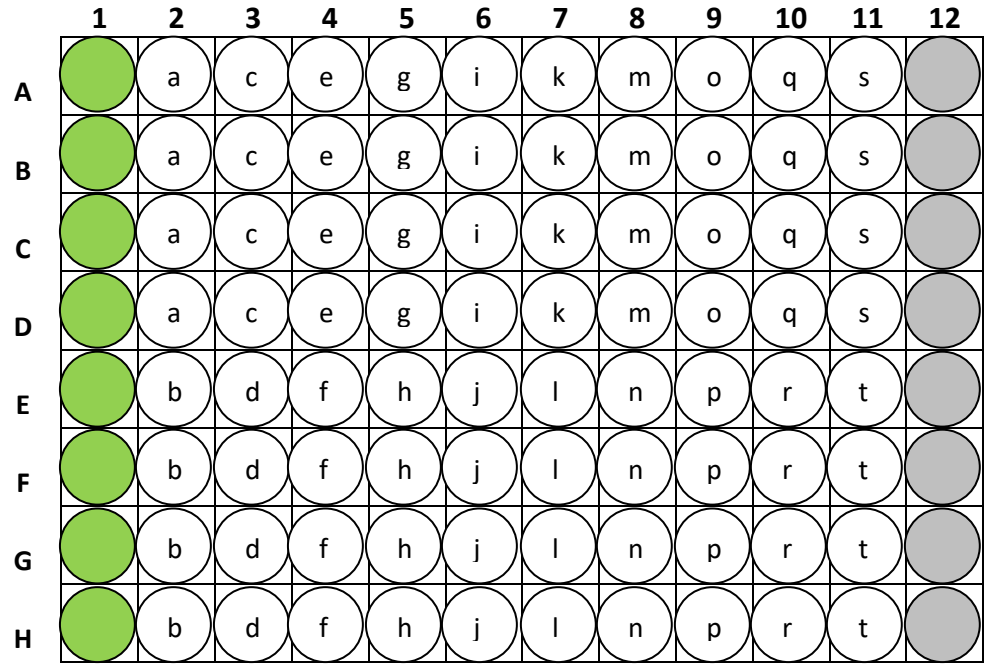
19. Step 18 is repeated for row E-H to make replicates.
20. A plate map is created corresponding to the plate bar-code, samples added and receptor assigned.
21. Steps 16-20 are repeated for each assay plate. (See Figure 1 for primary drug plate map).

B. Secondary Plate Preparation:

1. A "work list" is created by a senior staff member utilizing our PDSP Database.
2. The work list is down-loaded onto the ASM computer.
3. The "run control" program is opened by the user on the ASM server computer.
4. The assay "method" corresponding to the assay to be run is selected; the ASM server and storage module, de-capper and robotic arms perform self-checks.
5. The user is prompted to select the work list to be run.
6. The "run control" program verifies the work list, prompts the user to select start and thaw time variables or defaults.
7. Sample retrieval begins in the ASM storage module, and sends messages to the appropriate liquid handling station.
   A. The ASM storage module retrieves samples using the 2-dimensional bar code on each tube
   B. Samples are collected into an empty rack, inside the ASM storage module
   C. When all required samples are collected, the rack is sent to the ASM server via an automated trolley and the server's internal single-grip arm.
   D. Samples are thawed with forced hot air according to protocol with an option to enter thaw time.
   E. The rack is then delivered via the ASM Server single-grip arm and hand-off arm to the Capper/Decapper
   F. Sample tubes are uncapped and sent to the appropriate STAR deck
8. The user prepares the STAR liquid handler deck with proper plates, buffer and pipette tips. (Steps 8-14 are performed in parallel to step 7.)
9. The STAR computer run control program (this is a separate instance from the ASM run control) prompts the user for tip-counting verification; the user also confirms that the protocol and plate count are correct.

10. The STAR platform begins the plating procedure.
11. Assay plates are distributed to the STAR deck (1 dilution plate and 3 plates for replicates per set); the plate barcodes are recorded and are used throughout our process to verify the integrity of sample tracking.
12. 180 microliters of buffer are added to all wells of each assay plate with the 96CORE head.
13. 15 microliters of buffer are added to column 12, 40 microliters are aspirated from column 11.
14. The STAR platform pauses and waits for drug delivery from ASM (step 7f above).
15. Samples are mixed.
16. 5 microliters of sample (or control compound) is aspirated from the sample tubes and distributed into the first dilution plate in column 12 using the eight single-channel pipetters.
17. 40 microliters of buffer are removed from column 11.
18. After mixing, 60 microliters are aspirated from column 12 and dispensed into column 11.
19. A serial dilution is performed; 20 microliters is aspirated from/dispensed to col 12 to 10, col 10 to 8, col 8 to 6, col 6 to4, col 4 to 2, col 11 to 9, col 9 to 7, col 7 to 5 and, col 5 to 3.
20. The entire plate is mixed again and then three replicates with 25 micoliters per well are made.
21. Steps 16-20 are repeated for each additional set of four plates.
22. Plate maps are created corresponding to the plate barcode, samples added and receptor assigned for all plates on the deck. (See Figure 2 for secondary drug plate map).

Managing the workflow of the automated system is extremely important. First, the overall flow, i.e., what assays to select, when to make assay plates and, assigning assays to a researcher must be performed by a senior staff member. Second, the workflow for the system also requires careful planning and scheduling. Operating the system can be performed by a researcher. The operation of the system *requires* careful attention to detail and significant planning in order to achieve optimal performance. Preparing the liquid handling deck for a method, being attentive to tip usage, having all the reagents ready prior to start, having all consumables ready prior to start, as well as the sequence of primary versus secondary methods to minimize transition time between runs are all required to have the system operate at 35-plus hours per week (its current utilization).



Input Barcodes:

- BAGS33

Primary Binding Plates:

Barcode: BAGS33
Receptor: H3-0 Primary BAGS33 03
Date: 3-14-2013 09-13-18

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	27054	22578	22580	22581	22582	22583	22584	22585	26656	26657	Histamine
B	Empty	27054	22578	22580	22581	22582	22583	22584	22585	26656	26657	Histamine
C	Empty	27054	22578	22580	22581	22582	22583	22584	22585	26656	26657	Histamine
D	Empty	27054	22578	22580	22581	22582	22583	22584	22585	26656	26657	Histamine
E	Empty	26658	26772	26787	26788	26789	26790	26791	26793	26794	26796	Histamine
F	Empty	26658	26772	26787	26788	26789	26790	26791	26793	26794	26796	Histamine
G	Empty	26658	26772	26787	26788	26789	26790	26791	26793	26794	26796	Histamine
H	Empty	26658	26772	26787	26788	26789	26790	26791	26793	26794	26796	Histamine

Figure 1. 96-well drug plate setup diagram (up) and an actual barcoded plate (bottom) for primary radioligand binding assays. The drug plate is designed to contain 20 compounds (compound "a" to compound "t" in Columns 2 - 11, each in quadruplicate) and one reference compound (Column 12), 25 µl per well at 50 µM (5x of final assay concentration of 10 µM). Column 1 contains buffer only, and is therefore designated for total binding (0% inhibition). Column 12 contains reference compound (histamine in this case) is designated as nonspecific binding (100% inhibition).

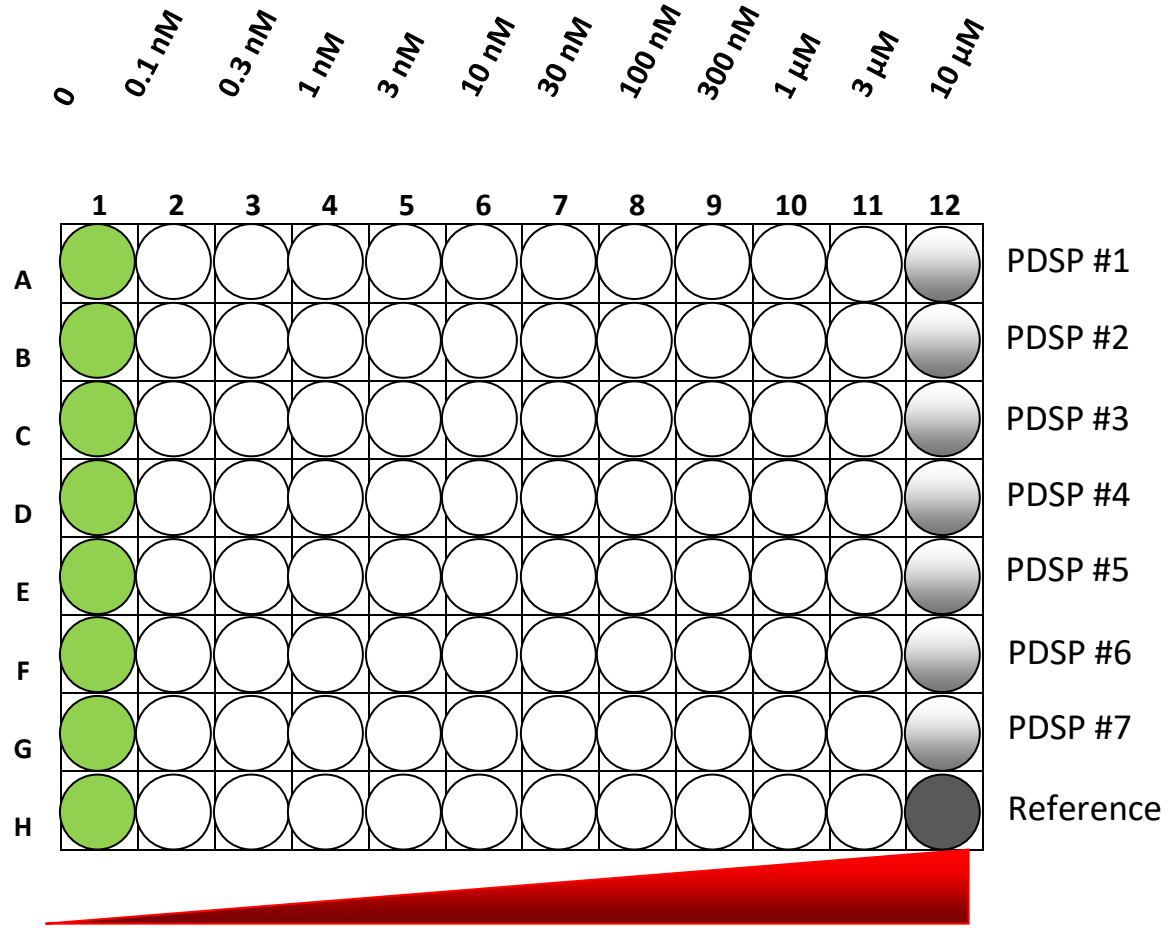


Figure 2a. 96-well drug plate map for secondary radioligand binding assays. The drug plate is designed to contain 7 PDSP compounds (Rows A to G) and 1 reference compound (Row H) as indicated at right of the plate, one compound per row and 25 µl per well at 5x of final concentrations as indicated on top of the plate. Well H12 is designated for nonspecific binding and column 1 is designated for total binding for the plate. Each secondary assay consists of 3 sets of identical 96-well plates.

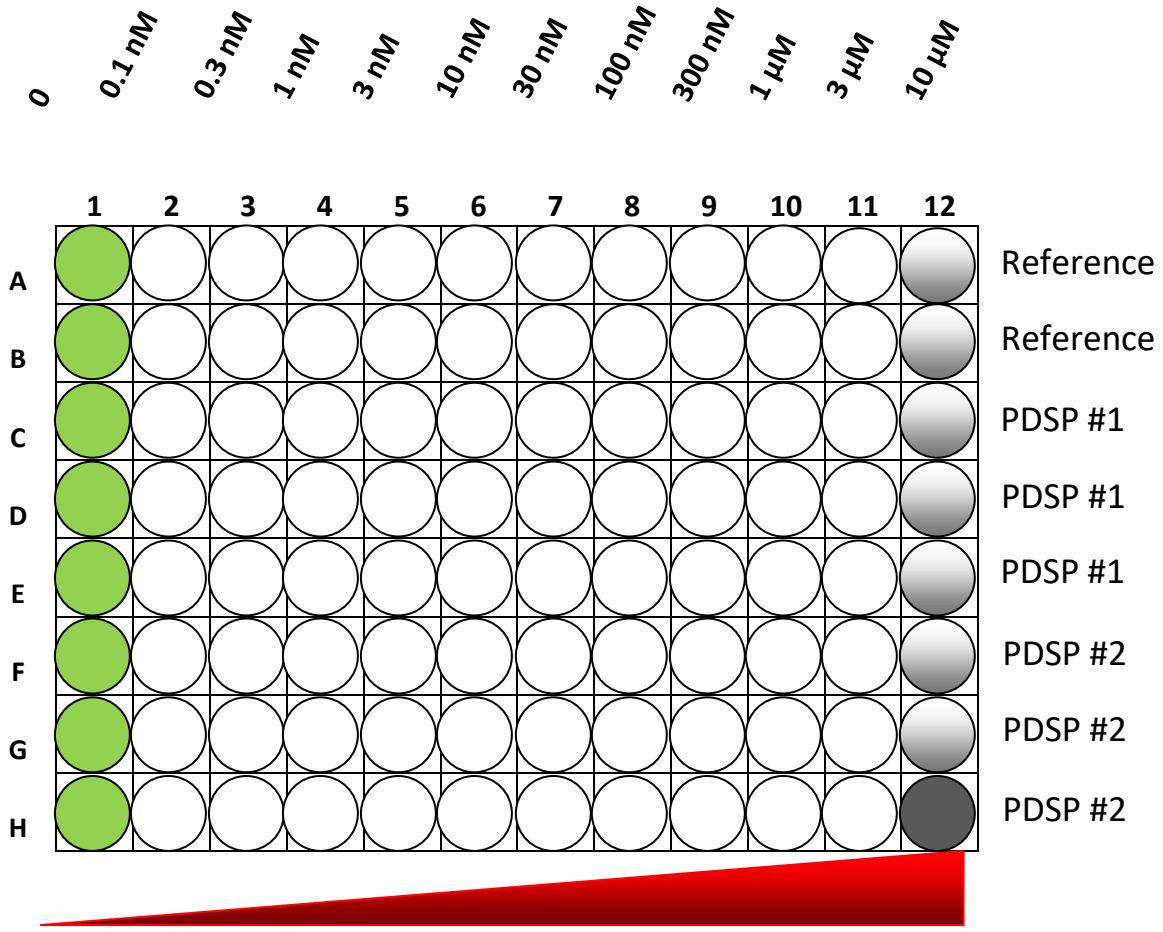


Figure 2b. 96-well drug plate map for secondary radioligand binding assays. The drug plate is designed to contain 2 PDSP compounds (Rows C to H) and 1 reference compound (Rows A and B) as indicated at right of the plate, therefore each PDSP compound is assayed in triplicate and the reference compound in duplicate. Each well contains 25 µl at 5x of final concentrations as indicated above the plate.

1.2. Cell culture and membrane fraction preparation

The majority of binding assays performed by the NIMH PDSP staff employ stably or transiently transfected cell lines expressing mainly human recombinant receptors, monoamine transporters, or ion channels. A detailed description of the cell lines, the culture and sub-culture conditions, and the appropriate media are listed in **Table 1**.

1.2.1. Calcium phosphate precipitation transfection for adherent HEK293 T cells.

2x HBS: 140 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH 7.05, room temperature.

The calcium phosphate precipitation method was adapted from Jordan et al (1996)(1). In brief, HEK293T cells are subcultured into 15-cm dishes at a density of 8 – 10 million cells per dish and incubated overnight. For each dish of 15-cm, 18 μg DNA, 100 μl of 2.5 M $CaCl_2$, 100 μl of TE (1 mM Tris HCl, 0.1 mM EDTA, pH 7.60) are diluted into a final volume of 1 ml water. The mixture is then quickly added to an equal volume of 2x HBS and added dropwise to cells. The cells are then incubated overnight and for another day or two in fresh growth medium. For transient transfections with 5-HT receptors, we use medium containing dialyzed FBS overnight before harvesting. Cells are usually harvested 48 – 72 hours after transfection.

1.2.2. Suspension HEK293 tranfection.

The PDSP also uses suspension HEK293 cells to obtain high levels of protein expression for binding assays (2). HEK293 suspension culture is maintained in flasks (115 rpm, 37°C, and 8% CO_2) with the Expi293 Expression Medium with GlutaMax-1 (Life Technologies) supplemented with Pen/Strep (such as 30 ml medium in a 125 ml flask), and passaged when they reach a density of 5 – 7 million cells per ml. When seeding for a new passage, cell density is maintained at 0.5 – 0.75 million cells per ml. For transfection, we use the ExpiFectamine 293 Transfection Kit (Life Technologies). Cells are first subcultured in the Expi293 medium without Pen/Strep at a density of 3 million cells per ml for 25.5 ml in a 125 ml flask. To make the transfection mix (for each 125 ml flask), 30 μg receptor DNA is mixed in 1.5 ml OptiMEM medium in one tube and 80 μl of ExpiFetamine reagent is mixed in 1.5 ml OptiMEM medium in another tube. Both tubes are incubated for 5 min separately at RT and then for

20-30 min after being mixed together at RT. The mixture is then added to cells for overnight incubation at 115 rpm, 37°C, and 8% CO_2. At 16-20 hr after the initial transfection, 0.15 ml Enhancer #1 and 1.5 ml Enhancer #2 are added and the culture are incubated for another 20-28 hrs to enhance protein expression (3). Cells are then collected for membrane preparation or kept at -80°C until membrane preparation (next section).

1.2.3. Membrane preparations from stable or transiently transfected cells. To make membrane fractions from stably transfected cell lines, cells are subcultured into 15-cm dishes and grown to 90% confluency. For 5-HT receptors, cells are incubated overnight in either serum-free medium or medium containing 1% dialyzed fetal bovine serum. The next day, the cells are rinsed with PBS, scraped off into 50 ml conical tubes, and pelleted by centrifugation (1000 x g, 10 min at 4°C). The cell pellet is resuspended in chilled (4°C) lysis buffer (50 mM Tris HCl buffer, pH 7.4) and triturated gently for hypotonic lysis. The suspension is then centrifuged at 21,000 x g for 20 min (4°C) to obtain a crude membrane fraction pellet. The fresh membrane pellet is then resuspended in cold lysis buffer with 3x volumes of the pellet size and is immediately subjected to the Bradford protein assay to determine protein concentration, followed by a saturation binding assay (see following section for details) to determine receptor expression level and the affinity of the selected radioligand. Based on the receptor expression level and the K_d value, fresh membrane suspensions are stored at -80 °C in small aliquots such that one aliquot is enough for one 96-well plate to have at least 500 cpm/well when assayed at 0.5 – 1.0x K_d value of the hot ligand.

1.2.4. Membrane preps from tissues. To make membrane fractions from tissues, crude membrane fractions are prepared from rodent (typically rat or guinea pig brain or kidney, purchased from PelFreeze Biologicals). Here is the general procedure for membrane preparations unless otherwise stated. Frozen tissue (maintained at -80°C) is thawed on ice, homogenized on ice in 10 volumes of cold lysis buffer (50 mM Tris HCl, pH 7.4, containing protease inhibitor cocktail from Roche) using a Polytron homogenizer (6 pulses and 10 seconds per pulse). The homogenate is centrifuged at 1,000 x g for 10 min at 4°C to obtain supernatant. The supernatant is then centrifuged at 40,000 x g for 20 min, and then the resulting supernatant is decanted and replaced with the same ice-cold lysis buffer. Two or three additional rounds of homogenization-centrifugation are performed to ensure thorough

homogenization and also to wash out endogenous ligands (particularly important for GABA assays). The final pellet is resuspended in the same buffer and homogenized one last time. The fresh suspension is subjected to protein concentration measurement, saturation binding, and aliquoted for storage at -80°C for future use. Aliquots are also made such that each aliquot is enough for one binding assay in one 96-well plate to have at least 500 cpm/well when assayed with a 0.5 – 1.0x K_d level of the corresponding hot ligand.

1.2.5. Membrane preps from rat brain for NMDA radioligand binding assays. The protocol is adapted from Chiu et al., 1999 (4). In brief, rat brain is thawed on ice in SHE buffer (300 mM Sucrose, 10 mM HEPES, 2 mM EDTA, pH 7.3) and homogenized on ice in 20 ml ice-cold SHE buffer per gram of wet tissue using glass homogenizer (at least 6 strokes). The homogenate is centrifuged at 1,000 x g for 20 min at 4°C to collect supernantant. The supernatant is then centrifuged at 16,000 x g for 1h at 4°C. The pellet is suspended in the same SHE buffer and stored in aliquots (10 mg per aliquot after protein concentration determination) at -80°C until use. Protein concentration is determined using the Bradford method. Immediately prior to assay, the aliquoted pellet is resuspended in 1 ml HE buffer (20 mM HEPES, 1 mM EDTA, pH 7.0) and centrifuged briefly in desktop centrifuge at 4°C to remove the sucrose. The pellet is then resuspended in 0.5 ml of HE buffer and incubated at 37°C for 30 min. The suspension is then centrifuged at 13,000 x g for 10 min at 4°C. The pellet is washed four times by resuspending it in 1 ml HE buffer, followed by centrifugation. The final pellet is resuspended to about 2 mg/ml concentration after protein concentration determination and is then used in binding assays at 100 ug per well, using the HE buffer supplemented with with 100 μM sodium glutamate and 100 μM glycine.

1.2.6. List of cell lines and targets that PDSP uses for binding assays.

Table 1. List of cell lines and targets that PDSP uses for making membrane pallets for binding assays. All clones are stable lines, while transient cells are marked with "*". Most clones are of human origin unless noted, such as (rat) behind receptor name. Detailed info about media is listed below the table.

Receptor	Note	Parental cells	Media (see details below the table)
Serotonin (5HT)			
5-HT_{1A}		stable CHO	500 G418
5-HT_{1B}		stable HEK	500 G418
5-HT_{1D}	*	HEKT	COS/HEK
5-HT_{1E}		stable HEK	500 G418
5-HT_{2A} (rat)		stable 3T3	500 G418
5-HT_{2A}	*	HEKT	COS/HEK
5-HT_{2B}		stable HEK	2 μg/ml Puromycin
5-HT_{2C}		HEK T	COS/HEK
5-HT_3	*	HEKT	COS/HEK
5-HT_4		HEK T	COS/HEK
5-HT_{5A}		Flp-In CHO	DMEM/F-12 200 μg/ml Hygromycin B
5-HT_6		stable HEK	500 G418
5-HT_{7A}		Stable HEK	2 μg/ml Puromycin
Dopamine			
D_1	*	HEKT	COS/HEK
D_2		stable fibroblast	COS/HEK
D_{2L}		stable CHO	F-12/10%FBS 400G418
D_3 (rat)	*	HEKT	COS/HEK
D_3	*	HEKT	COS/HEK
D_4		Stable HEK	DMEM/F12 10% CS Fe+
D_5	*	HEKT	COS/HEK
Opioid			
DOR		stable HEK	200 G418
MOR		stable HEK	200 G418
KOR (rat)		stable HEK	500 G418
KOR		stable HEK	500 G418
NOP	*	HEKT	COS/HEK
Neurotransmitter Transporters			
SERT		stable HEK	500 G418
NET		stable HEK	hNET (250 G418)
DAT		stable HEK	hDAT (350 G418)
Vasopressin and Oxytocin			
V_{1A}		stable CHO	V1A & OT media
V_{1B}		stable CHO	V2 & V1B media
V_2		stable CHO	V2 & V1B media
OT		stable CHO	V1A & OT media
Prostaglandin			
EP-3	*	HEKT	COS/HEK
EP-4	*	HEKT	COS/HEK
Adrenergic			
α_{1A}		stable	500 G418
α_{1B}	*	HEKT	

Receptor	Note	Parental cells	Media (see details below the table)
α_{1D}		stable	500 G418
α_{2A}		stable MDCK	500 G418
α_{2B}	*	HEKT	COS/HEK
α_{2C}		stable MDCK	500 G418
β_1		CHO Flp-In	DMEM/F12 200 μg/ml Hygromycin B
β_2		HEK Flp-In	DMEM 100 μg/ml Hygromycin B
β_3		HEK Flp-In	DMEM 100 μg/ml Hygromicin B
Muscarinic acetylcholine			
M_1		stable CHO	250 G418
M_2		stable CHO	500 G418
M_3		stable CHO	500 G418
M_3D (DREADD)		CHO Flp-In	DMEM/F12 100 μg/ml Hygromycin B
M_4		stable CHO	10% FBS F12
M_4D (DREADD)		HEKT	COS/HEK
M_5		stable CHO	250 G418
Nicotinic acetylcholine			
α2β3		HEK	500 G418
α2β4		HEK	500 G418
α3β2		HEK	500 G418
α3β4		HEK	500 G418
α4β2		HEK	500 G418
α4β4		HEK	500 G418
α7		HEK	500 G418
Histamine			
H_1		stable HEK	500 G418
H_2		Stable HEK	In Progress
H_3		HEK Flp-In	DMEM 100 μg/ml Hygromycin B
H_4		HEK T	COS/HEK
Cannabinoid			
CB_1		HEK	500 G418, In Progress
CB_1		HEK Flp-In	DMEM 100 μg/ml Hygomycin B
CB_2		HEK Flp-In	DMEM 100 μg/ml Hygomycin B
Adenosine			
A_1	*	HEKT	COS/HEK
A_2A	*	HEKT	COS/HEK
A_2A		HEK	500 G418
A_2B	*	HEKT	COS/HEK
A_3	*	HEKT	COS/HEK
Melanocortin			
MC_1	*	HEKT	COS/HEK
MC_2	*	HEKT	COS/HEK
MC_3	*	HEKT	COS/HEK

Receptor	Note	Parental cells	Media (see details below the table)
MC_4	*	HEKT	COS/HEK
MC_5	*	HEKT	COS/HEK
Purinergic P2Y			
P2Y1		Astrocyte line	500 G418
P2Y2		Astrocyte line	500 G418
P2Y4		Astrocyte line	500 G418
P2Y6		Astrocyte line	500 G418
P2Y11		Astrocyte line	500 G418
P2Y12		Astrocyte line	500 G418
Trace Amine			
TA-1	*	HEKT	COS/HEK
TA-3	*	HEKT	COS/HEK
TA-4	*	HEKT	COS/HEK
TA-5	*	HEKT	COS/HEK
Lysophospholipid (LPA)			
LPA1	*	HEKT	COS/HEK
LPA2	*	HEKT	COS/HEK
LPA3	*	HEKT	COS/HEK
Tachykinin (NK)			
NK_1		HEK	500 G418
NK_2		HEK	500 G418
NK_3		HEK	500 G418
mGluR			
$mGlu_1$	*	HEKT	In progress
$mGlu_2$	*	HEKT	In progress
$mGlu_3$	*	HEKT	In progress
$mGlu_4$	*	HEKT	In progress
$mGlu_5$		CHO	2 μg/ml Puromycin
$mGlu_5$	*	HEKT	In progress
$mGlu_6$	*	HEKT	In progress
$mGlu_7$	*	HEKT	In progress
$mGlu_8$	*	HEKT	In progress
Others			
Ghrelin		HEK Flp-In	DMEM 100 μg/ml Hygomycin B
PAR1		Lung Fibroblast, PAR1 KO	500 G418
SMO	*	HEKT	COS/HEK
SMO		HEK	500 G418, in progress
CCK_2		CHO	500 G418
Orexin-2	*	HEKT	COS/HEK
RXFP1	*	HEKT	
RXFP2	*	HEKT	
RXFP3	*	HEKT	
RXFP4	*	HEKT	

Receptor	Note	Parental cells	Media (see details below the table)
GPR39	*	HEKT	
GPR4	*	HEKT	
GPR40	*	HEKT	COS/HEK
GPR41	*	HEKT	COS/HEK
GPR43	*	HEKT	COS/HEK
GPR58	*	HEKT	COS/HEK
GPR61	*	HEKT	COS/HEK
GPR62	*	HEKT	COS/HEK
GPR65	*	HEKT	COS/HEK
GPR68	*	HEKT	COS/HEK
GPR88	*	HEKT	COS/HEK
Il-1 imidazoline	*	HEKT	COS/HEK
HERG-K^+ Channel		HEK	500ug/ml G418
PC12			COS/HEK
Cav1.2		HEK	
Sigma1	*	HEKT	
Sigma2	*	HEKT	

1.2.7. Media Recipes

Details of media composition for cells are as follows (please note that DMEM is purchased as 1X high D-glucose (4,500 mg/L) with L-glutamine and supplemented upon receipt with penicillin/streptomycin (100 U/ml).

1. **COS/HEK Medium (10% serum) – 1 L: also used for transient transfection**

1L DMEM, 100 ml FBS, 10 ml Pen/Strep

2. **Standard G418 Media (500 μg/ml; 10% serum) – 1L**

1 L DMEM, 500 mg G418, 100 ml FBS, 10 ml Pen/Strep

3. **Standard Puromycin Medium (2 μg/ml Puromycin:10% Serum) - 1L**

1 L DMEM, 2 aliquots (1mg/ml Puro in PBS), 100 ml FBS, 10 ml Pen/Strep

4. **Standard Puromycin Medium +G418 Selection Medium**

1L DMEM, 2 aliquots (1mg/ml Puro in PBS), 500 mg geneticin, 100 ml FBS, 10 ml Pen/Strep

5. **Dialyzed Medium (1% dialyzed serum) – 1 L**

1 L DMEM, 10 ml Dialyzed FBS, 10 ml Pen/Strep

6. hNET Medium (250 μg/mL G418; 10% serum) – 1 L

1 L DMEM, 250 mg geneticin, 100 ml FBS, 10 ml Pen/Strep

7. hDAT Medium (350 μg/ml G418; 10% serum) - 1L

1L DMEM, 350mg geneticin, 100ml FBS, 10ml Pen/Strep

8. V1A and OT Medium (400 μg/ml G418, 10% FSB, 15 mM HEPES) – 1L

1L Hams F12, 400 mg Geneticin, 100 ml Calf serum, 15 ml HEPES (1M stock), 10 ml Pen/Strep

9. V2 & V1B Medium (150 μg/mL Zeocin; 10% Serum; 15 mM Hepes) – 500 mL

1L Hams F12, 1.5 ml Zeocin (100mg/mL), 100ml Calf Serum, 15 ml Hepes (1M), 10 ml Pen/Strep

10. MOR/DOR selection medium (200 μg/ml G418, 10% serum) – 1L

1 L DMEM, 200 mg G418, 100 ml FBS, 10 ml Pen/Strep

11. Alpha 1A and 1D selection medium (500 μg/ml G418, 10% serum) – 1L

1 L DMEM, 500 mg G418, 100 ml FBS, 10 ml Pen/Strep

12. M4 medium (10% serum) - 1L

1L Hams F12, 100 ml FBS, 10 ml Pen/Strep

13. D4 medium (10% serum) – 1L

1L DMEM/F12, 15mM HEPES; with pyridoxine HCl, 100 ml Donor Calf Serum with Iron, 10 ml Pen/Strep

14. FLP-In CHO medium (10% serum) – 1L

1L DMEM/F12 50/50, 10% FBS, 200 μg/ml Hygromycin B, 10 ml Pen/Strep

15. FLP-In HEK medium (10% serum) – 1L

1L DMEM, 10% FBS, 100 ug/ml Hygromycin B, 10 ml Pen/Strep

16. PC-12 medium (10% serum) – 1L

1L DMEM, 5% Horse Serum, 5% FBS, 10 ml Pen/Strep

17. HTLA medium – 1L

1L DMEM, 100 ml FBS, 100 μg/ml Hygromycin B, 5 μg/ml Puromycin, 10 ml Pen/Strep

18. Inositol/Inositol-free medium

500 ml BME (BME phrchased from LONZA), 5 ml Pen/Strep, 5 ml L-Glutamine

19. McCoy medium for U2OS – 1L

1L McCoy, 100 ml dialyzed FBS, 50 μg/ml Hygromycin B, 200 μg/ml Zeocin, 100 μg/ml G418, 0.1 mM non-essential AA, 25 mM HEPES, 1 mM Sodium Pyruvate, 10 ml Pen/Strep.

20. Expi293 Expression Medium – 1L

1L Expi293 Expression Medium with GlutaMax-1, 10 ml Pen/Strep.

1.3. Saturation binding assays

Saturation binding assays, usually performed immediately after the membrane fraction is obtained and protein concentration is determined (see above section for membrane preparations), are routinely carried out to measure receptor expression level (B_{max}) and binding affinity (K_d) of a selected radioligand. **Tables 2-24** lists the identity of hot ligands, reference compounds, and buffers for each family of targets. Saturation binding assays are carried out in 96-well plates in a final volume of 125 µl per well. In brief, 25 µl of radioligand is added to a 96-well plate according to the setup in **Figure 3**; followed by addition of 25 µl binding buffer (for total binding) or 25 µl reference compound at a final concentration of 10 µM (for nonspecific binding). The reaction starts upon addition of 75 µl of fresh membrane protein (typically 25 to 50 µg per well) and the reaction is usually incubated in the dark at room temperature for 90 min. The reaction is stopped by vacuum filtration onto cold 0.3% polyethyleneimine (PEI) soaked 96-well filter mats using a 96-well Packard Filtermate harvester, followed by three washes with cold wash buffers (for details, see following **Tables 2 to 24**). Scintillation cocktail is then melted onto microwave-dried filters on a hot plate and radioactivity is counted in a Microbeta counter.

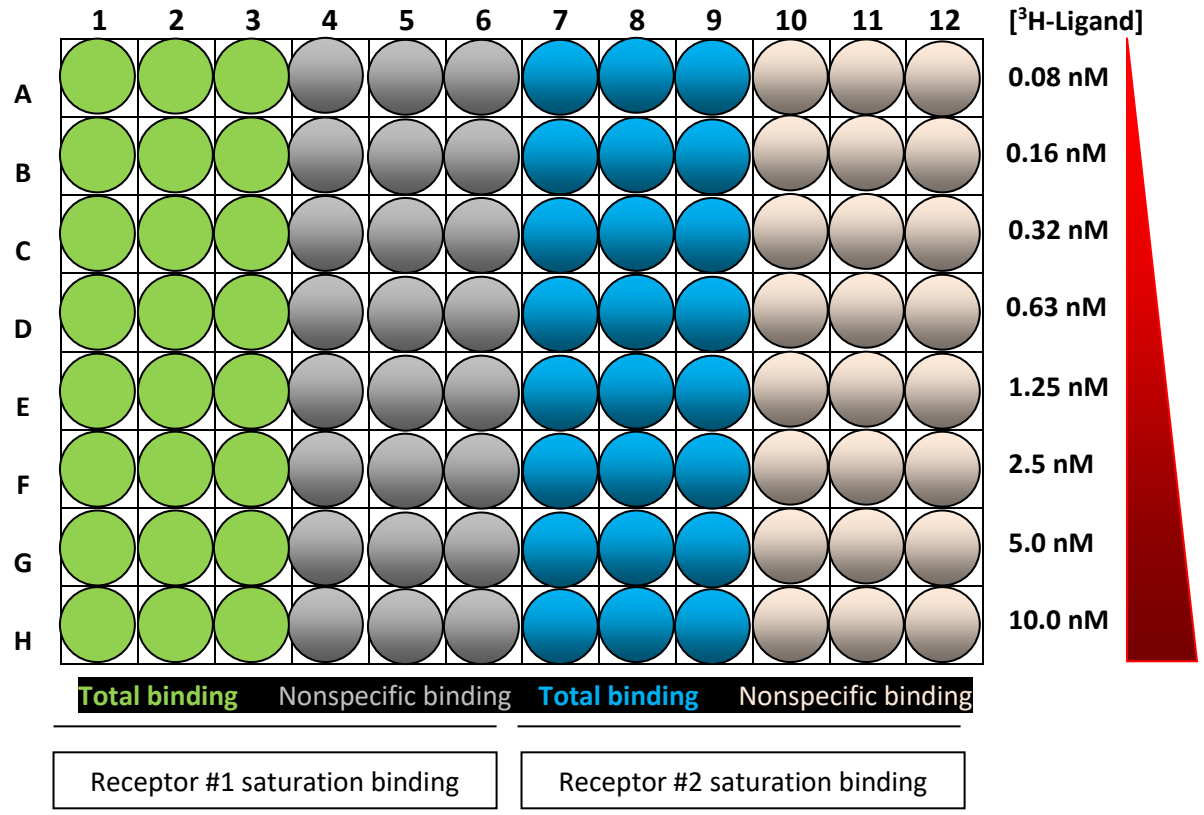


Figure 3. Radioligand saturation binding plate set up in a 96-well plate. Total and nonspecific binding are determined in the absence and presence of 10 µM of the appropriate reference compound, respectively, in the indicated final concentrations of hot ligand (nM). Final radioligand concentrations are shown as an example and different concentration ranges are used for different receptors, dependent on their K_d values for selected radioligand. The K_d value is usually in the middle, i.e. Row D or E. Amount of membrane protein (µg/well) are adjusted so that bound radioactivity is less than 10% of the total added radioactivity.

1.4. Primary and secondary radioligand binding assays

Compounds are typically subjected to primary radioligand binding assays at targets selected by PIs and approved by the PDSP Director, Dr Bryan Roth. In primary binding assays, compounds are usually tested at a single concentration (10 µM) and in quadruplicate in 96-well plates (see **Figure 1** for drug

plate map). Compounds showing a minimum of 50% inhibition at 10 µM are tagged for secondary radioligand binding assays to determine equilibrium binding affinity at specific targets. In secondary binding assays, selected compounds are usually tested at 11 concentrations (0.1, 0.3, 1, 3, 10, 30, 100, 300 nM, 1, 3, 10 µM) and in triplicate (3 sets of 96-well plates, see **Figure 2a** for drug plate map). An alternative drug plate set up is shown in **Figure 2b** which contains 2 PDSP test compounds and 1 reference compound. The drug map in **Figure 2a** is more efficient than the one in **Figure 2b**; the **Figure 2b** drug plate is usually made manually for urgent projects with a small number of high priority compounds to be tested immediately upon request.

Both primary and secondary radioligand binding assays are carried out in a final of volume of 125 µl per well in appropriate binding buffers (for details, see **Tables 2 to 24**). The hot ligand concentration is usually at a concentration close to the K_d (unless otherwise indicated). Total binding and nonspecific binding are determined in the absence and presence of 10 µM of the appropriate reference compound, respectively. In brief, plates are usually incubated at room temperature and in the dark for 90 min (unless otherwise indicated). Reactions are stopped by vacuum filtration onto 0.3% polyethyleneimine (PEI) soaked 96-well filter mats using a 96-well Filtermate harvester, followed by three washes with cold wash buffers (for details, see **Tables 2 to 24**). Scintillation cocktail is then melted onto the microwave-dried filters on a hot plate and radioactivity is counted in a Microbeta counter. A general procedure for binding assays is shown below (unless otherwise stated, such as radioligand binding assays for nAChRs; see details in the next section for nAChR binding assays).

1. Receive barcoded primary drug plate or secondary drug plates from Hamilton STAR®
2. Check out drug plates and mark them “in progress” in PDSP database
3. Prepare appropriate binding buffers and wash buffers
4. Check out membrane pellets
5. Check pellet box to obtain K_d and concentration to use for the radioligand
6. Prepare 2.5x of final concentration of radioligand working solution
7. Count 50 µl of radioligand working solution to confirm radioligand concentration and activity

8. Add 50 µl radioligand to each well
9. Add 50 µl membrane suspension to each well
10. Mix by gentle and brief shaking
11. Incubate the plates in the dark for desired time (usually 60 – 90 min at RT)
12. Soak filters in cold 0.3% PEI
13. Stop the reaction by vacuum filtration and washing
14. Dry the filters using microwave oven
15. Melt scintillation cocktail on top of filters
16. Wrap filters in plastic wrap
17. Count radioactivity
18. Download results, process and upload to PDSP database
19. Preview and submit results
20. Report progress in the PDSP database (completion or redo or repeat) after receiving approval email

1.5. Radioligand binding assays for nicotinic acetylcholine receptors (nAChRs). Radioligand binding assays with nAChRs follow slightly different protocols from those outlined in the above sections and are detailed in the following sections, modified from previously published procedures (5, 6).

1.5.1. Cell culture. The six stable cell lines expressing human $\alpha2\beta2$, $\alpha2\beta4$, $\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$, or $\alpha4\beta4$ subtype were established by stably co-transfecting HEK293 cells with a combination of one α nicotinic receptor subtype gene and one β subunit gene. The cell line expressing $\alpha7$ nAChRs were established by stably transfecting HEK293 cells with the rat $\alpha7$ nAChR subunit. Cells are grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, and selective antibiotics at 37°C with 5% CO_2 in a humidified incubator.

1.5.2. Radioligand binding assays. Radioligand binding assays of nAChRs (displacement of [^{3}H]-epibatidine) use the above stably expressed nAChRs as well as rat forebrain tissue. In brief, cells

stably expressing nAChRs are harvested in 50 mM Tris HCl (pH 7.4), washed, homogenized with a Brinkmann polytron homogenizer and centrifuged at 36,000 g. The resulting washed membranes are then incubated with [^{3}H] epibatidine for 4h at room temperature in a final volume of 0.5 ml. Nonspecific binding is assessed in parallel incubations in the presence of 300 μM nicotine. Bound and free ligands are separated by vacuum filtration through Whatman GF/C filters treated with 0.5% polyethylenimine. The filter-retained radioactivity is measured by liquid scintillation counting. Specific binding is defined as the difference between total binding and nonspecific binding.

1.5.3. Primary and secondary nAChR binding assays. Primary binding assays are performed with 100 pM [^{3}H]-epibatidine and a single concentration of a PDSP test compound (10 μM) in quadruplicate. Results are expressed as percentage inhibition of [^{3}H]-epibatidine specific binding. Compounds with a minimum of 25% inhibition are subjected to secondary binding assays to determine binding affinity. In the secondary binding assays, 0.5 nM [^{3}H]-epibatidine and 10 concentrations of the PDSP test compound are used to generate a competition binding curve. Results are analyzed in Prism 5.0 with nonlinear least-square regression to obtain K_i values. Nicotine at 300 μM is included in all assays to define nonspecific binding and a nicotine dose-response curve is included as a positive control in the secondary binding assays.

1.5.4. Nicotinic acetylcholine receptor binding data entry and reporting. Binding assay results with nAChRs are analyzed as indicated in above sections and K_i values are then manually entered into the PDSP database. Figures and raw data are available to corresponding investigators upon request.

1.6. General binding data entry, analysis, and quality control for targets other than nAChRs.
Except for radioligand binding results with nAChRs, whose analysis is outlined in the above sections, binding assays for targets other than nAChRs are analyzed and reported as outlined in the following sections. Raw cpm data from the Microbeta counter are uploaded into the PDSP database and analyzed online using built-in analysis tools in the PDSP database. If necessary, binding assay results are also analyzed in Microsoft Excel (for primary binding results) or Prism v5.0 (for saturation binding and secondary binding results). The following sections provide detailed procedures for binding result

analysis and reporting, using both built-in tools in the PDSP database or third party software (Microsoft Excel and GraphPad Prism).

1.6.1. Result analysis using built-in tools in the PDSP database. The PDSP database is programmed to analyze results from saturation binding and competition binding assays (primary and secondary binding assays). The code for analyzing saturation and competition binding results is written in PHP using external library JQuery UI. The non-linear regression calculation is computed by the R statistical programming language using an Automatic Differentiation Model Builder (ADMB) to support robust non-linear regression calculation. To use built-in tools in the PDSP database to analyze binding assay results, technicians can uploade raw counting results in Excel spreadsheets from the Microbeta counters directly into the PDSP database through corresponding reporting systems designed for saturation binding, primary binding, or secondary binding assays. The online reporting system and analysis tools are designed to optimize data processing and quality control.

1.6.1.1. Saturation binding result analysis. Saturation binding assays are carried out whenever a new membrane preparation is made. The Protein amount per well is adjusted so that less than 10% of the total added radioligand is bound. Upon completion of saturation binding assays, technicians can upload saturation binding results in Excel spreadsheets directly to the membrane pellet section of the PDSP database. During the uploading and reporting process, technicians must provide (1) receptor identity and species information; (2) stable line or transient transfection or animal tissue; (3) amount of protein per well used; (4) radioligand identity and concentration. An automatic email will be sent to the designated PDSP administrator when saturation binding results are submitted and results are placed in a list pending review and approval as indicated in **Figure 4**. The PDSP database is programmed to analyze saturation binding results and generate a hyperbolic curve using a non-linear least squares curve-fitting algorithm to determine B_{max} and K_d values. The PDSP administrator will review and analyze the binding results online with options to (1) reject the whole set of results; (2) exclude apparent outliers among replicates; (3) request a repeat; or (4) approve the data set. **Figure 5** shows a screen captured when a saturation binding assay is being reviewed. When the data set is approved, the PDSP administrator decides on (1) amount of protein per well and (2) radioligand

concentration to use for inhibition binding assays. Upon approval, the saturation binding curve is immediately available in approved membrane pellet section with a unique timestamp associated with each batch of membrane preparation. Technicians make aliquots accordingly so that each aliquot is enough for one binding assay plate at the decided concentration of radioligand. Aliquots are stored in 1.5 ml eppendorf tubes at -80°C until their use at the specified concentration of radioligand and one aliquot per plate.

1.6.1.2. Primary binding assay result analysis. Upon completion of primary binding assays, technicians upload raw results in Excel spreadsheets directly into the PDSP database via the primary binding assay reporting menu. When uploading primary binding results, technicians must identify plates by barcodes and match those generated by the Hamilton STAR liquid handling system when the drug plates were made. A designated PDSP administrator double checks the plate identity and sample order before making the results available online for reviewing. The PDSP database is programmed to calculate the percentage inhibition for each assay plate with total binding (with buffer) as 0% inhibition and nonspecific binding (in the presence of 10 μM of reference compound) as 100% inhibition. It also calculates an average value from 4 replicates for each sample. The PDSP database automatically marks those entries with 50% or higher inhibition for secondary binding assays; also highlights those with variances greater than 20% among the quadruplicate determinations for further inspection.

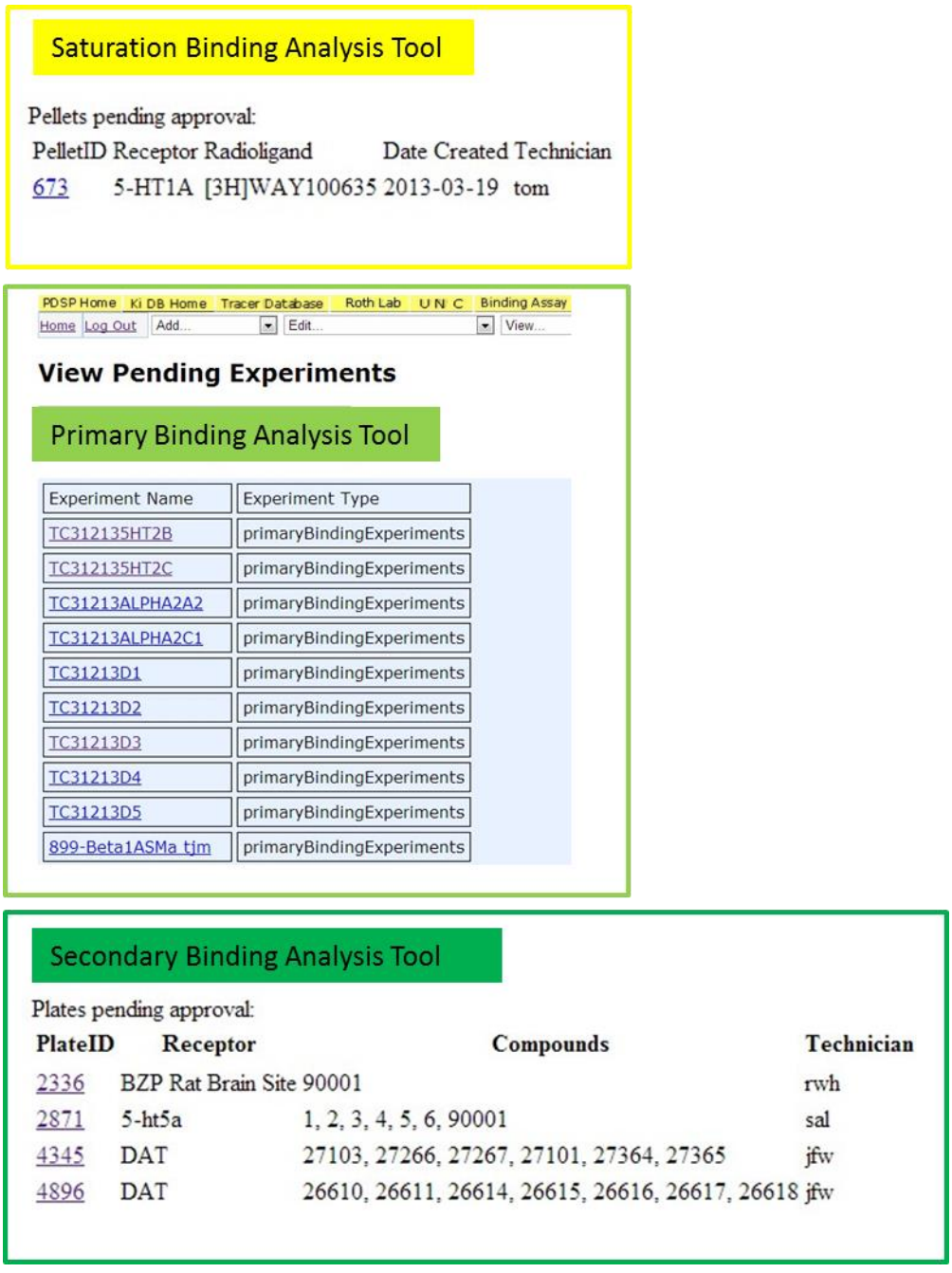


Figure 4. Representative pending lists for Saturation binding (Top), Primary binding (Middle), and Secondary binding (bottom) results. When binding assay results are reported and uploaded into the PDSP database, they are placed in separate pending lists.

Alternatively, compounds with negative inhibition (more binding in the presence of test compound) by over 20% are also highlighted for inspection. For any compound, there are four options available: "Redo" would delete the data from database and put the compound back on the "scheduled" list; "Repeat" would accept the results and put the compound back on the "scheduled" list for a repeat; "Force Secondary" would accept the primary results and add the compound on the "scheduled" list for secondary assays, even if the primary inhibitory percentage is less than 50%; "Approve" would accept the results, and database puts the compound on secondary "schedule" list if primary inhibition is over 50%. Either "Repeat", or "Force Secondary", or "Approve" immediately makes primary binding results available online to investigators. A representative screen capture (**Figure 6**) shows a reviewing screen for primary binding assay results.

1.6.1.3. Secondary binding assay result analysis. Upon completion of secondary binding results, technicians can upload raw results in Excel spreadsheets directly into the PDSP database via the secondary binding assay reporting menu. Differently from primary binding results, the PDSP database analysis tools can accept two types of 96-well drug plate layouts as indicated in **Figures 2a and 2b**, either manually made or made by the Hamilton STAR®. When uploading and submitting secondary binding results, technicians must match drug plate maps and barcodes with those generated by the Hamilton STAR® liquid handling system when the drug plates were made. They also need to chose buffers, radioligand identity, and reference compounds from pull-down menus and enter the total radioligand activity (dpm), specific activity, and K_d values (from saturation binding assay, see above) for the radioligand; the latter values will be used by the PDSP database to calculate the radioligand concentration used for the assay and to convert IC_{50} to K_i values. A representative screen capture (**Figure 7**) is shown below as technicians are uploading secondary binding results for submission. Technicians can view and examine the preliminary curves per plate or individually before submission.

After secondary binding results are submitted, the PDSP database treats results from 3 replicates of 96-well plates as a single file, assigns it a serial number with extra information such as receptor identity and PDSP compound numbers, and places the results in a pending list for review (**Figure 4**).

When PDSP administrators review and analyze the results for approval by clicking on any set of results in the pending list, the PDSP database will parse the uploaded file, and generate an editable table along with a preliminary graph for the purpose of analysis (**Figure 8**). Clicking on individual PDSP compounds leads to a new screen (**Figure 9**) showing a curve of the selected compound along with the reference curve. The raw data for both the selected compound and the reference are shown in tables at left. The PDSP database is also programmed to run a **R**obust regression and **Out**lier removal **(ROUT**) algorithm to identify and highlight potential outliers. PDSP administrators can either exclude or include the potential outliers when reviewing each data set. The raw data tables are also editable and allow for excluding points that are known to be in error, e.g., when a mistake is noticed in pipetting radioligand or membrane by a technician.

As an internal control, each secondary binding assay contains a reference dose-response curve, also in triplicate. The reference K_i value is automatically compared with its historical average value and/or that in our K_i database. If the reference **K_i** is larger than 3 fold different than its historical average value, the entire data set is flagged for further inspection. Senior PDSP scientists work with technicians to determine underlying causes and arrive at solutions. Typically, the secondary binding assay is repeated or the old reference stock is replaced with a fresh one, if the same reference has a low K_i value in multiple data sets.

Each curve has its K_i value listed at the right side and can be marked as either “Redo” or “Accept”. If a curve is marked as “Accept”, a new graph is generated for the PDSP compound, together with a reference curve and the status of the assay is flagged as “completed”; the result is then immediately available online to the investigator. Otherwise, “Redo” puts the compound back on to PDSP “scheduled” list. When an assay is approved, the PDSP database checks whether that compound has been previously tested at the same receptor. If so, database compares the results and generates a warning email if it finds that one K_i value is at least 4x different (0.6 log unit) from another one. All the secondary assays with the same compound will then be put into the “Experiments under review” list. PDSP administrators will review and decide what to do next with the compounds. Options include (1) one more repeat with the same stock; (2) one more repeat with a fresh stock; (3) approval

with no more action since lowest K_i value could be 4x away from highest K_i value, but they are less than 3x away from mean value. If the PDSP database has multiple accepted entries for the same compound, the database will calculate an average from all previous repeats. If a compound shows allosteric potentiation in radioligand binding assays, the result is usually extracted and analyzed separately using Prism v5.0 (See representative figures for 5-HT_{1E} secondary binding below).

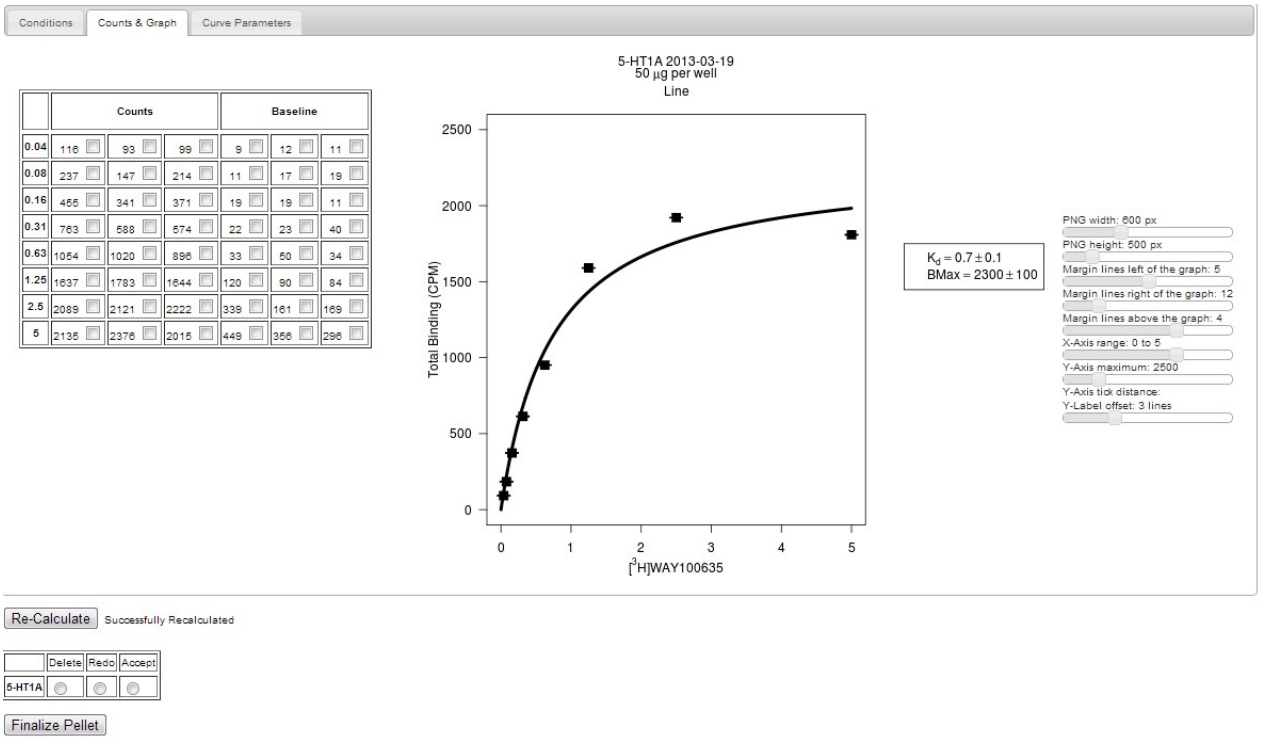


Figure 5. A representative screen capture showing the saturation binding result reviewing process. Both total and nonspecific binding (cpm/well) are shown in the tables on the left. Specific binding is fitted to a hypobolic curve to determine B_{max} (cpm/well) and K_d (nM); both values are listed to the right of the curve. The pellet information is listed on top of the figure, showing that this is a 5-HT_{1A} membrane pellet, made from a stable line, assayed at 50 µg/well protein on March 19, 2013.

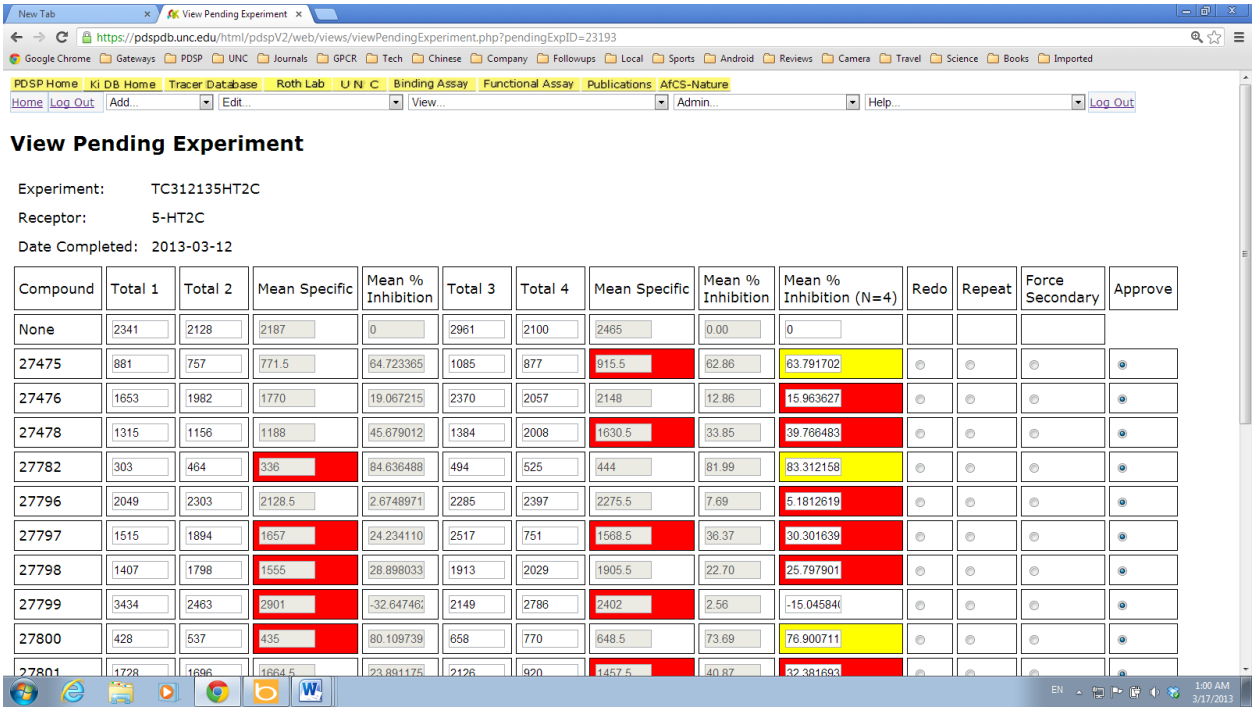
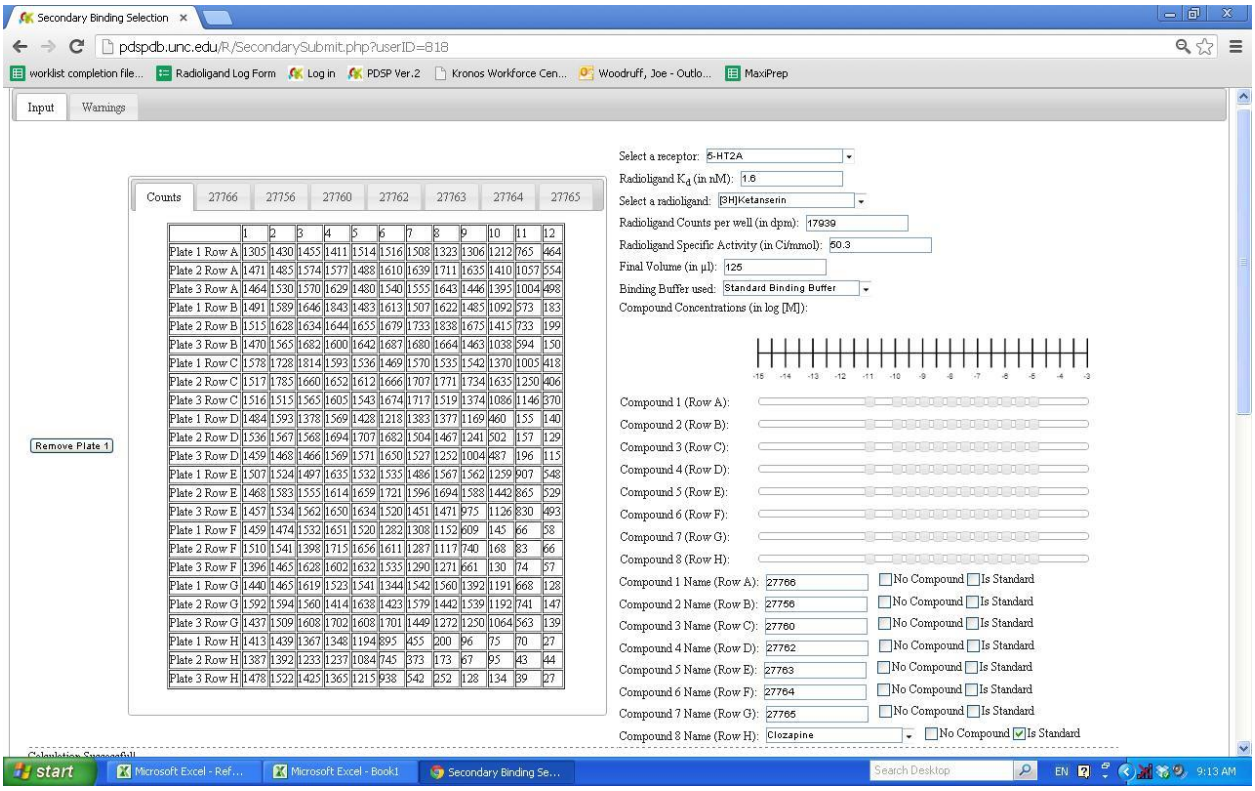


Figure 6. A representative screen capture showing a reviewing screen of primary binding assay results. The barcoded plate is for 5-HT_{2B} binding assays carried out on March 12, 2013. Total binding is shown in the top row and PDSP compounds are shown below. Nonspecific binding is at the bottom of the screen and not shown in the figure. Highlighted in yellow are the the samples with more than 50% inhibition; these samples will be tagged for secondary binding assays automatically. Highlighted in red are samples with more 20% variation among the 4 replicates. There are four options for each compound: “Redo”, “Repeat”, “Force Secondary”, and “Approve”, as indicated at the right side of the screen capture.



	1	2	3	4	5	6	7	8	9	10	11	12
Plate 1 Row A	1305	1430	1455	1411	1514	1516	1508	1323	1306	1212	765	464
Plate 2 Row A	1471	1485	1574	1577	1488	1610	1639	1711	1635	1410	1057	554
Plate 3 Row A	1464	1530	1570	1629	1480	1540	1555	1643	1446	1395	1004	498
Plate 1 Row B	1491	1589	1646	1843	1483	1613	1507	1622	1485	1092	573	183
Plate 2 Row B	1515	1628	1634	1644	1655	1679	1733	1838	1675	1415	733	199
Plate 3 Row B	1470	1565	1682	1600	1642	1687	1680	1664	1463	1038	594	150
Plate 1 Row C	1578	1728	1814	1593	1536	1469	1570	1535	1542	1370	1005	418
Plate 2 Row C	1517	1785	1660	1652	1612	1666	1707	1771	1734	1635	1250	406
Plate 3 Row C	1516	1515	1565	1605	1543	1674	1717	1519	1374	1086	1146	370
Plate 1 Row D	1484	1593	1378	1569	1428	1218	1383	1377	1169	460	155	140
Plate 2 Row D	1536	1567	1568	1694	1707	1682	1504	1467	1241	502	157	129
Plate 3 Row D	1459	1468	1466	1569	1571	1650	1527	1252	1004	487	196	115
Plate 1 Row E	1507	1524	1497	1635	1532	1535	1486	1567	1562	1259	907	548
Plate 2 Row E	1468	1383	1555	1614	1659	1721	1596	1694	1588	1442	865	529
Plate 3 Row E	1457	1534	1562	1650	1634	1520	1451	1471	975	1126	830	493
Plate 1 Row F	1459	1474	1532	1651	1520	1282	1308	1152	609	145	66	58
Plate 2 Row F	1510	1541	1398	1715	1656	1611	1287	1117	740	168	83	66
Plate 3 Row F	1396	1465	1628	1602	1632	1535	1290	1271	661	130	74	57
Plate 1 Row G	1440	1465	1619	1523	1541	1344	1542	1560	1392	1191	668	128
Plate 2 Row G	1592	1594	1560	1414	1638	1423	1579	1442	1539	1192	741	147
Plate 3 Row G	1437	1509	1608	1702	1608	1701	1449	1272	1250	1064	563	139
Plate 1 Row H	1413	1439	1367	1348	1194	895	455	200	96	75	70	27
Plate 2 Row H	1387	1392	1233	1237	1084	745	373	173	67	95	43	44
Plate 3 Row H	1478	1522	1425	1365	1215	938	542	252	128	134	89	27

Figure 7. A representative screen capture showing the final stage in an actual secondary binding result submission. On the left, one complete set of raw data are pooled together from 3 96-well plates (Plate 7, Plate 8, and Plate 9) and organized from Rows A to G from top to bottom with PDSP compound # showing at the top of the table. On the upper right, detailed information for the binding assay is shown, including target receptor identity (5-HT_{2A} receptor in this case), K_d value (1.6 nM) for the radioligand [^{3}H]-ketanserin, total radioactivity (17939 dpm) added to each well, specific activity of the radioligand (50.3 Ci/mmol), and final volume of the assay (125 µl/well) in standard binding buffer. On the lower right, detailed information on the tested compounds (PDSP numbered compounds and reference with corresponding concentrations) isare shown. The concentrations for each compound are set at default values, but can be changed individually by sliding the corresponding bar button, if necessary.

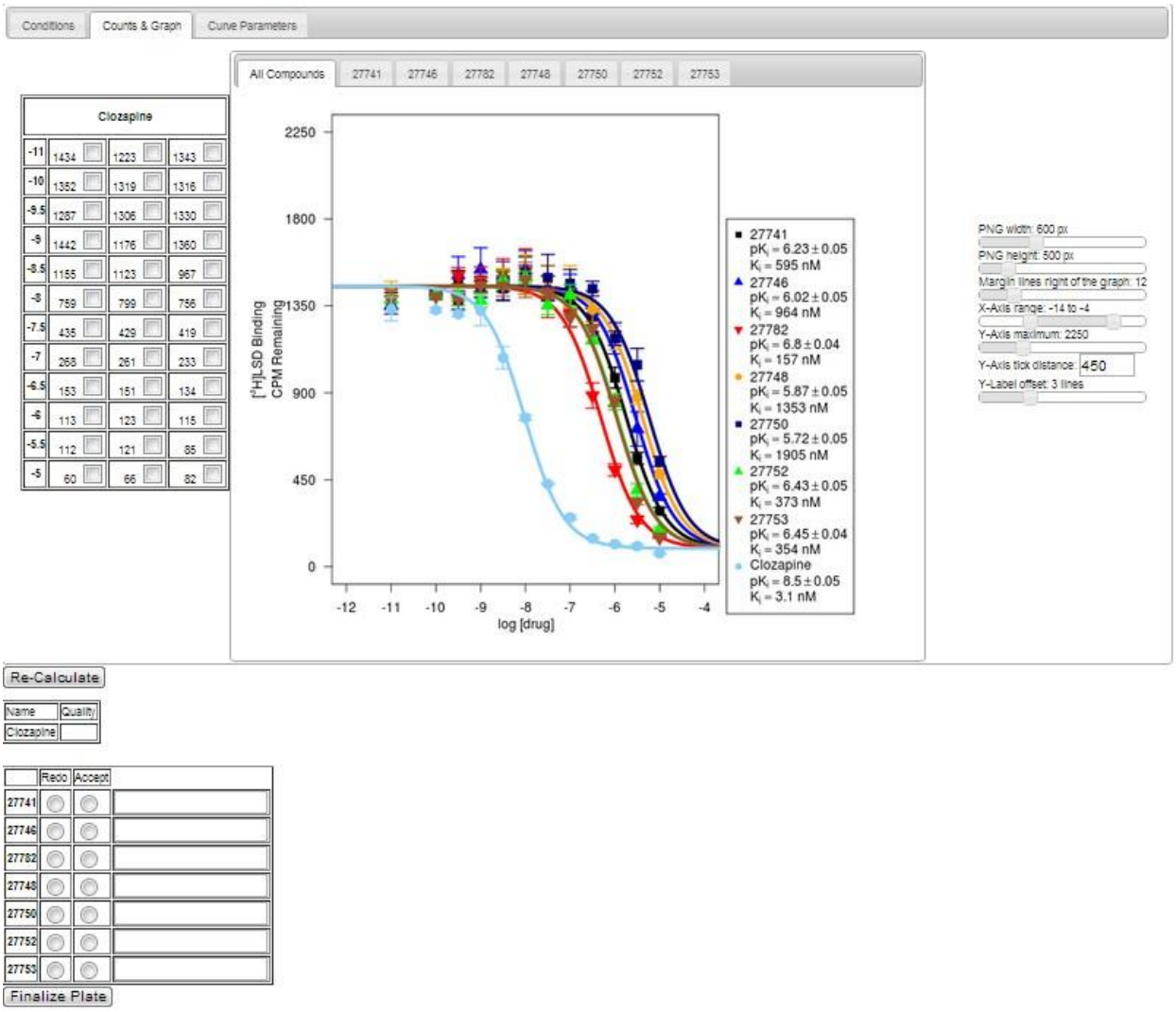


Figure 8. A screen capture showing a representative overview of a set of secondary binding results. On the left, detailed counts (cpm/well) for the reference compounds are shown in a table. In the middle, up to 8 curves are shown on the same graph with pre-determined color scheme with PDSP numbers on top of the graph (one on each tab) and corresponding pK_i and K_i values on the right. Clicking on any PDSP number brings up a graph showing the selected compound and reference, see Figure 9.

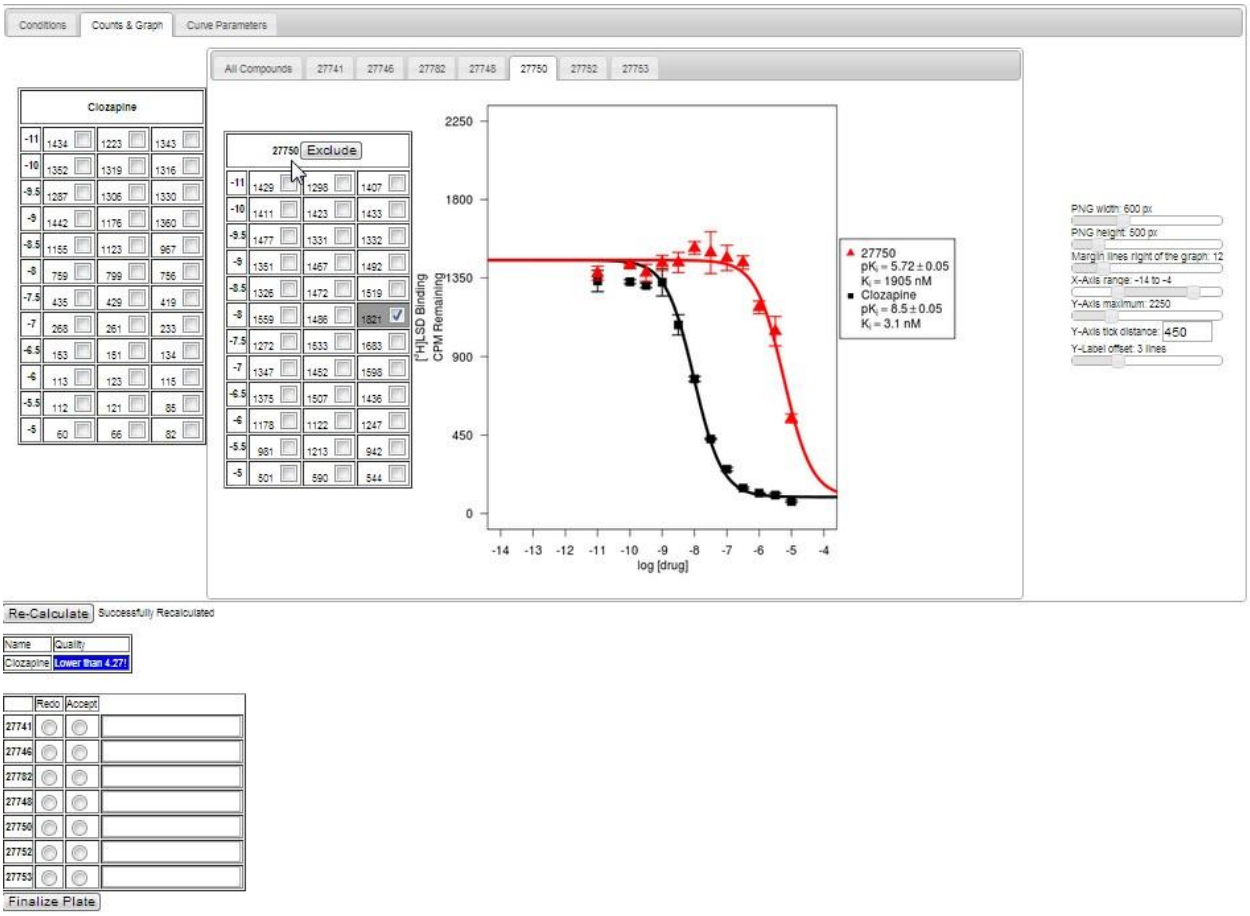


Figure 9. A screen capture showing a representative reviewing process. This screen shows when a PDSP compound is clicked on its tab in **Figure 8**. On the right, detailed counts (cpm/well) for both reference compound and the selected PDSP compound are shown in corresponding tables. One potential outlier has been highlighted (in dark gray with a checkmark "√") by the PDSP database using a robust outlier identification algorithm. In the middle, the selected compound is plotted side by side with the reference compound, with corresponding pK_i and K_i values listed on the right. At the lower left corner, each compound can be marked either "Redo" or "Accept" and there is a box for a brief note.

1.6.2. Binding assay result analysis using Prism v5.0: Binding assay results are also analyzed in Prism v5.0, if necessary, using Prism built-in functions for corresponding assay types. The following sections provide detailed procedures for binding assay data analysis.

1.6.2.1. Saturation binding result analysis. For saturation binding results, total binding and nonspecific binding results are analyzed in Prism v5.0 by fitting results to the following equations to determine B_{max} and K_d values. To do this, total binding values in cpm must be entered in column A and nonspecific binding values in cpm must be entered in column B, corresponding to concentrations of the free radioligand.

$$Nonspecific\ binding = NS * X + Background$$

$$Total\ binding = Nonspecific\ binding + \frac{B_{max} * X}{(X + K_d)}$$

in which "***Nonspecific binding***" and "***Total binding***" are measured radioactivity (cpm per well) in the absence and presence of 10 μM reference compound, respectively, at corresponding concentration [**X**] in nM. **B_{max}** is receptor expression level in cpm/well or fmol/mg protein; **K_d** is the equilibrium binding affinity, corresponding to the radioligand concentration at which point 50% of **B_{max}** is bound to the radioligand; NS and Background are two fitting values for nonspecific binding and background counts.

1.6.2.2. Primary binding result analysis. For primary binding results, non-specific binding in the presence of 10 μM of an appropriate reference compound is set as 100% inhibition; total binding in the absence of test compound or reference compound is set as 0% inhibition. The radioactivity in the presence of test compound is calculated with the following equation and expressed as a percentage inhibition:

$$\%\ Inhibition = 100 - \frac{Sample\ cpm - Nonspecific\ cpm}{Total\ cpm - nonspecific\ cpm} \times 100$$

1.6.2.3. Secondary binding result analysis. For secondary binding results, counts (cpm/well) are pooled and fitted to a three-parameter logistic function for competition binding in Prism v 5.0 to determine IC_{50} values,

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{X - LogIC_{50}}}$$

in which **Y** is the total binding in the presence of corresponding concentration of test compound (**X**); **Top** and **Bottom** are the total and nonspecific binding in the absence and presence of 10 μM reference antagonist; **IC_{50}** is the concentration at which 50% observed binding was inhibited and is converted to K_i according to the Cheng-Prusoff equation (7),

$$K_i = \frac{IC_{50}}{1 + \frac{L}{K_d}} \text{ or } LogK_i = LogIC_{50} - Log(1 + \frac{L}{K_d})$$

in which **L** is the radioligand concentration used in the competition binding assay; **K_d** is the radioligand equilibrium binding affinity determined in above saturation binding assays. In the curve-fitting analysis, top and bottom values are shared among all binding curves from the same plate if necessary.

1.7. Tables of binding assay conditions and representative figures

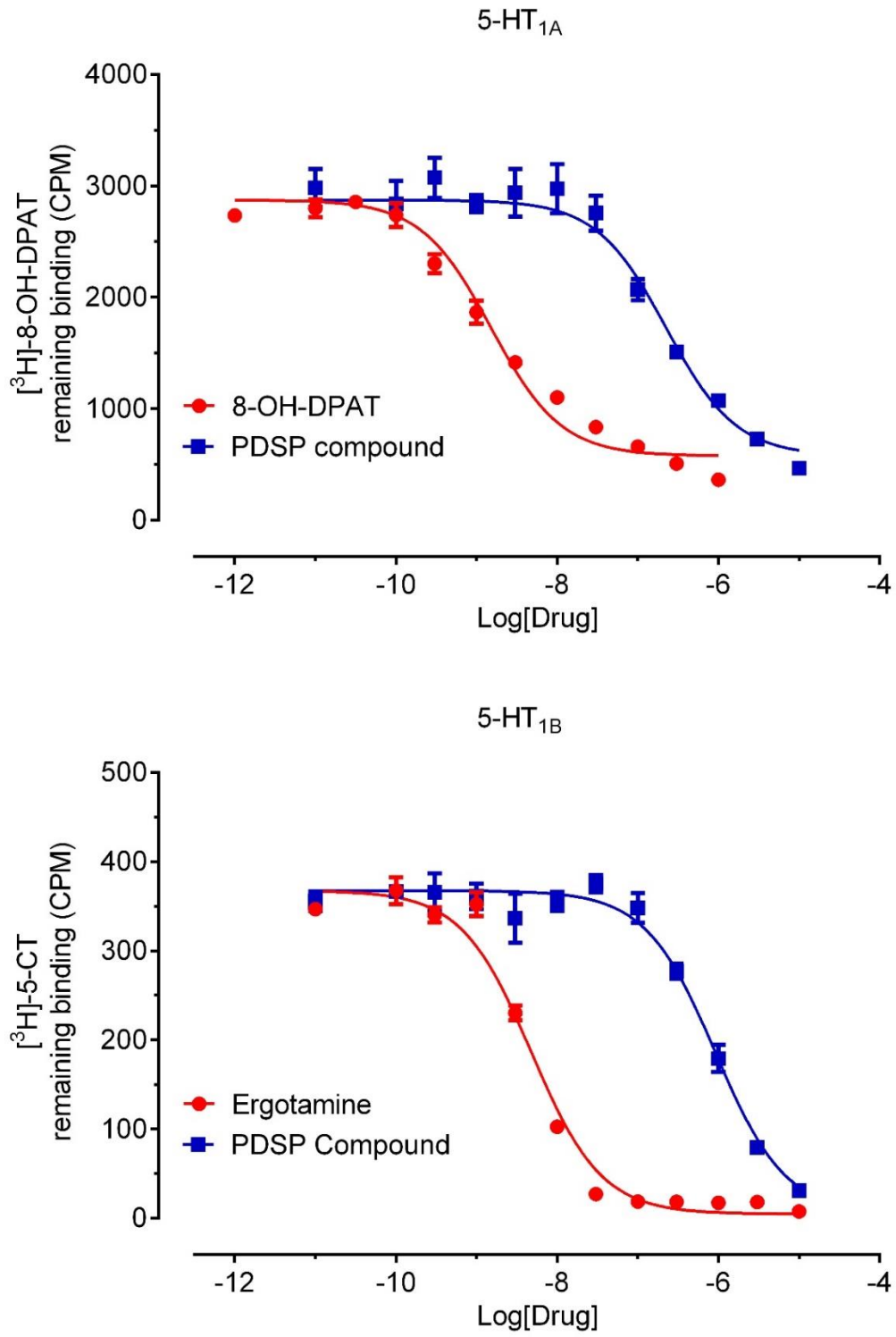
Table 2. 5-HT receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. BB for binding buffer; WB for wash buffer. Historical reference K_i values from the last 2 years are also listed.

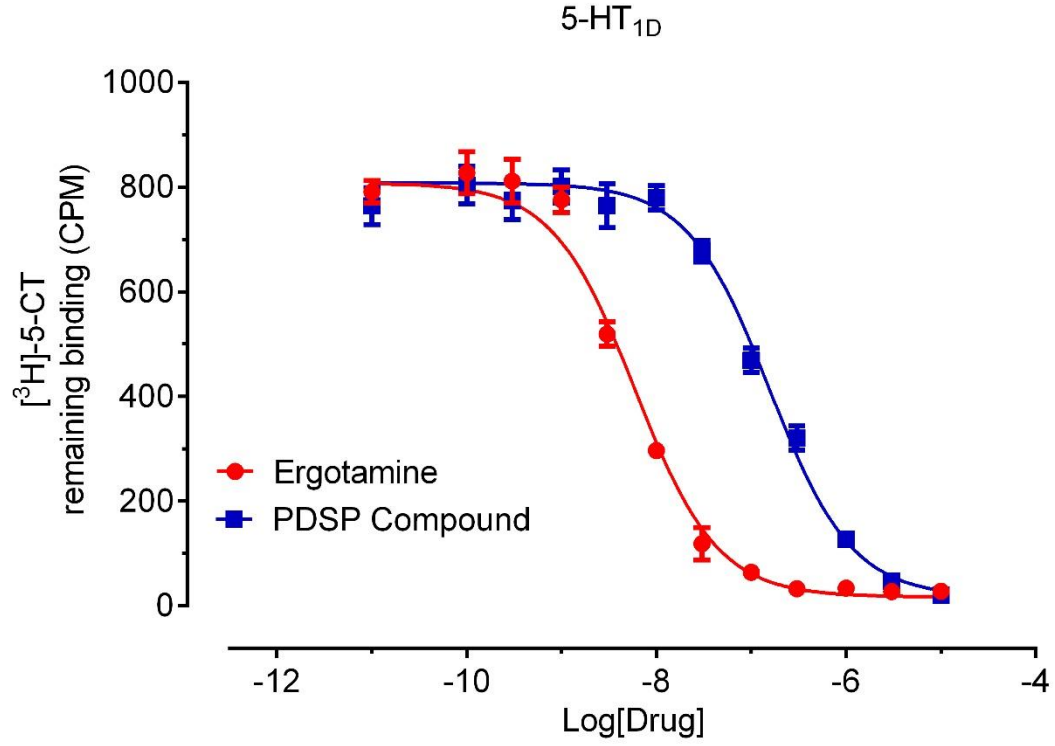
5-HT receptors

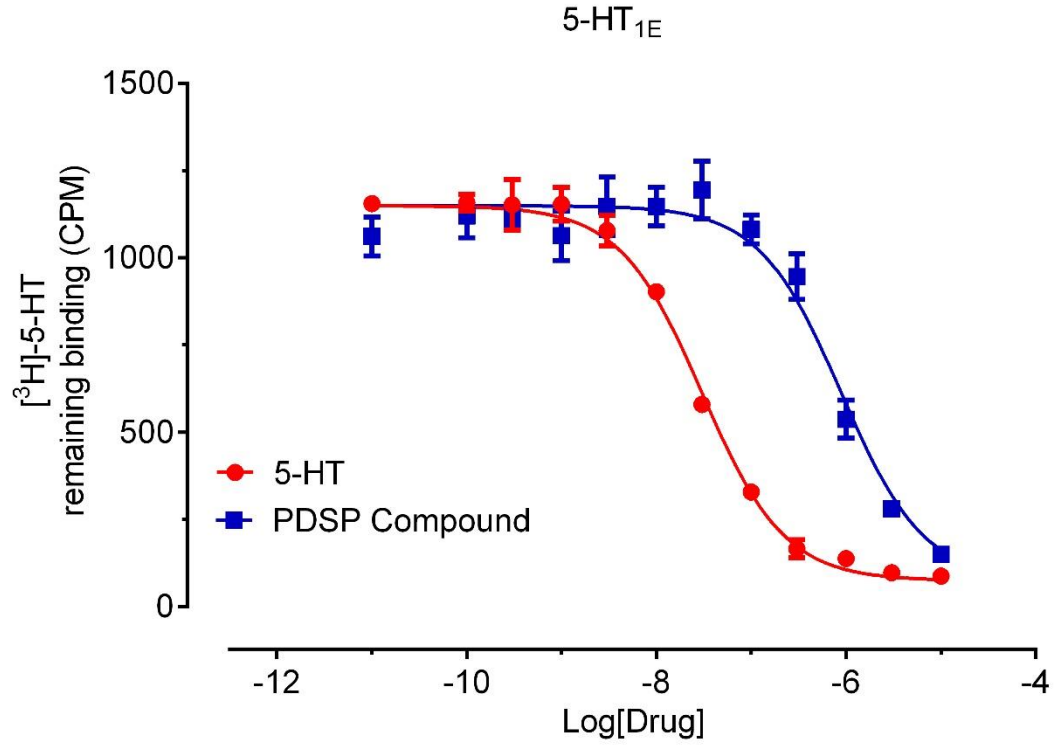
Standard BB (SBB): 50 mM Tris HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4, RT
Standard WB (SWB): 50 mM Tris HCl, pH 7.4, cold

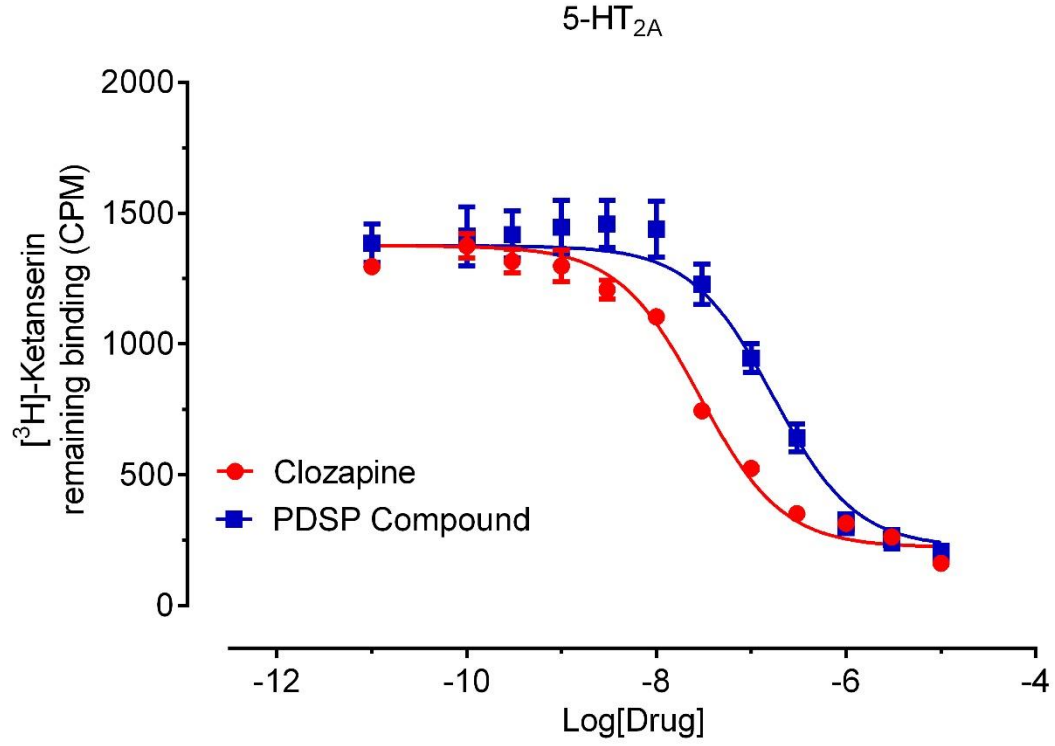
Target	Radioligand $pK_d \pm SEM$ (K_d, nM)	Radioligand used (nM)	Reference Ligand $pK_i \pm SEM$ (K_i, nM)	Literature
5-HT_{1A}	[^{3}H]-Way100635 9.30 ± 0.04 (0.50)	0.5 – 1.0	8-OH-DPAT 9.18 ± 0.02 (0.66)	(8)
5-HT_{1B}	[^{3}H]5-CT 9.06 ± 0.09 (0.87)	0.6 – 1.5	Ergotamine 8.84 ± 0.03 (1.44)	(9, 10)
5-HT_{1D}	[^{3}H]5-CT 9.06 ± 0.06 (0.86)	1.0 – 2.0	Ergotamine 8.32 ± 0.02 (4.83)	(9, 10)
5-HT_{1E}	[^{3}H]5-HT 8.42 ± 0.06 (3.82)	2.1 – 5.0	5-HT 8.05 ± 0.03 (8.82)	(11–13)
5-HT_{1F}				
5-HT_{2A}	[^{3}H]-Ketanserin 8.92 ± 0.04 (1.20)	1.2 – 2.4	Clozapine 8.23 ± 0.01 (5.91)	(14–16)
5-HT_{2B}	[^{3}H]-LSD 8.93 ± 0.03 (1.19)	1.0 – 2.0	SB206553 7.84 ± 0.01 (14.39)	(17–19)
5-HT_{2C}	[^{3}H]-Mesulergine 5.85 ± 0.06 (2.66)	1.0 – 2.5	Ritanserin 8.84 ± 0.02 (1.44)	(17, 20)
5-HT_3	[^{3}H]GR65630 8.46 ± 0.14 (3.48)	1.0 – 2.0	Zacopride 9.23 ± 0.02 (0.58)	(21, 22)
5-HT_4	[3H]GR113808 8.66 ± 0.17 (2.19)	0.5 – 2.0	SDZ205557 7.94 ± 0.09 (11.44)	(23–27)
5-HT_{5A}	[^{3}H]-LSD 8.72 ± 0.03 (1.91)	2.0 - 3.3	Ergotamine 7.61 ± 0.03 (24.60)	(28, 29)
5-HT_6	[^{3}H]-LSD 8.37 ± 0.04 (4.27)	2.0 – 4.0	Clozapine 8.28 ± 0.02 (5.27)	(30)
5-HT_{7A}	[^{3}H]-LSD 8.13 ± 0.06 (7.48)	5.0 – 6.0	Clozapine 7.99 ± 0.02 (10.33)	(31)

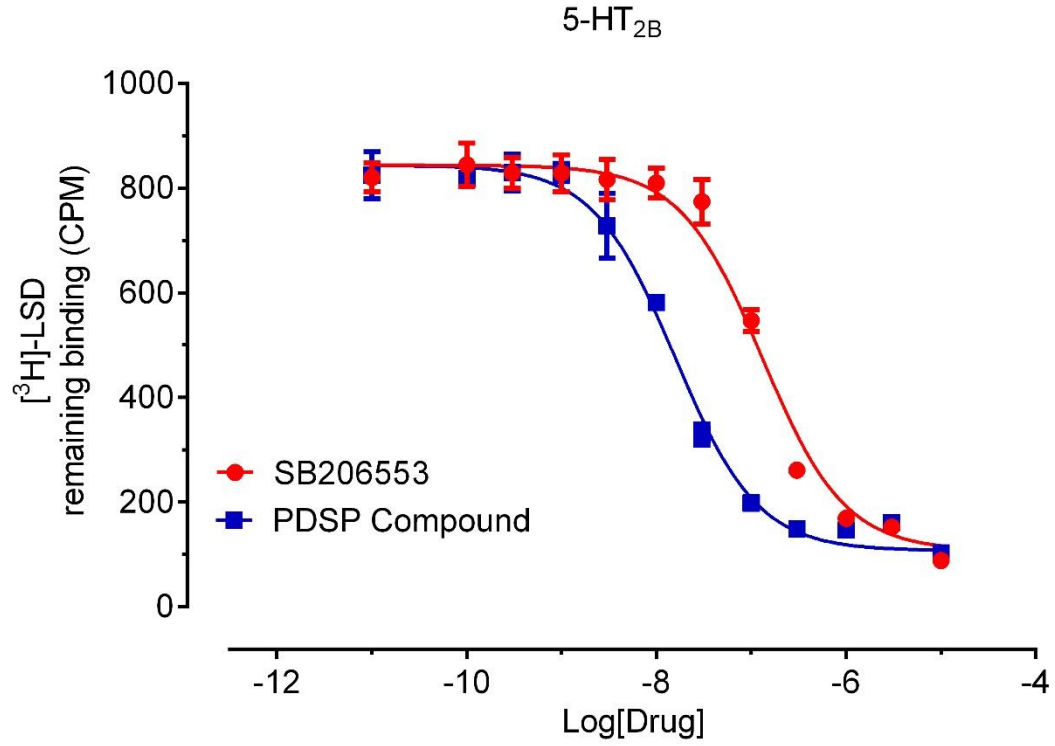
Figure 10. Representative competition binding curves with 5-HT receptors

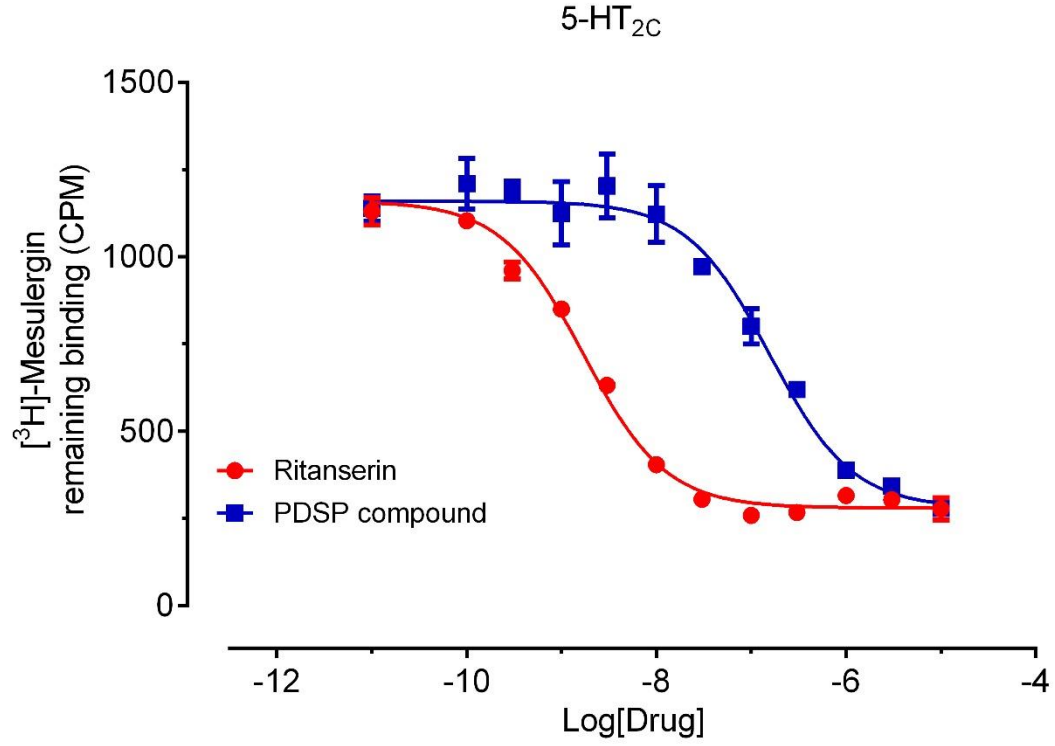


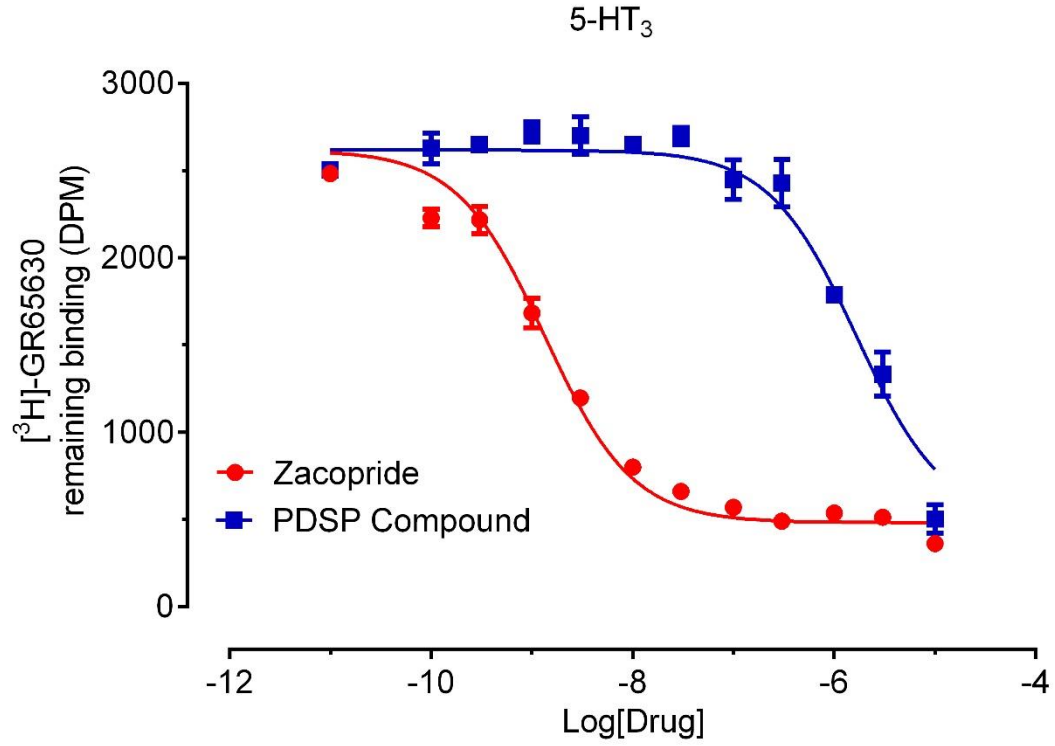

5-HT1D
[3H]-5-CT
remaining binding (CPM)
1000
800
600
400
200
0
Ergotamine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

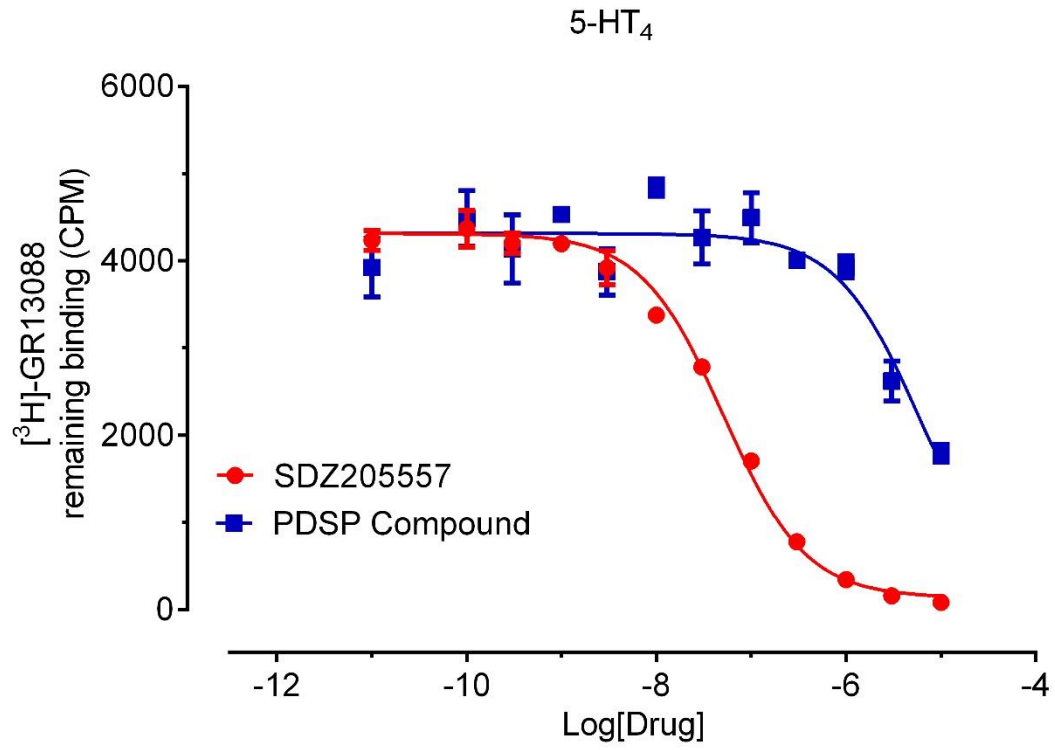

5-HT1E
[3H]-5-HT
remaining binding (CPM)
1500
1000
500
0
5-HT
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

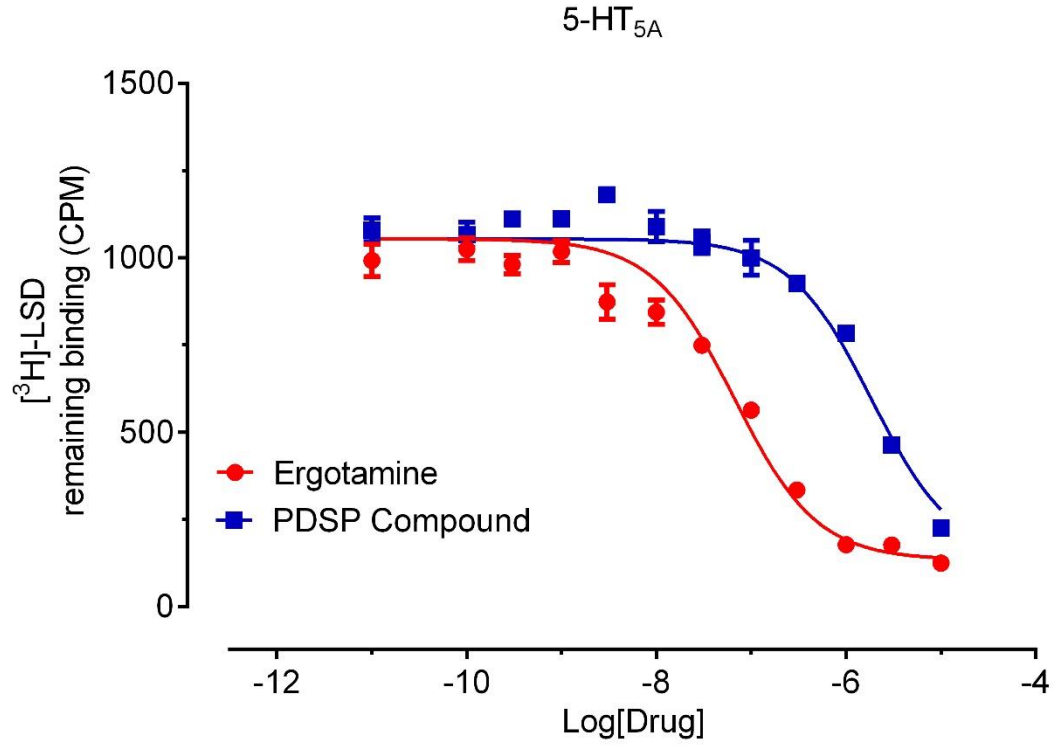

5-HT2A
[3H]-Ketanserin
remaining binding (CPM)
2000
1500
1000
500
0
Clozapine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

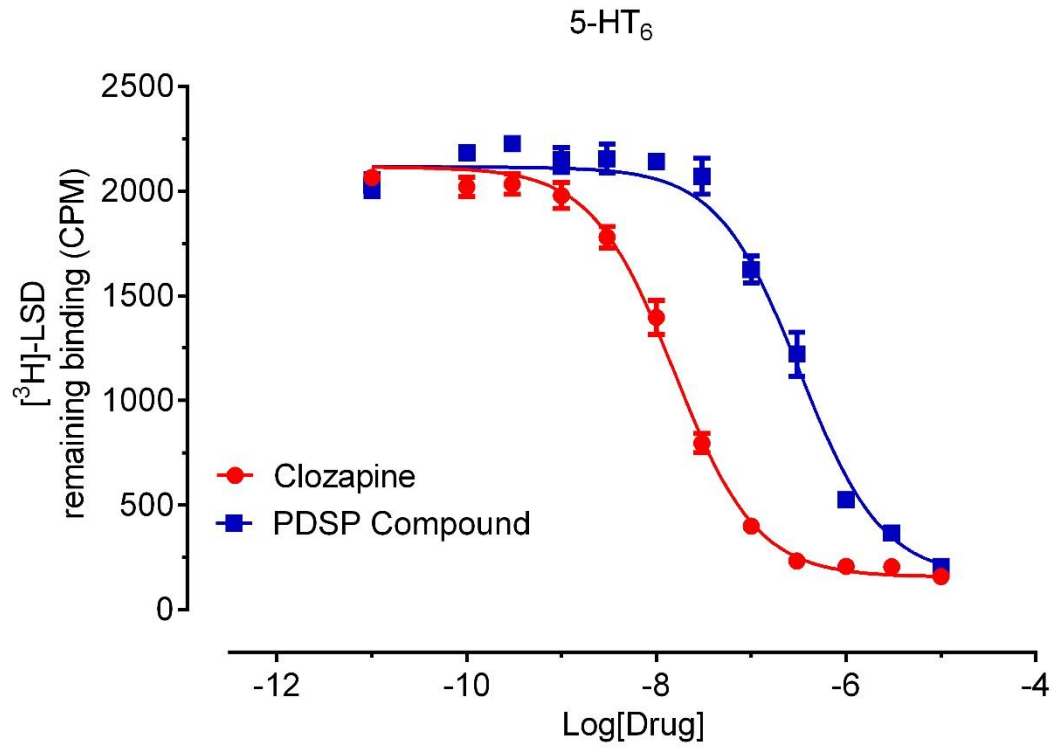

5-HT2B
[3H]-LSD
remaining binding (CPM)
1000
800
600
400
200
0
SB206553
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


5-HT2C
1500
1000
500
0
[3H]-Mesulergin
remaining binding (CPM)
Ritanserin
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]


5-HT3
3000
2000
1000
0
[3H]-GR65630
remaining binding (DPM)
Zacopride
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


5-HT4
6000
4000
2000
0
[3H]-GR13088
remaining binding (CPM)
SDZ205557
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


5-HT5A
1500
1000
500
0
[3H]-LSD
remaining binding (CPM)
Ergotamine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


5-HT6
2500
2000
1500
1000
500
0
[3H]-LSD
remaining binding (CPM)
Clozapine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

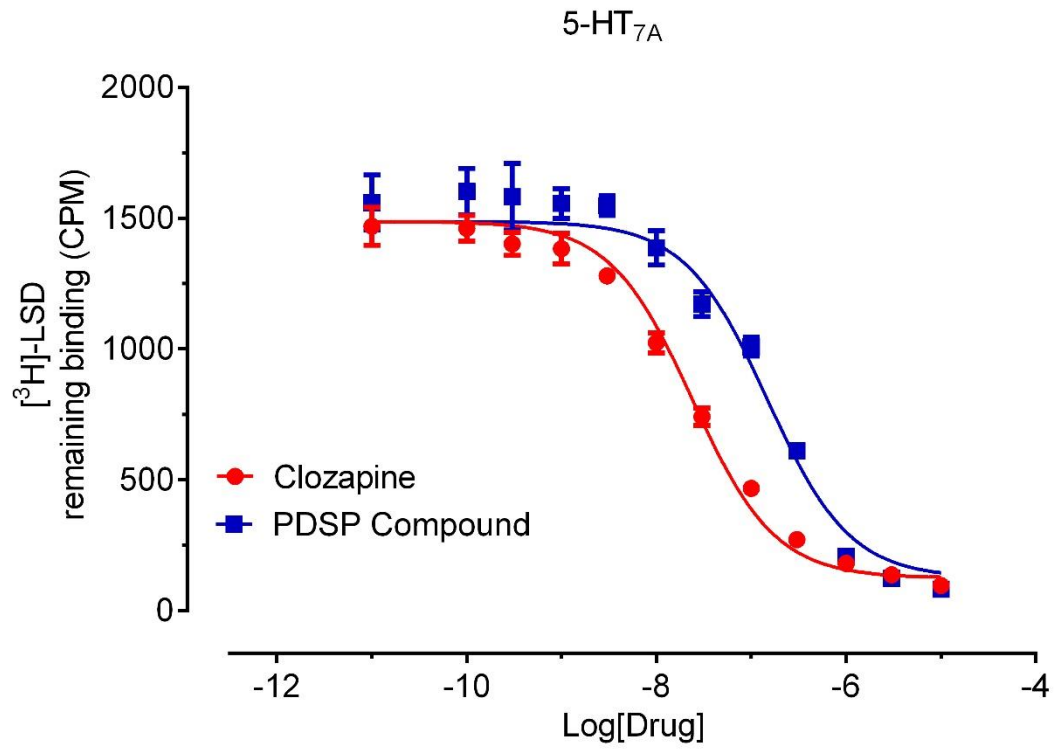

5-HT7A
2000
1500
1000
500
0
[3H]-LSD
remaining binding (CPM)
Clozapine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

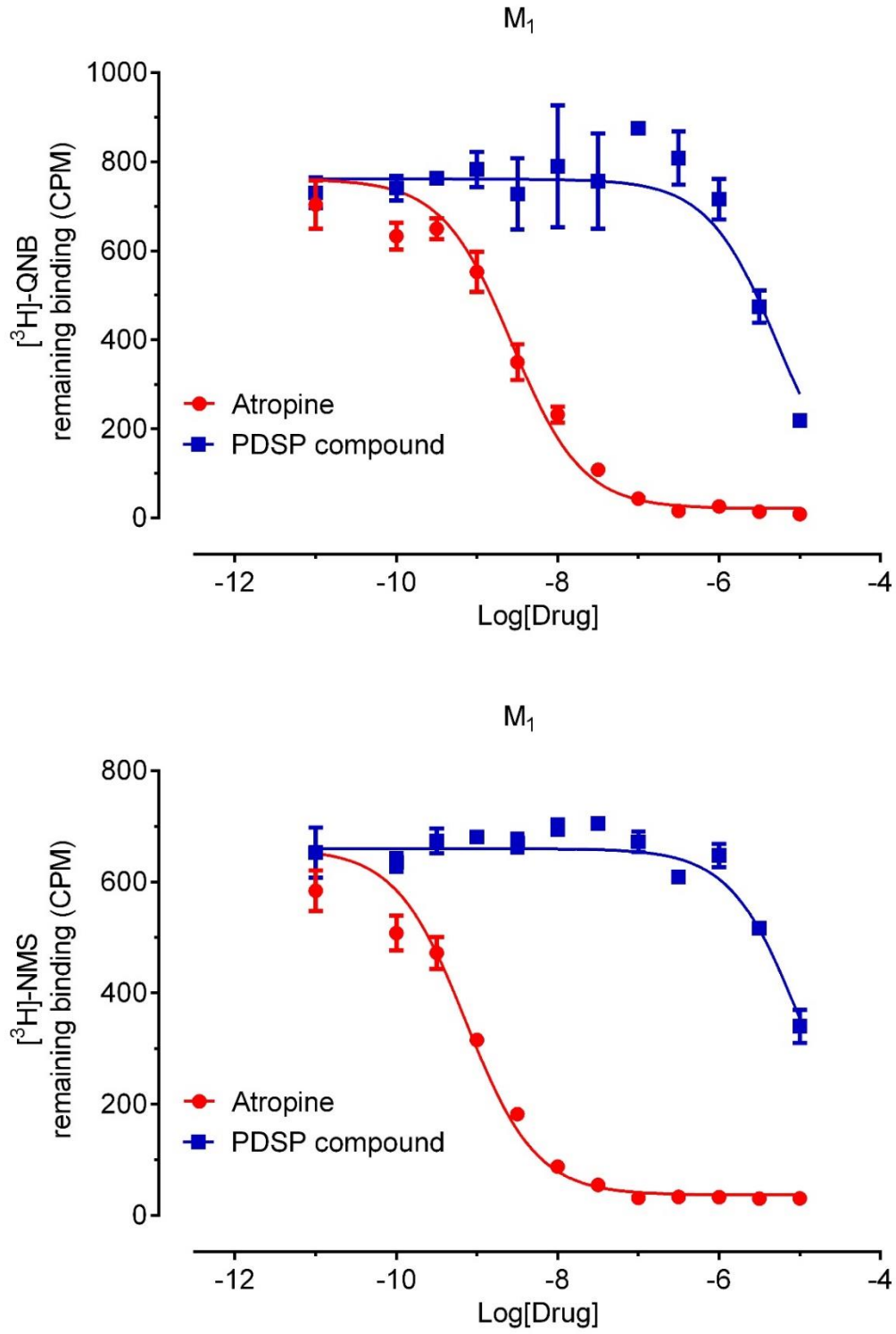
Table 3. Muscarinic acetylcholine receptors, radioligands and corresponding K_d values, reference compounds, and buffers for primary and secondary radioligand binding assays. BB for binding buffer; WB for wash buffer. Historical reference K_i values from over last years are also included.

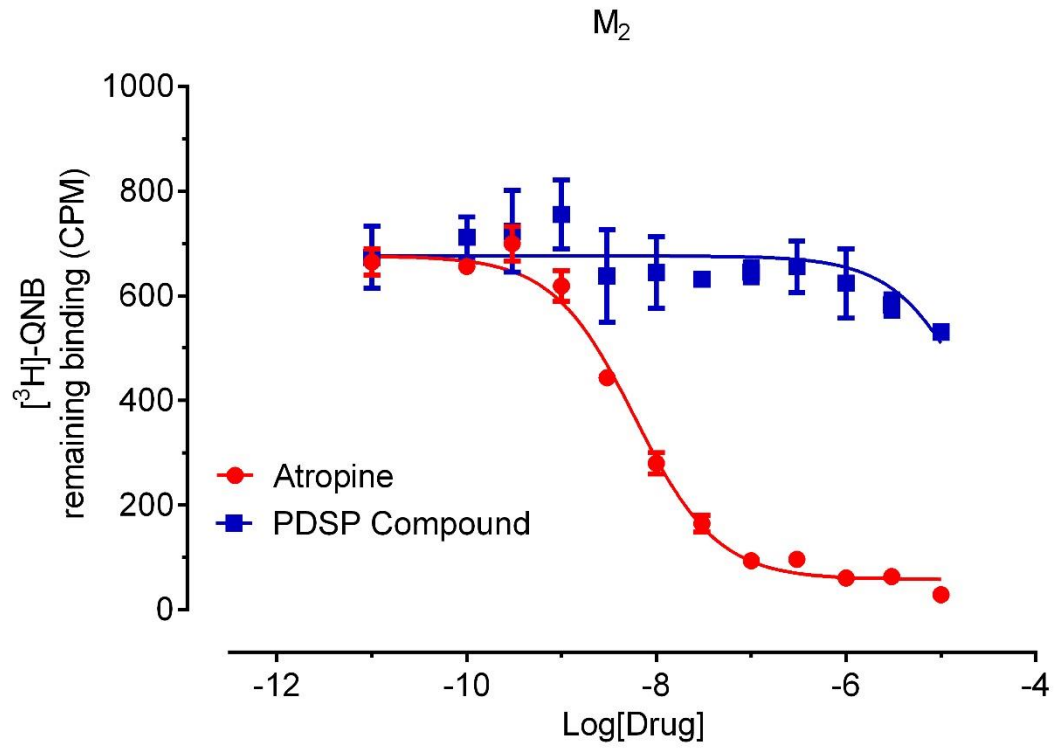
Muscarinic acetylcholine receptors

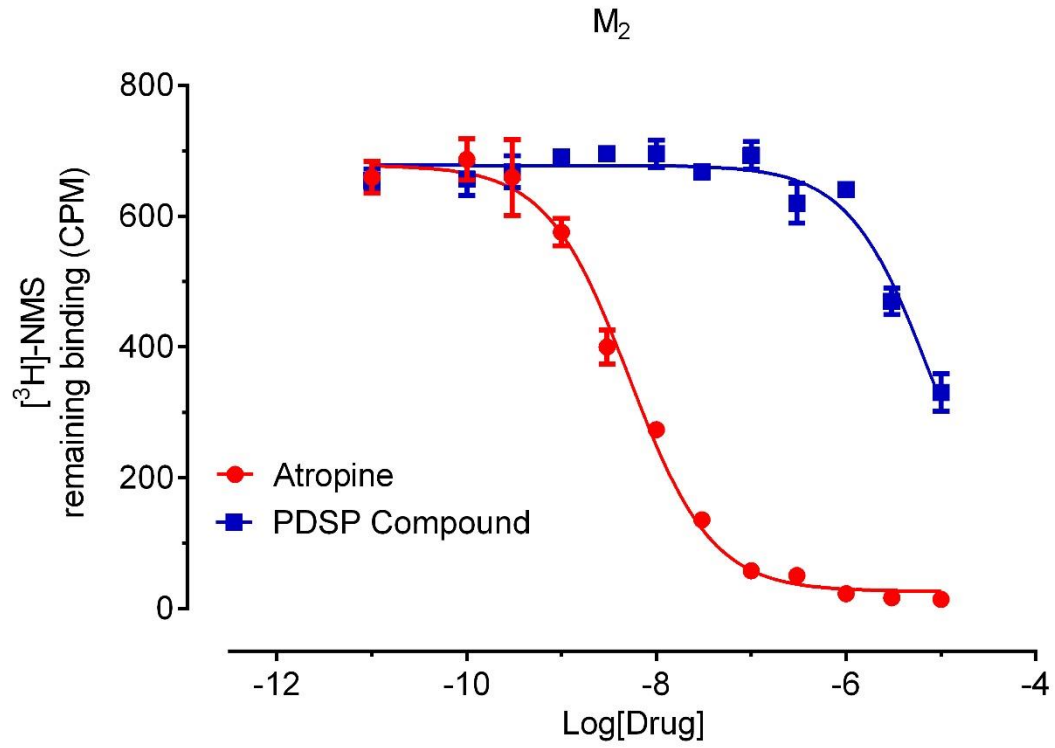
Muscarinic BB (MBB) #1: 50 mM Tris HCl, pH 7.7, RT + SWB
Muscarinic BB (MBB) #2: 25 mM Sodium Phosphate, 5 mM $MgCl_2$, pH 7.4, RT (cold for washing)
Muscarinic wash buffer #2: Same as muscarinic binding buffer #2, cold

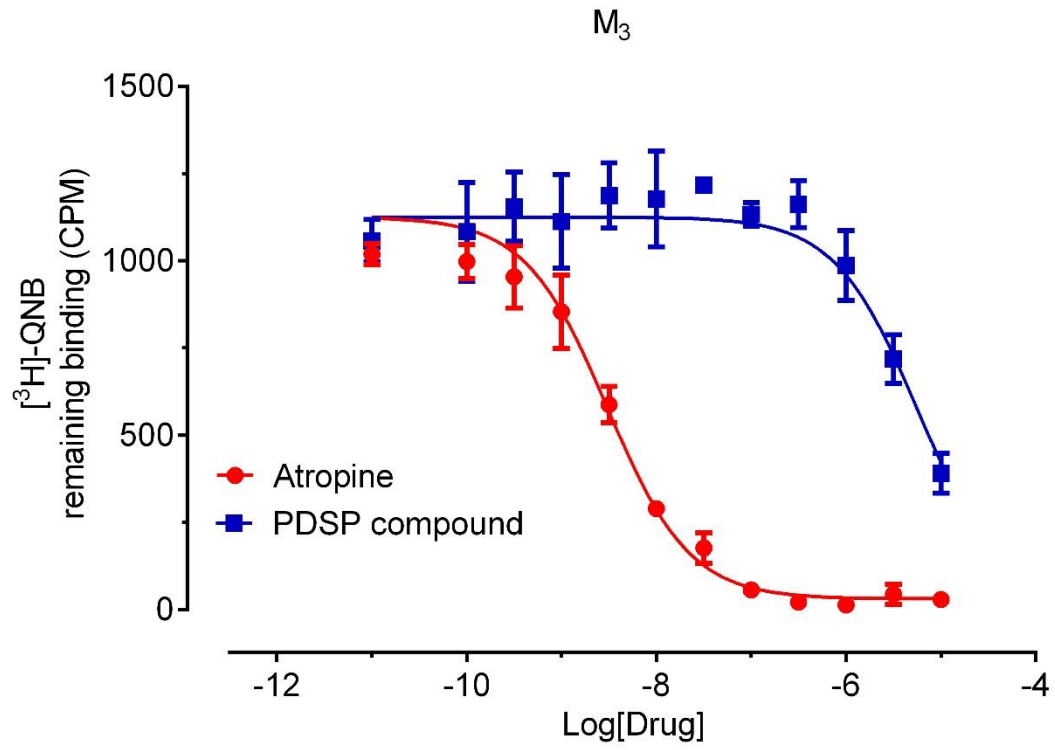
Target	Radioligand $pK_d \pm SEM$ (K_d, nM)	Radioligand used (nM)	Reference Ligand $pK_i \pm SEM$ (K_i, nM)	Literature
M_1	[^{3}H]-QNB 9.21 ± 0.18 (0.62)	0.3 – 1.2	Atropine 8.92 ± 0.03 (1.21)	(32, 33)
M_1	[^{3}H]-NMS 9.26 ± 0.21 (0.54)	0.5 – 0.9	Atropine 8.86 ± 0.08 (1.39)	(34, 35)
M_2	[^{3}H]-QNB 9.57 ± 0.05 (0.27)	0.2 – 1.0	Atropine 8.63 ± 0.03 (2.36)	(32)
M_2	[^{3}H]-NMS 9.67 ± 0.10 (0.21)	0.2 - 1.0	Atropine 9.11 ± 0.34 (0.77)	(34)
M_3	[^{3}H]-QNB 9.37 ± 0.06 (0.43)	0.3 – 1.0	Atropine 9.30 ± 0.03 (0.50)	(32)
M_3	[^{3}H]-NMS 9.53 ± 0.08 (0.30)	0.3 – 1.0	Atropine 9.19 ± 0.12 (0.64)	(34)
M_3D	[^{3}H]-QNB 9.13 ± 0.19 (0.74)	0.5 – 1.0	Atropine 8.30 ± 0.12 (4.97)	(36, 37)
M_4	[^{3}H]-QNB 9.64 ± 0.06 (0.23)	0.3 – 1.0	Atropine 9.25 ± 0.04 (0.56)	(32, 33)
M_4	[^{3}H]-NMS 9.72 ± 0.11 (0.19)	0.3-1.0	Atropine 8.89 ± 0.07 (1.28)	(33, 34)
M_4D	[^{3}H]-QNB 8.74 ± 0.14 (1.80)	1.0 – 2.5	Atropine 8.46 ± 0.08 (3.47)	(37, 38)
M_5	[^{3}H]-QNB 9.31 ± 0.06 (0.49)	0.2 – 1.0	Atropine 9.26 ± 0.02 (0.55)	(34)
M_5	[^{3}H]-NMS 9.49 ± 0.05 (0.32)	0.2 – 1.0	Atropine 8.99 ± 0.08 (1.03)	(34, 39)

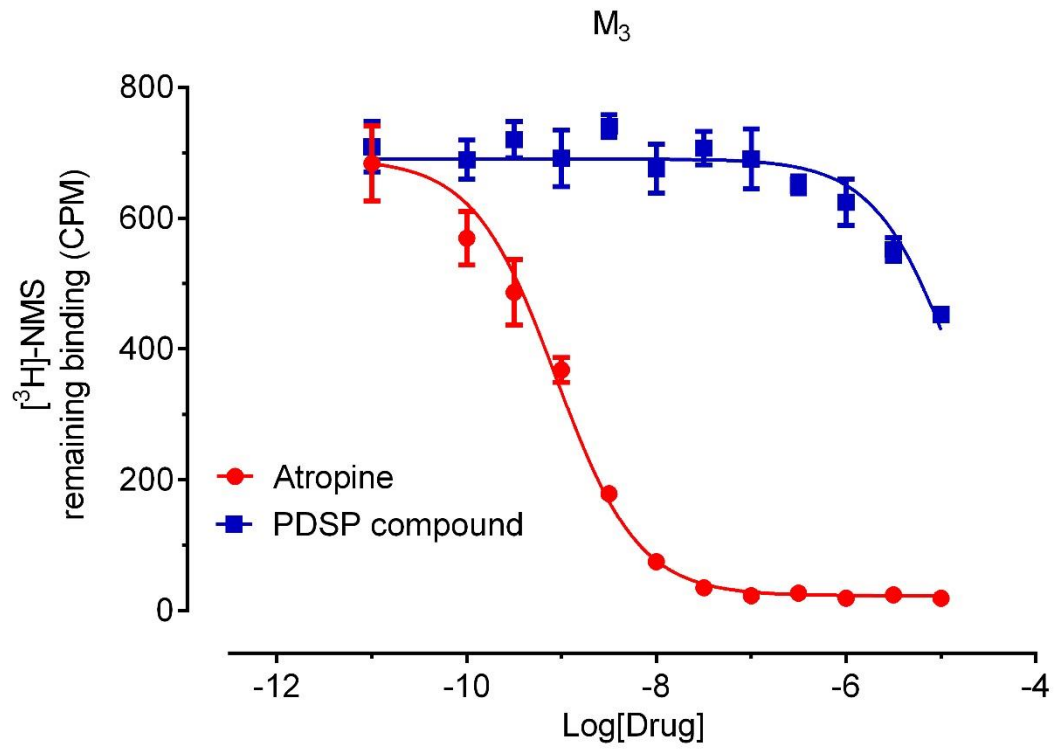
Figure 11. Representative competition binding curves with muscarinic acetylcholine receptors

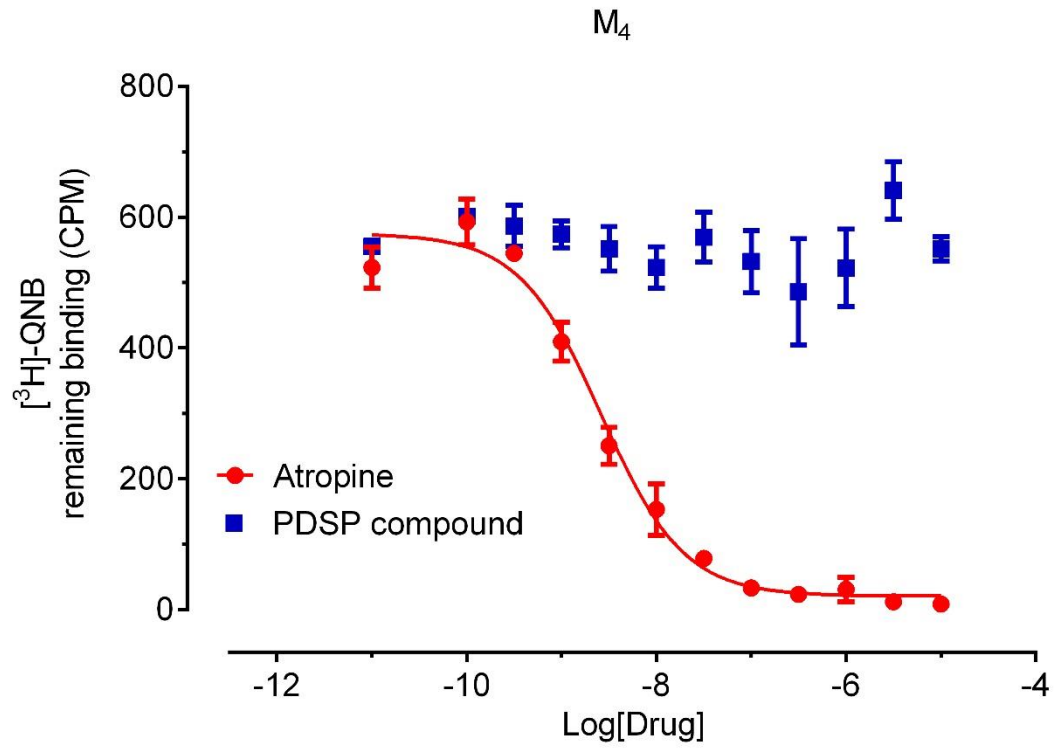


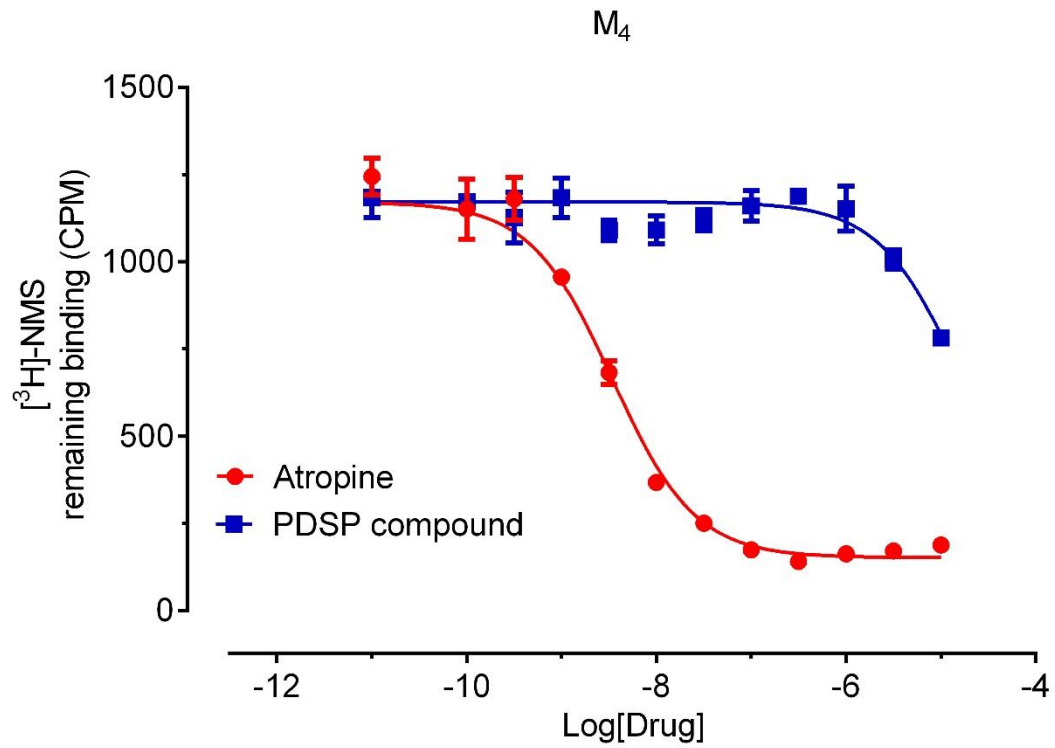

M2
1000
800
600
400
200
0
[3H]-QNB
remaining binding (CPM)
Atropine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

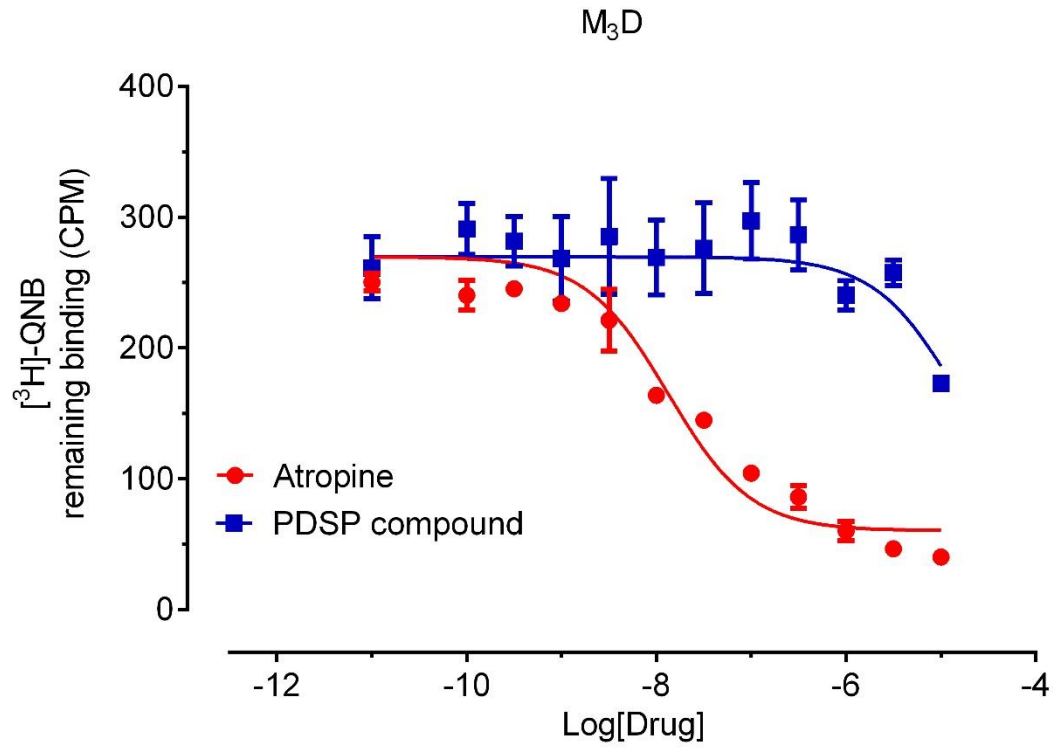

M2
800
600
400
200
0
[3H]-NMS
remaining binding (CPM)
Atropine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

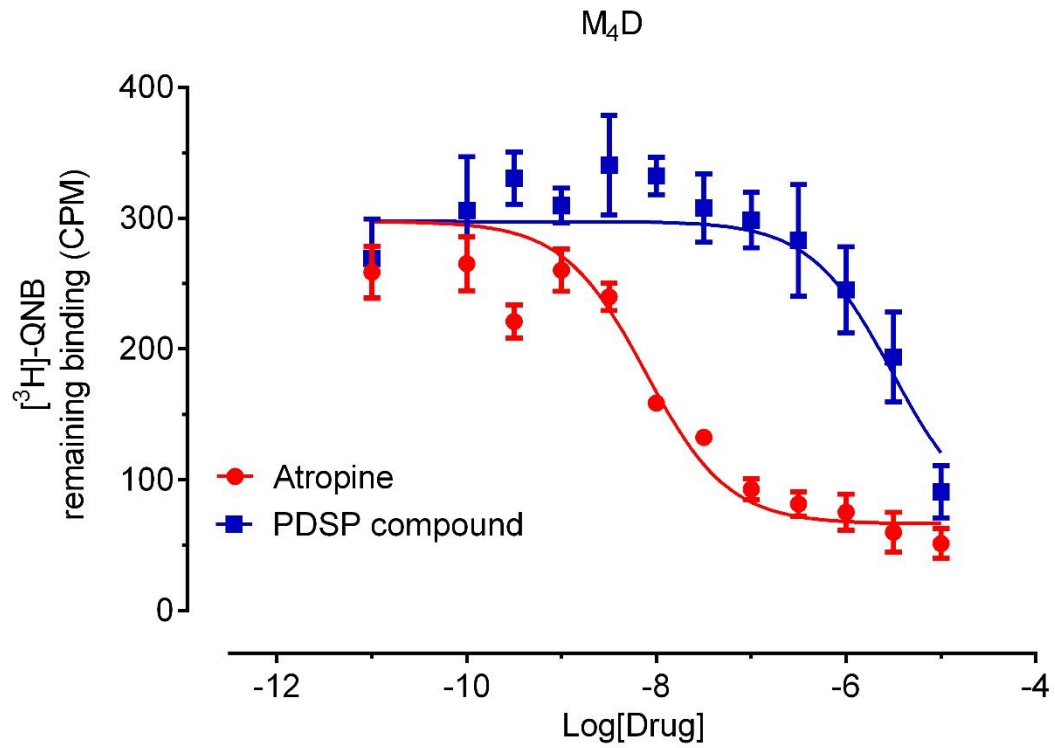

M3
1500
1000
500
0
[3H]-QNB
remaining binding (CPM)
Atropine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

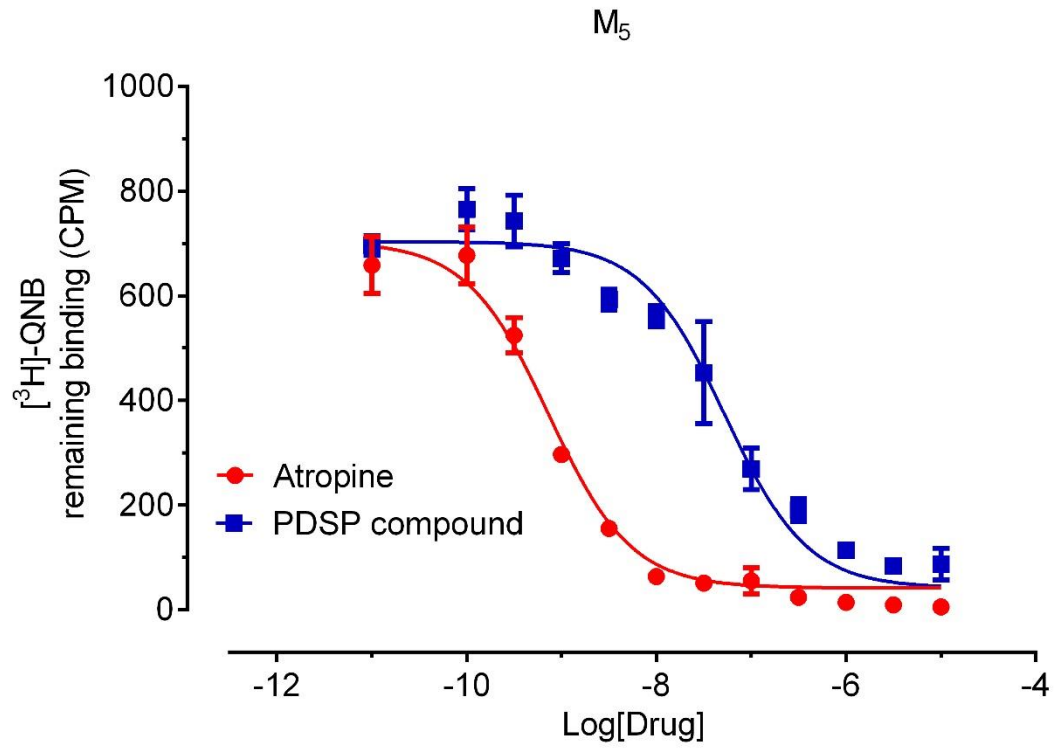

M3
800
600
400
200
0
[3H]-NMS
remaining binding (CPM)
Atropine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]


M4
800
600
400
200
0
[3H]-QNB
remaining binding (CPM)
Atropine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]


M4
1500
1000
500
0
[3H]-NMS
remaining binding (CPM)
Atropine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]


M3D
400
300
200
100
0
[3H]-QNB
remaining binding (CPM)
Atropine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]


M4D
400
300
200
100
0
[3H]-QNB
remaining binding (CPM)
Atropine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]


M5
[3H]-QNB
remaining binding (CPM)
1000
800
600
400
200
0
Atropine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

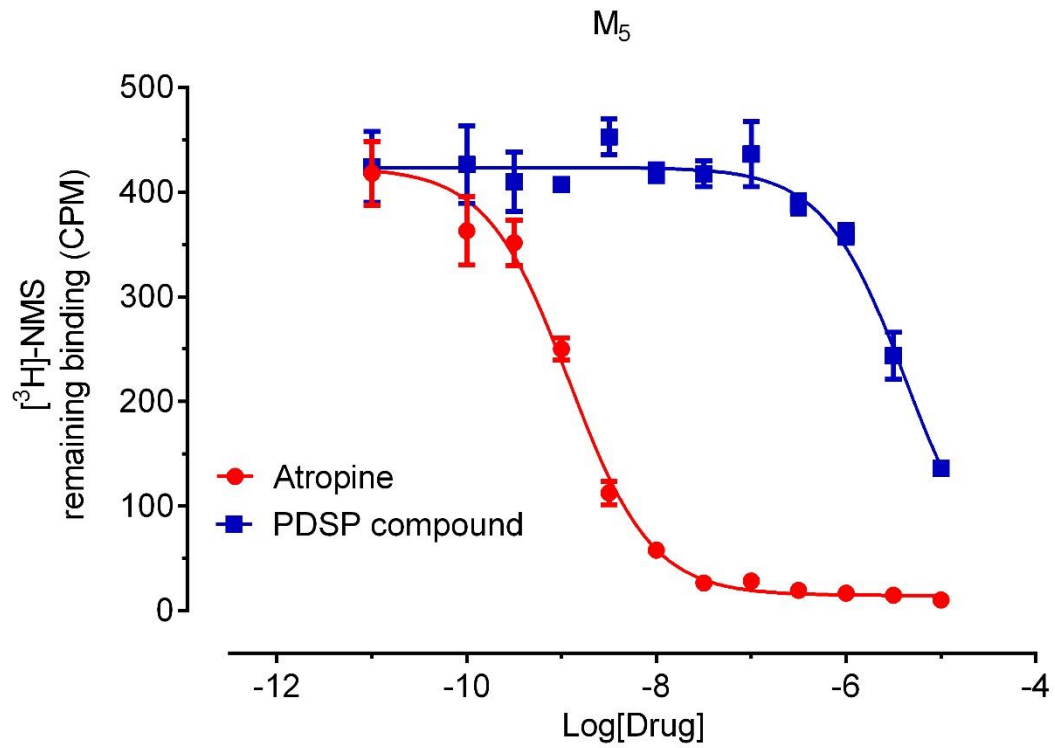
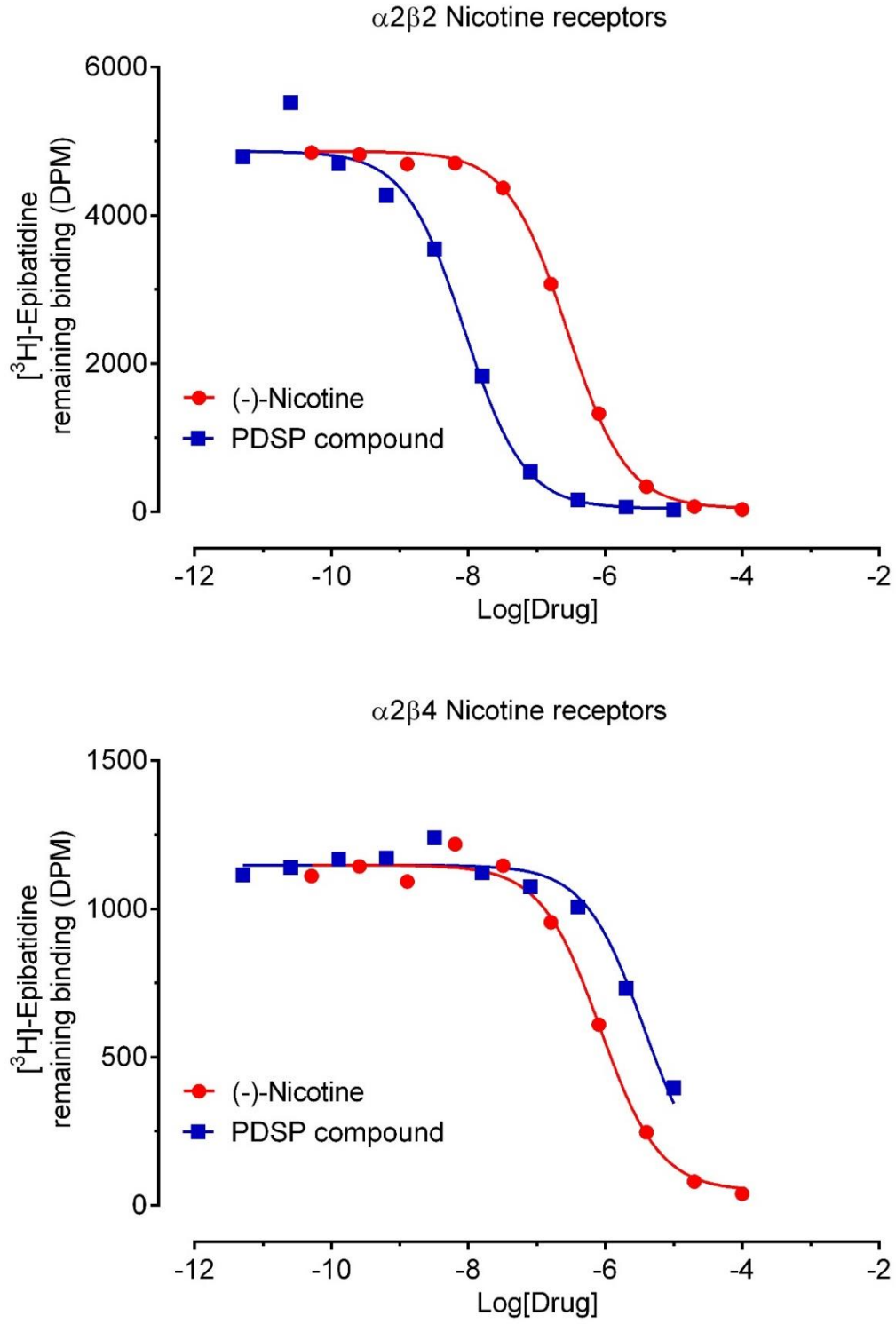

M5
[3H]-NMS
remaining binding (CPM)
500
400
300
200
100
0
Atropine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

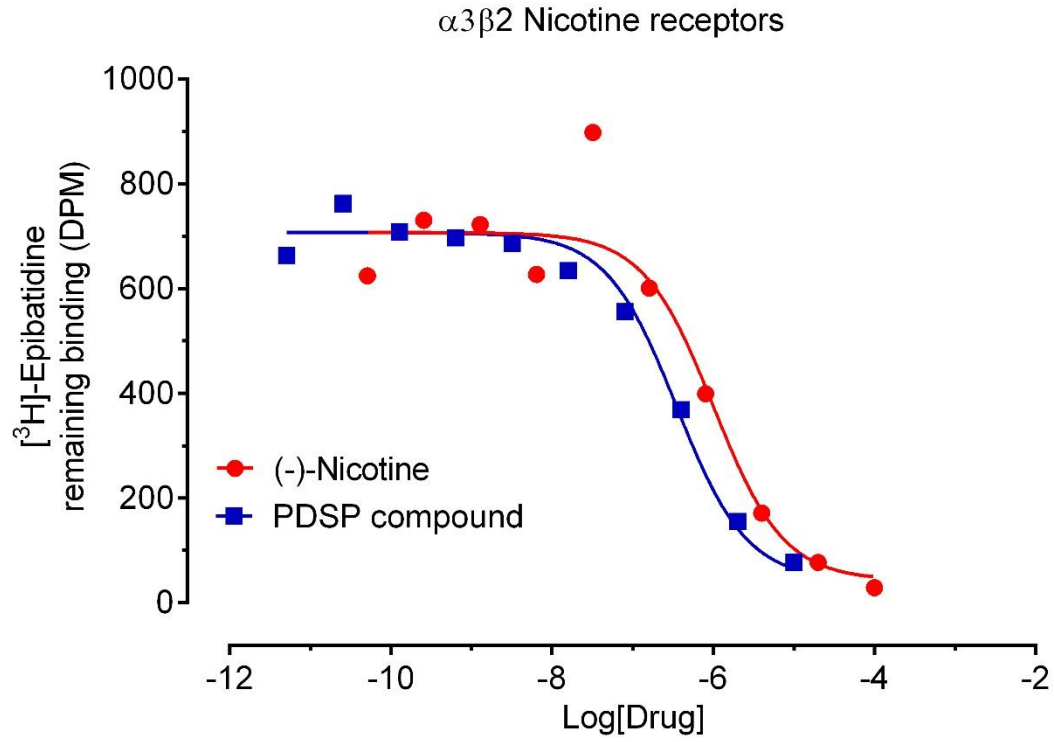
Table 4. Radioligand binding assays for nAChRs. Primary binding assays used 100 pM of [^{3}H]-epibatidine and secondary binding assays used 0.5 -2.3 nM [^{3}H]-epibatidine. Historical reference K_i values from last > 2 years are also included for quality control.

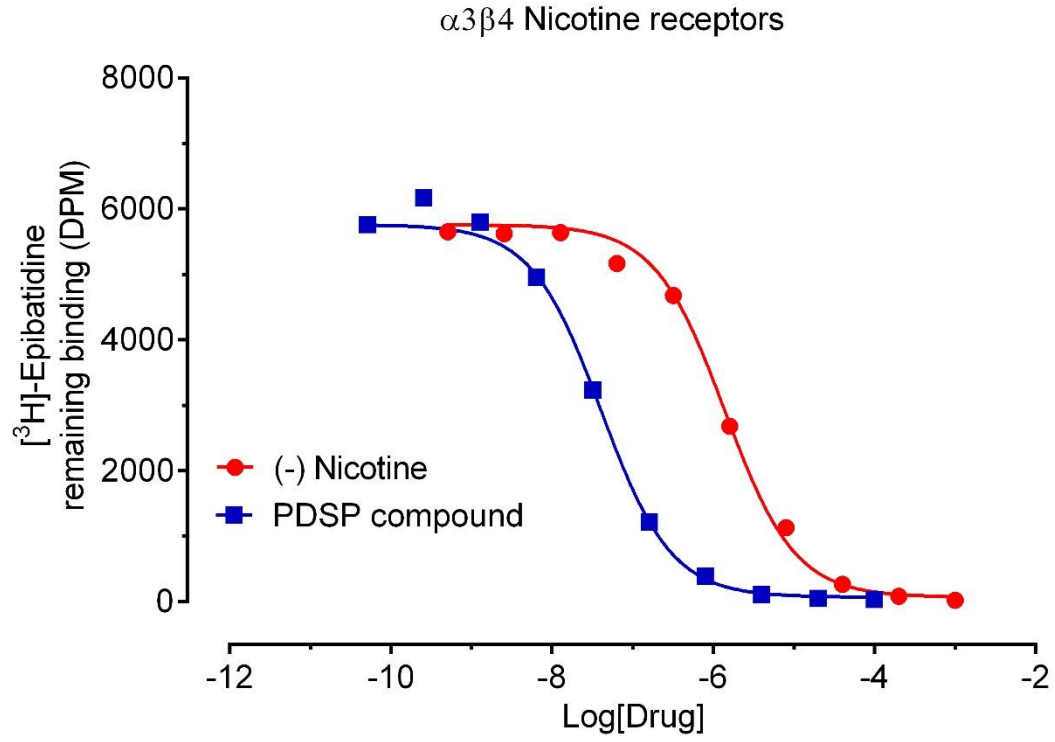
Nicotinic acetylcholine receptors (nAChRs) Nicotinic acetylcholine receptor binding buffer: 50 mM Tris HCl, pH 7.4, RT Standard wash buffer: 50 mM Tris HCl, pH 7.4, cold				
Target	**Radioligand Avg K_d (nM)**	**Radioligand used (nM)**	**Reference pK_i ± SEM (K_i,nM)**	**Literature**
α2ß2	[^{3}H]-Epibatidine 0.010	0.46 – 0.53	Nicotine 8.25 ± 0.06 (5.60)	(5, 6)
α2ß4	[^{3}H]-Epibatidine 0.040	0.46 – 0.53	Nicotine 7.12 ± 0.03 (76.5)	
α3ß2	[^{3}H]-Epibatidine 0.015	0.46 – 0.53	Nicotine 8.12 ± 0.02 (7.59)	
α3ß4	[^{3}H]-Epibatidine 0.080	0.46 – 0.53	Nicotine 6.51 ± 0.03 (311)	
α4ß2	[^{3}H]-Epibatidine 0.030	0.46 – 0.53	Nicotine 8.18 ± 0.04 (6.65)	
α4ß2*	[^{3}H]-Epibatidine 0.050	0.46 – 0.53	Nicotine 8.04 ± 0.03 (9.03)	
α4ß4	[^{3}H]-Epibatidine 0.080	0.46 – 0.53	Nicotine 7.56 ± 0.02 (27.5)	
α7, rat	[^{3}H]-Epibatidine 1.36	1.79 – 2.31	Nicotine 6.50 ± 0.05 (316)	
Cortex, rat	[^{3}H]-Epibatidine 0.04	0.46 – 0.53	Nicotine 8.20 ± 0.03 (6.27)	

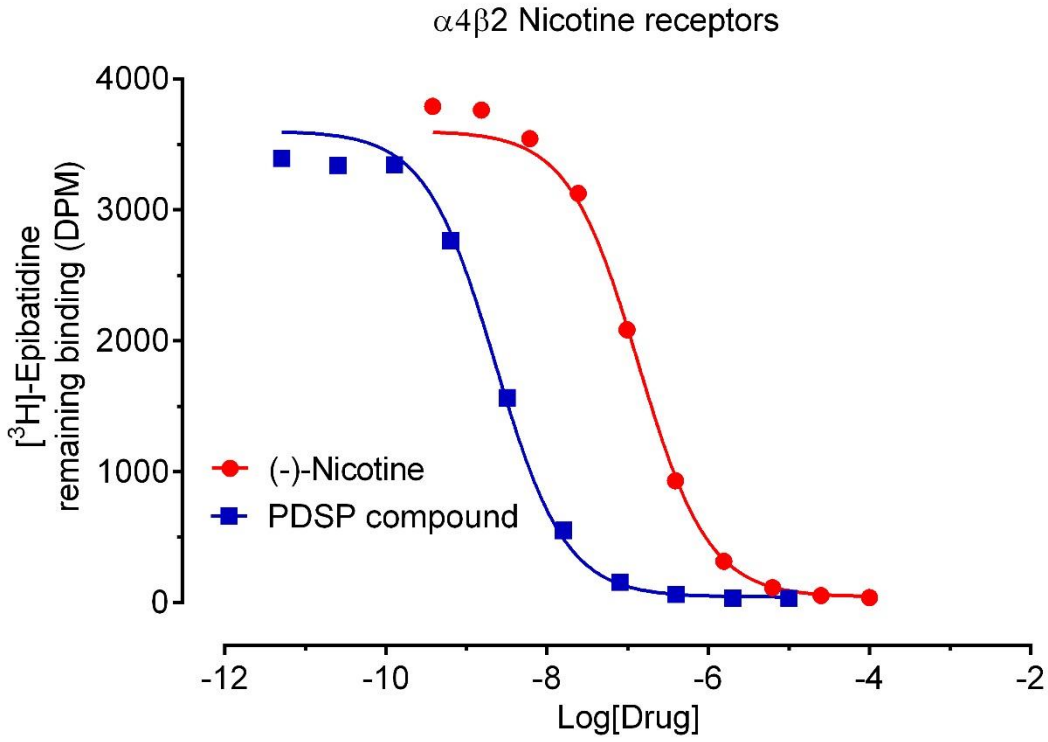
* Rat forebrain

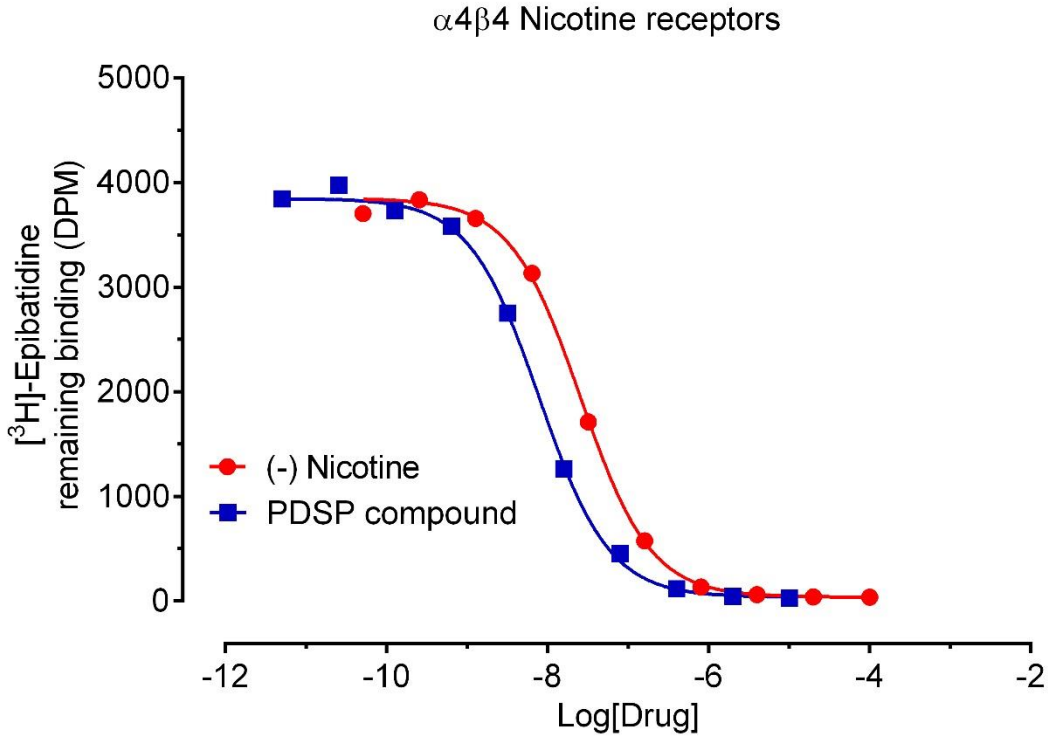
Figure 12. Representative competition binding curves with nicotinic acetylcholine receptors

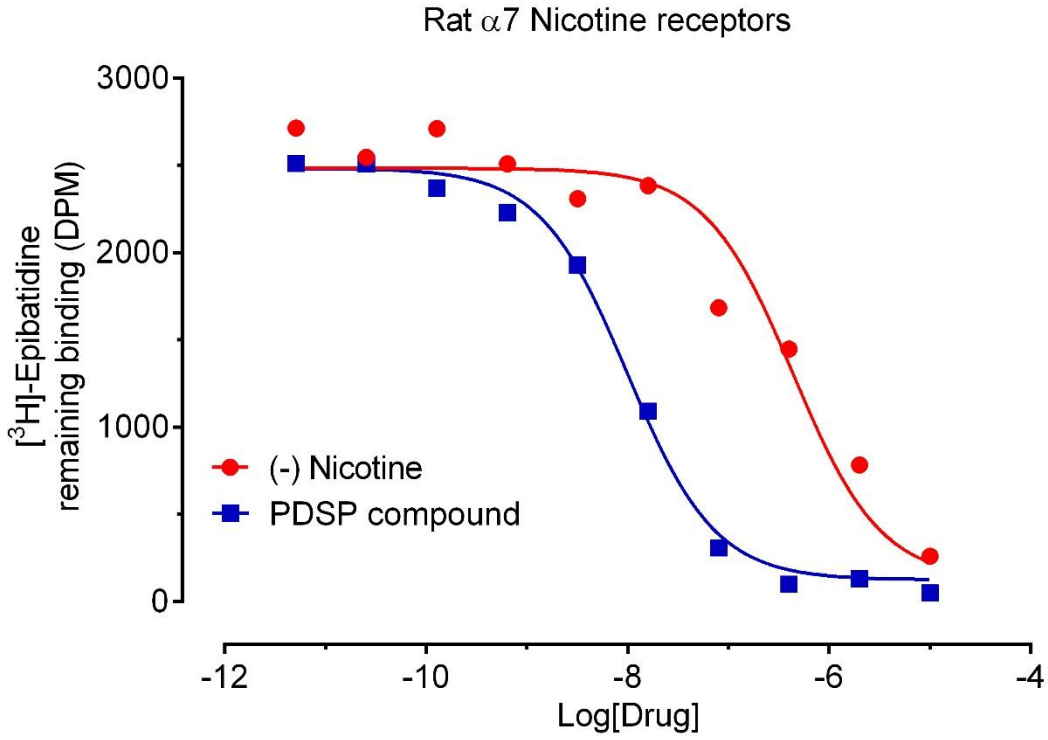



α3β2 Nicotine receptors
[3H]-Epibatidine remaining binding (DPM)
1000
800
600
400
200
0
(-)-Nicotine
PDSP compound
-12
-10
-8
-6
-4
-2
Log[Drug]


α3β4 Nicotine receptors
[3H]-Epibatidine remaining binding (DPM)
8000
6000
4000
2000
0
(-) Nicotine
PDSP compound
-12
-10
-8
-6
-4
-2
Log[Drug]


α4β2 Nicotine receptors
[3H]-Epibatidine remaining binding (DPM)
4000
3000
2000
1000
0
(-)-Nicotine
PDSP compound
-12
-10
-8
-6
-4
-2
Log[Drug]


α4β4 Nicotine receptors
[3H]-Epibatidine remaining binding (DPM)
5000
4000
3000
2000
1000
0
(-) Nicotine
PDSP compound
-12
-10
-8
-6
-4
-2
Log[Drug]


Rat α7 Nicotine receptors
[3H]-Epibatidine remaining binding (DPM)
3000
2000
1000
0
(-) Nicotine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

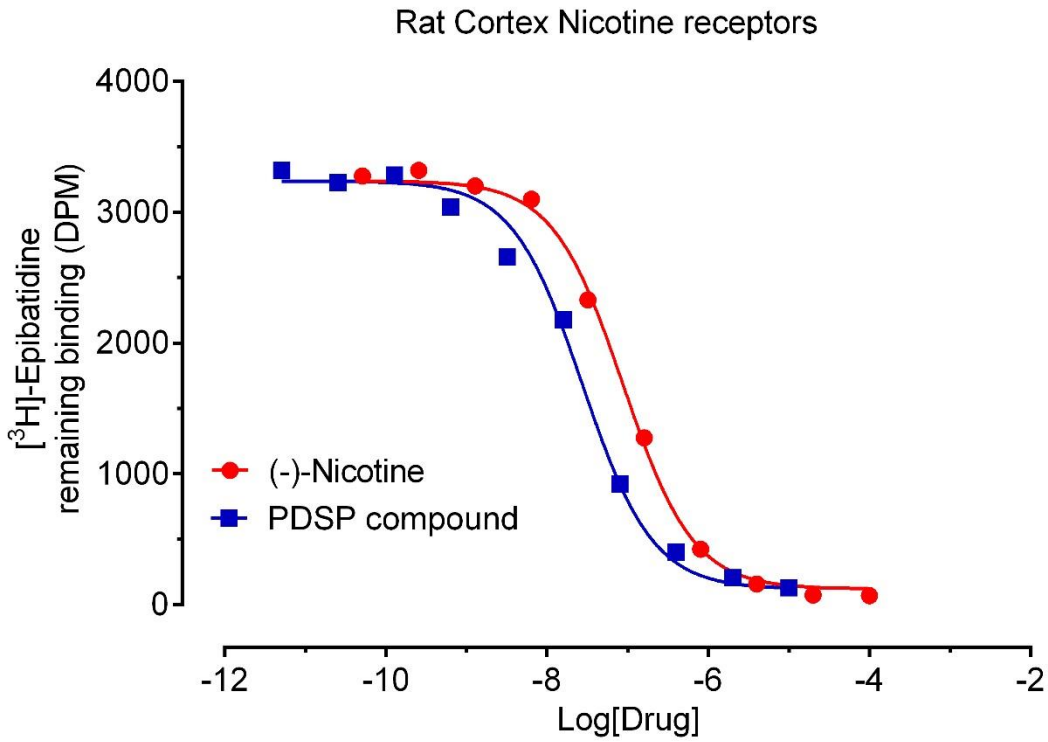
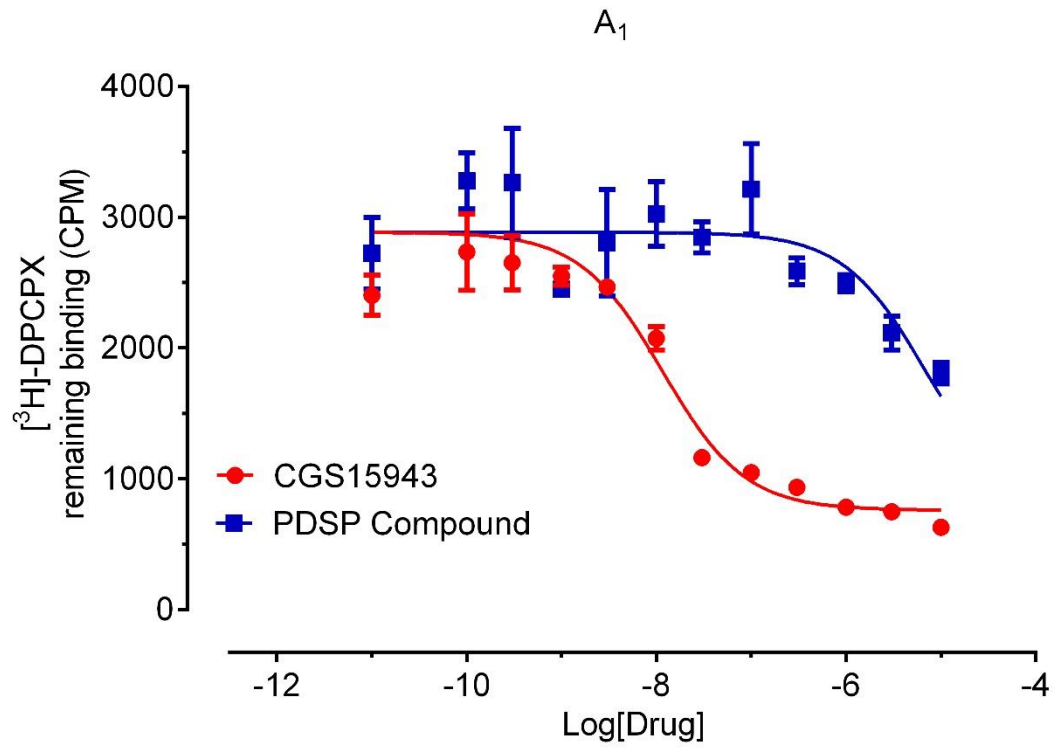

Rat Cortex Nicotine receptors
[3H]-Epibatidine remaining binding (DPM)
4000
3000
2000
1000
0
(-)-Nicotine
PDSP compound
-12
-10
-8
-6
-4
-2
Log[Drug]

Table 5. Adenosine receptors, radioligand and corresponding concentrations, reference compound, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from the last > 2 years are also included for quality control.

Adenosine receptors Adenosine Binding Buffer: 50 mM Tris HCl, 1U/ml adenosine deaminase, pH 7.4, RT Standard Washing Buffer: 50 mM Tris HCl, pH 7.4, cold				
Target	**Radioligand pK_d ± SEM (K_d, nM)**	**Radioligand used (nM)**	**Reference Ligand pK_d ± SEM (K_d, nM)**	**Literature**
A_1	[^{3}H] DPCPX 8.31 ± 0.16 (4.95)	5.0	CGS15943 7.88 ± 0.26 (13.3)	(40, 41)
A_{2A}	[^{3}H] ZM241385 8.34 ± 0.20 (4.60)	2.0 - 5.0	ZM-241385 8.11 ± 0.19 (7.85)	(42, 43)
			NECA 6.24 ± 0.07 (575)	
A_{2B}	Being developed			
A_3	Being developed			

Figure 13. Representative competitive binding curves for adenosine receptors


A1
4000
3000
2000
1000
0
[3H]-DPCPX
remaining binding (CPM)
CGS15943
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

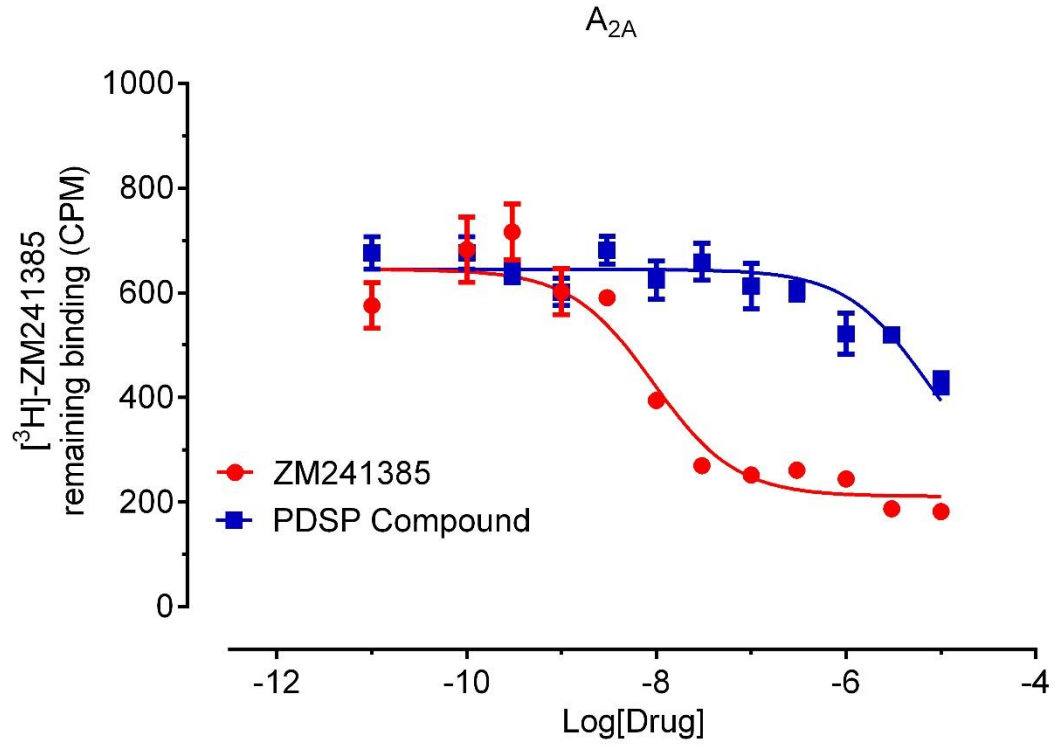

A2A
1000
800
600
400
200
0
[3H]-ZM241385
remaining binding (CPM)
ZM241385
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

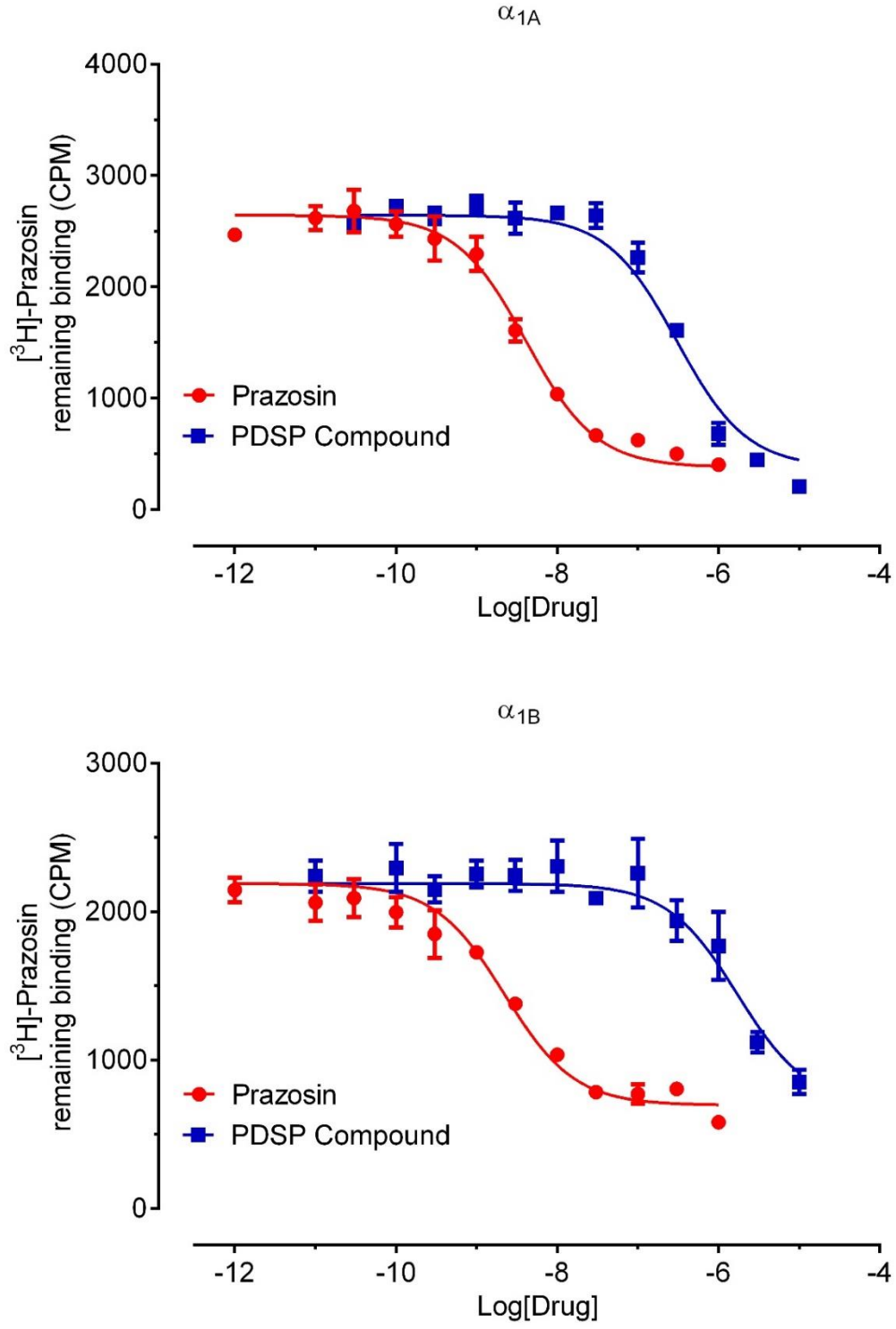
Table 6. Adrenergic receptors, radioligands and corresponding K_d values, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from the last >2 years are also listed.

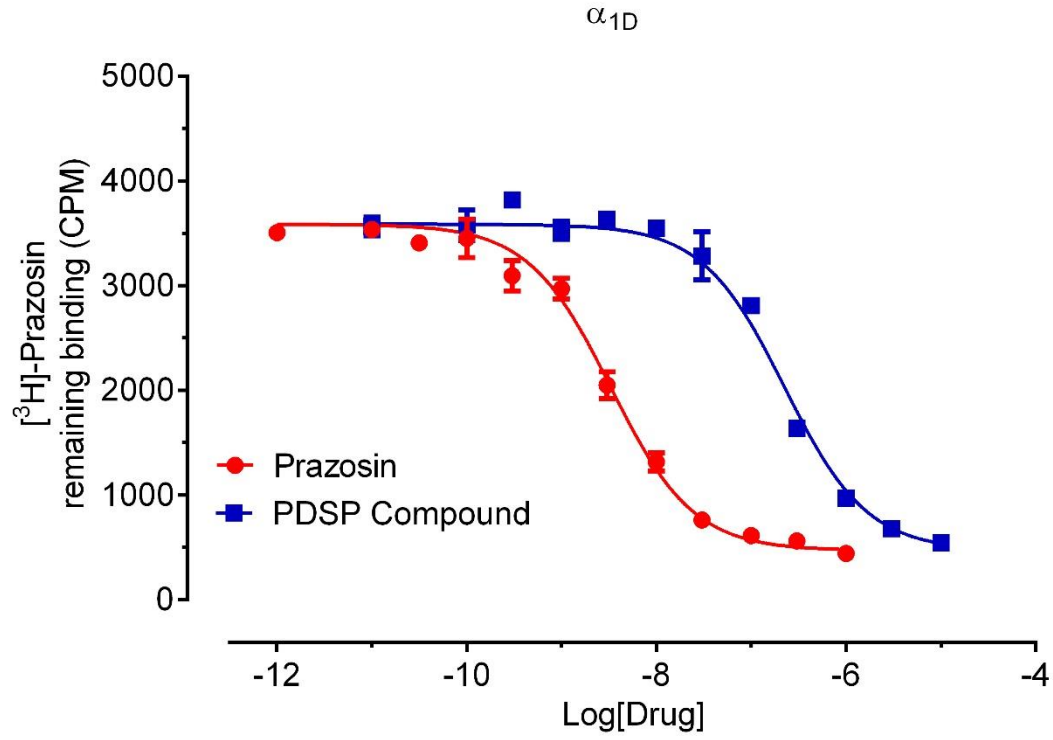
Adrenergic receptors

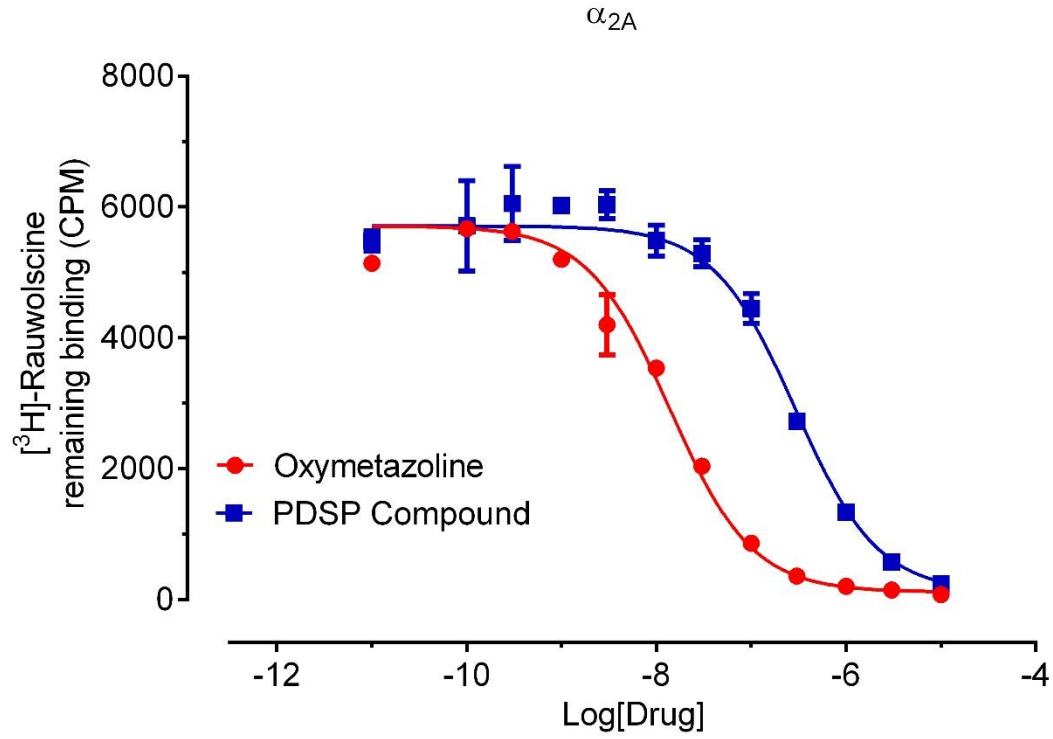
α1 Binding Buffer: 20 mM Tris HCl, 145 mM NaCl, pH 7.4, RT
α2 Binding Buffer: 50 mM Tris HCl, 5 mM $MgCl_2$, pH 7.7, RT
ß Binding Buffer: 50 mM Tris HCl, 3 mM $MgCl_2$, pH 7.7, RT
Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold

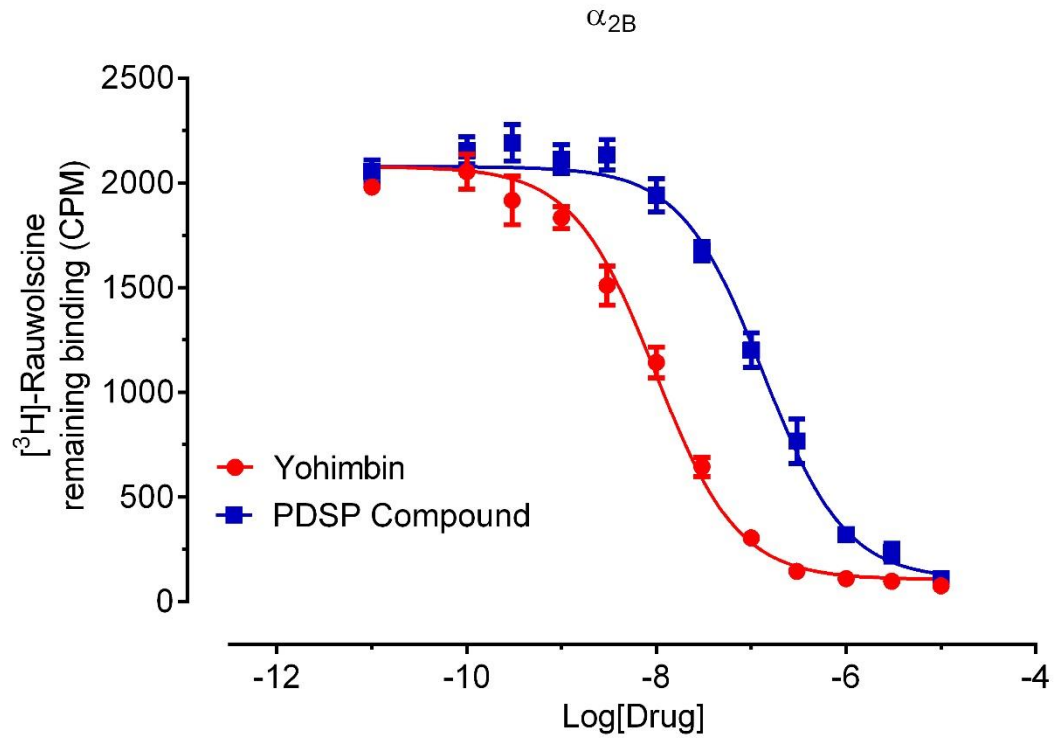
Target	Radioligand pK_d ± SEM (K_d, nM)	Radioligand used (nM)	Reference ligand pK_i ± SEM (K_i, nM)	Literature
α_{1A}	[^{3}H]-Prazosin 9.36 ± 0.07 (0.43)	0.2 - 1.0	Prazosin 9.16 ± 0.03 (0.70)	(44, 45)
α_{1B}	[^{3}H]-Prazosin 9.23 ± 0.29 (0.58)	0.3 – 1.0	Prazosin 9.05 ± 0.03 (0.88)	
α_{1D}	[^{3}H]-Prazosin 9.21 ± 0.07 (0.62)	0.3 – 1.0	Prazosin 9.20 ± 0.03 (0.63)	
α_{2A}	[^{3}H]-Rauwolscine 8.46 ± 0.16 (3.47)	1.0 – 3.0	Oxymetazoline 8.35 ± 0.02 (4.51)	(46–48)
α_{2B}	[^{3}H]-Rauwolscine 8.74 ± 0.13 (1.81)	1.5 – 2.0	Yohimbine 8.24 ± 0.02 (5.81)	
α_{2C}	[^{3}H]-Rauwolscine 9.02 ± 0.10 (0.96)	0.5 – 1.0	Oxymetazoline 7.38 ± 0.02 (41.6)	
ß$_1$	[^{125}I]-Pindolol 10.06 ± 0.09 (0.10)	0.1 – 0.2	Alprenolol 8.73 ± 0.03 (1.85)	(49–52)
ß$_2$	[^{3}H]-CGP12177 9.15 ± 0.16 (0.71)	0.5 - 1.0	Alprenolol 8.79 ± 0.03 (1.61)	
ß$_3$	[^{3}H]-CGP12177 7.50 ± 0.16 (31.8)	20		
ß$_3$	[^{125}I]-Pindolol 9.45 ± 0.15 (0.36)	0.2 – 0.5	Alprenolol 7.64 ± 0.03 (23.0)	

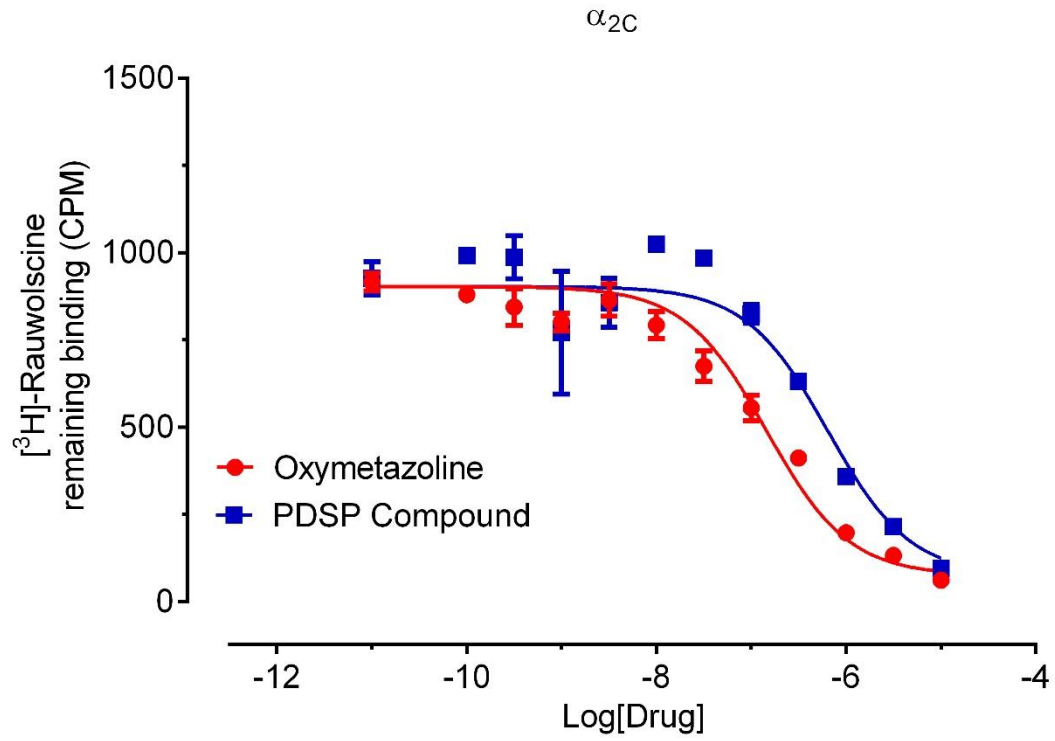
Figure 14. Representative competition binding curves with adrenergic receptors

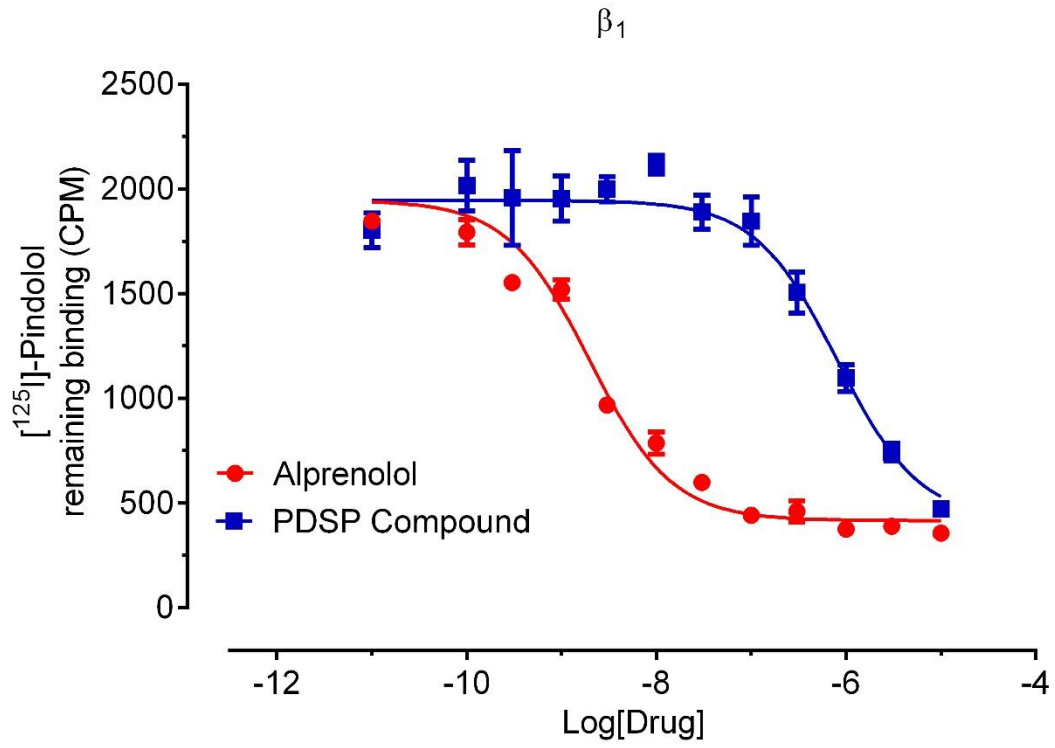


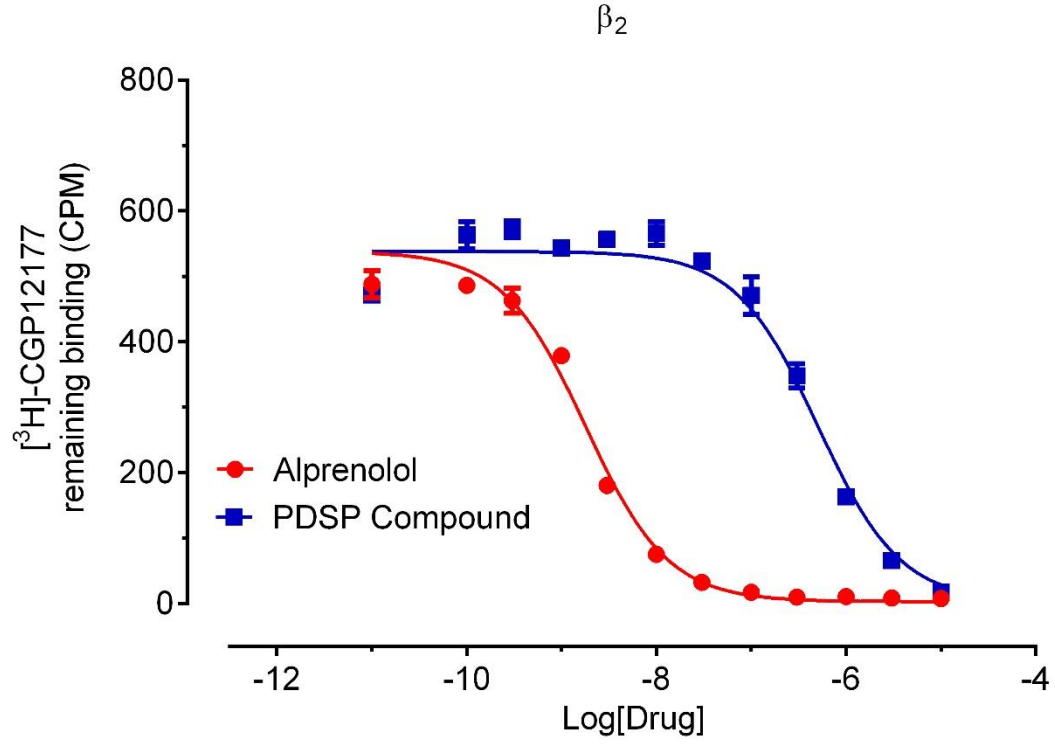

α1D
5000
4000
3000
2000
1000
0
[3H]-Prazosin
remaining binding (CPM)
Prazosin
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

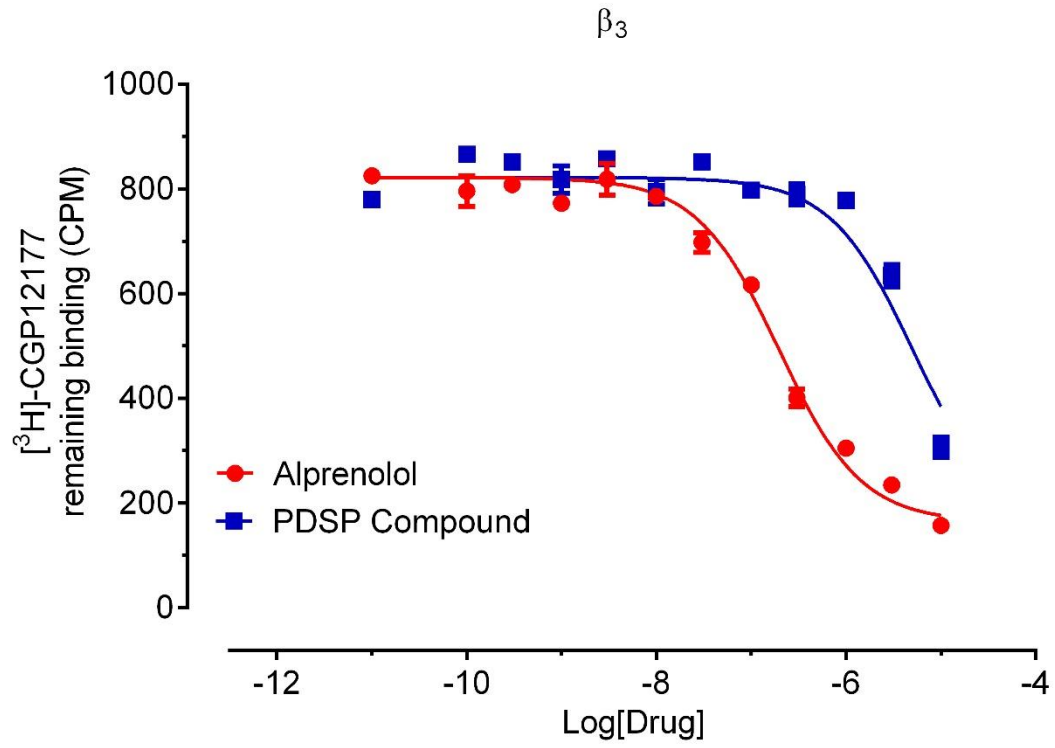

α2A
8000
6000
4000
2000
0
[3H]-Rauwolscine
remaining binding (CPM)
Oxymetazoline
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


α2B
2500
2000
1500
1000
500
0
[3H]-Rauwolscine
remaining binding (CPM)
Yohimbin
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


α2C
1500
1000
500
0
[3H]-Rauwolscine
remaining binding (CPM)
Oxymetazoline
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


β1
2500
2000
1500
1000
500
0
[125I]-Pindolol
remaining binding (CPM)
Alprenolol
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


β2
800
600
400
200
0
[3H]-CGP12177
remaining binding (CPM)
Alprenolol
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


β3
[3H]-CGP12177
remaining binding (CPM)
1000
800
600
400
200
0
Alprenolol
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

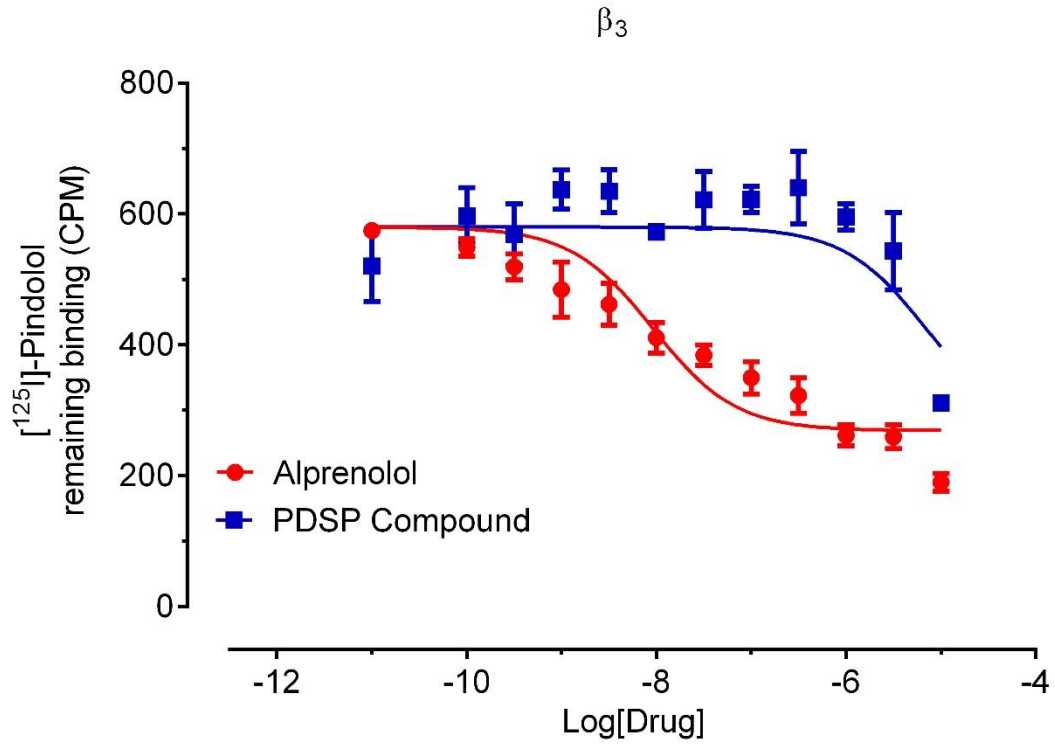

β3
[125I]-Pindolol
remaining binding (CPM)
800
600
400
200
0
Alprenolol
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

Table 7. Cannabinoid receptors, radioligand and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from the last >2 years are also included.

Cannabinoid receptors Cannabinoid Binding Buffer: 50 mM Tris HCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mg/ml BSA, pH 7.4, RT Cannabinoid Wash Buffer: cannabinoid binding buffer + 1 mg/ml BSA, pH 7.4, cold				
Target	**Radioligand $pK_d \pm SEM$ (K_d, nM)**	**Radioligand used (nM)**	**Reference Ligand $pK_i \pm SEM$ (K_i, nM)**	**Literature**
CB_1 (rat brain)	$[^3H]$-CP55940 8.73 ± 0.22 (1.85)	0.5 – 2.0	CP-55940 8.23 ± 0.05 (5.87)	(53–55)
CB_2	$[^3H]$-CP55940 8.47 ± 0.11 (3.37)	1.0 – 3.0	CP-55940 8.06 ± 0.05 (8.77)	

Figure 15. Representative competitive binding curves for cannabinoid receptors.

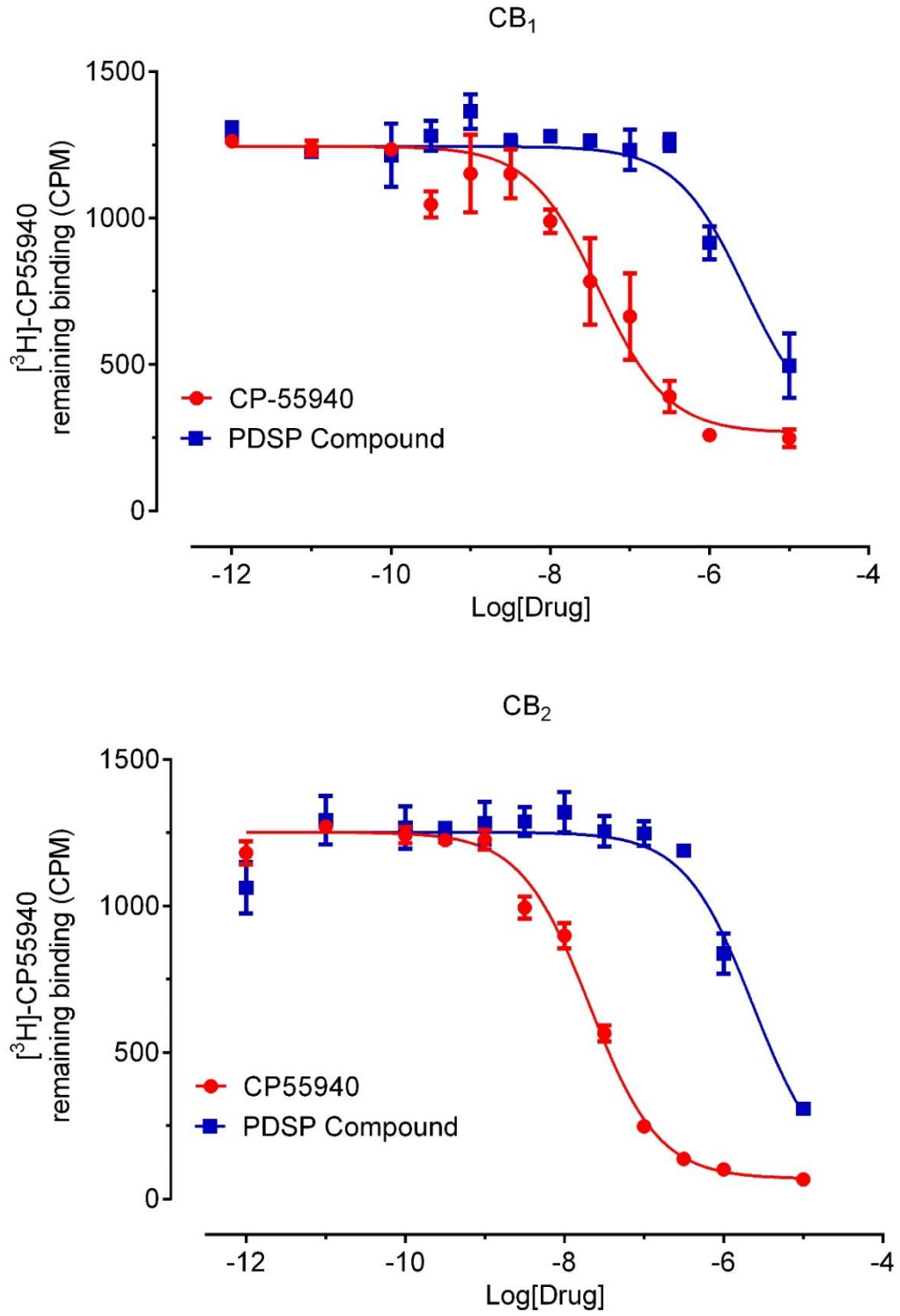
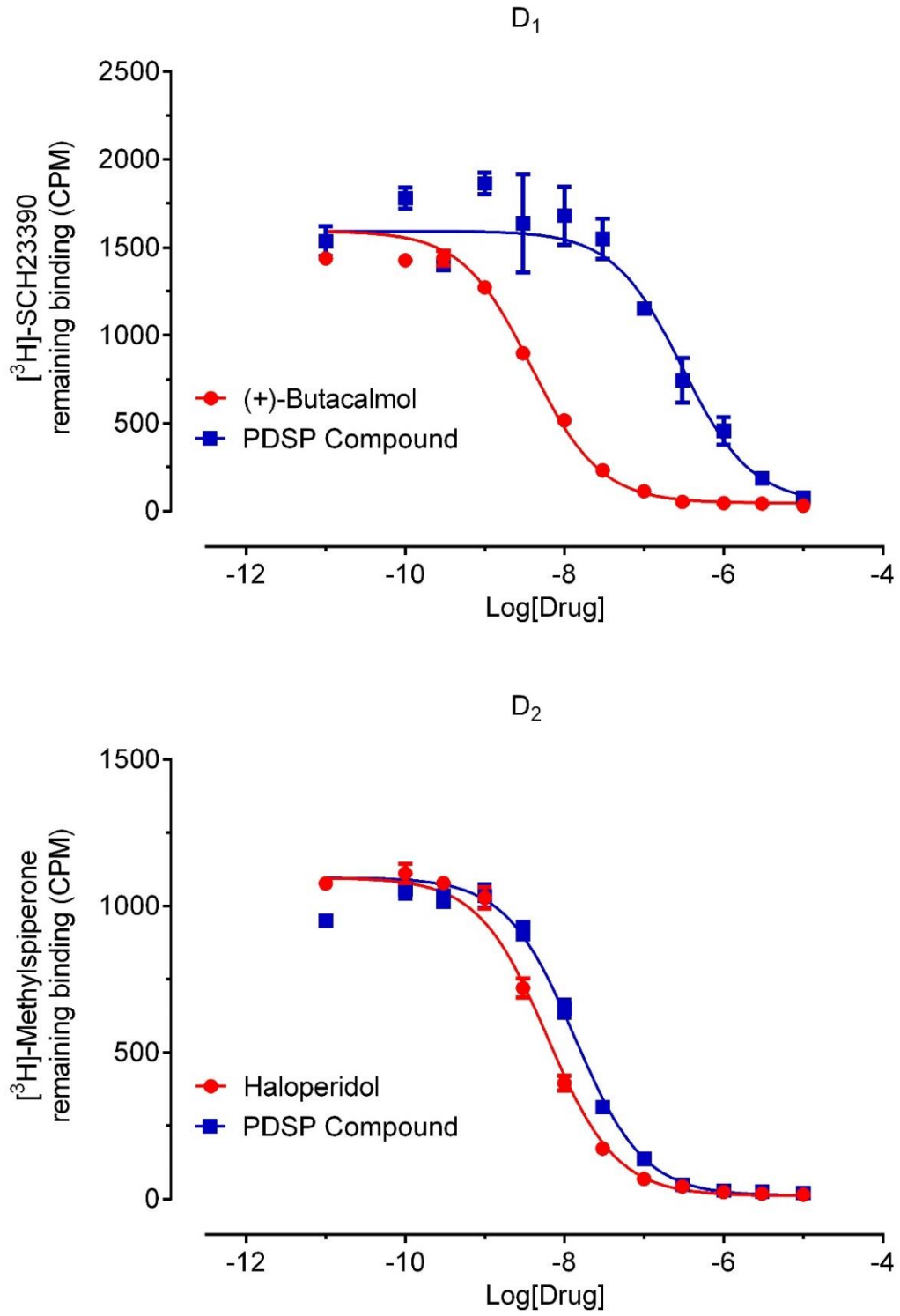
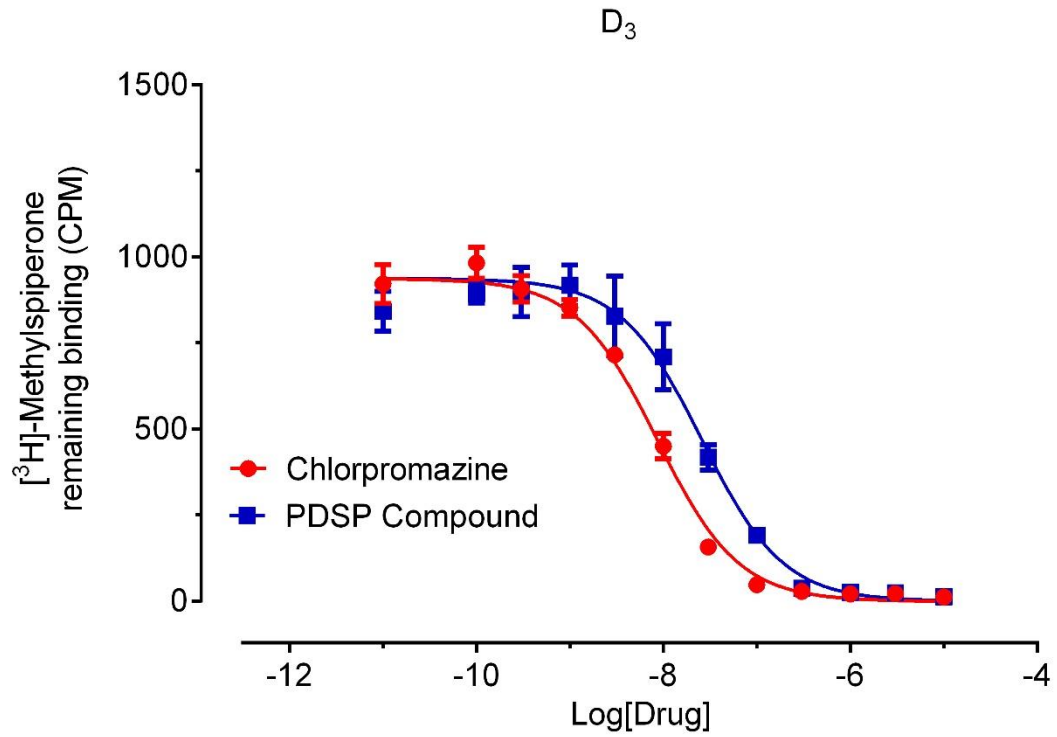


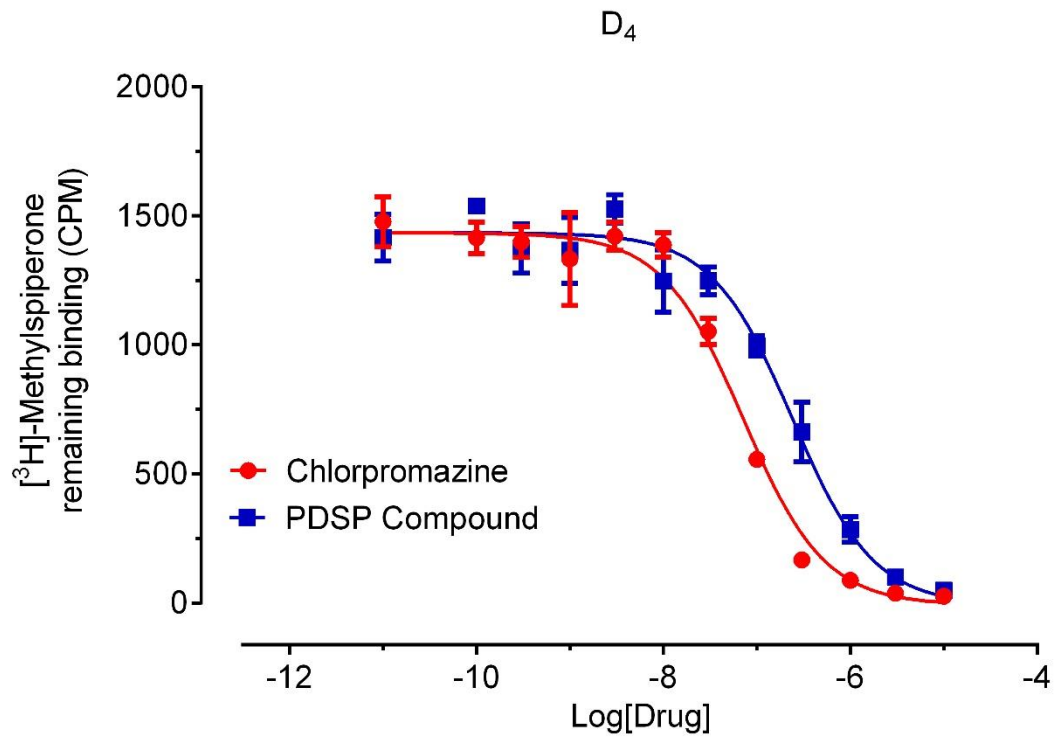
Table 8. Dopamine receptors, radioligands and corresponding K_d values, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from >2 years are also included.

Dopamine receptors Dopamine Binding Buffer: 50 mM HEPES, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4, RT Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold				
Target	**Radioligand pK_d ± SEM (K_d, nM)**	**Radioligand used (nM)**	**Reference Ligand pK_i ± SEM (K_i, nM)**	**Literature**
D_1	[^{3}H]-SCH23390 9.13 ± 0.05 (0.74)	0.6 – 1.3	(+)-Butaclamol 8.51 ± 0.02 (3.07)	(56)
D_2	[^{3}H]-N-methylspiperone 9.33 ± 0.06 (0.47)	0.4 – 1.0	Haloperidol 8.15 ± 0.02 (7.15)	(57, 58)
D_3	[^{3}H]-N-methylspiperone 9.44 ± 0.09 (0.36)	0.5 – 1.8	Chlorpromazine 7.99 ± 0.02 (10.30)	
D_4	[^{3}H]-N-methylspiperone 9.07 ± 0.06 (0.86)	0.6 – 1.7	Chlorpromazine 7.49 ± 0.02 (32.62)	
D_5	[^{3}H]-SCH23390 8.69 ± 0.05 (2.03)	2.0 – 3.0	SKF38393 8.59 ± 0.02 (2.59)	(59)

Figure 16. Representative competitive binding curves with Dopamine receptors.




D3
1500
1000
500
0
[3H]-Methylspiperone
remaining binding (CPM)
Chlorpromazine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


D4
2000
1500
1000
500
0
[3H]-Methylspiperone
remaining binding (CPM)
Chlorpromazine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

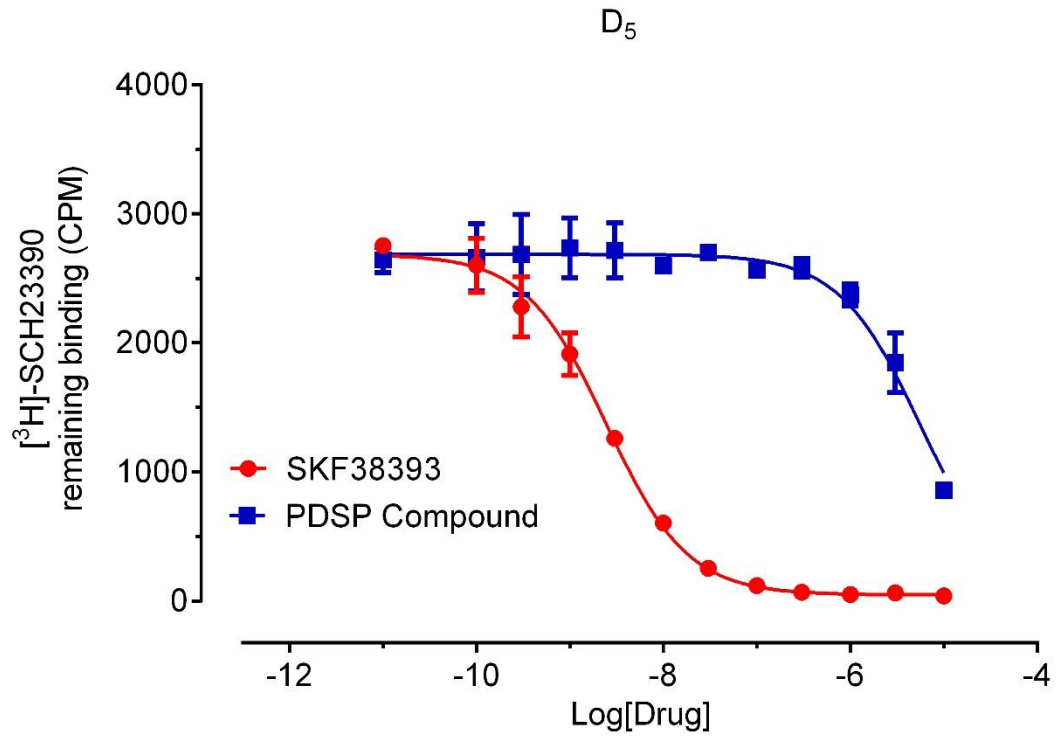

D5
4000
3000
2000
1000
0
[3H]-SCH23390
remaining binding (CPM)
SKF38393
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

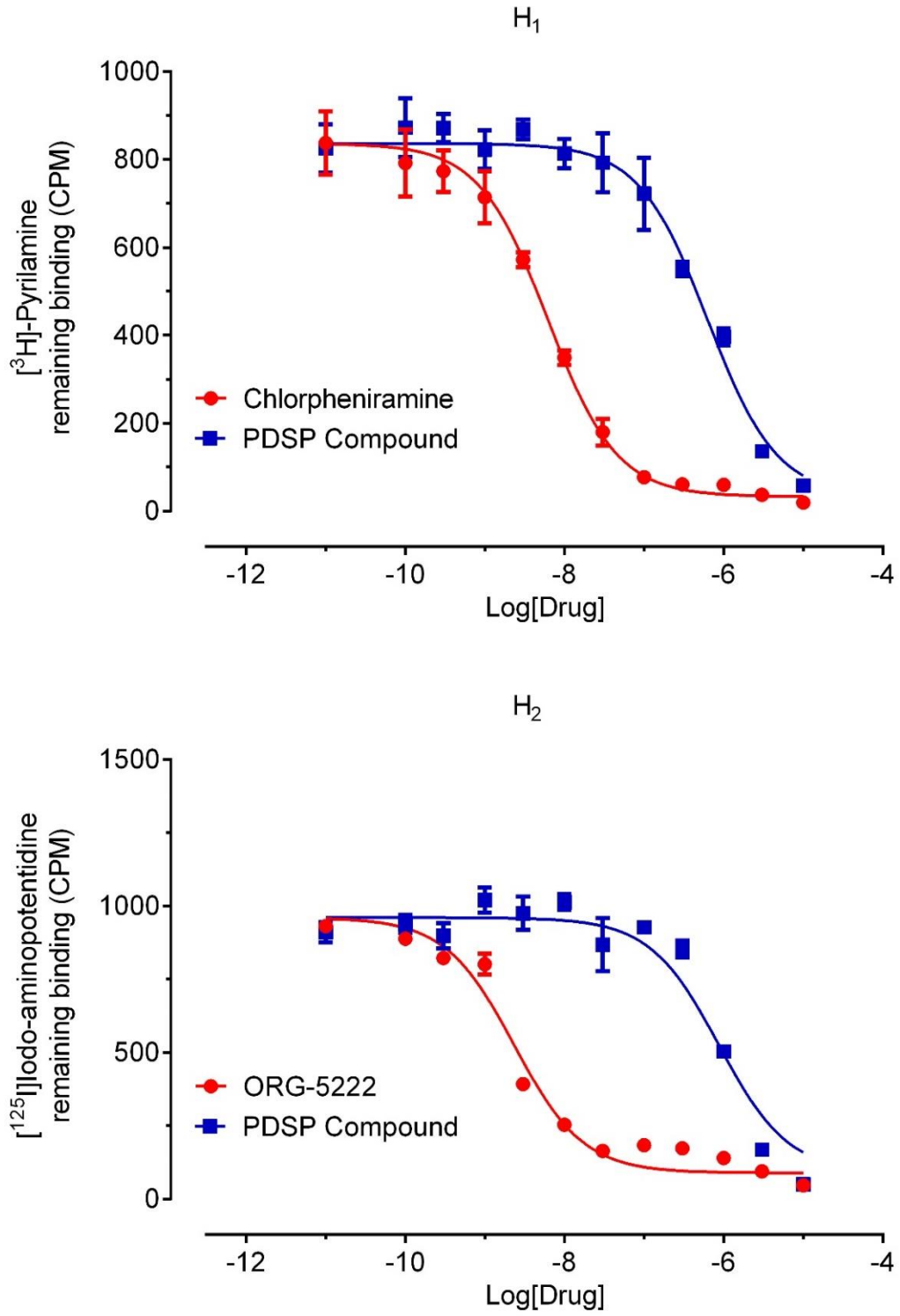
Table 9. Histamine receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from >2 years are also included.

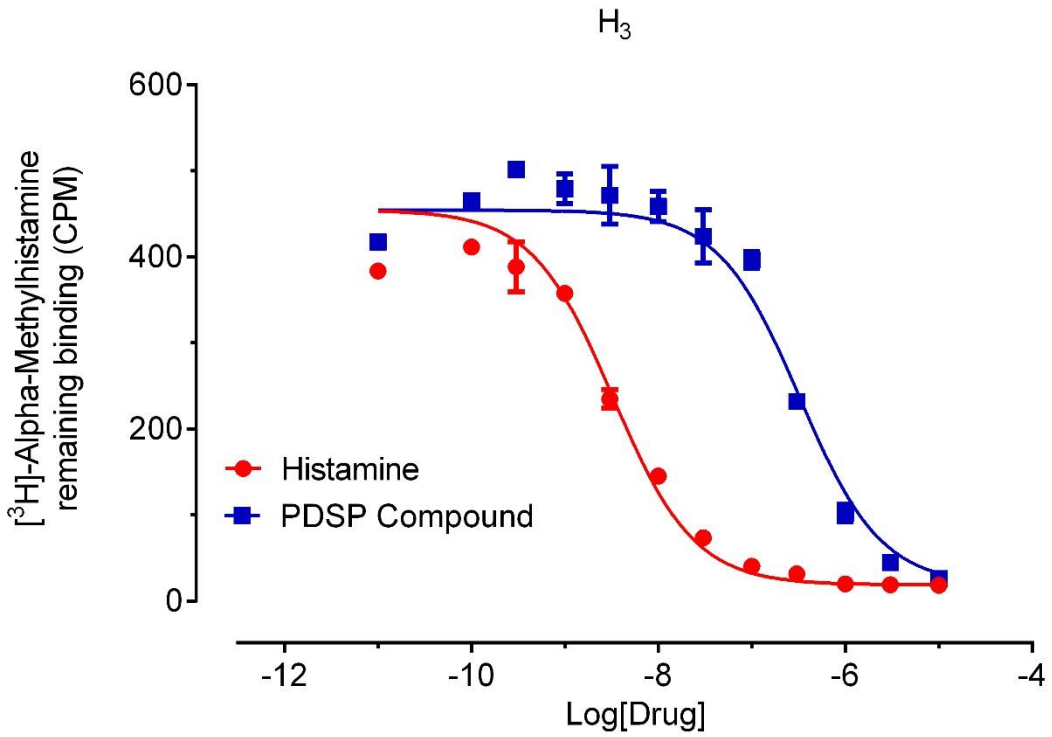
Histamine receptors

Histamine Binding Buffer: 50 mM Tris HCl, 0.5 mM EDTA, pH 7.4, RT
Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold
Filter: GF/B

Target	Radioligand $pK_d \pm SEM$ (K_d, nM)	Radioligand used (nM)	Reference Ligand $pK_i \pm SEM$ (K_i, nM)	Literature
H_1	[^{3}H]-Pyrilamine 9.01 ± 0.05 (0.97)	0.6 – 2.0	Chlorpheniramine 8.78 ± 0.02 (1.64)	(60, 61)
H_2	[^{125}I]-Iodo-aminopotentidine 10.47 ± 0.12 (0.03)	0.02 – 0.05	ORG-5222 9.05 ± 0.04 (0.90)	(62)
H_3	[^{3}H]-α-methylhistamine 9.11 ± 0.07 (0.78)	0.5 – 1.0	Histamine 8.30 ± 0.03 (4.99)	(63)
H_4	[^{3}H]-Histamine 8.26 ± 0.08 (5.46)	1.0 – 5.0	Clozapine 6.60 ± 0.06 (250)	(64, 65)

Figure 17. Representative competitive binding curves with Histamine receptors.




H3
600
400
200
0
[3H]-Alpha-Methylhistamine
remaining binding (CPM)
Histamine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

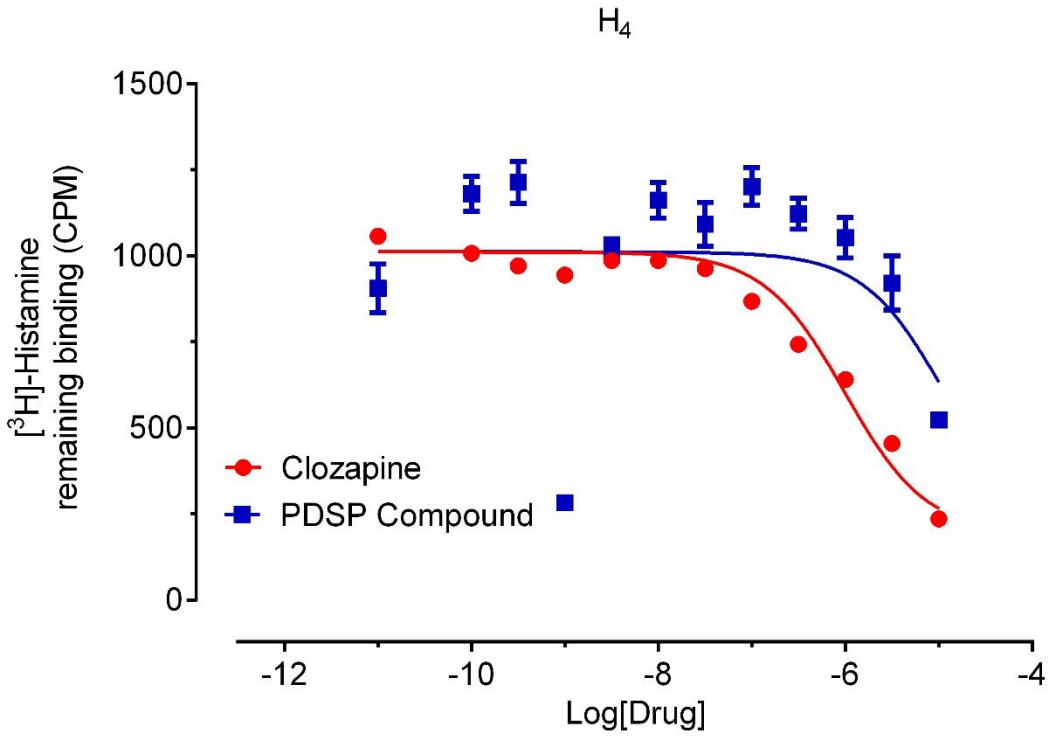

H4
1500
1000
500
0
[3H]-Histamine
remaining binding (CPM)
Clozapine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

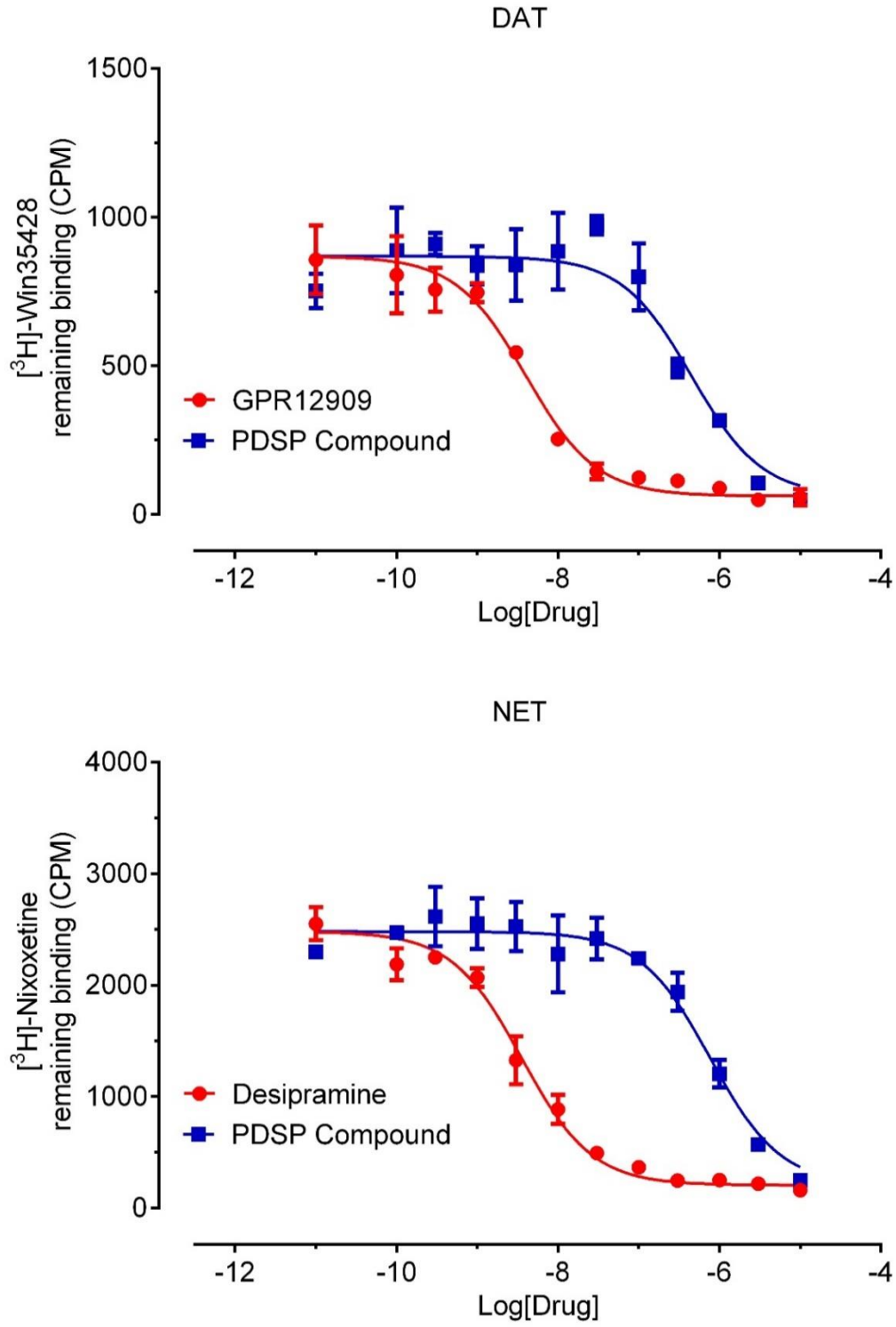
Table 10. Neurotransmitter transporters, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from >2 years are also included.

Neurotransmitter transporters

Transporter Binding Buffer: 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1 mM ETGA, pH 7.4, RT
Transporter Wash Buffer: Transporter binding buffer, pH 7.4, cold

Target	Radioligand $pK_d \pm SEM$ (K_d, nM)	Radioligand used (nM)	Reference Ligand $pK_i \pm SEM$ (K_i, nM)	Literature
DAT	[^{3}H]-Win35428 8.02 ± 0.04 (9.47)	3.6 – 16.0	GBR12909 8.33 ± 0.02 (4.73)	(66, 67)
NET	[^{3}H]-Nisoxetine 8.42 ± 0.06 (3.83)	1.3 – 5.0	Desipramine 8.65 ± 0.01 (2.24)	(68, 69)
SERT	[^{3}H]-Citalopram 8.58 ± 0.11 (2.63)	1.5 – 2.0	Amitriptyline 8.26 ± 0.02 (5.53)	(70, 71)

Figure 18. Representative competitive binding curves with neurotransmitter transporters.



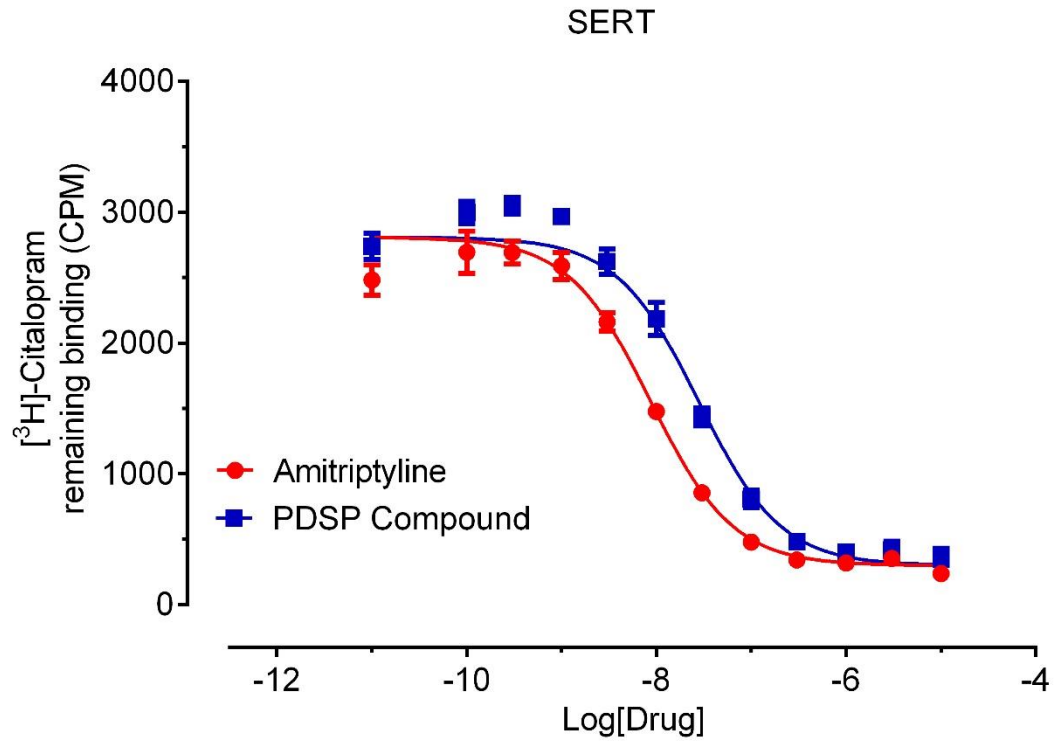
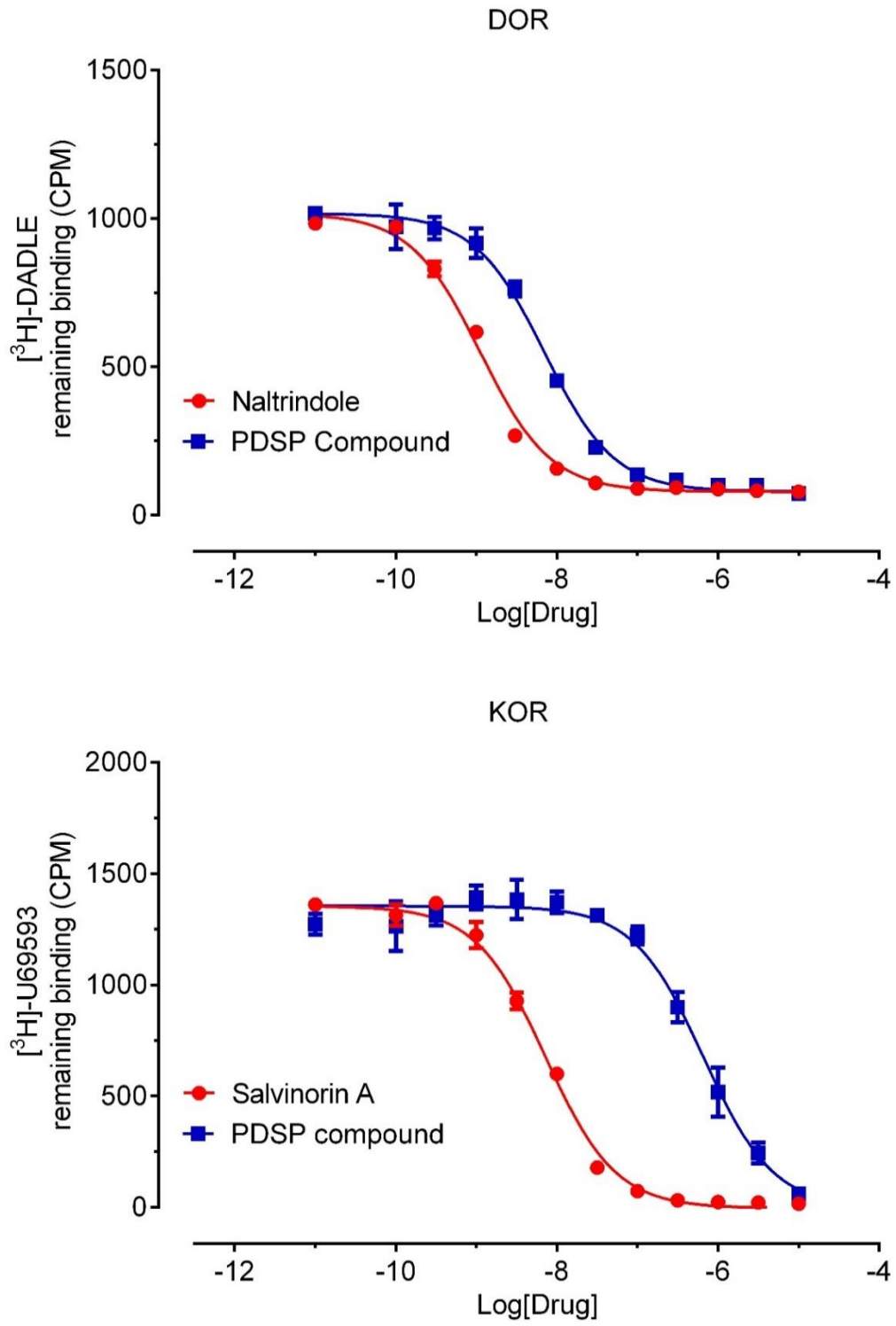
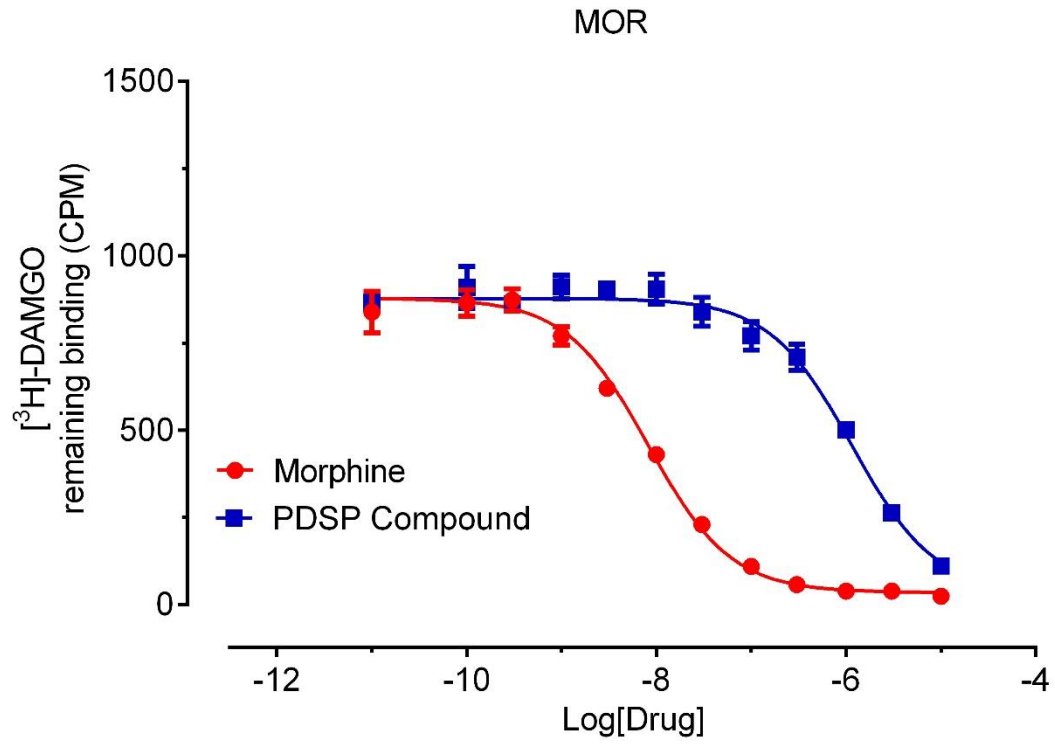

SERT
4000
3000
2000
1000
0
[3H]-Citalopram
remaining binding (CPM)
Amitriptyline
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

Table 11. Opioid receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from >2 years are also included.

Opioid receptors Standard Binding Buffer: 50 mM Tris HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4, RT Standard Wash Buffer: 50 mM Tris HCl, pH. 7.4, 4 °C to 8 °C				
Target	**Radioligand pK_d ± SEM (K_d, nM)**	**Radioligand used (nM)**	**Reference Ligand pK_i ± SEM (K_i, nM)**	**Literature**
DOR	[^{3}H]-DADLE 8.57 ± 0.05 (2.69)	1.0 – 2.0	Naltrindole 9.30 ± 0.02 (0.50)	(72, 73)
KOR	[^{3}H]-U69593 9.08 ± 0.04 (0.83)	0.6 – 1.2	Salvinorin A 8.53 ± 0.02 (2.98)	(74, 75)
MOR	[^{3}H]-DAMGO 8.92 ± 0.05 (1.20)	1.0 – 2.0	DAMGO 8.75 ± 0.02 (1.76)	(76, 77)
NOP	[^{3}H]-Nociceptin 9.11 ± 0.12 (0.78)	0.5 – 2.0	JDTiC 7.94 ± 0.06 (11.56) SB612111 8.59 ± 0.10 (2.59)	(78, 79)

Figure 19. Representative competitive binding curves with opioid receptors.




MOR
1500
1000
500
0
[3H]-DAMGO
remaining binding (CPM)
Morphine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

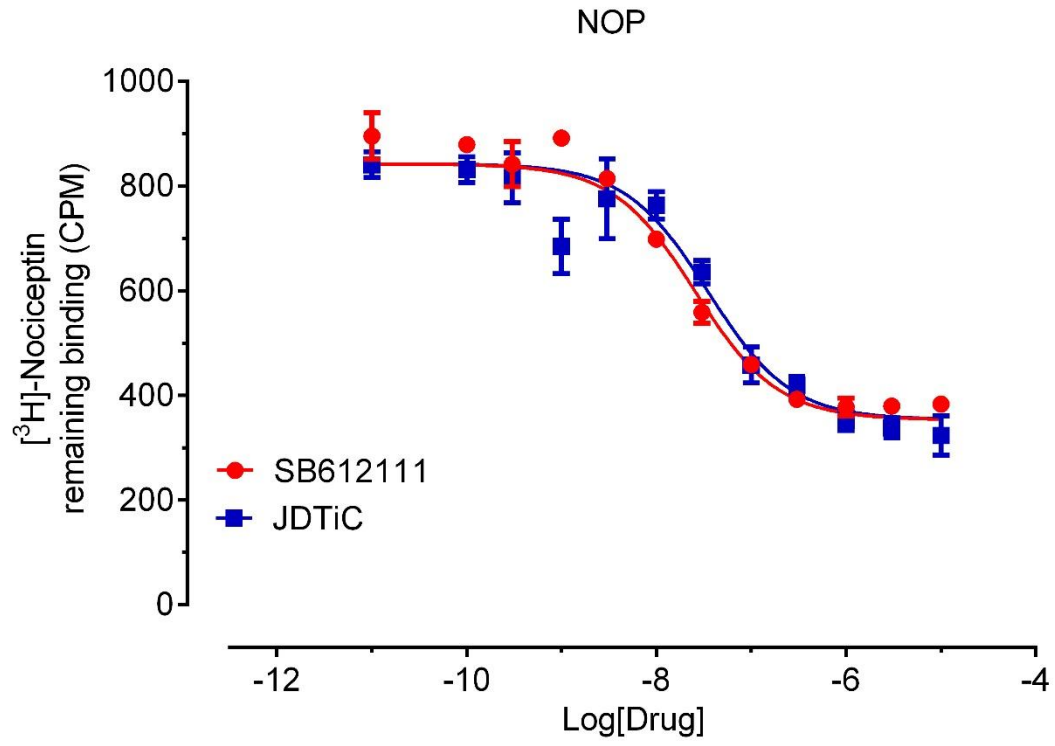

NOP
1000
800
600
400
200
0
[3H]-Nociceptin
remaining binding (CPM)
SB612111
JDTiC
-12
-10
-8
-6
-4
Log[Drug]

Table 12. HCA_2 receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from > 6 months are also included.

HCA_2 receptors HCA2 Binding Buffer: 50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4 Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold				
Target	**Radioligand pK_d ± SEM (K_d, nM)**	**Radioligand used (nM)**	**Reference Ligand pK_i ± SEM (K_i, nM)**	**Literature**
HCA_2	$[^3H]$-Niacin 7.90 ± 0.13 (12.47)	4.0 – 20.0	Niacin	(80–82)

Figure 20. Representative competitive binding curves with HCA_2 receptors.

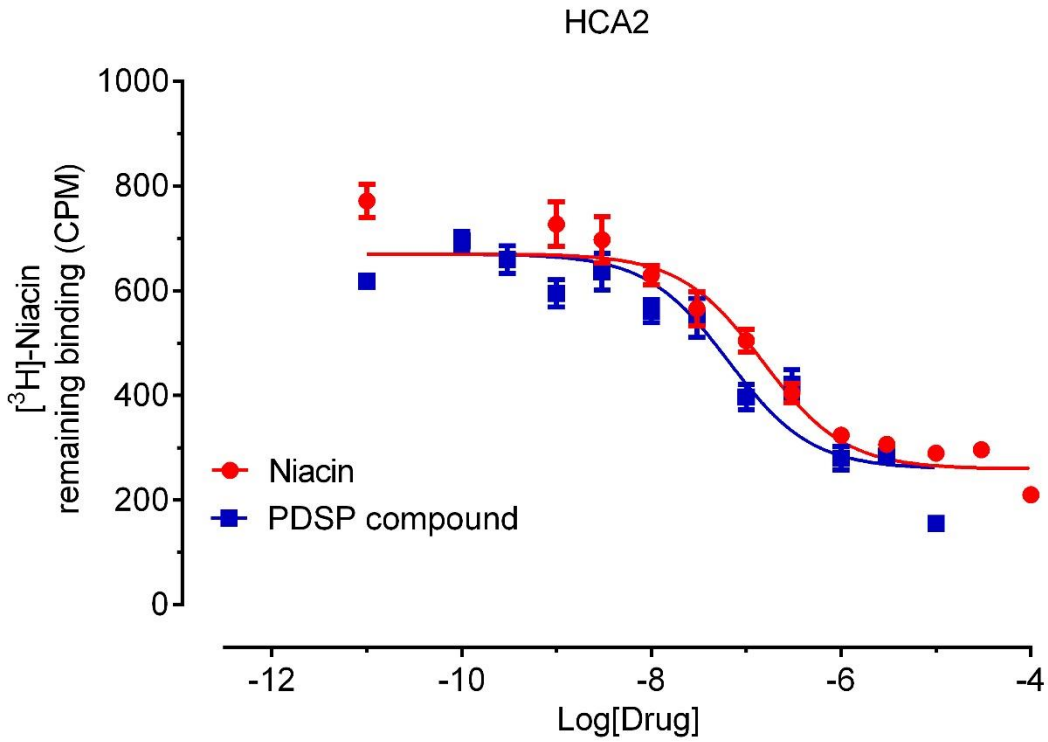


Table 13. Oxytocin and Vasopressin receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from > 2 years are also included.

Oxytocin and Vasopressin receptors

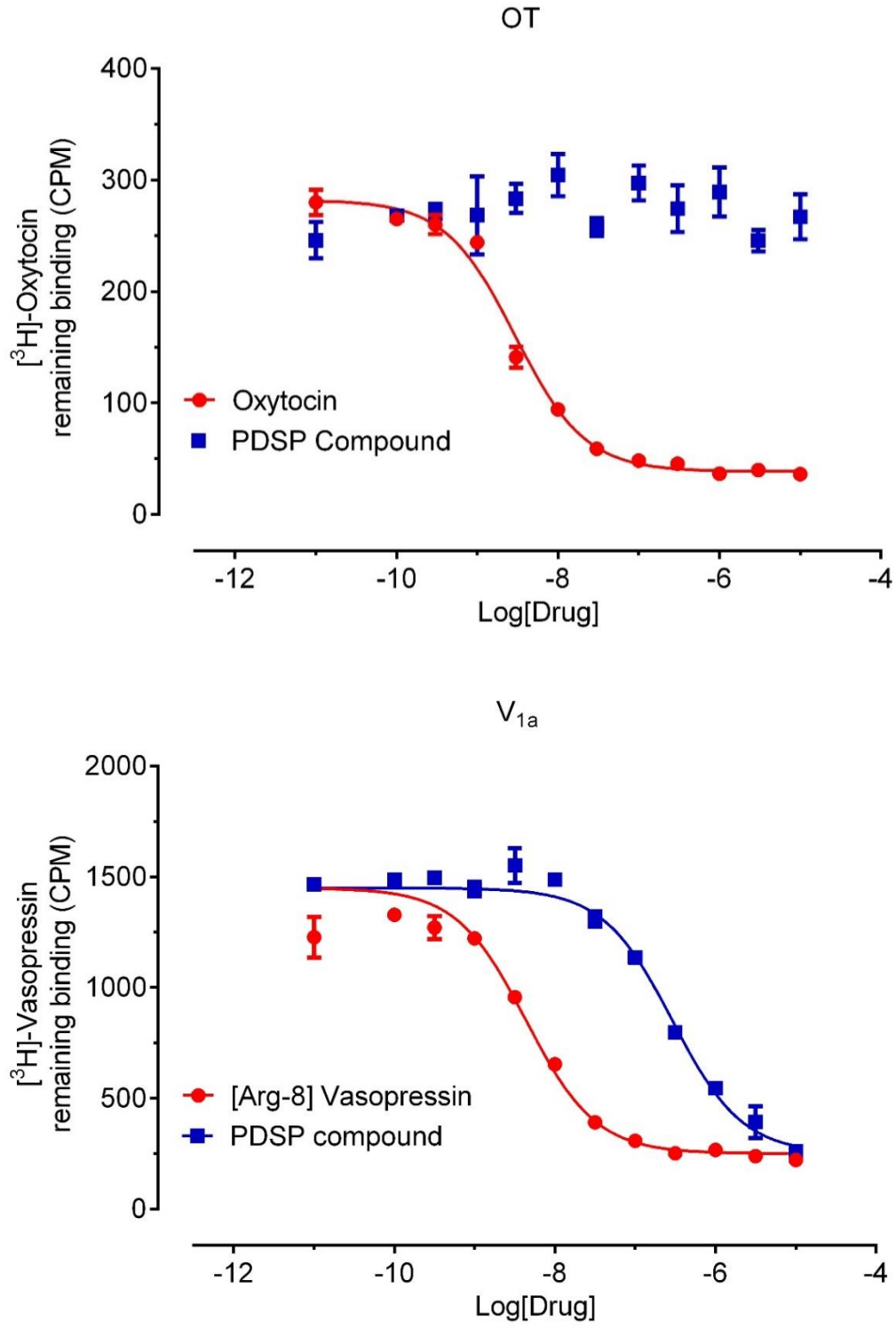
Oxytocin Binding Buffer: 50 mM HEPES, 10 mM $MgCl_2$, pH 7.4

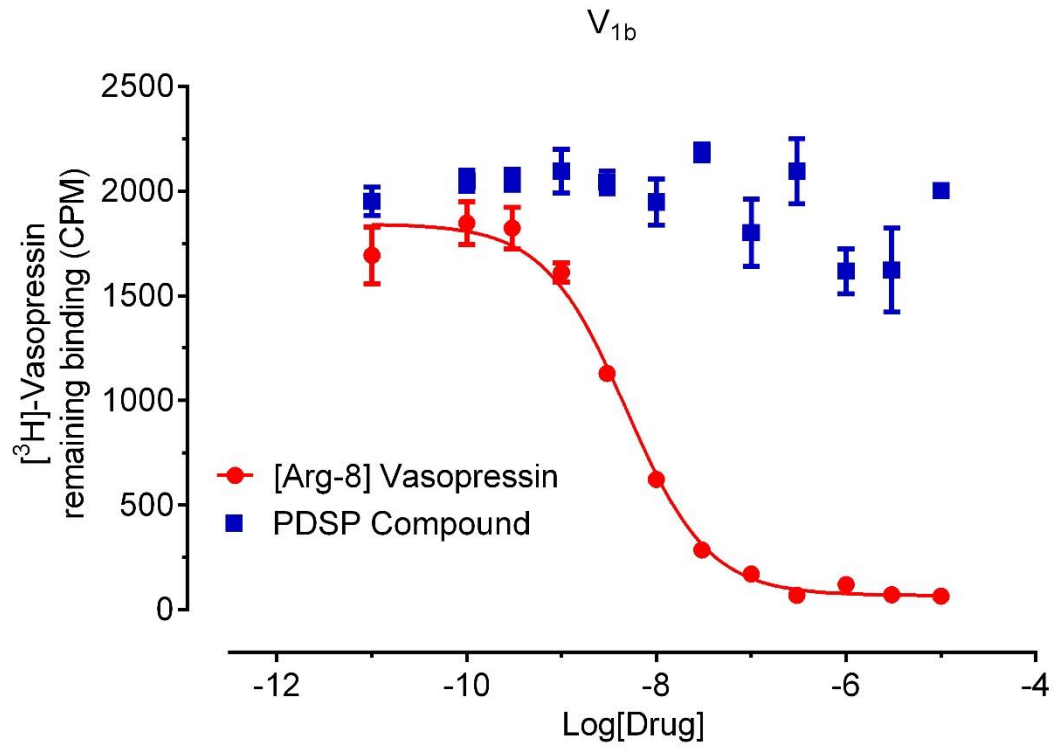
Vasopressin Binding Buffer: 20 mM Tris HCl, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/ml bacitracin, 1 mg/ml BSA, pH 7.4, RT

Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold

Target	Radioligand $pK_d \pm SEM$ (K_d, nM)	Radioligand used (nM)	Reference Ligand $pK_i \pm SEM$ (K_i, nM)	Literature
Oxytocin	[^{3}H]-Oxytocin 8.89 ± 0.06 (1.28)	1.5 – 2.0	Oxytocin 8.56 ± 0.04 (2.73)	(83–85)
V_{1a}	[^{3}H]-Vasopressin 9.17 ± 0.06 (0.68)	0.4 – 1.5	Vasopressin 8.76 ± 0.05 (1.73)	(86, 87)
V_{1b}	[^{3}H]-Vasopressin 9.00 ± 0.05 (0.99)	0.5 – 2.0	Vasopressin 8.88 ± 0.05 (1.32)	
V_2	[^{3}H]-Vasopressin 8.54 ± 0.05 (2.91)	0.6 – 1.7	Vasopressin 8.61 ± 0.05 (2.44)	

Figure 21. Representative competitive binding curves with Oxytocin and Vasopressin receptors.




V1b
2500
2000
1500
1000
500
0
[3H]-Vasopressin
remaining binding (CPM)
[Arg-8] Vasopressin
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

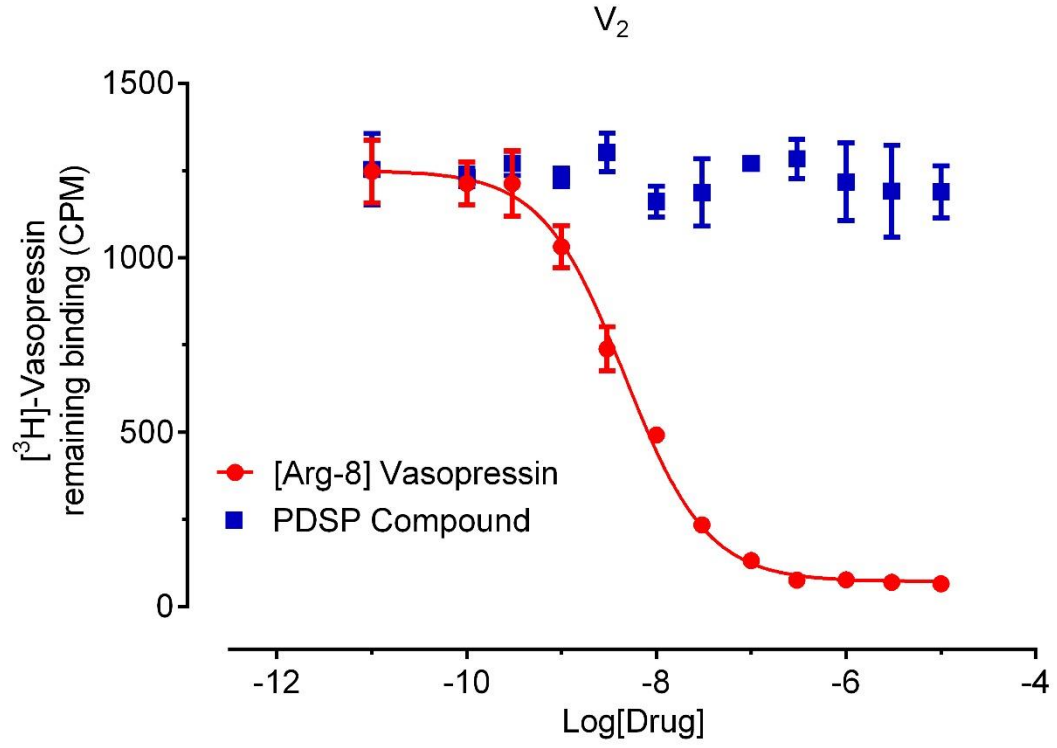

V2
1500
1000
500
0
[3H]-Vasopressin
remaining binding (CPM)
[Arg-8] Vasopressin
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

Table 14. Smoothened receptor, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from >2 years are also included.

Smoothened receptor SMO Binding Buffer: 50 mM HEPES, 3 mM $MgCl_2$, Protease inhibitors, 0.1mg/ml BSA, pH 7.2, RT SMO Wash Buffer: PBS, pH 7.2, cold				
Target	**Radioligand $pK_d \pm SEM$ (K_d, nM)**	**Radioligand used (nM)**	**Reference Ligand $Pk_i \pm SEM$ (K_i, nM)**	**Literature**
SMO	[^{3}H]-Cyclopamine 8.42 ± 0.08 (3.81)	2.6 – 5.0	SAG 8.05 ± 0.28 (8.93) LY2490680 7.76 ± 0.07 (17.57) Cyclopamine 785 ± 0.09 (13.98)	(88, 89)

Figure 22. Representative competitive binding curves with SMO receptors.

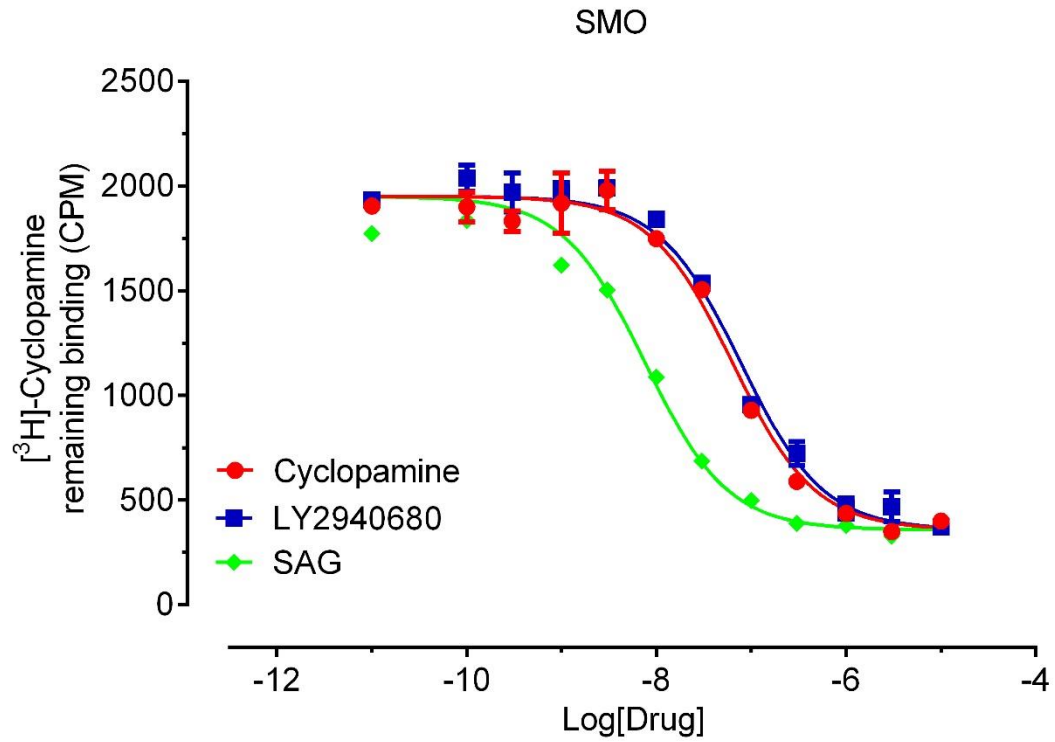


Table 15. Prostanoid receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. These assays are seldom requested and were not carried out in the last 5 years.

Prostanoid receptors Prostanoid binding buffer: 25 mM Tris HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4, RT Standard wash buffer: 50 mM Tris HCl, pH 7.4, cold			
Target	**Radioligand**	**[Radioligand] used (nM)**	**Literature**
EP3	[^{3}H]-PGE2	10	(90, 91)
EP4	[^{3}H]-PGE2	10	

Table 16. PKC subunits, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. These assays are seldom requested and were not carried out in the last 5 years.

PKC PKC Binding Buffer: 50 mM Tris HCl, 1 mM $CaCl_2$, 4 mg/ml BSA, 100 µg/ml phosphatidylserine, pH 7.4, RT Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold			
Target	**Radioligand**	**[^{3}H] in nM**	**Literature**
PKCα	[^{3}H]-PDBU	3	(92–94)
PKCβ	[^{3}H]-PDBU	3	
PKCγ	[^{3}H]-PDBU	3	
PKCδ	[^{3}H]-PDBU	3	
PKCε	[^{3}H]-PDBU	3	

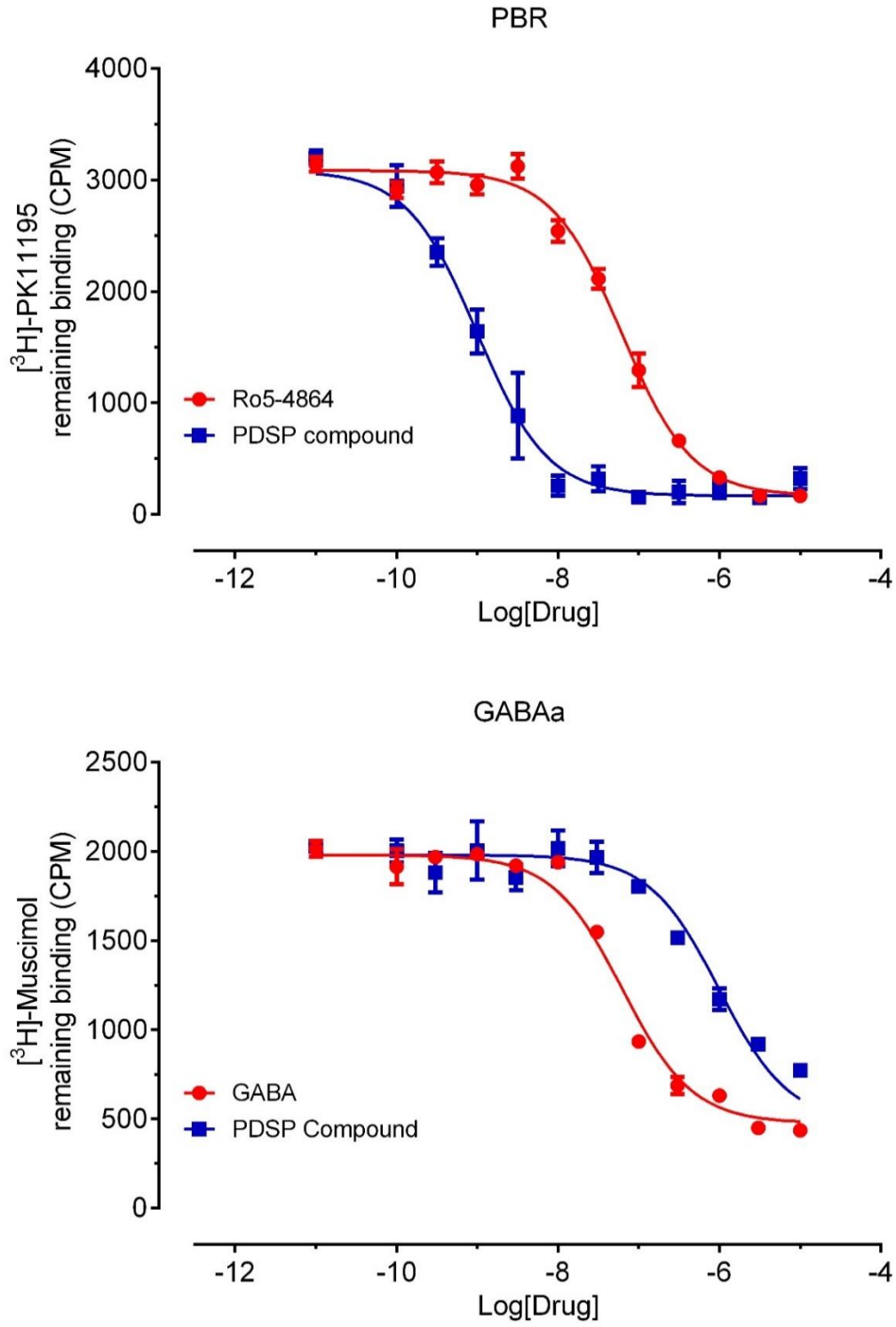
Table 17. GABAA receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from >2 years are also included.

GABA receptors

GABA/PBR Binding Buffer: 50 mM Tris Acetate, pH 7.4, RT
Benzodiazepin (BZP) Binding Buffer: 50 mM Tris HCl, 2.5 mM $CaCl_2$, pH 7.4, RT
Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold

Target	Radioligand $pK_d \pm SEM$ (K_d, nM)	Radioligand used (nM)	References	Literature
GABA/PBR (rat brain)	[^{3}H]-PK11195 9.00 (1.0)	0.3 – 2.0	Ro5-4864 7.82 ± 0.02 (14.97)	(95, 96)
GABAA (rat brain)	[^{3}H]-Muscimol 8.11 ± 0.06 (7.70)	9.50 – 25.0	GABA 6.86 ± 0.08 (137.5)	(97, 98)
GABAA/BZP (rat brain)	[^{3}H]-Flunitrazepam 8.79 ± 0.14 (1.64)	0.6 – 4.0	Clonazepam 8.99 ± 0.03 (1.03)	(99, 100)

Figure 23. Representative competitive binding curves with GABA receptors.



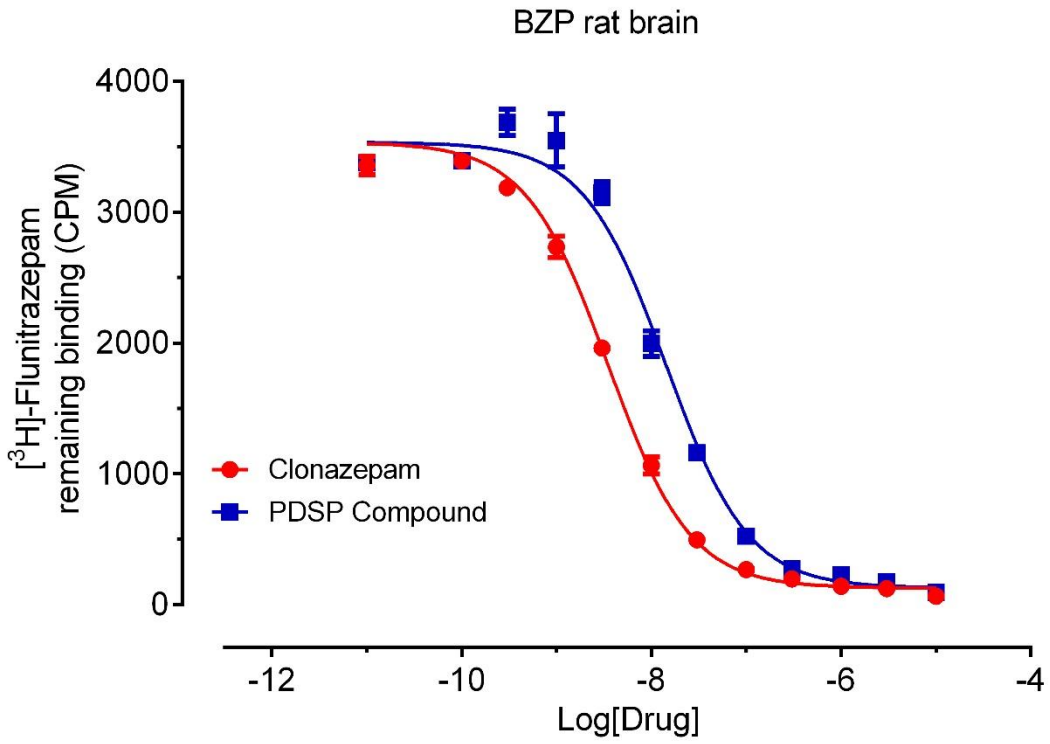
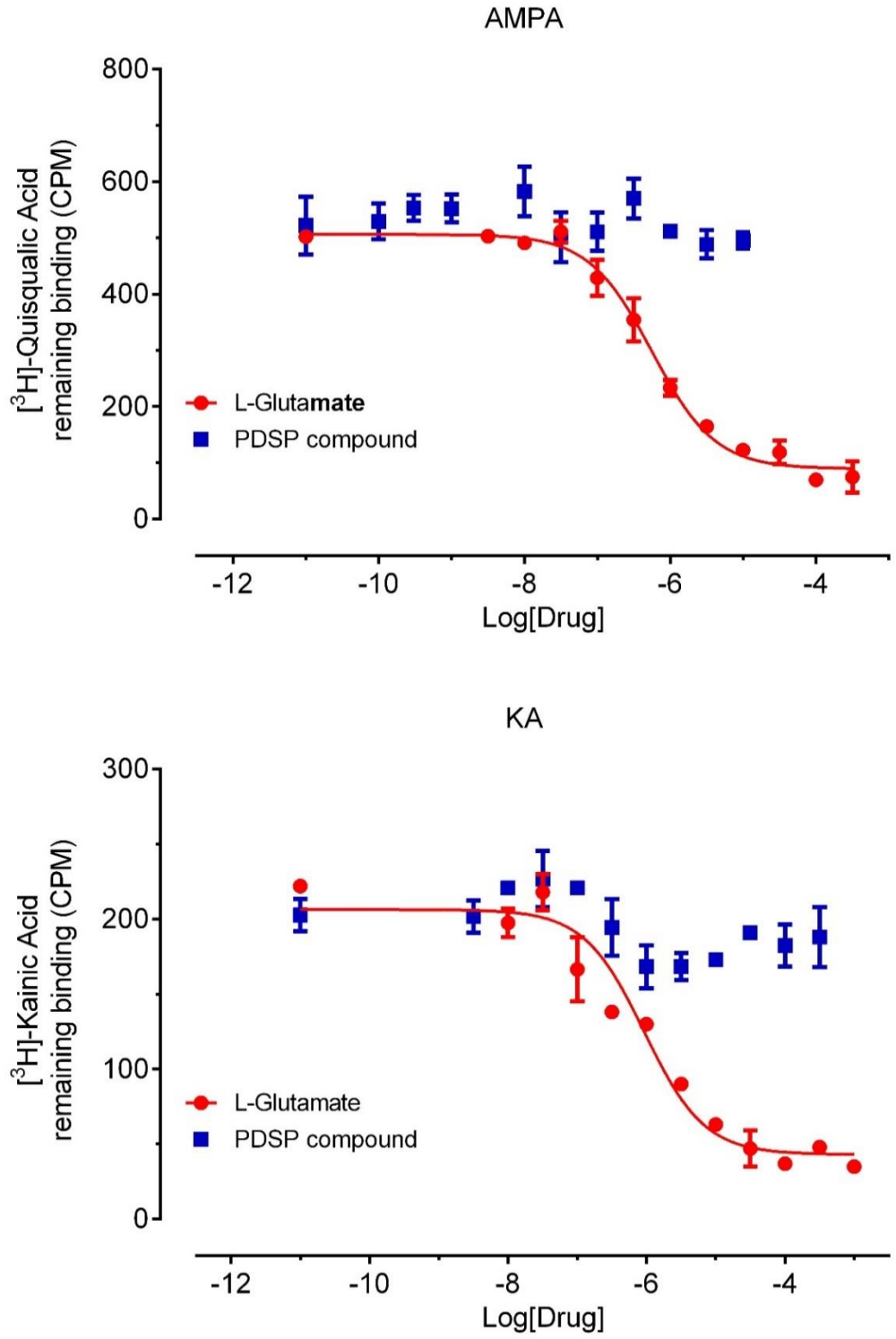
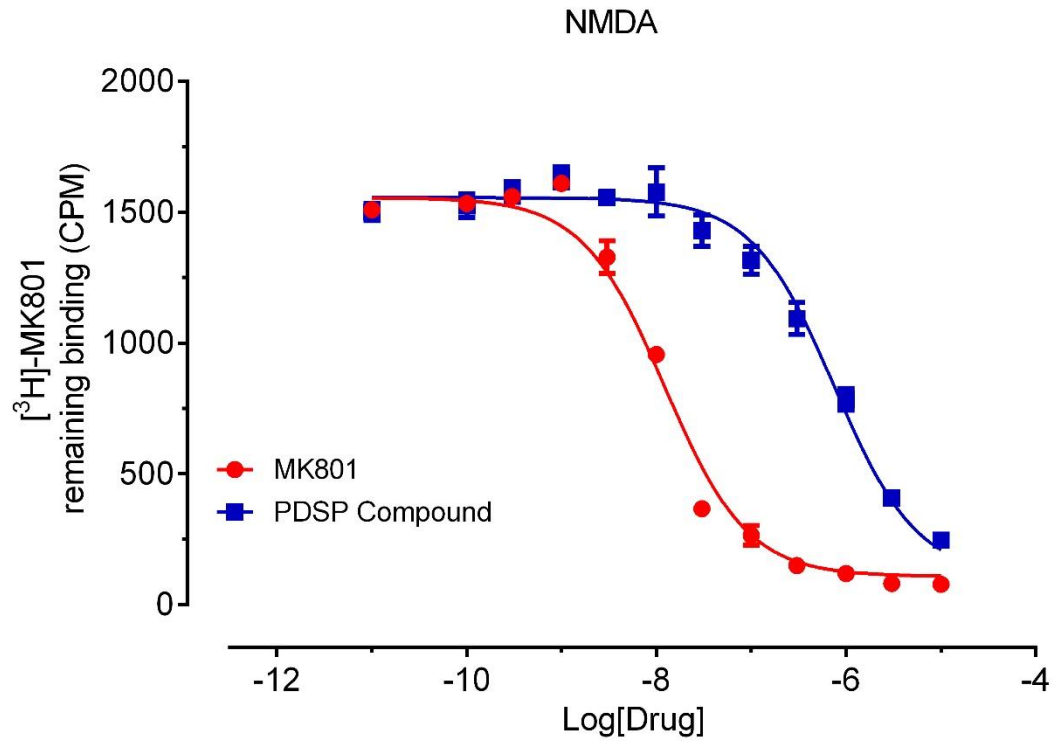

BZP rat brain
[3H]-Flunitrazepam remaining binding (CPM)
4000
3000
2000
1000
0
Clonazepam
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

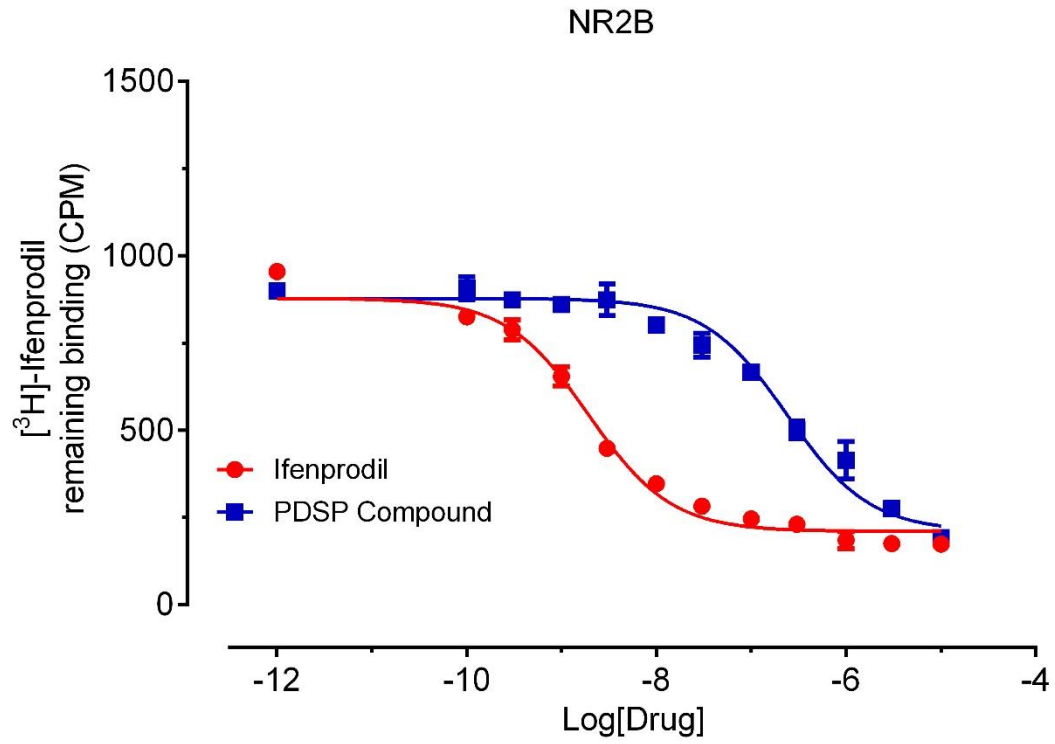
Table 18. Glutamate receptors radioligands, corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical affinity results from the last >2 years are listed.

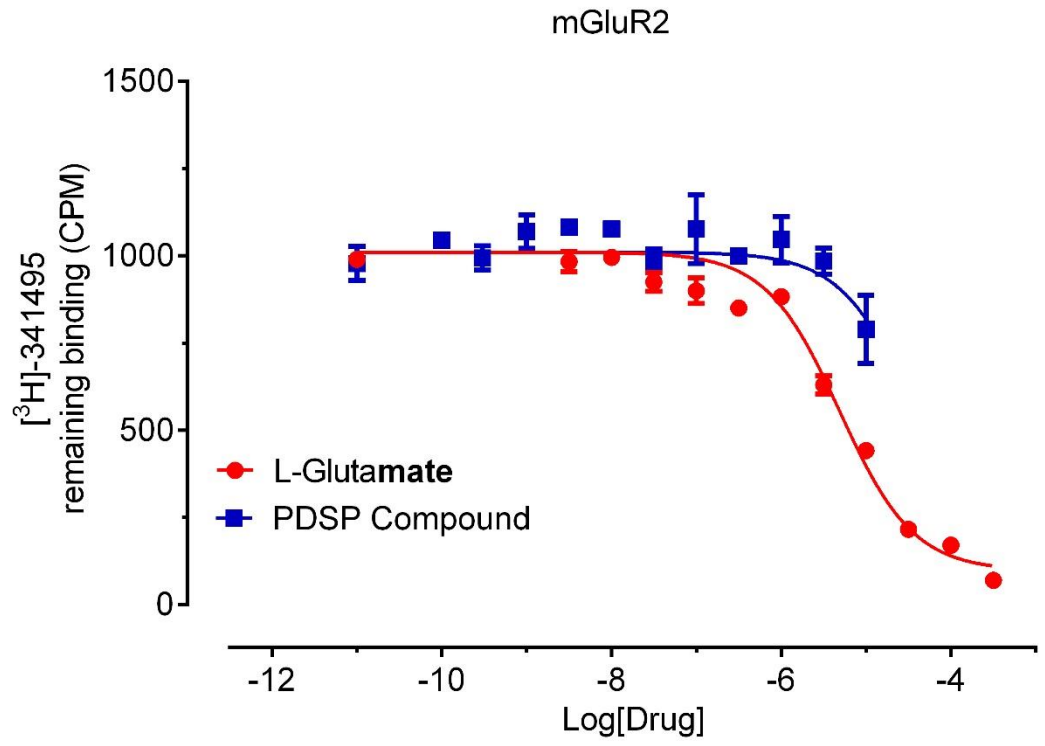
Glutamate receptors NMDA Binding Buffer: 20 mM HEPES, 1 mM EDTA, 100 μM L-Glutamate, 100 μM Glycine, pH 7.0, RT mGluR Binding Buffer: 50 mM Tris HCl, 10 mM MgCl2, 0.1 mM EDTA, pH 7.4, RT Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold				
Target	**Radioligand pK_d ± SEM (K_d, nM)**	**Radioligand used (nM)**	**Reference Ligand pK_i ± SEM (K_i, nM)**	**Literature**
NMDA (rat brain)	[^{3}H]-MK801 8.47 ± 0.07 (3.38)	1.4 – 13.0	MK801 8.41 ± 0.04 (3.88)	(101–103)
NR2B	[^{3}H]-Ifenprodil (3.27)	3.0 – 5.0	Ifenprodil 8.96 ± 0.03 (1.10)	(104–107)
AMPA	[^{3}H]-Quisqualic Acid (30)	25.0 – 32.0	L-Glutamate 6.19 ± 0.09 (647)	
KA	[^{3}H]-Kainic Acid 8.02 ± 0.15 (9.58)	8.0 – 12.0	L-Glutamate 6.22 ± 0.14 (596)	(108, 109)
mGluRs				
$mGlu_1$	Being developed			
$mGlu_2$	[^{3}H]-LY341495 8.66 ± 0.07 (2.17)	2.1 – 4.6	L-Glutamate 5.51 ± 0.05 (3090)	(110–112)
$mGlu_3$	[^{3}H]-LY341495 8.43 ± 0.17 (3.71)	1.8 – 2.5	L-Glutamate 6.48 ± 0.10 (3340)	
$mGlu_4$	Being developed			
$mGlu_5$	[^{3}H]-MPEP 8.47 ± 0.16 (3.40)	1.0 – 5.0	Fenobam 7.85 ± 0.08 (14.0)	(113)
$mGlu_6$	[^{3}H]-LY341495 8.47 ± 0.16 (3.40)	3.0 – 5.0	L-Glutamate 7.45 ± 0.37 (35.5)	(114)
$mGlu_7$	Being developed			
$mGlu_8$	Being developed			

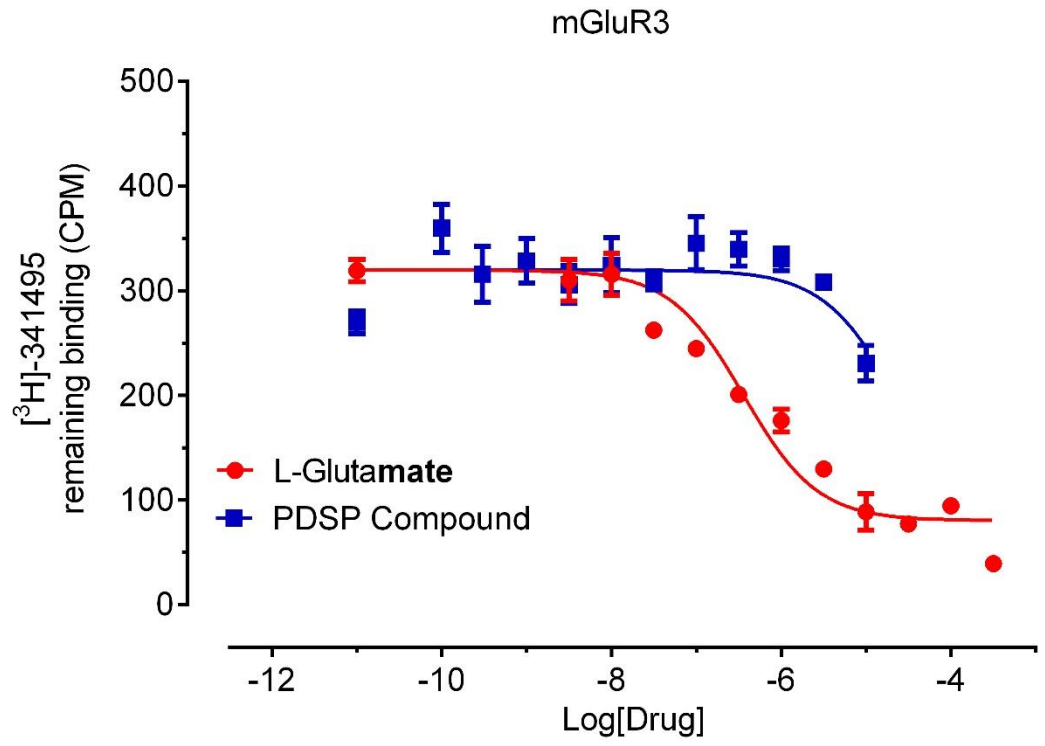
Figure 24. Representative competitive binding curves for NMDA and mGluR5 receptors.

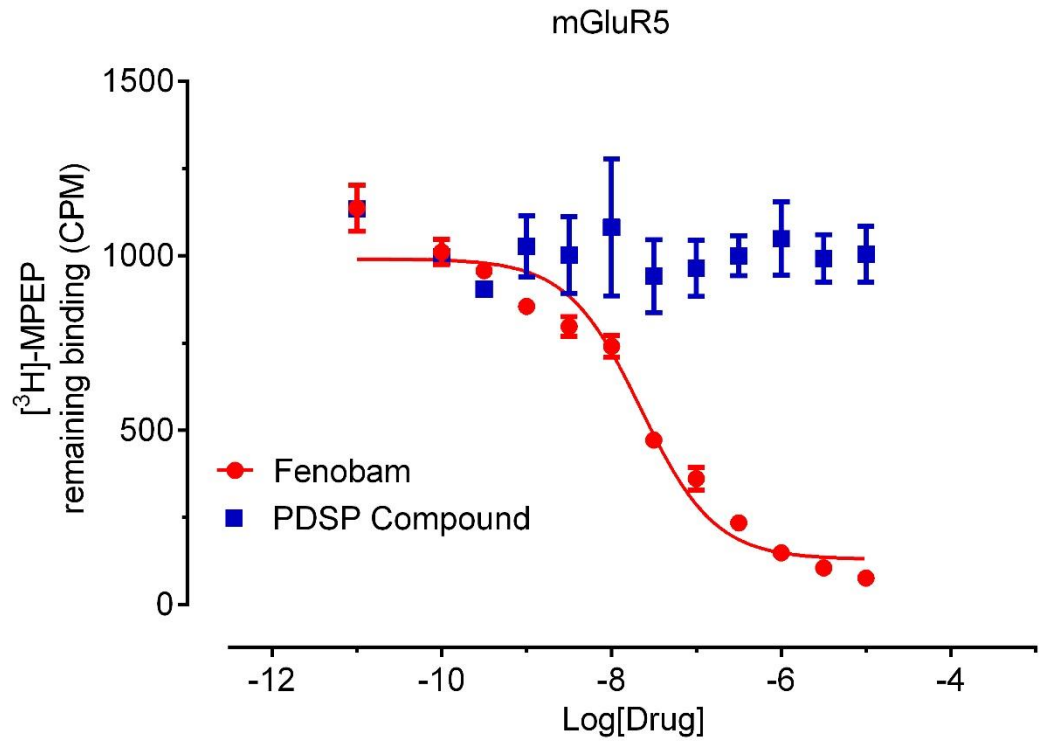



NMDA
2000
1500
1000
500
0
[3H]-MK801
remaining binding (CPM)
MK801
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


NR2B
1500
1000
500
0
[3H]-Ifenprodil
remaining binding (CPM)
Ifenprodil
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


mGluR2
1500
1000
500
0
[3H]-341495
remaining binding (CPM)
L-Glutamate
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


mGluR3
500
400
300
200
100
0
[3H]-341495
remaining binding (CPM)
L-Glutamate
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


mGluR5
1500
1000
500
0
[3H]-MPEP
remaining binding (CPM)
Fenobam
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

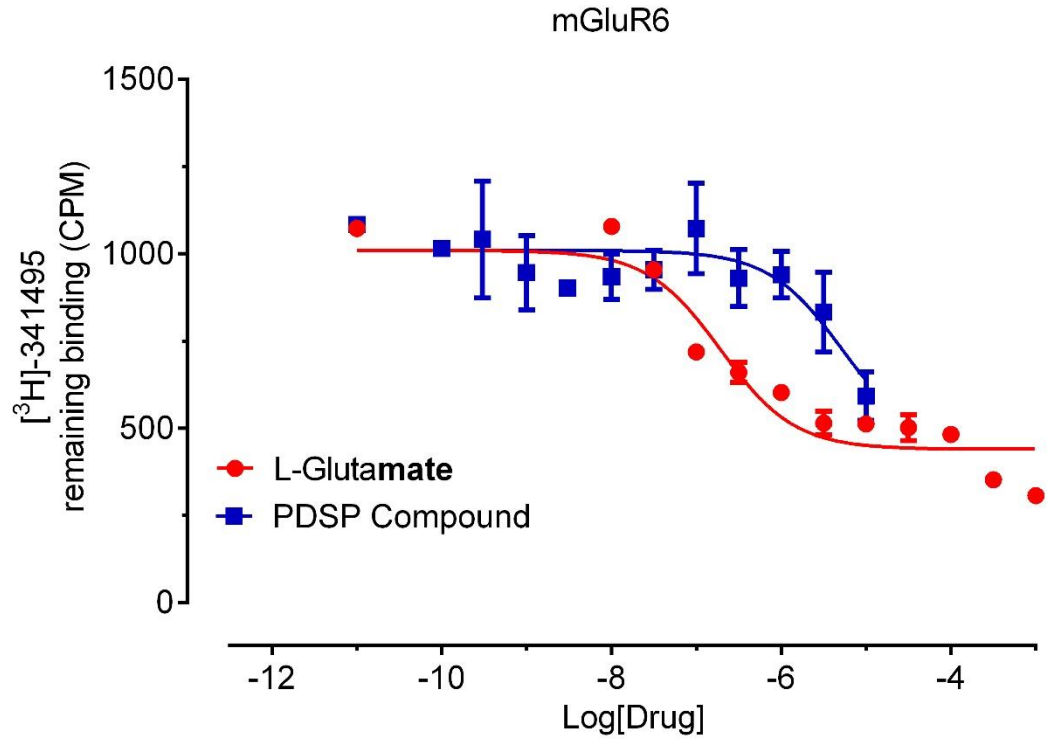
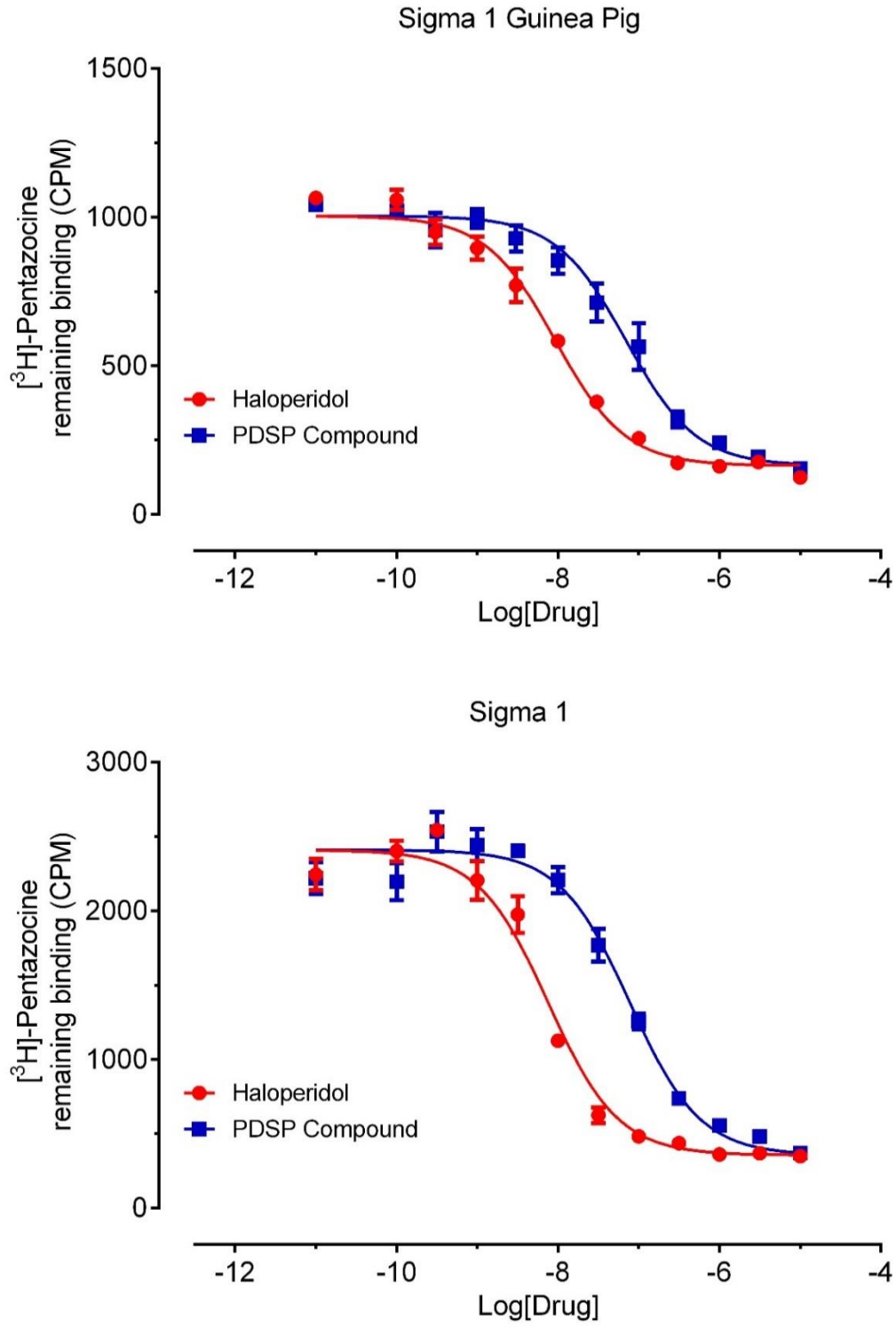
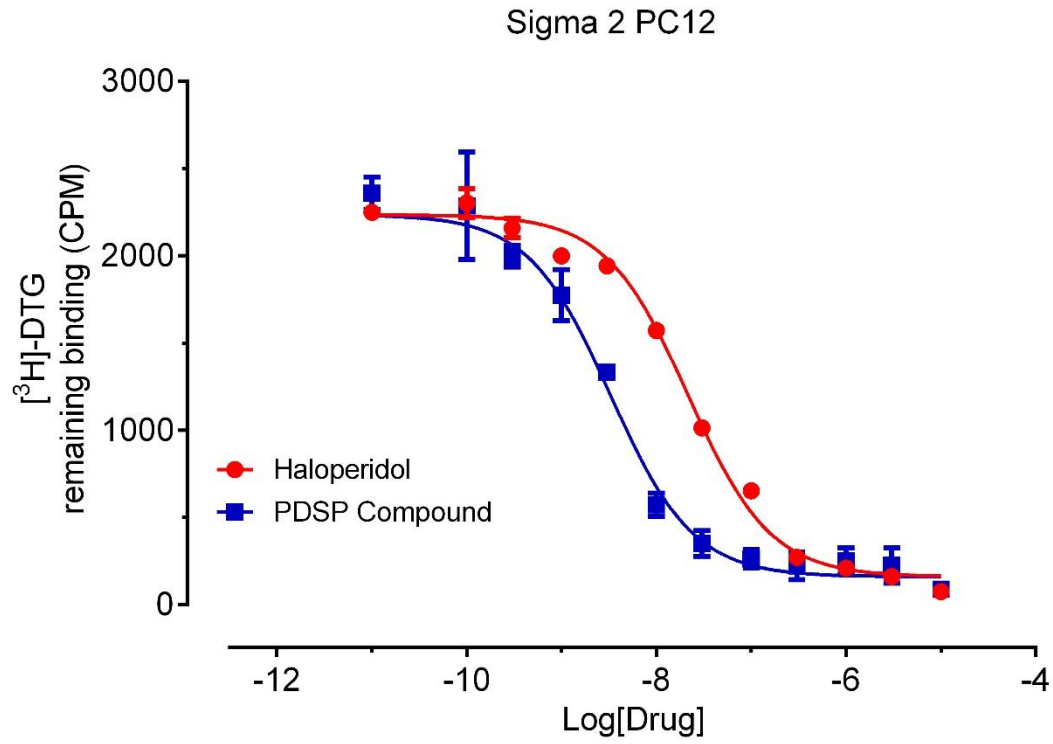

mGluR6
1500
1000
500
0
[3H]-341495
remaining binding (CPM)
L-Glutamate
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

Table 19. Sigma receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from > 6 months are also included.

Sigma receptors Sigma Binding Buffer: 50 mM Tris HCl, pH 8.0, RT Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold				
Target	**Radioligand pK_d ± SEM (K_d, nM)**	**Radioligand used (nM)**	**Reference Ligand pK_i ± SEM (K_i, nM)**	**Literature**
Sigma 1 (Guinea pig)	[^{3}H]-Pentazocine 8.19 ± 0.18 (6.50)	2.0 – 10.0	Haloperidol 8.45 ± 0.01 (3.54)	(115–117)
Sigma 1 (Human)	[^{3}H]-Pentazocine 8.08 ± 0.12 (8.32)	4.0 – 5.0	Haloperidol 8.14 ± 0.04 (6.77)	
Sigma 2 (PC12)	[^{3}H]-DTG 8.00 ± 0.03 (9.90)	5.0 – 7.0	Haloperidol 7.86 ± 0.01 (13.91)	
Sigma 2 (Human)	[^{3}H]-DTG 7.55 ± 0.18 (27.93)	5.0	Haloperidol 7.80 ± 0.04 (15.87)	

Figure 25. Representative competitive binding curves with Sigma receptors.




Sigma 2 PC12
3000
2000
1000
0
[3H]-DTG
remaining binding (CPM)
Haloperidol
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

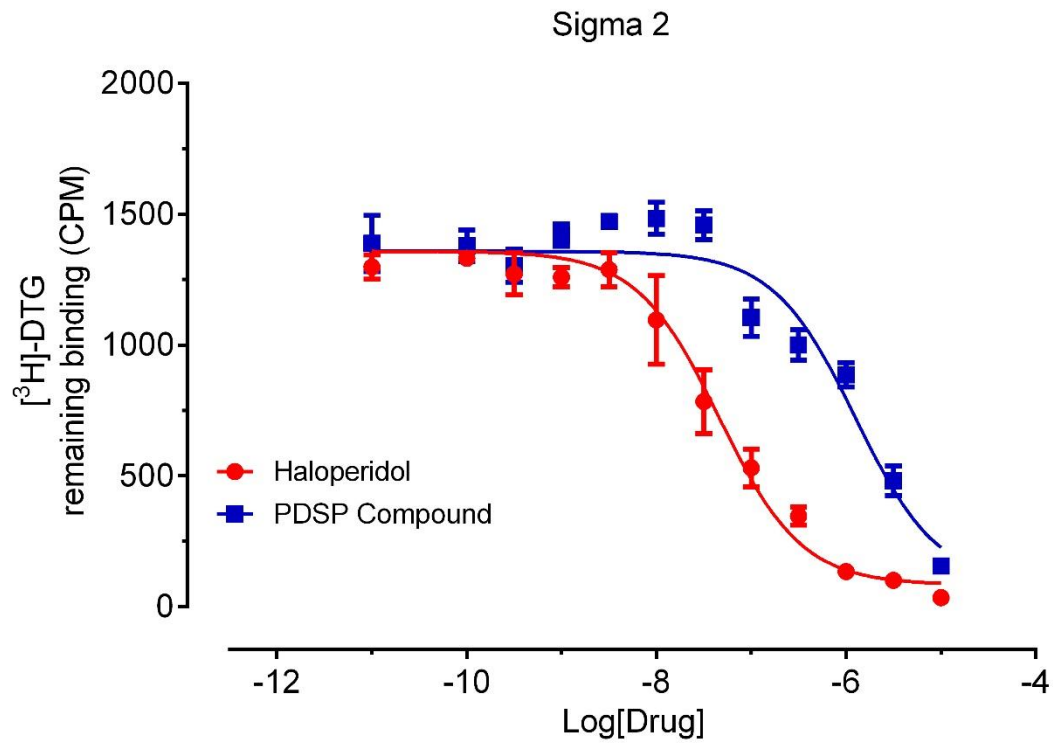

Sigma 2
2000
1500
1000
500
0
[3H]-DTG
remaining binding (CPM)
Haloperidol
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

Table 20. Angiotensin II receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values (if available) from > 6 months are also included.

Angiotensin receptors Angiotensin Binding Buffer: 50 mM Tris HCl, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mg/ml BSA, 100 mM Bacitracin, pH 7.4, RT Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold				
Target	**Radioligand** **pK_d ± SEM (K_d, nM)**	**[^{3}H] in nM**	**Reference Ligand** **pK_i ± SEM (K_i, nM)**	**Literature**
AT_1	[^{3}H]-Angiotensin II	0.1	Candesartan	(118, 119)
AT_2	[^{3}H]-Angiotensin II 8.19 ± 0.28 (6.52)	3.50	PD123319 7.52 ± 0.06 (30.0)	

Figure 26. Representative binding curve for AT_2 receptors.

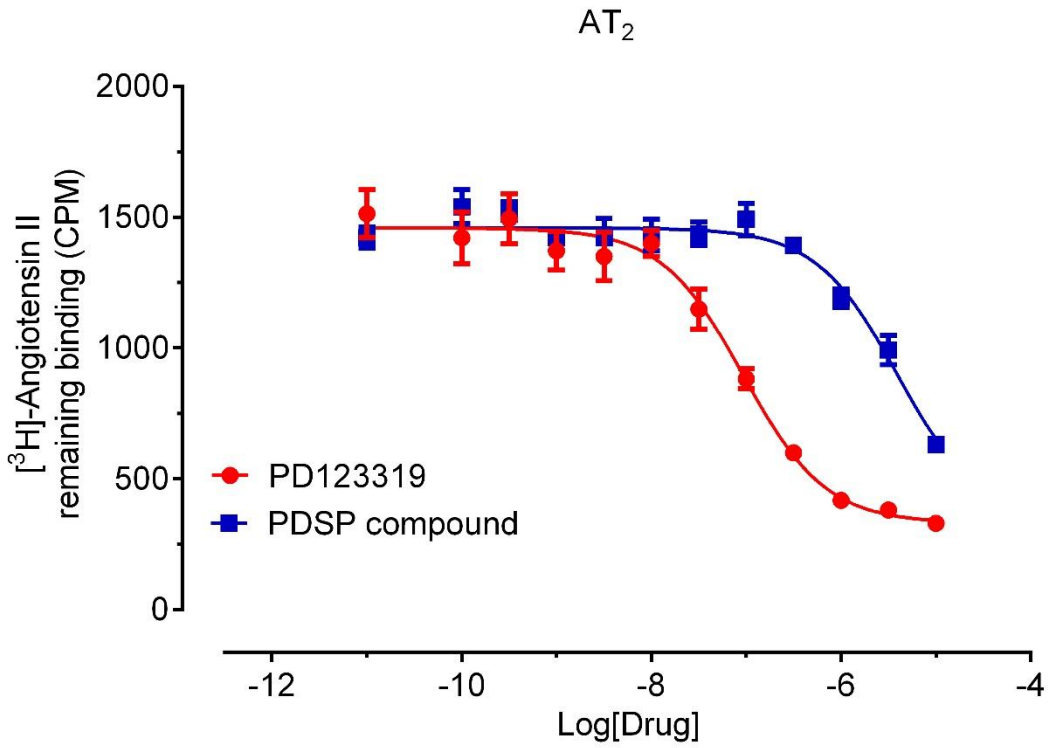
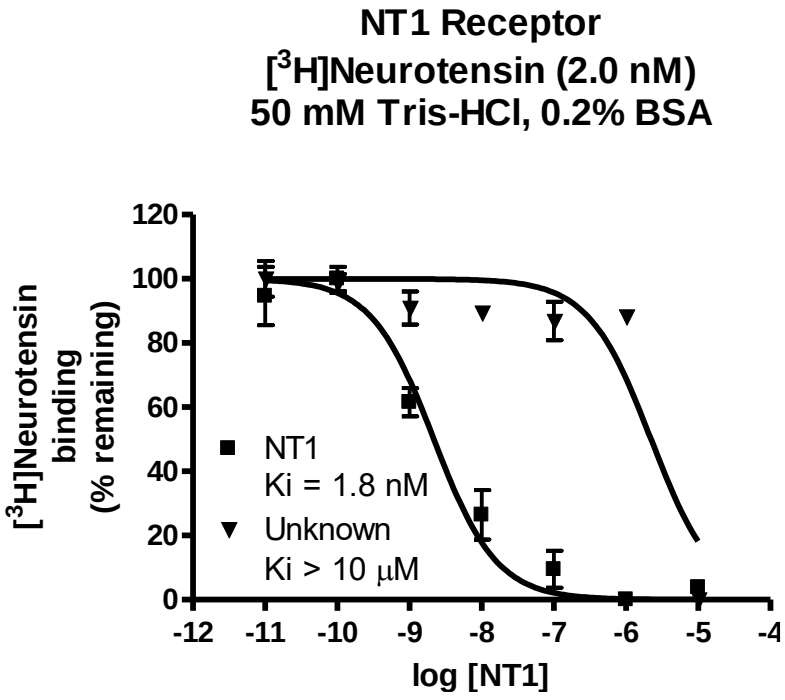

AT2
2000
1500
1000
500
0
[3H]-Angiotensin II
remaining binding (CPM)
PD123319
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

Table 21. Neurotensin receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values (if available) from > 6 months are also included.

Neurotensin receptors Neurotensin binding buffer: 50 mM Tris HCl, 0.2% BSA Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold				
Target	**Radioligand pK_d ± SEM (K_d, nM)**	**Radioligand used (nM)**	**Reference Ligand pK_i ± SEM (K_i, nM)**	**Literature**
NTS_1	[^{3}H]-Neurotensin 8.25 ± 0.15 (5.65)	1.0 – 4.0	Neurotensin 8.40 ± 0.07 (3.95)	(120–122)
NTS_2	[^{3}H]-Neurotensin	1.2	Neurotensin	

Figure 27. Representative binding curve for Neurotensin receptors.



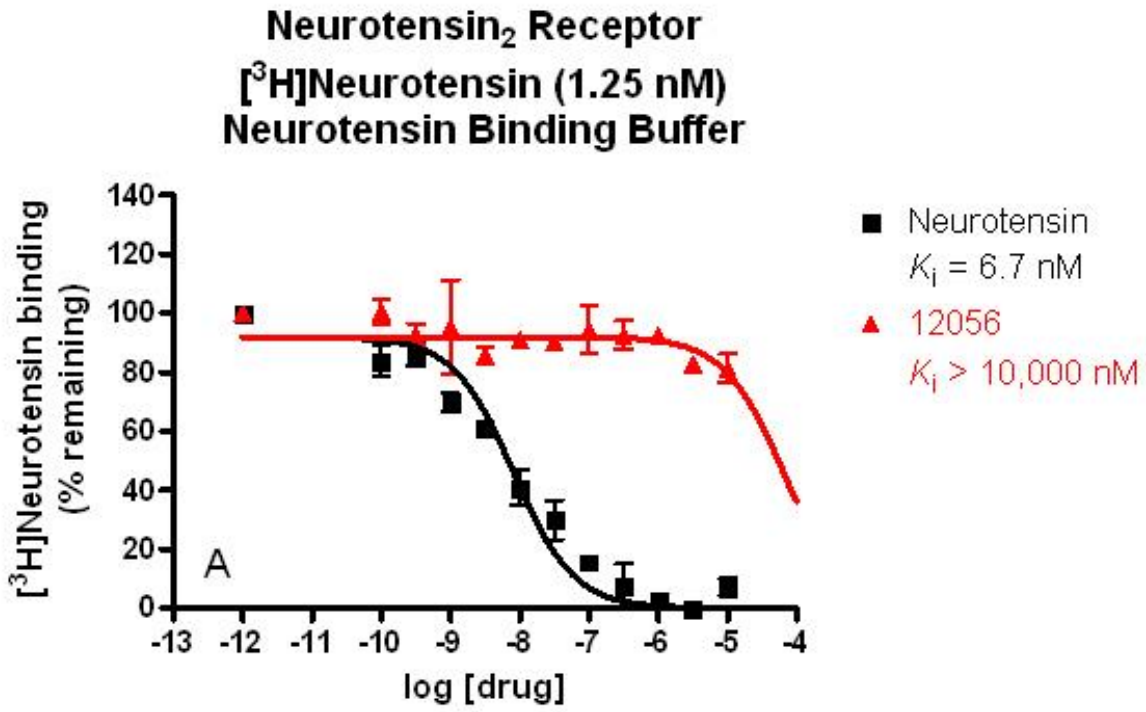


Table 22. VMAT2 transportor, radioligand and corresponding concentration, reference compound, and buffers for primary and secondary radioligand binding assays.

VMAT2 transporter VMAT2 binding buffer: 50 mM Tris HCl, 0.2% BSA Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold				
Target	**Radioligand**	**Radioligand used (nM)**	**Reference Ligand**	**Literature**
VMAT2	[^{3}H]-Tetrabenazine	1.5	Reserpine	(123)

Figure 28. Representative binding curve for VMAT2 transporters.

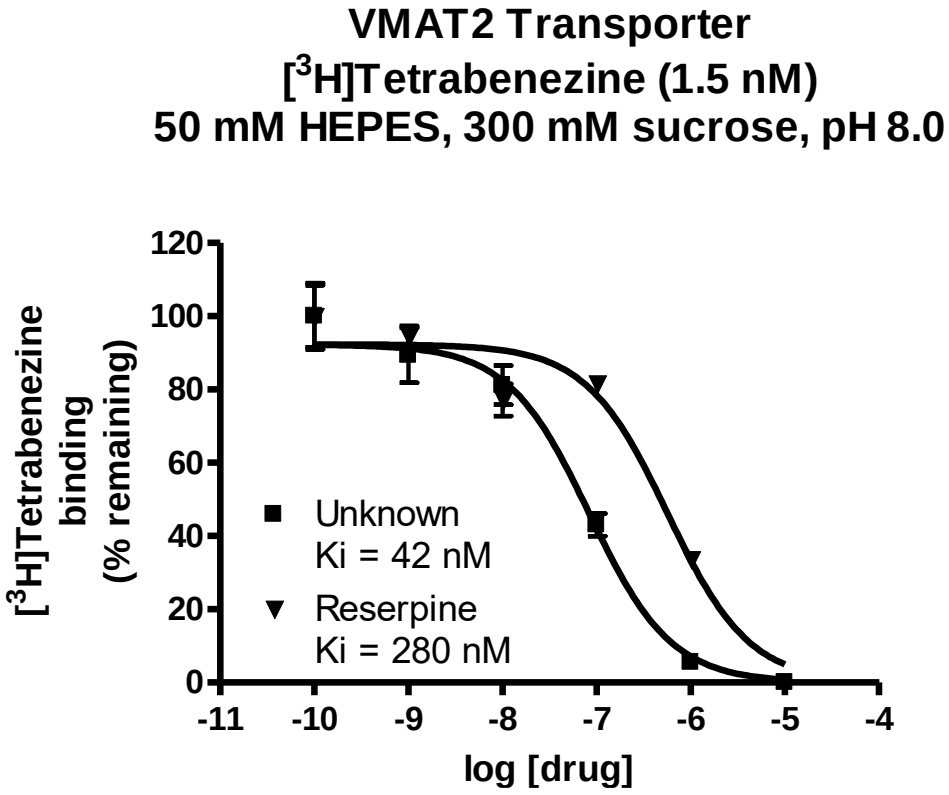


Table 23. Calcium and sodium channels, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values (if available) from > 6 months are also included.

Calcium and Sodium channels

Calcium channel Binding Buffer: 50 mM Tris HCl, 50 mM NaCl, 1 mM $CaCl_2$, pH 7.4, RT
Sodium channel Binding Buffer: 50 mM HEPES, 130 mM Choline Cl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM Glucose, 1 µM tetrodotoxin, 1 mg/ml BSA, 30 µg/well scorpion verom, pH 7.4, 37°C.
Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold

Target	Radioligand pK_d ± SEM (K_d, nM)	[Radioligand] used (nM)	Reference Ligand pK_i ± SEM (K_i, nM)	Literature
Ca^{2+} channel	[^{3}H]-PN200110 8.52 ± 0.12 (3.04)	2.0 – 3.0	Nifendipine 8.05 ± 0.17 (8.85)	(124, 125)
	[^{3}H]-Nitrendipine 8.21 ± 0.25 (6.20)	3.0 – 5.0	Nifendipine	(126, 127)
Na^+ channel	[^{3}H]-Batrachotoxin		Veratridine	(128, 129)

Figure 29. Representative figure for Ca^{2+} channel binding assay.

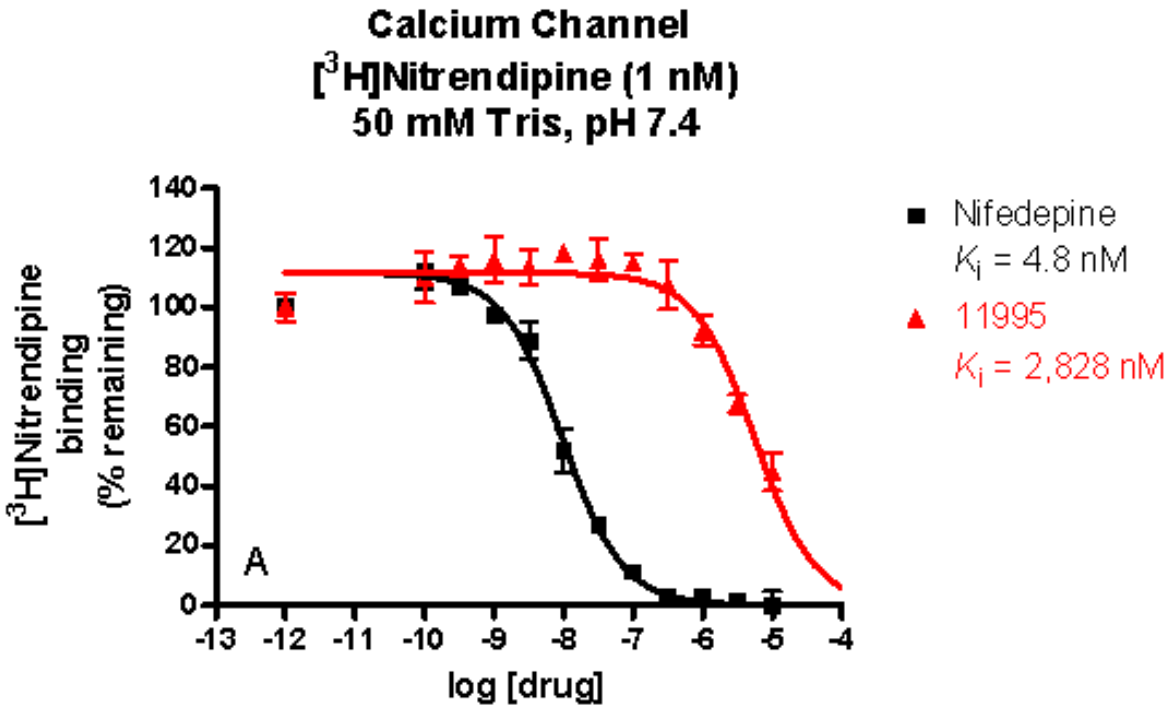


Table 24. HERG potassium channel, radioligand and corresponding concentration, reference compound, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually near the K_d value, or as listed. Historical reference K_i values from >2 years are also included.

hERG channel hERG binding buffer: 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1 mM EGTA, 1 mg/ml BSA, pH 7.4, RT hERG wash buffer: hERG binding buffer, pH 7.4, cold				
Target	**Radioligand** **pK_d ± SEM (K_d, nM)**	**[Radioligand] used (nM)**	**Reference Ligand** **pK_i ± SEM (K_i, nM)**	**Literature**
hERG	[^{3}H]-Dofetilide 8.39 ± 0.03 (4.07)	3.0 – 5.0	Dofetilide 8.37 ± 0.08 (4.29)	(130, 131)

Figure 30. Representative binding curve with hERG potassium channel.

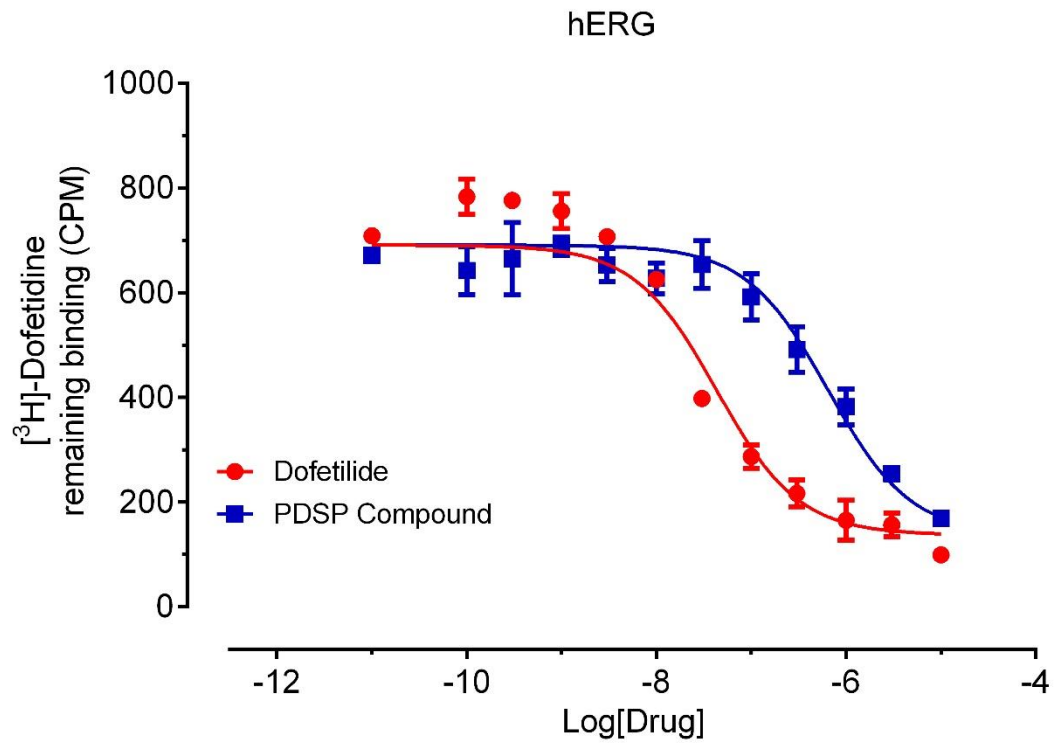


Table 25. Imidazoline receptor, radioligand and corresponding concentration, reference compound, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually near the K_d value, or as listed.

Imidazoline

Imidazoline binding buffer: 5 mM Tris HCl, 5 mM HEPES, 0.5 mM EGTA, 0.5 mM EDTA, 0.5 mM $MgCl_2$, pH 8.0, RT
Standard wash buffer: 50 mM Tris HCl, pH 7.4, cold

Target	Radioligand	Radioligand used (nM)	Reference Ligand	Literature
Imidazoline 1	[^{3}H]-Clonidine	0.1 nM	Naphazoline	(132, 133)

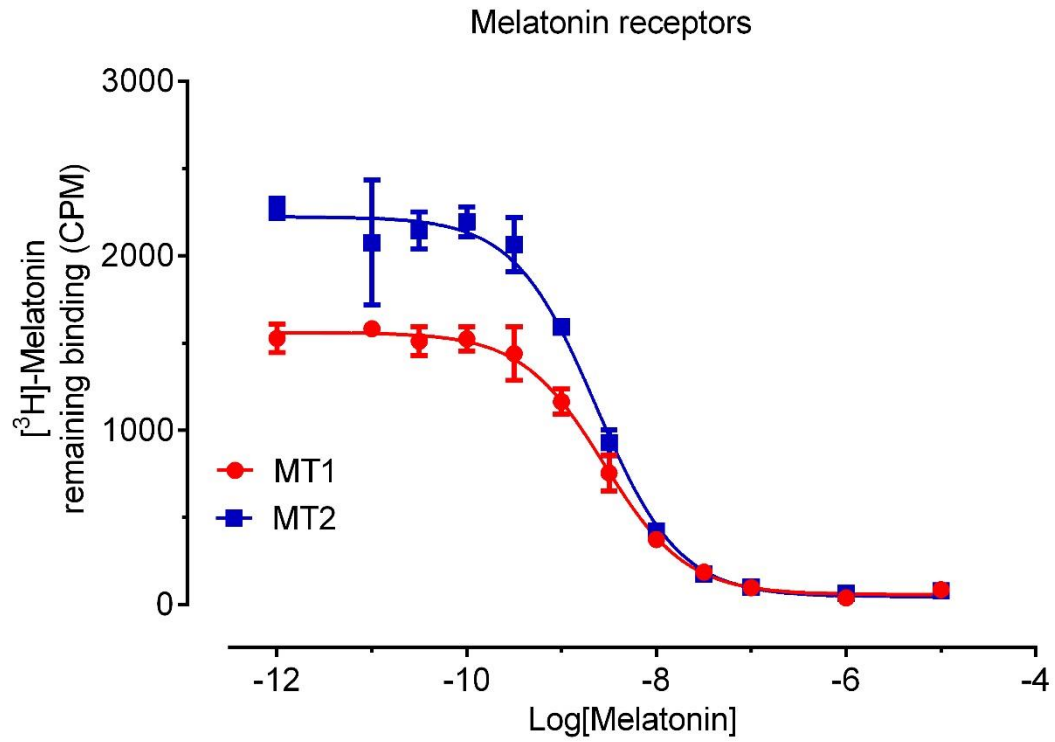
Table 26. Melatonin receptors, radioligand and corresponding concentration, reference compound, and buffers for primary and secondary radioligand binding assays. These are new binding assays, and thus have no legacy data.

Melatonin receptors
(Being developed and optimized)

Standand binding buffer: 50 mM Tris HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4, RT
Standard wash buffer: 50 mM Tris HCl, pH 7.4, cold

Target	Radioligand $pK_d \pm SEM$ (K_d, nM)	[Radioligand] used (nM)	Reference Ligand $pK_i \pm SEM$ (K_i, nM)	Literature
MT_1	[^{3}H]-Melatonin	0.5 - 1.0	Melatonin	(134–136)
MT_2	[^{3}H]-Melatonin	0.5 – 1.0	Melatonin	

Figure 31. Representative binding curves with melatonin receptors.



Section 2: Functional assays

2.1. Drug plate preparations for functional assays: Drug plates for functional assays are either manually prepared or made by STAR robotics. The following drug plate maps are the ones routinely used for functional assays.

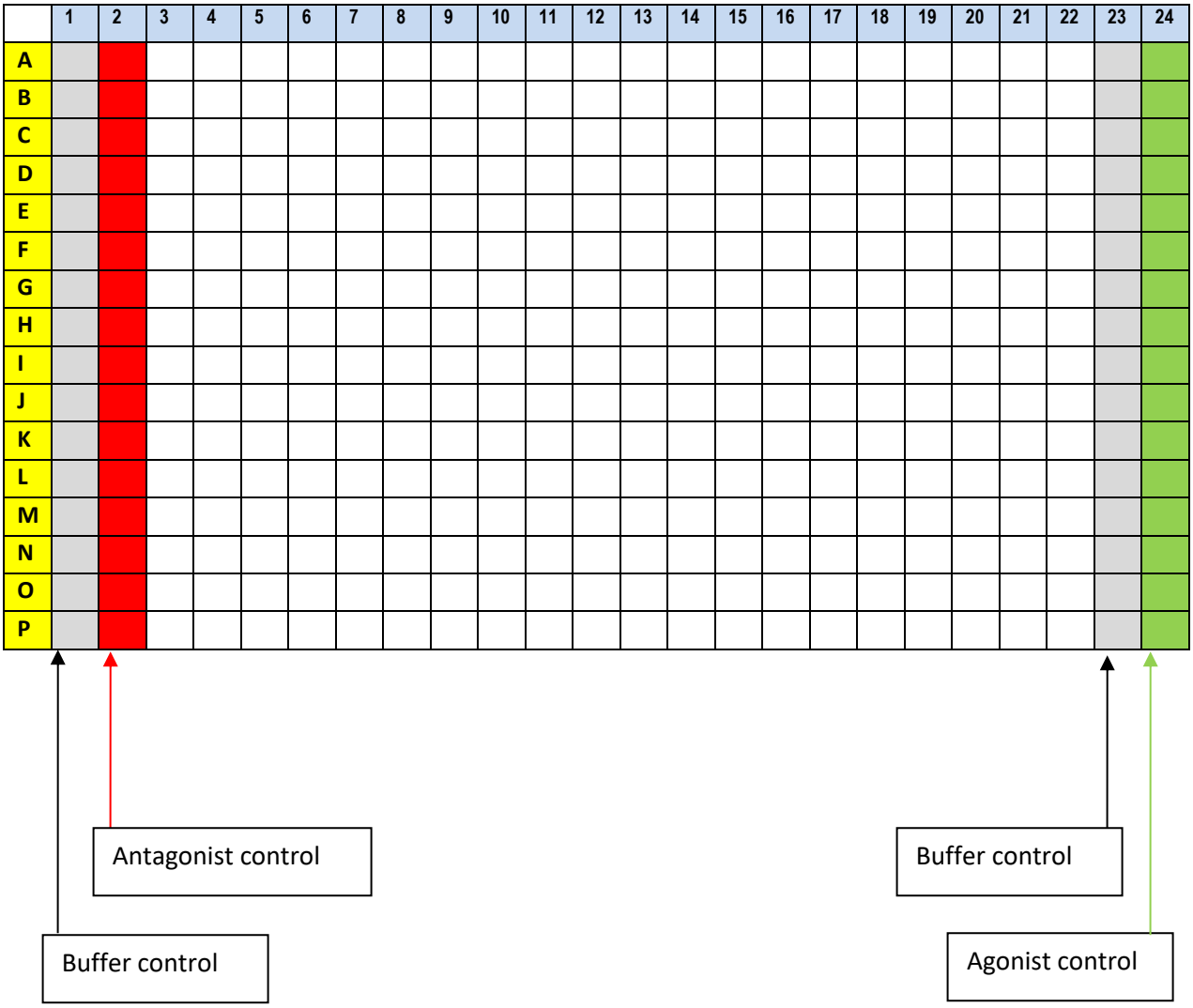


Figure 32. 384-well drug plate map #1 for primary screening: Singlet format (first addition). A total of 320 different compounds can be plated in singlet format in a 384-well drug plate. Columns 1, 2, 23, and 24 are reserved for positive and negative controls.

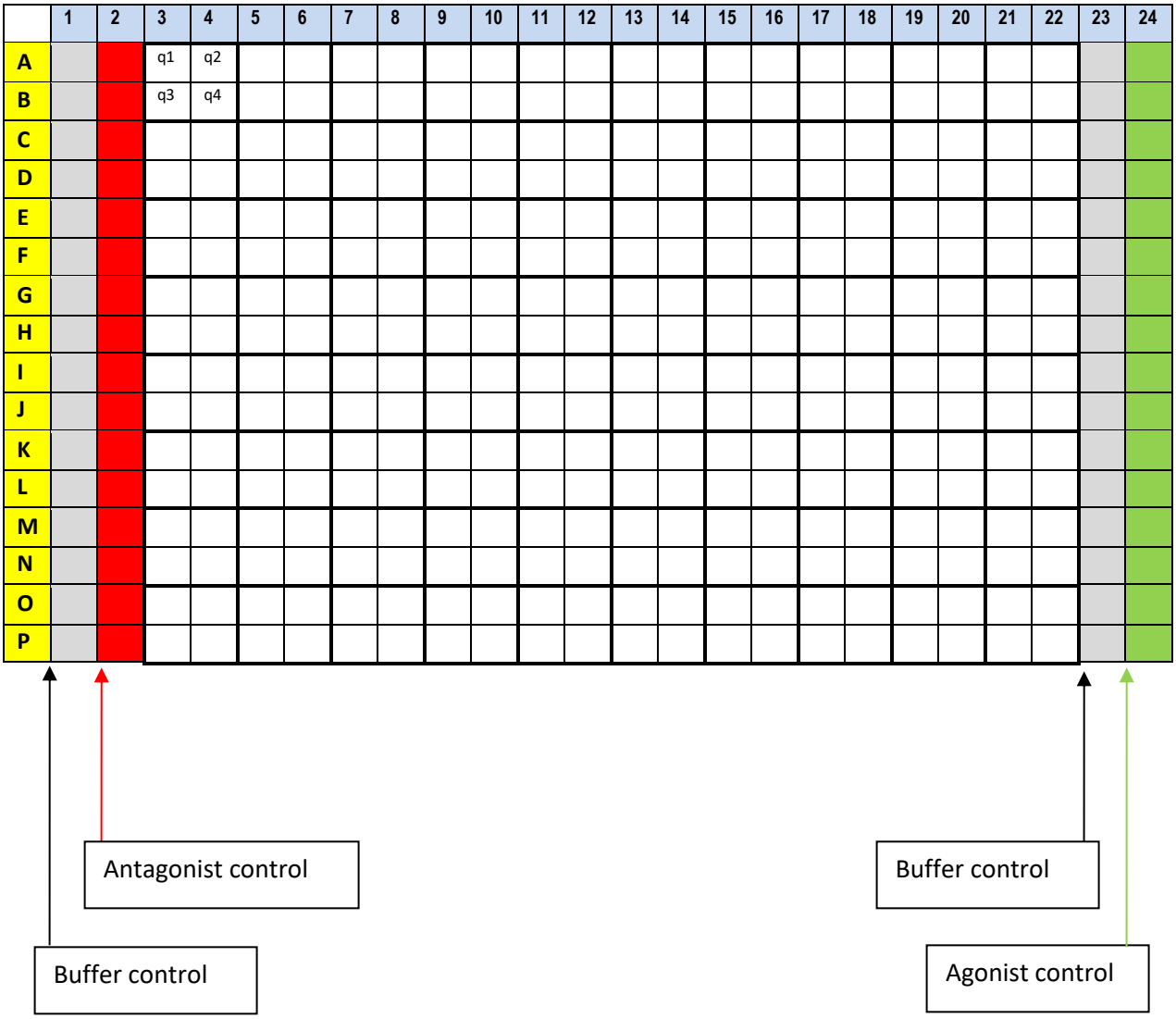


Figure 33. 384-well drug plate map #2 for primary screening: quadrant format (first addition). A total of 80 different compounds can be plated in quadrant format (q1, q2, q3, and q4) in a 384-well drug plate. Columns 1, 2, 23, and 24 are reserved for positive and negative controls.

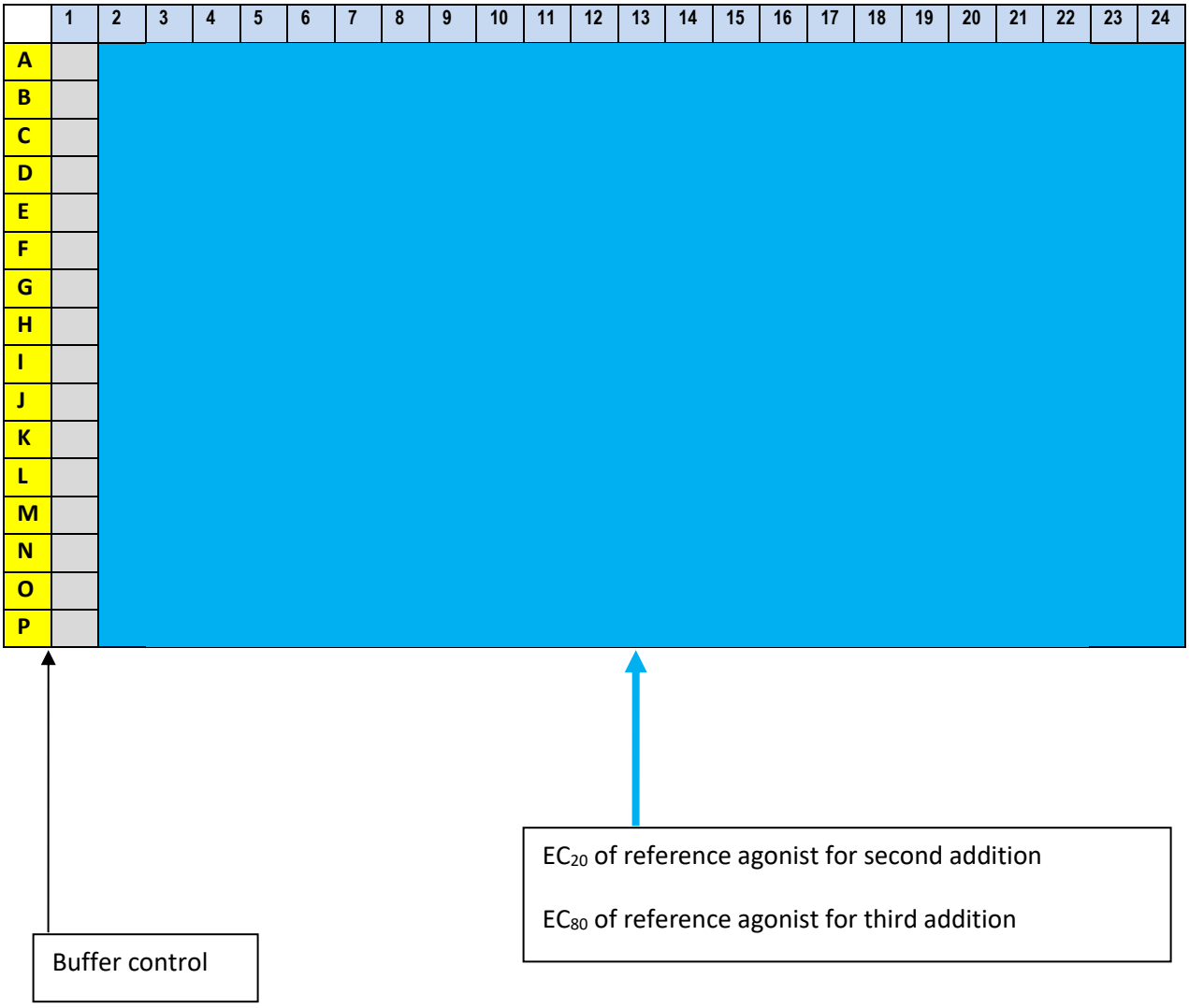


Figure 34. 384-well drug plate map #3: second or third addition in primary screening assays (second addition). Except for Column 1 (which serves as negative control with assay buffer for all additions), all the other wells receive EC_{20} or EC_{80} of the reference agonist to determine allosteric potentiator or antagonist activity.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	[X] (M)
A																									0
B																									3E-12
C																									1E-11
D																									3E-11
E																									1E-10
F																									3E-10
G																									1E-09
H																									3E-09
I																									1E-08
J																									3E-08
K																									1E-07
L																									3E-07
M																									1E-06
N																									3E-06
O																									1E-05
P																									3E-05

8	Drug A	Drug B	Drug C	Drug D	Drug E	Drug F	Drug G	Drug H

6	Drug A’	Drug B’	Drug C’	Drug D’	Drug E’	Drug F’

Figure 35. 384-well drug plate map #4: agonist or antagonist dose-responses for secondary screenings (first addition): Serial dilutions of eight compounds can be made in triplicate (Drug A to H) or six in quadruplicate (Drug A’ to F’) (indicated below the plate template) from high to low concentrations (final concentrations are indicated to the right of plate template). Either format is used; one of the compounds is a positive control with a known agonist and/or antagonist, such as acetylcholine and/or atropine for muscarinic receptors.

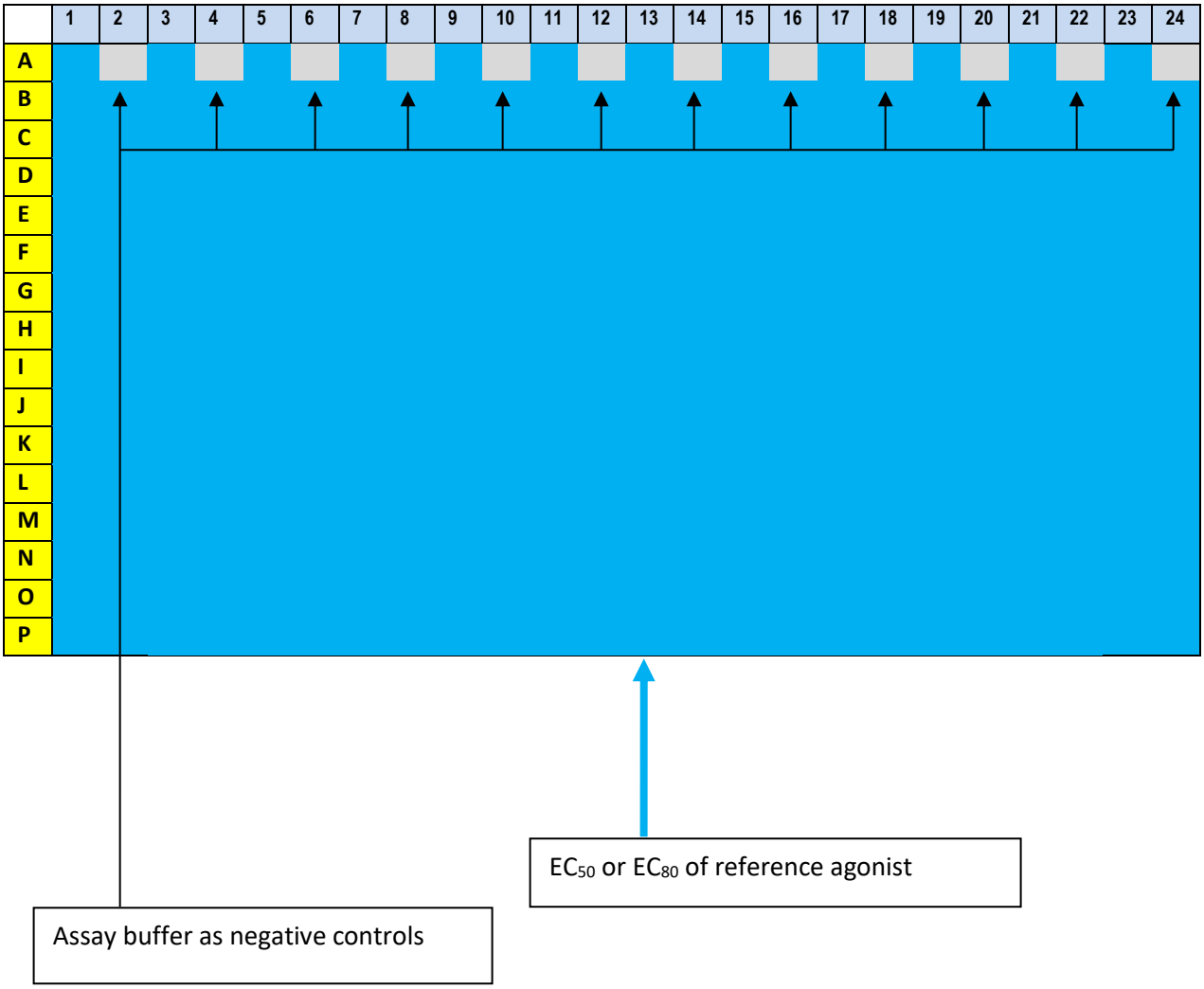


Figure 36. 384-well drug plate map #5: EC_{50} or EC_{80} of reference agonist as second addition for antagonist activity (second addition). The even-numbered wells in row A serve as negative controls with assay buffer; while the other wells on the plate contain EC_{50} or EC_{80} concentrations of the reference agonist.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								

X	Buffer	30 nM	100 nM	300 nM	1 µM	3 µM	10 µM	30 µM

Figure 37. 384-well drug plate map #6 for Schild plot analysis (first addition): Antagonist or allosteric modulator is made in 7 concentrations as indicated below the drug plate template. Each concentration (including the buffer control) has three columns.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	[X] (M)
A																									0
B																									3E-12
C																									1E-11
D																									3E-11
E																									1E-10
F																									3E-10
G																									1E-09
H																									3E-09
I																									1E-08
J																									3E-08
K																									1E-07
L																									3E-07
M																									1E-06
N																									3E-06
O																									1E-05
P																									3E-05

Figure 38. 384-well drug plate map #7 for Schild plot analysis (second addition). Reference agonist is made in the following serial dilutions (as indicated to the right of the drug plate template). Each concentration (including the buffer control) has one complete row.

2.2. General procedures for PDSP functional assays:

In general, PDSP functional assays are carried out in two steps, primary and secondary assays (unless otherwise stated). In primary screening assays, compounds are tested in triplicate or quadruplicate at final concentration of 10 μM (or 10 μg/ml for crude extracts) or specific concentrations upon request for agonist and antagonist activities (see 384-well drug plate maps #1, #2, and #3 for detailed setups). Results are normalized and transformed to percentage values. For agonists, the reference agonist activity at 10 μM is set as 100% and the basal activity with buffer as 0%. **For orphan GPCRs without a known agonist as a reference, activity is expressed as percentage value of basal (with buffer).** For antagonists, the basal activity with buffer is set as 100% inhibition and the activity of an EC_{80} concentration of the reference agonist as 0% inhibition. For allosteric potentiators, the activity of the EC_{20} concentration of the reference agonist is set as 0% potentiation. Compounds with a minimum of 30% agonist activity, or 50% antagonist activity, or 30% potentiation above control are flagged for secondary screening (dose-response) assays.

In secondary functional assays, potential agonist hits are tested in full dose-response curves to determine efficacy and potency (see 384-well drug plate map #4). Potential antagonist hits are tested in full dose-response curves to determine IC_{50} values against EC_{50} to EC_{80} concentrations of reference agonist (see 384-well drug plate map #5 for detailed setup). Potential antagonist hits and positive allosteric modulators are further characterized, if necessary, in Schild plot analysis to obtain pK_B values (see 384-well drug plate maps #6 and #7 for detailed setup). Schild plot analysis is designed to obtain full agonist dose-response curves in the absence and presence of increasing concentrations of potential antagonists or allosteric modulators. For those antagonists shifting agonist dose-response curves to the right and also reducing Emax, a modified Lew and Angus analysis is applied to obtain pK_B values. For allosteric modulators, secondary dose-response curves are also analyzed using the allosteric operational model to obtain modulation parameters (such as α and ß). For studies of ligand functional selectivity and bias among multiple signaling pathways, dose-response results are analyzed using the Black and Leff operational model to quantify bias. Detailed procedures for these secondary assays are given in the following data analysis sections.

Secondary functional assays provide the following parameters for the PDSP dabatase: maximum activation or inhibition after normalization (therefore in percentage values), concentration range used in the assay (lowest and highest concentrations), Hill slope of the dose-response curve, and corresponding potency.

2.3. Data analysis for functional assays

Unless otherwise stated, all functional results are analyzed using GraphPad Prism v5.0 using its built-in functions.

2.3.1: Agonist activity: Functional assay results are plotted against concentrations and analyzed with following 4-parameter built-in agonist dose-response function in GraphPad Prism v5.0.

$$Response = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogEC_{50} - X)n}}$$

In which, **Top** and **Bottom** are the maximum response ($\mathbf{E_{max}}$) and basal level, respectively; **X** is the agonist concentration and **n** is the Hill slope; $\mathbf{EC_{50}}$ is the concentration that generated a 50% response.

2.3.2: Antagonist activity: To determine antagonist activity, agonist responses are measured at a fixed EC_{80} concentration of the reference agonist and in the presence of serial dilutions of the antagonist. Results are fitted with the following inhibitory dose-response function to determine the IC_{50}.

$$Response = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogIC_{50} - X)n}}$$

The IC_{50} is then converted to K_i using Cheng-Prusoff equation (7):

$$K_i = \frac{IC_{50}}{1 + \frac{L}{EC_{50}}}$$

in which **K_i** is the ligand binding affinity determined from antagonist dose-response assay; **IC_{50}** is the antagonist concentration at which point 50% inhibition is reached; **L** is the reference agonist concentration used in the assay (usually EC_{50} to EC_{80} concentration of the reference agonist); **EC_{50}** is the predetermined potency of the reference agonist.

2.3.3. Schild plot analysis: The PDSP has adopted nonlinear regression analysis with the modified Lew and Angus method to estimate antagonist potency pA_2 value. For Schild plot analysis, agonist dose-responses are designed and performed in the absence and presence of 7 concentrations of antagonist in a 384-well plate (see above). Each agonist dose-response is in triplicate. Eight agonist dose-responses share the same X-axis and each set of measured responses is arranged in a Y column titled antagonist concentrations (M). Results are analyzed using the agonist dose-response function as above to determine Bottom and Top values for each dose-response curve. To normalize using Prism's built-in normalization function, the data set is transformed into percentage values with the shared ***bottom*** as 0% and ***Top*** of the reference agonist under control conditions as 100%. An equiactive agonist concentration (such as an agonist concentration to generate 20 – 50% response) is selected for all or most curves if possible and corresponding agonist concentrations in the absence and presence of increasing concentrations of antagonist are obtained. Prism 5 has a built-in feature that provides equiactive agonist concentrations if the radio buttom for "Interpolate unknowns from standard curve" is selected. This feature is at the bottom of the Analyze>Fit tab. The equiactive agonist concentrations are plotted in –log format (i.e., pEC_{20} or pEC_{50}) against corresponding antagonist (B) concentrations (M). The results are fitted with following equation to obtain $LogK_B$ and n values.

$$Y = -Log(X^n + 10^{LogK_B}) - LogC$$

In which Y is the equiactive agonist concentration in –log format (such as pEC_{25} or other equally-active agonist concentration values) at corresponding antagonist concentration (X); n is the Schild slope; K_B is the apparent binding affinity of the tested antagonist. The pA_2 value can be calculated from pK_B/n. If n = 1 or is not significantly different from 1, $pA_2 = pK_B$, and the tested antagonist is

concluded to be competitive with the agonist; otherwise, antagonist may not be competitive with the agonist.

Representative figures and examples are shown on Pages 169, 170, and 171.

2.3.4. Quantifying bias and functional selectivity analysis (see Pages 171-174). To quantify functional selectivity and bias factors, we analyze functional dose-esponse results using the Black and Leff operation model as outlined by Kenakin et al., 2012 (137).

$$Response = Basal + \frac{(E_m - Basal)[A]^n \tau^n}{[A]^n \tau^n + ([A] + K_A)^n}$$

Functional dose-response results are analyzed using the above equation in GraphPad Prism V5.0, in which $\mathbf{E_m}$ is the maximal possible response the system can have, and **basal** is the response in the absence of test drugs (i.e., buffer only). The $\mathbf{E_m}$ and **Basal** values are usually 100 and 0 if the responses are normalized to percentage of reference activity (100%). $\mathbf{K_A}$ is the equilibrium dissociation constant of the agonist (**A**), $\boldsymbol{\tau}$ is the operational agonist efficacy of the agonist (**A**) and is defined as $\mathbf{R_T/K_E}$ (where $\mathbf{R_T}$ is the receptor density and $\mathbf{K_E}$ is the intrinsic efficacy of the agonist (**A**) in a particular signaling pathway, **n** is the transducer slope for the function between agonist occupancy and measured responses. The fitting parameters $\mathbf{E_m}$ and **n** are cell-specific and should be shared by all agonists that are being tested at the same pathway. Log($\boldsymbol{\tau/K_A}$) is defined as the "transduction coefficient" for a particular agonist at a measured signaling pathway.

To quantify ligand bias (see following equations and steps), drug activity is measured in two or more pathways for a group of ligands (e.g., agonists) to determine corresponding $\log(\tau/K_A)$ values. For each pathway, $\Delta\log(\tau/K_A)$ values are calculated by subtracting the reference agonist's $\log(\tau/K_A)$ (usually the endogenous agonist). The same reference agonist should be used for the different pathways tested. For each agonist, $\Delta\Delta\log(\tau/K_A)$ values are calculated by subtracting $\Delta\log(\tau/K_A)$ for pathway I with the corresponding $\Delta\log(\tau/K_A)$ for pathway II. Bias is quantified as $10^{\Delta\Delta\log(\tau/KA)}$. If the value is 1, the agonist has no bias at all; if the value is larger than 1, it is biased towards pathway I; otherwise, it is biased towards pathway II.

Step 1: dose-responses for 2 or more pathways to determine 'transduction coefficient' Log(τ/K_A) values for each ligand at each pathway;

Step 2: calculate transduction coefficient difference ΔLog(τ/K_A) between different ligands in the same pathway by subtracting transduction coefficient of the tested sample from that of the reference, usually endogenous, ligand, such as 5-HT for serotonin receptors;

$$\Delta Log\left(\frac{\tau}{K_A}\right) = Log\left(\frac{\tau}{K_A}\right) of\ sample - \ Log\left(\frac{\tau}{K_A}\right) of\ reference$$

Step 3: calculate transduction coefficient difference $\Delta\Delta$Log(τ/K_A) between the different pathways for the same tested ligand by subtracting the ΔLog(τ/K_A) for one pathway from that of the other pathway;

$$\Delta\Delta Log\left(\frac{\tau}{K_A}\right) = \Delta Log\left(\frac{\tau_1}{K_{A1}}\right) - \ \Delta Log\left(\frac{\tau_2}{K_{A2}}\right)$$

Step 4: calculate bias factor as below:

$$Bias = 10^{\Delta\Delta log(\frac{\tau}{K_A})}$$

Step 5: to determine if the calculated bias is statistically significant, a statistical analysis is done with $\Delta\Delta$Log(τ/K_A) values. In addition to the comprehensive methods to estimate SEM values as outlined by Kenakin (137), an alternative (and simpler) method to estimate SEM values is given below, as suggested by Christopoulos et al. (138).

To calculate sem for Δlog(τ/K_A):

$$\boldsymbol{SEM = \sqrt{Sample\ sem^2 + Reference\ sem^2}}$$

To calculate sem for $\Delta\Delta$log(τ/K_A):

$$\boldsymbol{SEM = \sqrt{pathway\ I\ sem^2 + pathway\ II\ sem^2}}$$

2.3.5. Allosteric operational analysis (see Figure 44 on p. 175 for an example). For ligands with allosteric modulator activity, we apply the allosteric operational model to analyze functional results. The functional assay is carried out in the same way as for Schild plot analysis (**Secton 2.3.3**), in which the orthosteric agonist dose-response is measured in the absence and presence of increasing concentrations of a potential allosteric modulator. Results are then analyzed with the following equation as described by Leach et al., 2007 (139).

$$Effect = Basal + \frac{(E_{Max} - Basal)\ (\tau_A[A](K_B + \alpha\beta[B]) + \tau_A[B]K_A)^n}{([A]K_B + K_AK_B + K_A[B] + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}$$

In which ***Effect*** is the measured functional readout in the presence of the orthosteric agonist [**A]** and the allosteric modulator [**B]**; $\mathbf{E_{max}}$ is the maximal possible system activity; $\mathbf{K_A}$ and $\mathbf{K_B}$ are the equilibrium binding affinity for the orthosteric agonist (**A**) and the allosteric modulator (**B**), respectively; α and β are the allosteric effects on ligand binding (mutual effect between **A** and **B**) and agonist efficacy (with α = 1 for neutral cooperativity; α >1 for positive cooperativity; α <1 for negative cooperativity); τ_A and τ_B are the capacity of the orthosteric agonist (**A**) and the allosteric ligand (**B**) to exhibit agonism, respectively; **n** is a slope fitting factor.

When doing non-linear least-square regression curve-fitting in Prism v5.0, the $\mathbf{E_{max}}$ values should be shared for all assays carried out under the same conditions, and should be set to a fixed value which is the maximal system efficacy. $\mathbf{K_A}$ should be the equilibrium binding affinity and can be estimated by a radioligand binding assay. If the tested allosteric modulator itself has no agonist activity, then τ_B = 0. See **Figure 41** on p. 148 for a representative figure.

2.3.6. List of different functional assays PDSP carries out routinely:

- Calcium mobilization assay with FLIPR^{TETRA} for G_q coupled GPCRs.
- Intracellular inositol phosphate accumulation assay.
- Split luciferase-based biosensor cAMP assay for G_i or G_s coupled GPCRs.
- GPCR Tango assay for G-protein independent ß-arrestin translocation.
- PRESTO-Tango GPCRome screening
- FluxOR assay for hERG potassium channel function.
- PatchXpress automated patch clamp assay for hERG potassium channels.
- Neurotransmitter transporter assays for DAT, NET, and SERT.
- $^{86}Rb^+$ efflux assays for nAChRs
- Multidrug resistance transporter-1 (MDR-1) assay
- Enzyme activity assays
    - HDAC assay
    - MAO A and B assay
    - PKC assay
    - CHK2 assay
- Nicotinic acetylcholine receptors (nAChRs) activity assay ($^{86}Rb^+$ efflux)

2.4. Functional assays for G_q coupled GPCRs

2.4.1. Calcium mobilization assays (with FLIPR^{TETRA})

Main equipment: FLIPR^{TETRA} from Molecular Devices (Sunnyvale, CA)

Main reagents: Fluo-4 Direct® from Invitrogen (Carlsbad, CA)

FLIPR Drug buffer: 20 mM HEPES, 1x HBSS, 2.5 mM Probenecid, pH 7.40, room temperature

2.4.1.1. Cell culture: Cells, either stably expressing target receptors or transiently transfected with target receptor DNA (see below for transfection protocol) are grown overnight, and then plated into Poly-L-Lysine (PLL) coated 384-well black clear bottom cell culture plates with DMEM supplemented with 1% dialyzed FBS (dFBS) and at density of 15 – 20K cells in a volume 40 µl per well. The plates are cultured for a minimum of 6 hours, or overnight, before assays.

2.4.1.2. Calcium Precipitation transfection: The calcium phosphate transfection method (1, 140, 141) is used for most transfections in PDSP assays, mainly with HEK 293T cells. HEK 293T cells are subcultured into either 10-cm dishes (3 million cells per dish) or 15-cm dishes (8 million cells per dish) and incubated overnight. Alternatively, HEK 293T cells are seeded at density of 6 million per 10-cm dish 4 hours prior to transfection. For each 10-cm dish of HEK 293T cells, 10 µg receptor DNA construct in 440 µl distilled water is mixed with 60 µl of 2 M $CaCl_2$; the DNA/$CaCl_2$ solution is then added dropwise into 500 µl 2x HBS solution (50 mM HEPES, 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, pH 7.00) while shaking. The mixture is incubated at room temperature for 10 min, then added to cells dropwise, which are then incubated overnight. For transfections in 15-cm dishes, reagents and DNA amounts are increased by 2.5 fold per dish.

2.4.1.3. Calcium mobilization assays with FLIPR^{TETRA}. The protocol for calcium release assays with the FLIPR^{TETRA} is modified from previously published procedures (142, 143). On the day of assay, medium is removed and cells are loaded with 20 µl/well of 1x Fluo-4 Direct Calcium dye (prepared in FLIPR drug buffer). Plates are incubated for 60 min at 37°C, followed by 10 min incubation at room temperature in the dark, and then loaded into the FLIPR. The FLIPR is programmed to take 10

readings (1 read per second) initially as a baseline before addition of 10 µl of 3x drug solutions. The fluorescence intensity is recorded for 2 minutes after drug addition (first addition, see 384-well drug plate maps #1 and #2 for detailed setup) for agonist activity. To measure antagonist activity, drug stocks are prepared at 4x final concentration, added as above for potential effects on basal levels for 2 minutes first, followed by a 10 min incubation before addition of 10 µl of 4x of reference agonist at final concentration equal to the EC_{80} (see 384-well drug plate map #3) to measure remaining agonist activity. To detect positive allosteric modulator activity, the second addition is a 4x concentration of reference agonist at final concentration equal to the EC_{20} (usually endogenous agonist, see 384-well drug plate map #3) to determine effects on agonist activity. The EC_{20} or EC_{80} concentrations are predetermined separately using the same batch of transfected cells. Before the second and/or third additions, FLIPR is programmed to wash tips, first with 10% EtOH, and then with distilled water while recording fluorescence intensity. The incubation time between each addition can be adjusted accordingly to accommodate any preincubation requirements.

2.4.1.4. Schild analysis: To further examine antagonist activity at a particular receptor, we also carry out agonist dose-response studies in the absence and presence of 7 concentrations of selected PDSP compounds. In brief, cell plates are prepared in the same way as for primary or secondary functional assays using PLL-coated 384-well black clear bottom cell culture plates with DMEM + 1% dFBS for a minimum of 6 hours, or overnight. Selected antagonist drug plates are prepared as shown in the above 384-well drug map #6, and are used for the first addition to determine if the testing drug has any effect on basal levels. Reference agonist drug plates are prepared as shown in above 384-well drug map #7, and are used for the second addition to measure effect of antagonist on remaining agonist activity.

2.4.1.5. FLIPR data processing: In calcium mobilization assays with the $FLIPR^{TETRA}$, every well of the 384-well plate has its own basal activity, defined as the average value of the 10 readings before corresponding drug addition. We take maximal fluorescence intensity readings (RFU, Relative Fluorescence Units) within a minute after drug addition as the agonist activity and these values are exported in the format of "fold of basal" using FLIPR's ScreenWorks® built-in batch export function. Normalization, when needed, is carried out using Prism's built-in normalization function, in which

basal is transformed to 0% and the Emax of the reference agonist in the same assay plate is transformed to 100%.

2.4.2. Intracellular inositol phosphate accumulation for G_q coupled GPCRs

2.4.2.1. Primary screening assays – Single concentration and data analysis: Each new compound is tested on all receptors at a single concentration (10 μM) for activity as an agonist or an antagonist. Testing for antagonism is performed in presence of the EC_{50} concentration of a typical agonist (as described above). Each compound is tested in duplicate in two separate experiments performed on different lots of cells. In addition to the tested compounds, each 96-well plate contains wells for determination of basal activity, maximal agonist stimulation, agonist EC_{50} concentrations (i.e., concentration-response isotherm), and the IC_{50} concentration of a known antagonist for purposes of positive control and for activity calculations. The reported results for each compound are calculated for agonists as the % of maximal activity (as obtained with maximal agonist concentrations) and for antagonist as the percent of inhibition of receptor activity (in presence of an EC_{50} concentration of the agonist). Results are expressed as means ± SEM from four replicates.

2.4.2.2. Secondary screening assays: Dose-responses and data analysis. Compounds determined to be active as agonists or antagonists may be tested for their potency in concentration-response experiments. Six-point concentration-response curves are done in duplicate twice on two separate lots of cells (somethimes a third curve may be needed if, in the first experiment, the range of concentrations used is outside of the active range). For antagonists, these curves are performed in the presence of the EC_{50} concentration of the agonist. For each compound, the results from four replicates are averaged, and then either EC_{50} or IC_{50} values are calculated by non-linear regression using the 4-parameter logistic equation as indicated in **Section 2.3**. Results are reported as EC_{50} or IC_{50} values for each tested compound (and receptor), and also include the EC_{50} or IC_{50} values of a known agonist or antagonist for comparison purposes.

2.4.2.3. The following table lists GPCRs for which the PDSP has validated functional assays to measure G_q protein activation via calcium mobilization using FLIPR^{TETRA} or intracellular inositol phosphate accumulation. Representative results are analyzed using Prism v5.0.

Table 27. List of GPCRs for which the PDSP has validated G_q protein mediated calcium mobilization or inositol phosphate (IP) accumulation assays, and their pharmacological parameters. The PDSP will also develop additional calcium mobilization assays for other GPCRs upon request and approval. Assays are carried out according to the above procedures and results are analyzed in Prism. Representative results are from single assays done either in triplicate or quadruplicate.

Receptor	Cell line	Ligands * (references)	Emax (fold)	pEC_{50} (EC_{50} nM) pIC_{50} (IC_{50} nM)	Hill slope
M_1	CHO	Acetylcholine	3.4	8.69 (2.1)	1.58
M_1D	CHO	CNO (37)	1.5	7.66 (21.8)	0.84
M_3	CHO	Acetylcholine	2.8	8.91 (1.2)	0.95
M_3D	Flp-In CHO	CNO	2.7	7.44 (36.1)	1.01
M_5	CHO	Acetylcholine	3.4	8.25 (5.6)	1.03
M_5D	CHO	CNO	2.1	7.44 (36.5)	0.82
5-HT_{2A}	Flp-In HEK	5-HT	2.4	8.82 (1.5)	1.19
		Ketanserin (antagonist)	2.5	6.80 (159)	-2.92
5-HT_{2B}	Flp-In HEK	5-HT	4.8	8.85 (1.4)	1.25
		Clozapine (antagonist)	2.3	7.43 (37.1)	-1.65
5-HT_{2C} INI	Flp-In HEK	5-HT	3.3	10.11 (0.7)	1.89
		Clozapine (antagonist)	3.3	6.37 (432)	-3.31
5-HT_{2C} VNV	Flp-In HEK	5-HT	2.5	9.26 (0.5)	1.53
5-HT_{2C} VSV	Flp-In HEK	5-HT	5.7	9.08 (0.8)	1.87
α_{1A}	Fibroblast, rat	Norepinephrine	2.3	8.34 (4.5)	1.89
α_{1B}	Fibroblast, rat	Norepinephrine	1.6	8.76 (1.7)	0.86
α_{1D}	Fibroblast, rat	Norepinephrine	4.1	8.61 (2.4)	1.14
AT_{1A}	HEK	Angiotensin II	1.8	9.39 (0.4)	0.72
BB_1	HEK T	Bombesin	5.6	7.82 (15.3)	1.36
		BIM 187	5.7	7.21 (61.1)	1.73
		Neuromedin B	5.5	8.40 (4.0)	1.40
BB_2	HEK T	Bombesin	4.4	8.76 (1.7)	0.67
		BIM 187 (144)	4.7	8.49 (3.3)	0.95
		Neuromedin B	4.6	6.58 (262)	1.29
B_2	HEK T	Bradykinin	1.7	9.57 (0.3)	0.88
CCK_1	HEK T	Gastrin	4.1	9.83 (0.15)	1.29
CCK_2	HEK T	Gastrin	4.2	9.84 (0.14)	1.17
Ghrelin	HEK	L-692,585	1.7	9.05 (0.9)	0.80

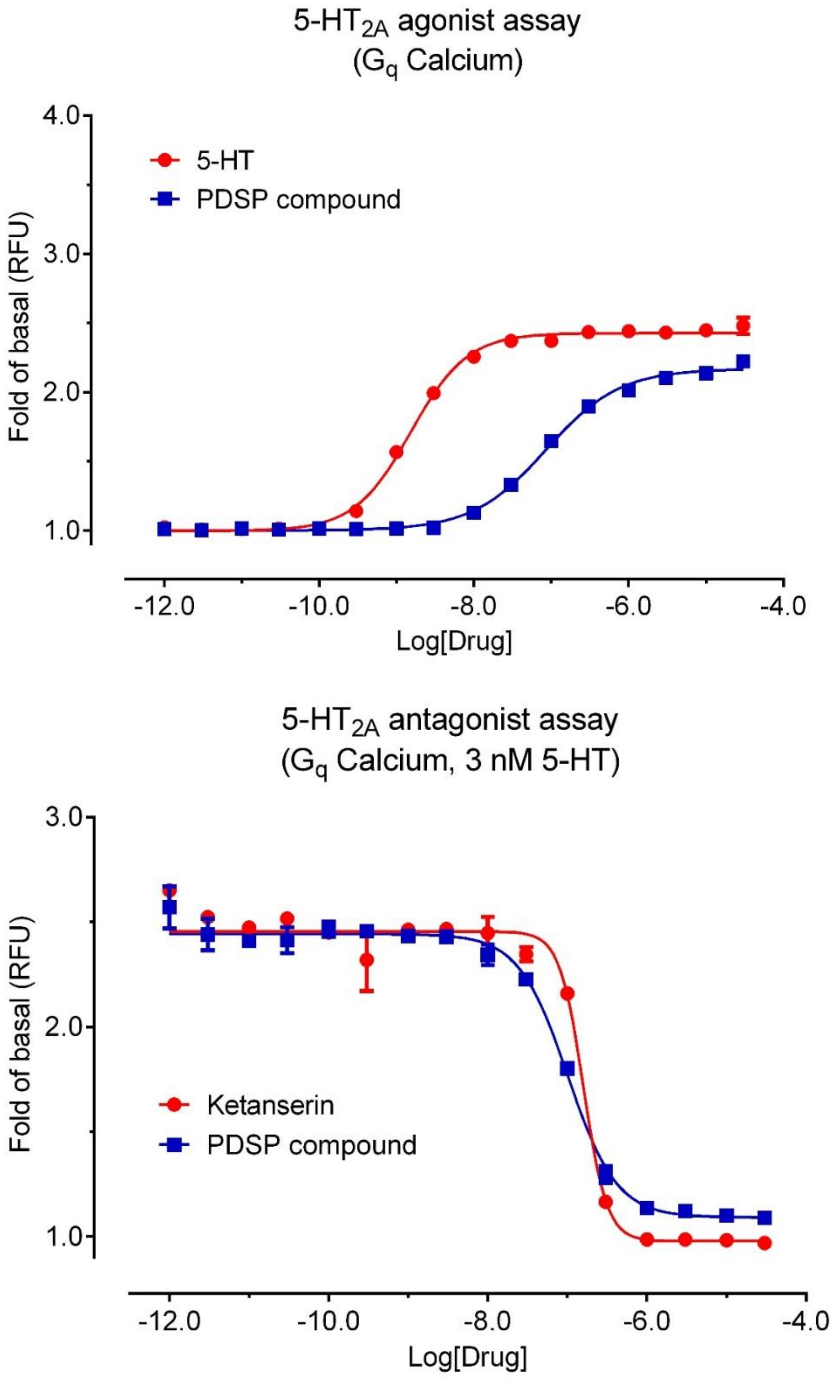
Receptor	Cell line	Ligands * (references)	Emax (fold)	pEC_{50} (EC_{50} nM) pIC_{50} (IC_{50} nM)	Hill slope
H_1	HEK	Histamine	2.4	7.77 (16.9)	1.22
H_2	HEK T	Histamine	1.5	6.24 (577)	1.42
$mGlu_1$	HEK	L-Glutamate	3.8	6.02 (964)	2.20
		FTIDC (antagonist)	3.6	7.93 (11.7)	-1.82
$mGlu_5$	HEK	L-Glutamate	2.3	5.89 (1397)	2.27
		MTEP (antagonist)	2.3	8.03 (9.4)	-0.63
NK_1	HEK	Substance P	2.7	7.64 (22.8)	0.79
NK_2	HEK	Neurokinin A	4.7	7.56 (27.6)	0.66
NK_2	HEK	Neurokinin B	4.4	6.90 (123)	0.79
NK_3	HEK	Neurokinin B	4.0	8.41 (3.9)	0.83
NTS_1	HEK	Neurotensin	3.5	7.99 (10.1)	0.78
		JMV-449 (145)	3.5	8.76 (1.8)	0.93
$P2Y_1$	1321N1	ADP	3.1	6.65 (226)	1.31
		UTP	2.0	5.94 (1140)	1.10
$P2Y_2$	1321N1	ATP	2.1	6.88 (132)	1.26
		ADP	2.1	5.48 (3306)	1.59
		UTP	1.9	8.03 (9.5)	1.54
		UDP	1.9	5.47 (3400)	1.50
$P2Y_4$	1321N1	UTP	2.4	7.65 (22.3)	1.60
		UDP	2.4	5.22 (6088)	1.63
$P2Y_6$	1321N1	ATP	1.7	4.59 (25470)	3.17
		ADP	3.5	5.26 (5517)	2.04
		UTP	3.6	7.33 (46.8)	1.22
$P2Y_{11}$	1321N1	ATP	3.6	5.70 (2009)	1.78
		UTP	1.9	5.80 (1579)	1.12
PAR1	KOLF, mouse	TRAP	3.9	6.86 (138)	1.17
PAF	HEK	PTAF	1.9	8.06 (87.4)	0.65
V_{1A}	CHO	Vasopressin	3.7	8.09 (8.1)	0.77
		Oxytocin	3.2	5.66 (2193)	1.55
V_{1B}	CHO	Vasopressin	3.3	8.20 (6.3)	0.99
		Oxytocin	3.1	5.42 (3849)	2.03
V_2	CHO	Vasopressin	2.7	7.42 (38.2)	1.57
OT	CHO	Oxytocin	3.1	7.29 (51.0)	1.21
GPR39	HEK T	GPR39-C3 (146–148)	1.4	6.08 (83.1)	2.72
GPR40	HEK T	GW9508 (149, 150)	1.6	6.53 (294)	0.54
		AS2034178 (151)	1.6	5.85 (1423)	0.72
		AM4668 (152)	1.7	6.93 (118)	0.42
GPR41	HEK T	Propionate	4.1	7.18	
GPR43	HEK T	Propionate		6.43	

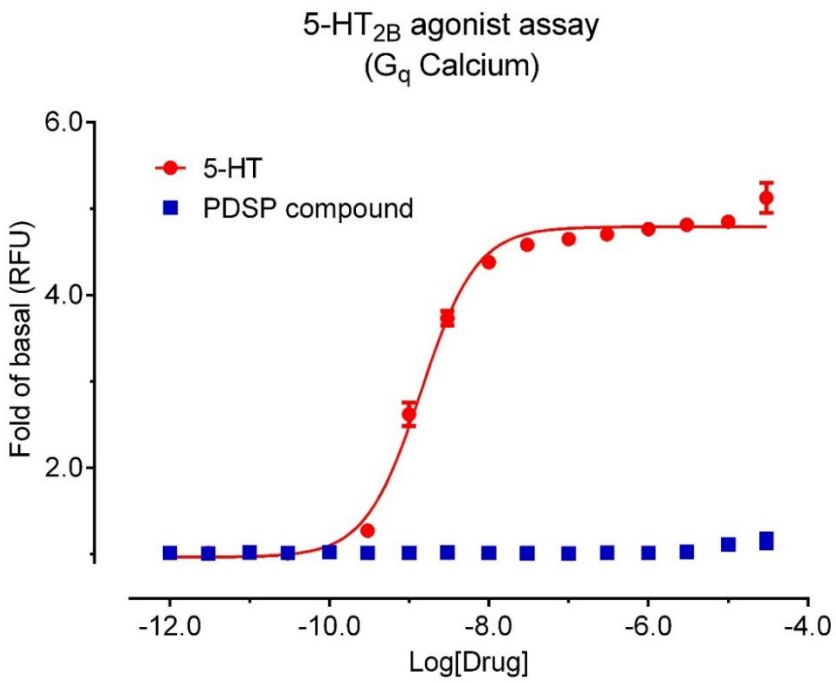
Receptor	Cell line	Ligands * (references)	Emax (fold)	pEC_{50} (EC_{50} nM) pIC_{50} (IC_{50} nM)	Hill slope
MRGPRX2	Flp-In HEK	TAN-67 (153)	4.5	6.25 (557)	1.02
MRGPRX4	Flp-In HEK	Nateglinide (154)	4.3	5.66 (2208)	3.00

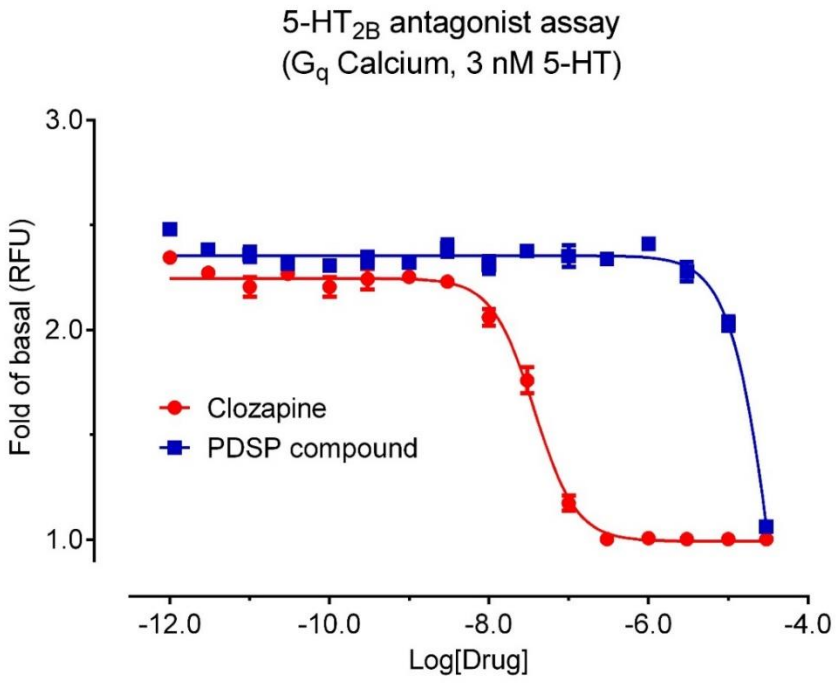
Notes

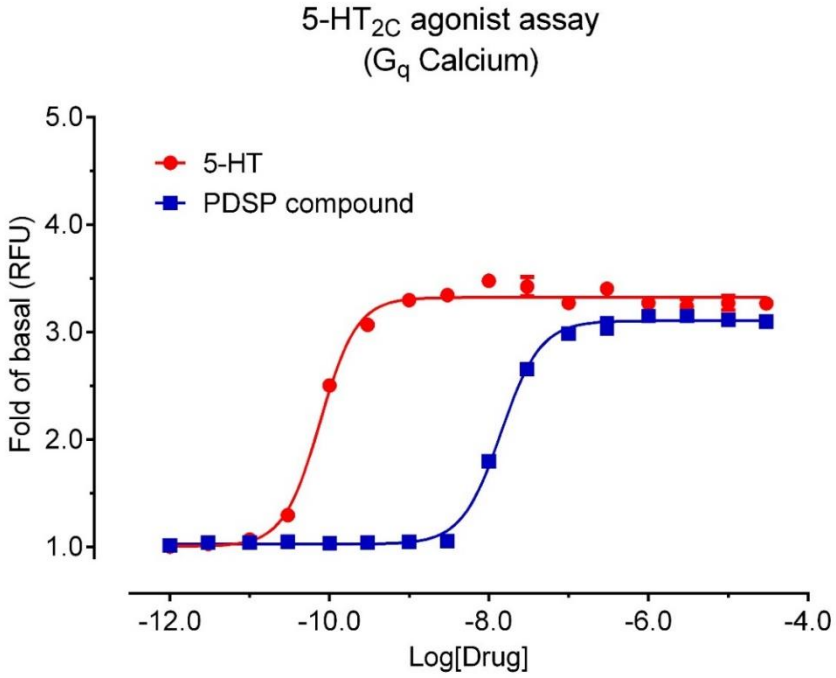
*: Reference antagonist in antagonist assays is indicated individually

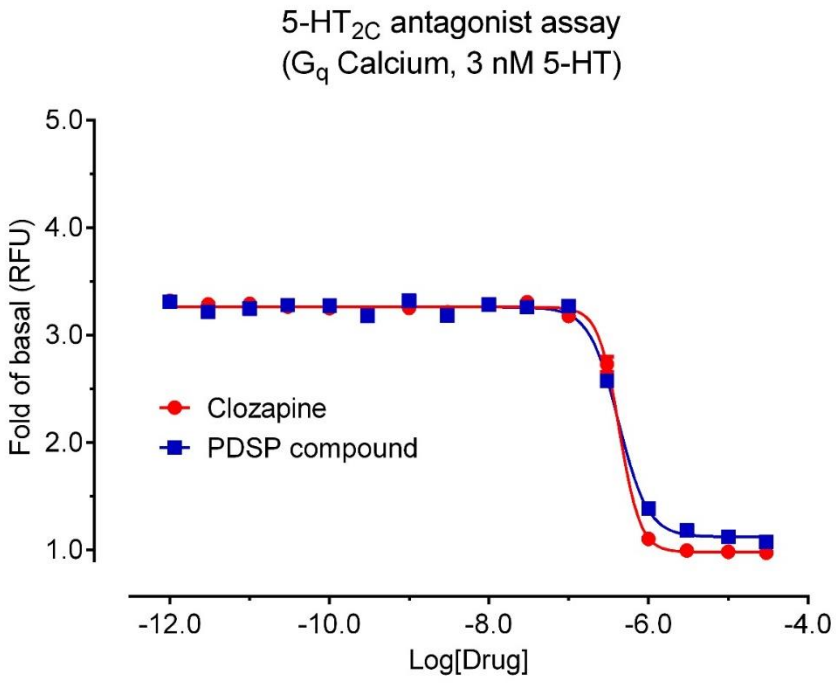
Figure 39. Representative dose-response curves of calcium mobilization for G_q-coupled GPCRs, obtained from the FLIPR^{TETRA}. Some curves are presented after normalization with reference agonist activity as 100% and basal as 0%.

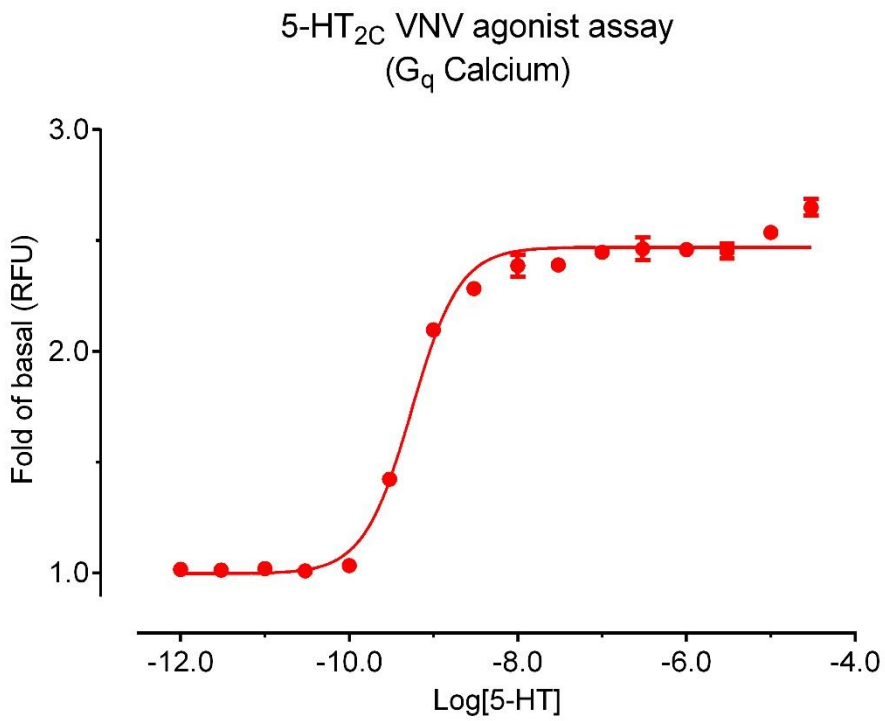


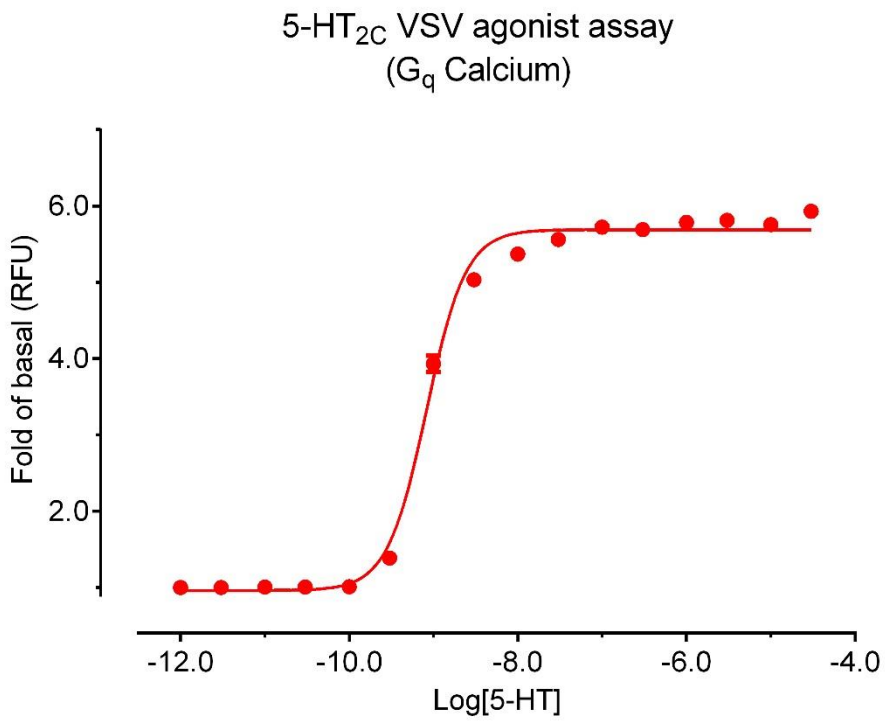

5-HT2B agonist assay
(Gq Calcium)
6.0
4.0
2.0
Fold of basal (RFU)
5-HT
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

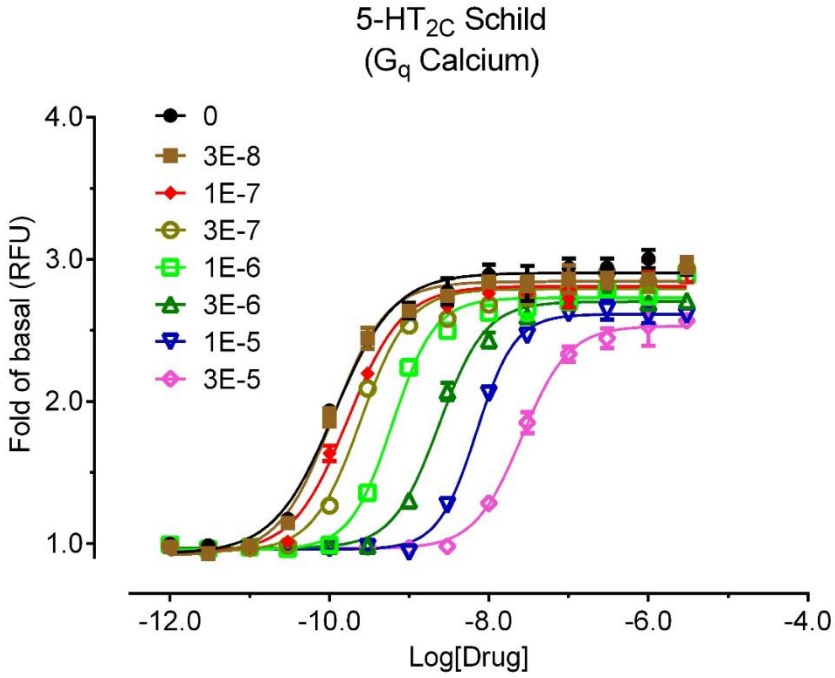

5-HT2B antagonist assay
(Gq Calcium, 3 nM 5-HT)
3.0
2.0
1.0
Fold of basal (RFU)
Clozapine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

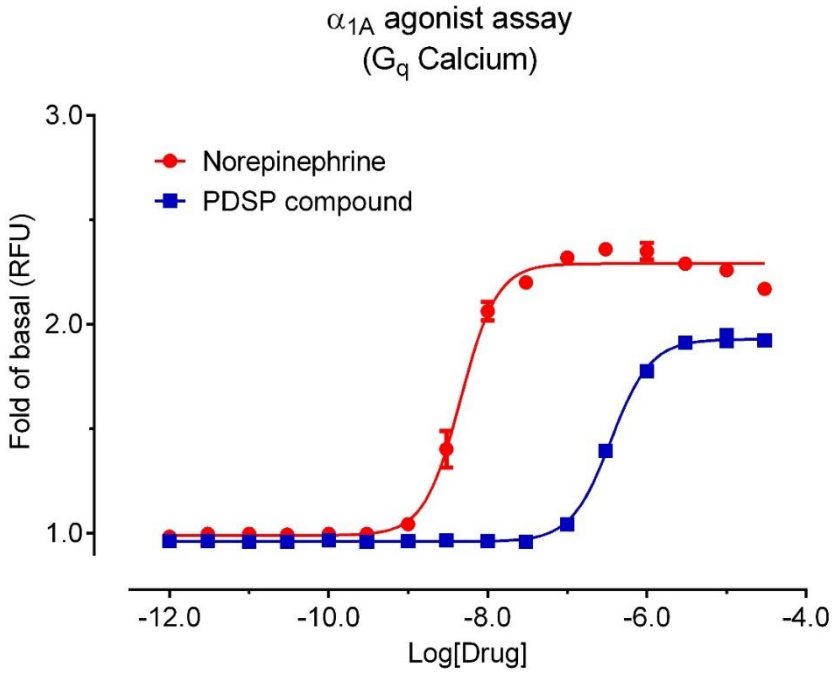

5-HT2C agonist assay
(Gq Calcium)
5.0
4.0
3.0
2.0
1.0
Fold of basal (RFU)
5-HT
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

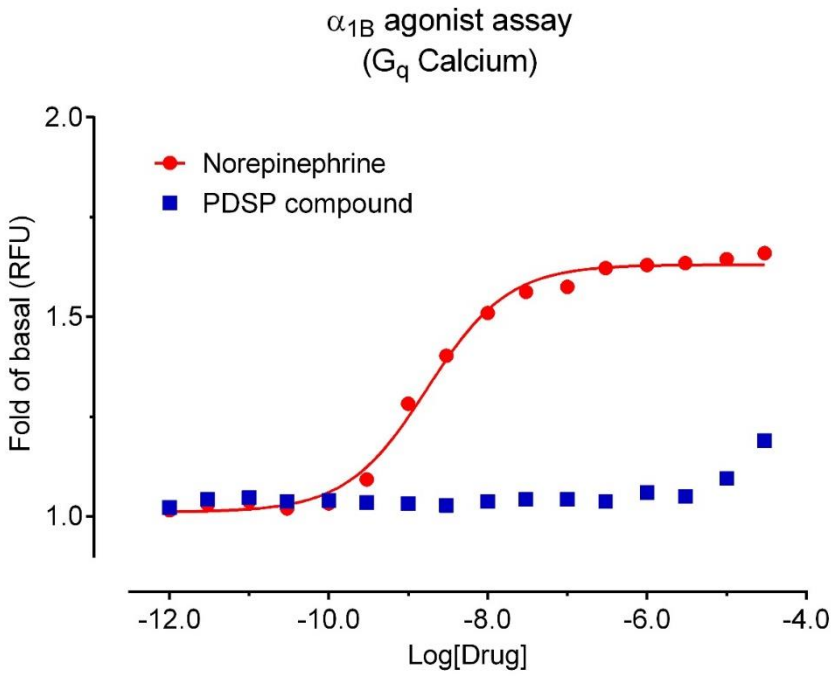

5-HT2C antagonist assay
(Gq Calcium, 3 nM 5-HT)
5.0
4.0
3.0
2.0
1.0
Fold of basal (RFU)
Clozapine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

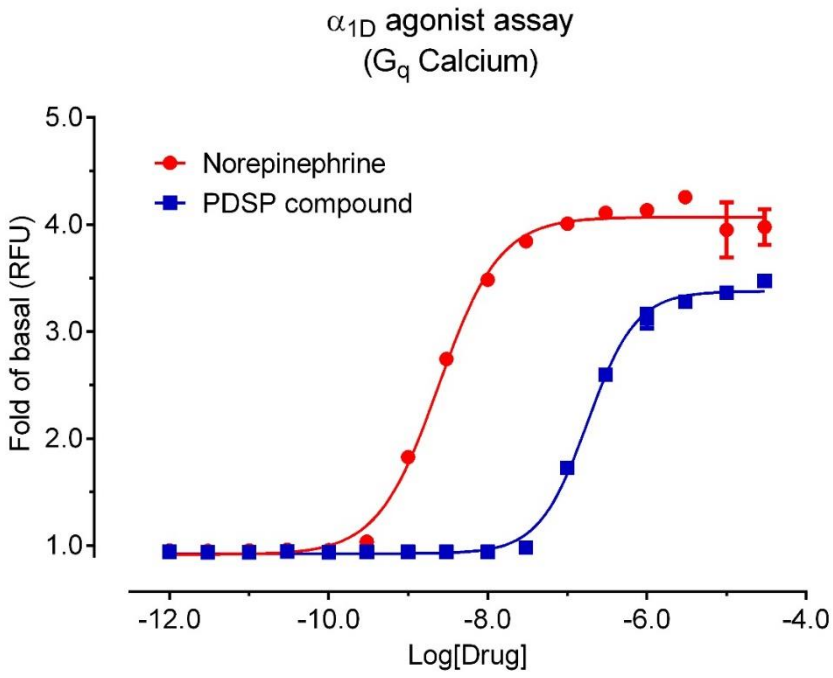

5-HT2C VNV agonist assay
(Gq Calcium)
Fold of basal (RFU)
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[5-HT]


5-HT2C VSV agonist assay
(Gq Calcium)
Fold of basal (RFU)
6.0
4.0
2.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[5-HT]


5-HT2C Schild
(Gq Calcium)
0
3E-8
1E-7
3E-7
1E-6
3E-6
1E-5
3E-5
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


α1A agonist assay
(Gq Calcium)
Norepinephrine
PDSP compound
Fold of basal (RFU)
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


α1B agonist assay
(Gq Calcium)
Norepinephrine
PDSP compound
Fold of basal (RFU)
2.0
1.5
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


α1D agonist assay
(Gq Calcium)
Norepinephrine
PDSP compound
Fold of basal (RFU)
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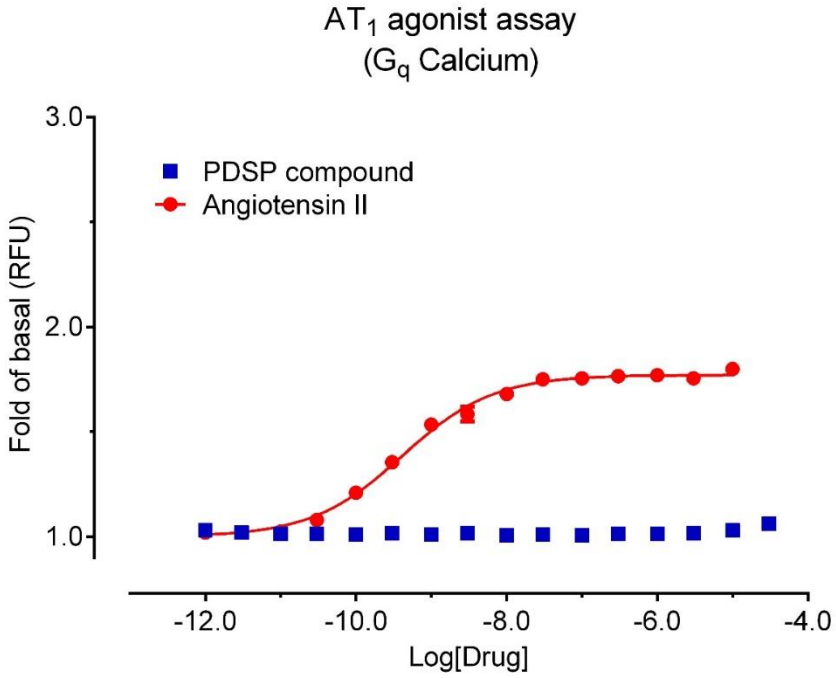
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


AT1 agonist assay
(Gq Calcium)
PDSP compound
Angiotensin II
Fold of basal (RFU)
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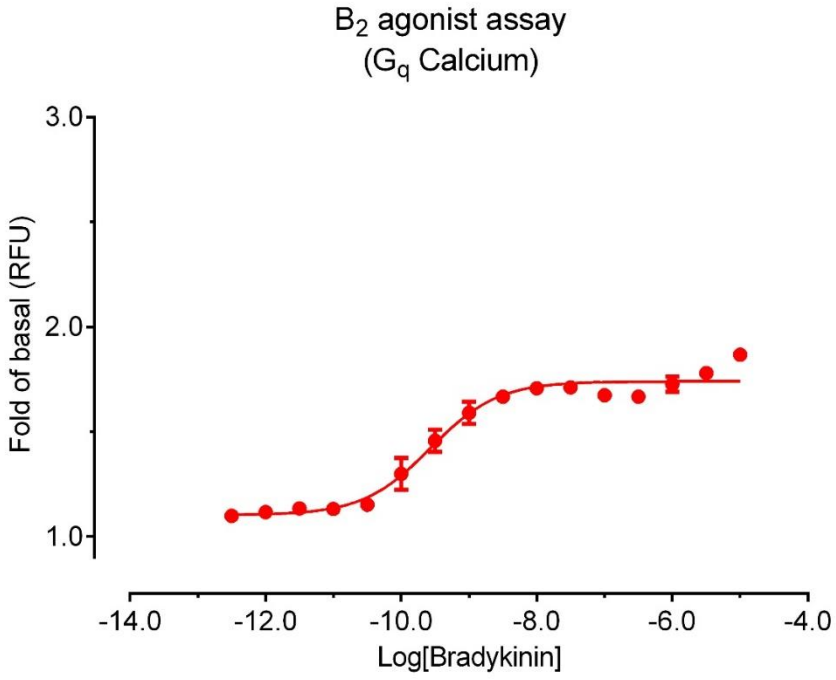
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


B2 agonist assay
(Gq Calcium)
Fold of basal (RFU)
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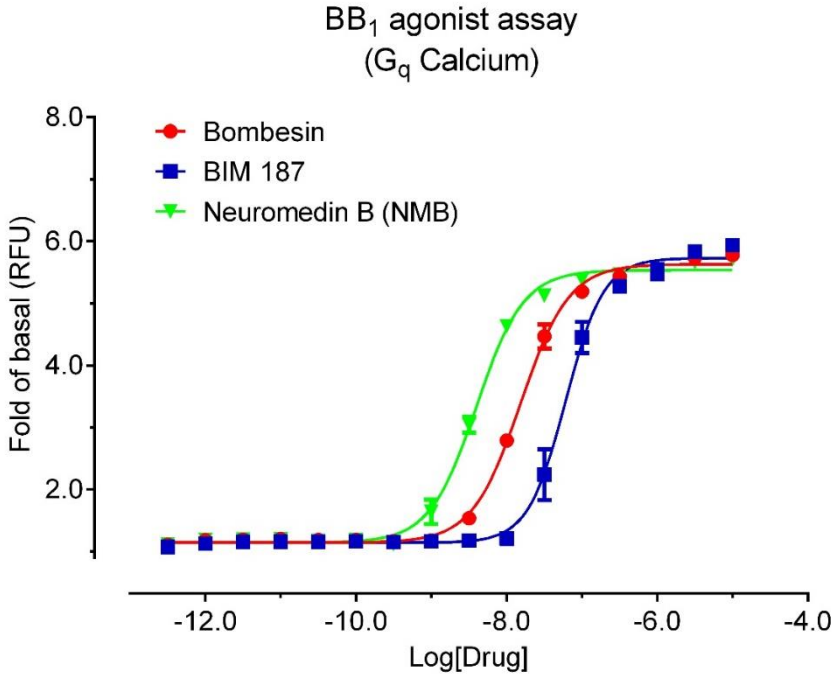
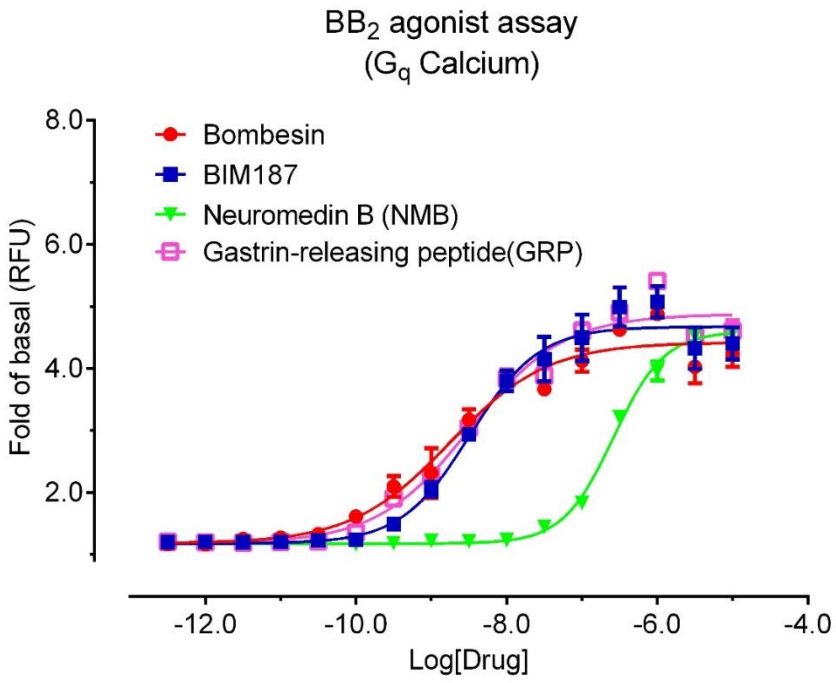
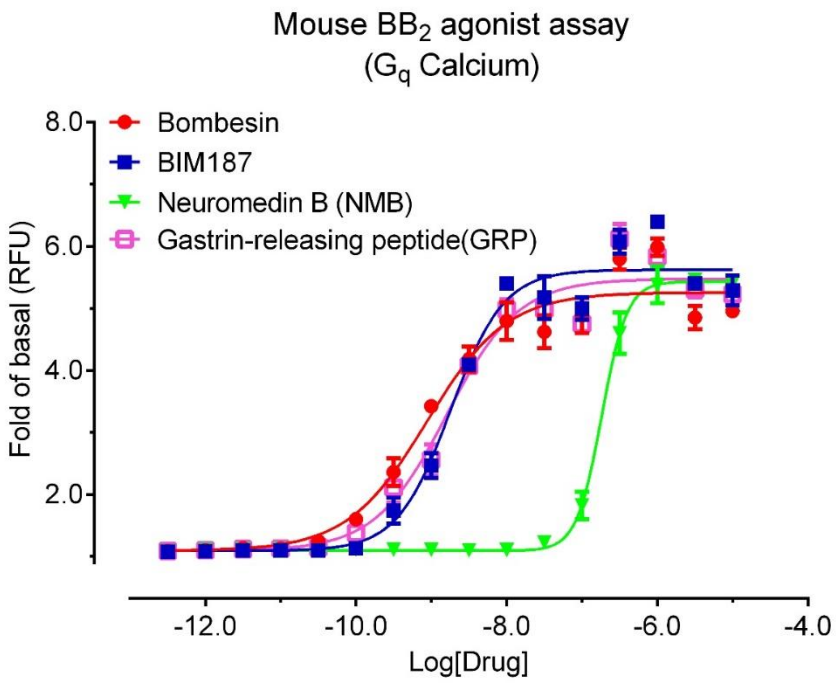
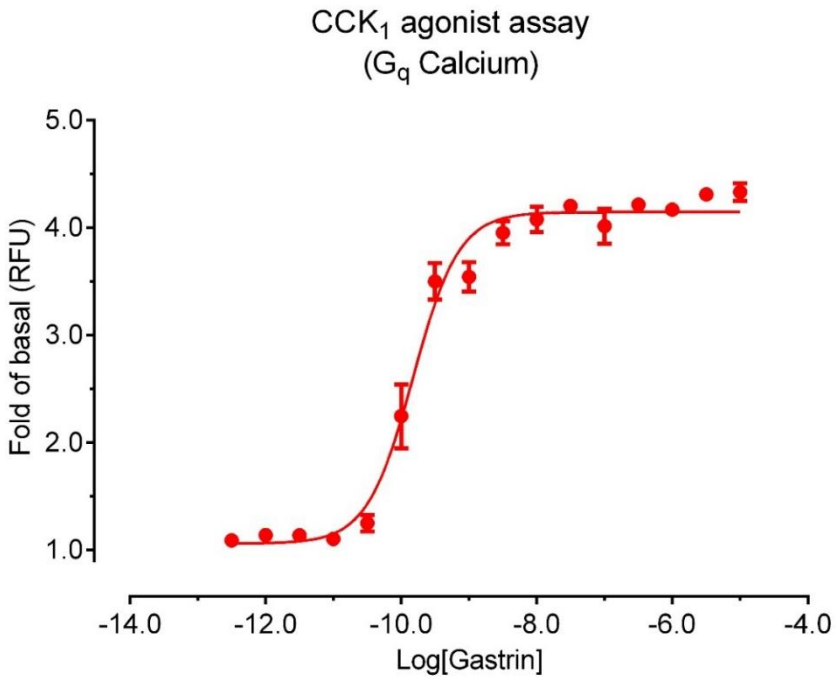
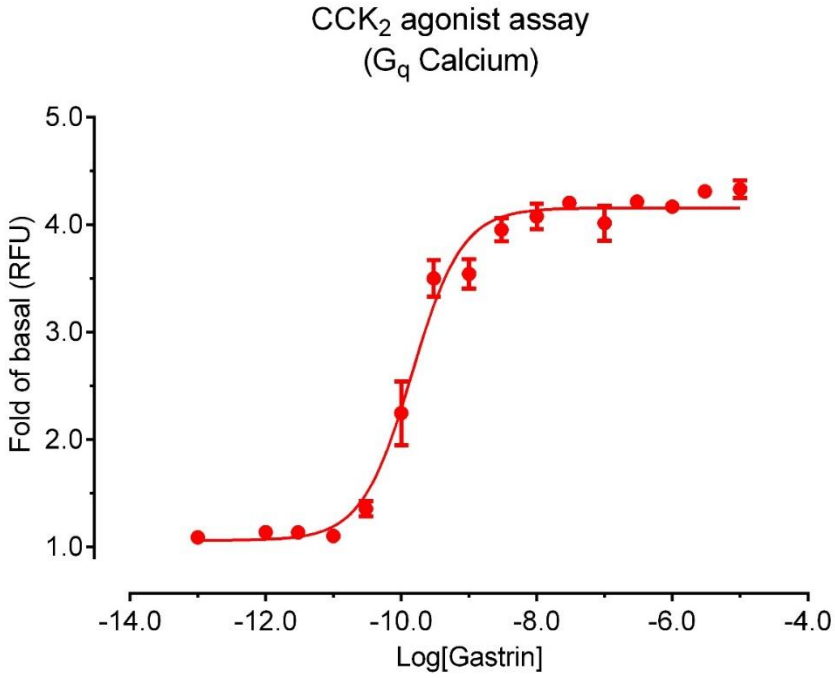
2.0
1.0
-14.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Bradykinin]

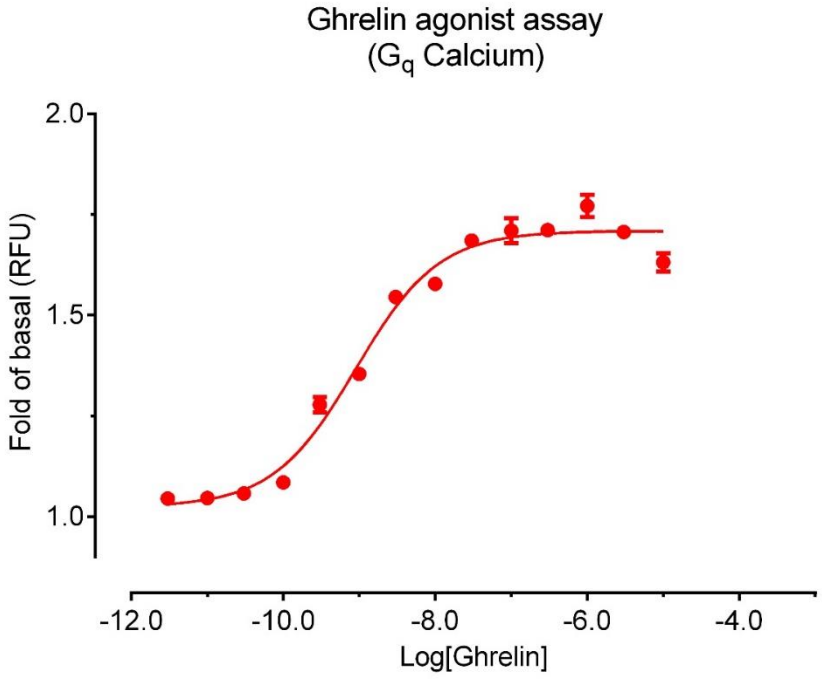

BB1 agonist assay
(Gq Calcium)
Bombesin
BIM 187
Neuromedin B (NMB)
Fold of basal (RFU)
8.0
6.0
4.0
2.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

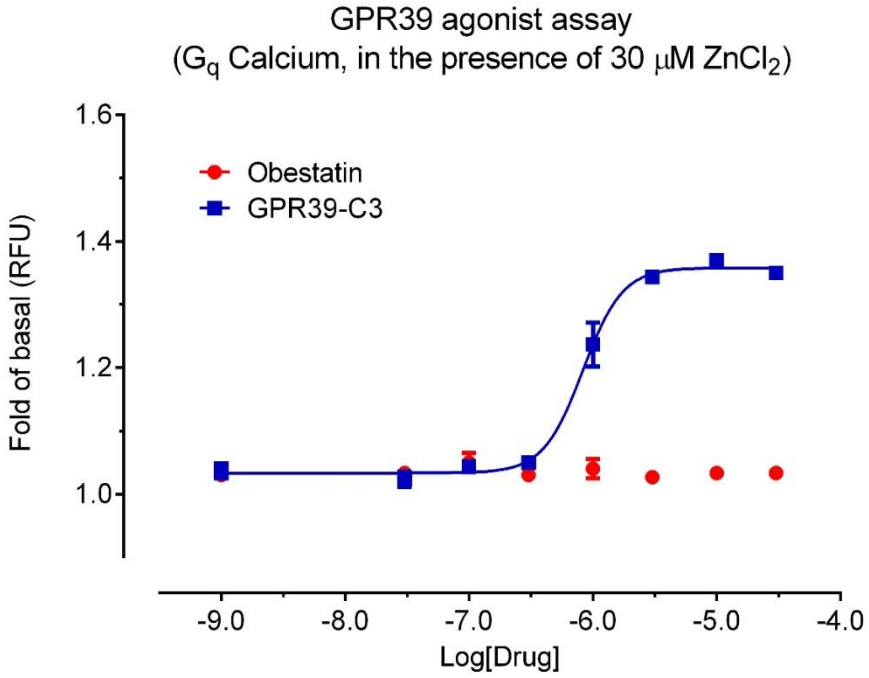

BB2 agonist assay
(Gq Calcium)
Bombesin
BIM187
Neuromedin B (NMB)
Gastrin-releasing peptide(GRP)
Fold of basal (RFU)
8.0
6.0
4.0
2.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

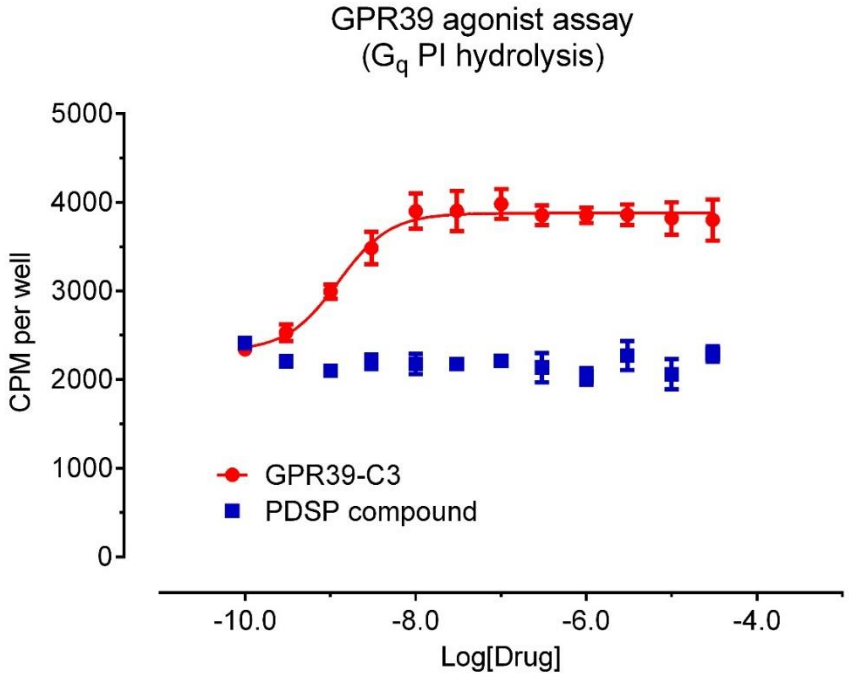

Mouse BB2 agonist assay
(Gq Calcium)
Bombesin
BIM187
Neuromedin B (NMB)
Gastrin-releasing peptide(GRP)
Fold of basal (RFU)
8.0
6.0
4.0
2.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

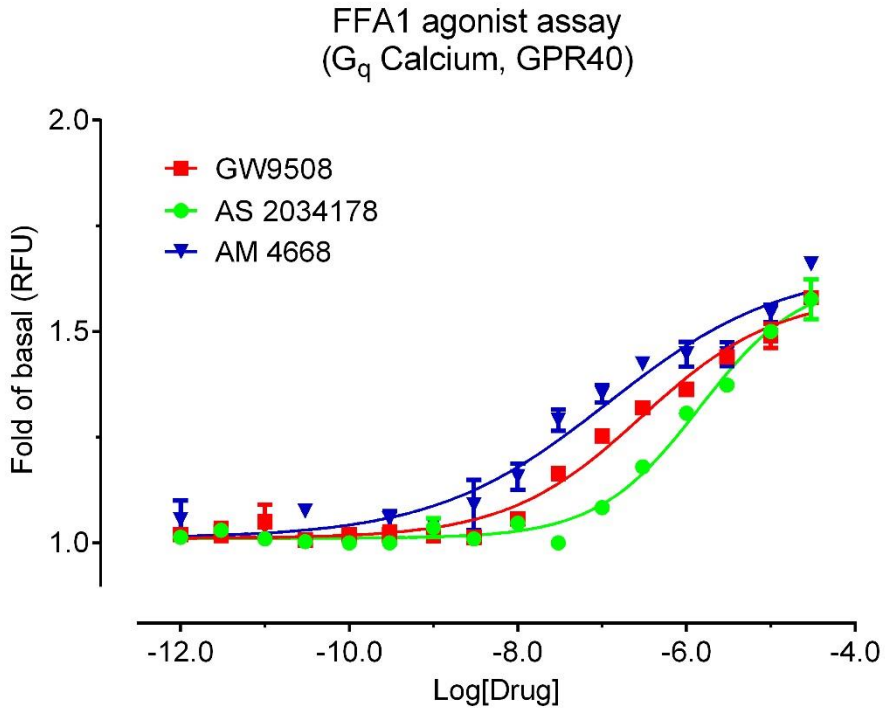

CCK1 agonist assay
(Gq Calcium)
Fold of basal (RFU)
5.0
4.0
3.0
2.0
1.0
-14.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Gastrin]


CCK2 agonist assay
(Gq Calcium)
Fold of basal (RFU)
5.0
4.0
3.0
2.0
1.0
-14.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Gastrin]

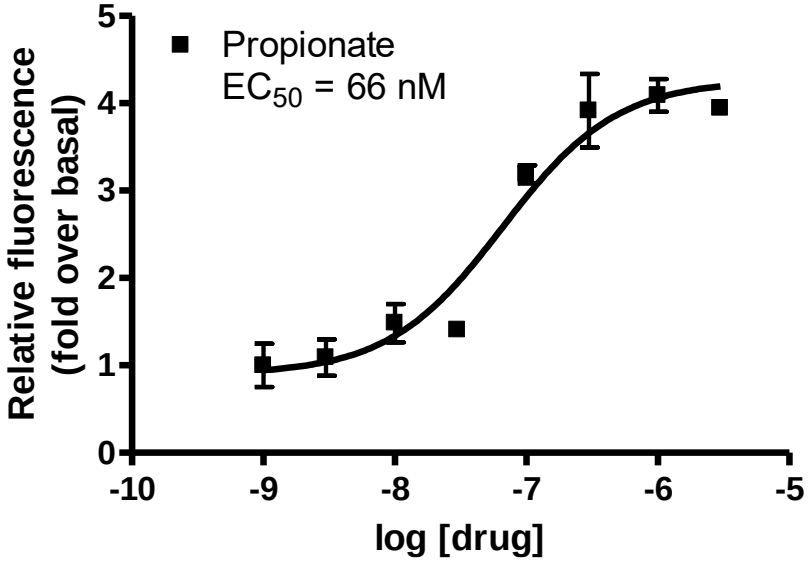

Ghrelin agonist assay
(Gq Calcium)
Fold of basal (RFU)
2.0
1.5
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Ghrelin]

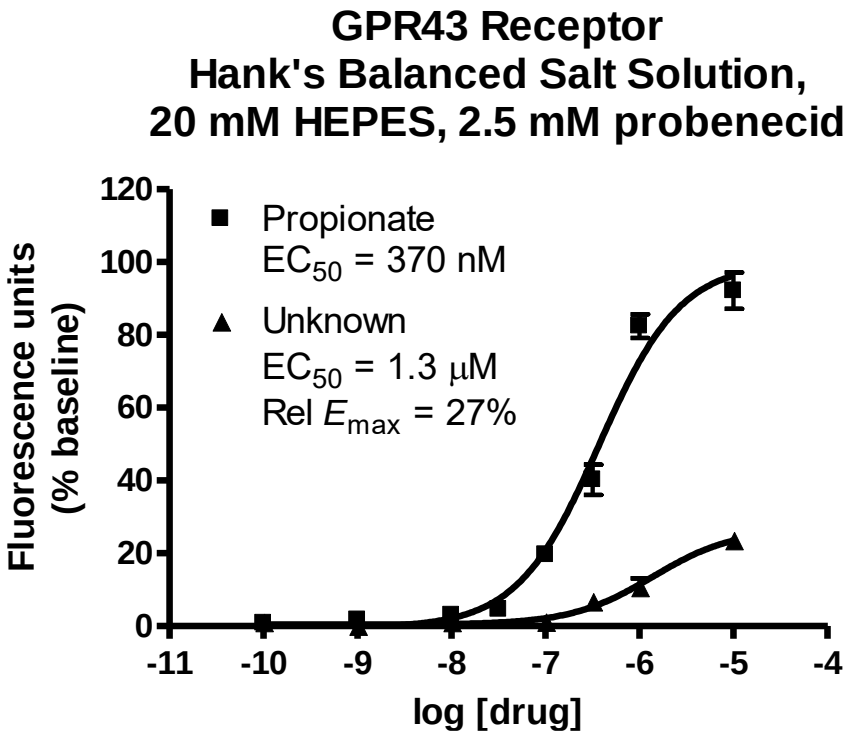

GPR39 agonist assay
(G_q Calcium, in the presence of 30 μM $ZnCl_2$)
Fold of basal (RFU)
1.6
1.4
1.2
1.0
Obestatin
GPR39-C3
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
Log[Drug]

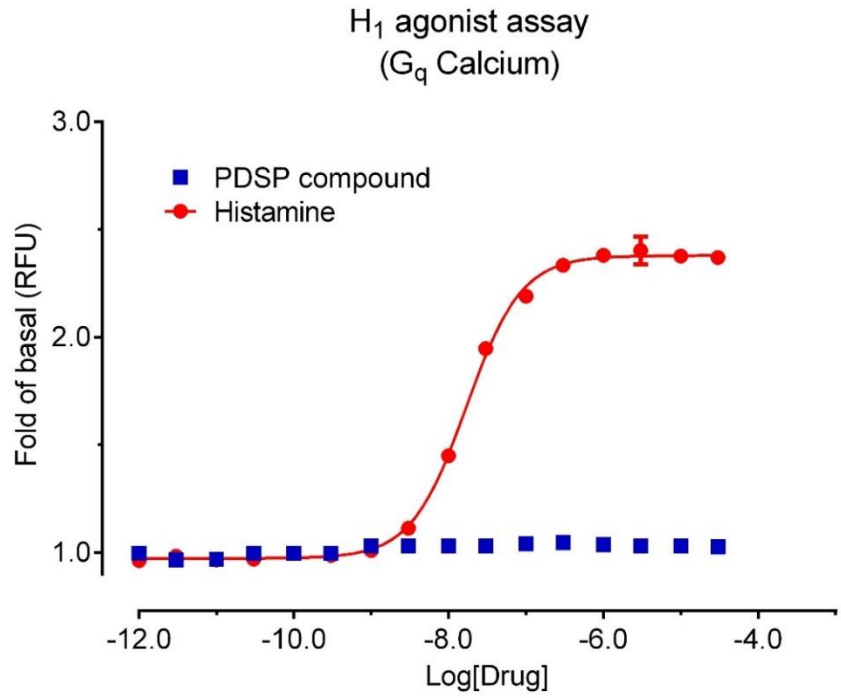

GPR39 agonist assay
(G_q PI hydrolysis)
CPM per well
5000
4000
3000
2000
1000
0
GPR39-C3
PDSP compound
-10.0
-8.0
-6.0
-4.0
Log[Drug]

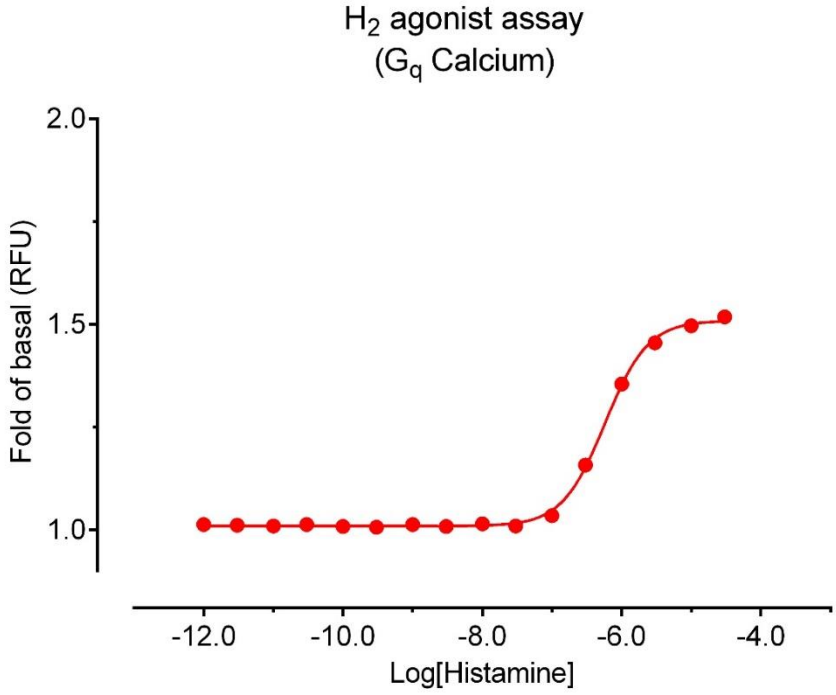


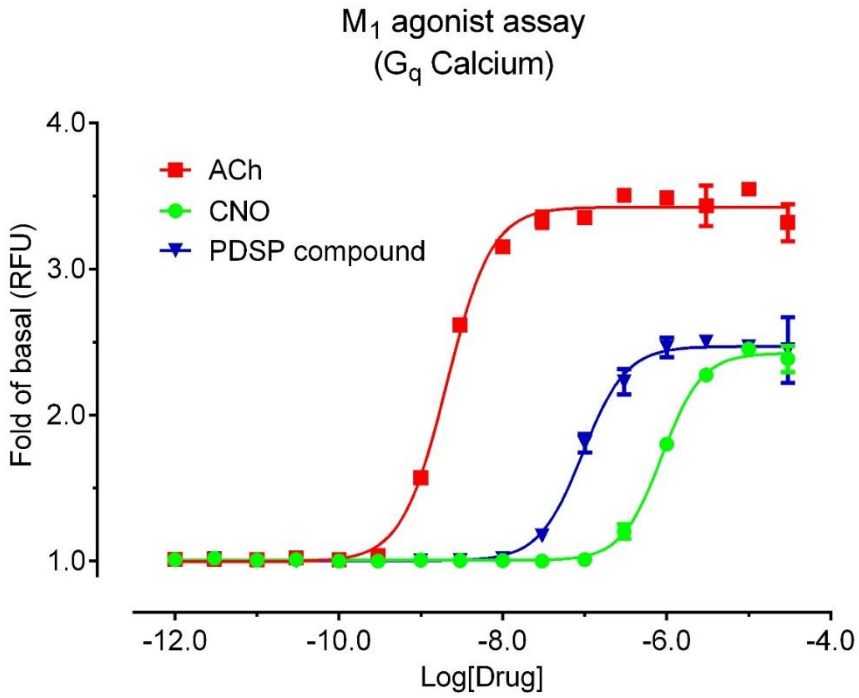
GPR41 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid

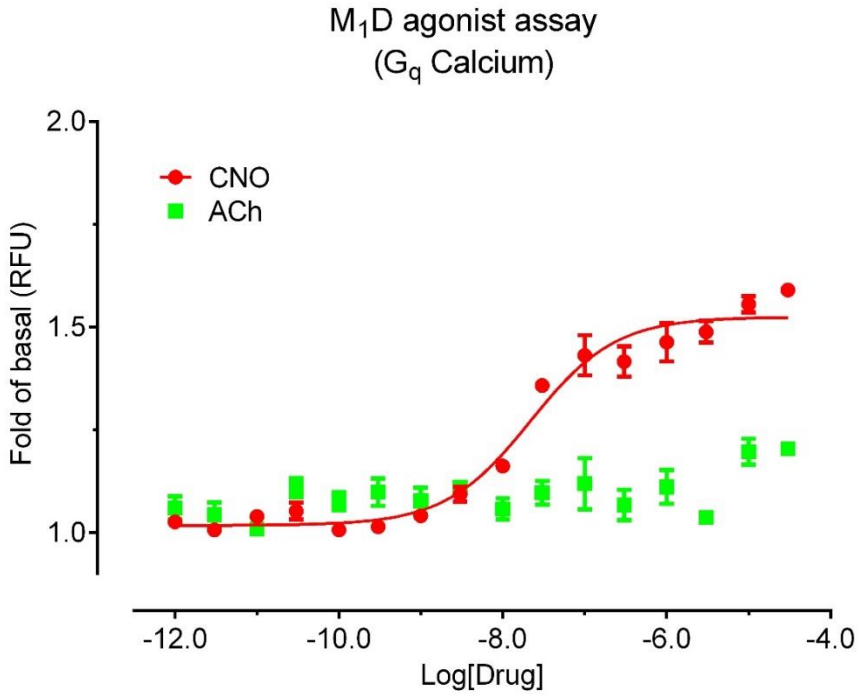


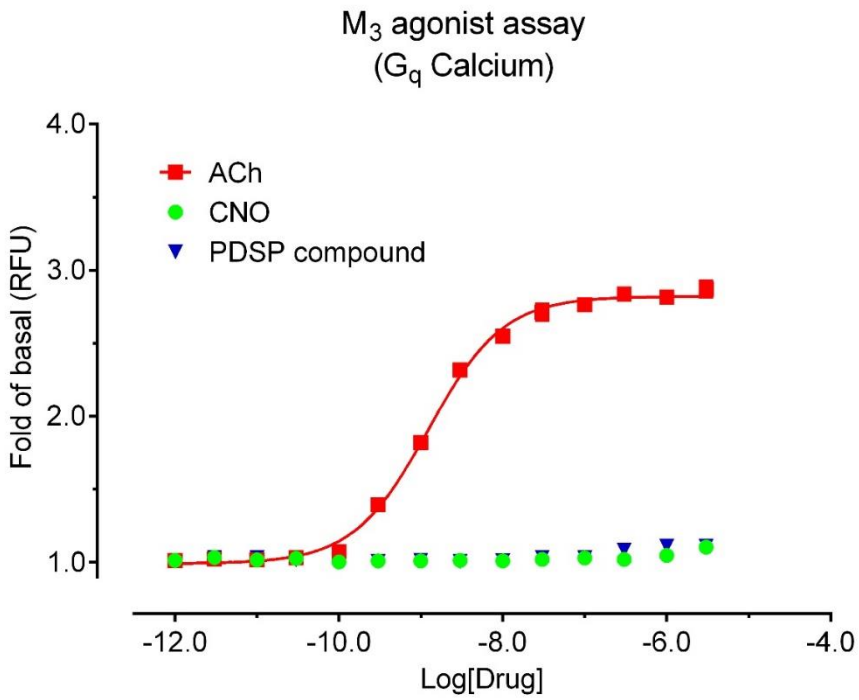

GPR43 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Fluorescence units
(% baseline)
120
100
80
60
40
20
0
Propionate
EC50 = 370 nM
Unknown
EC50 = 1.3 μM
Rel Emax = 27%
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]

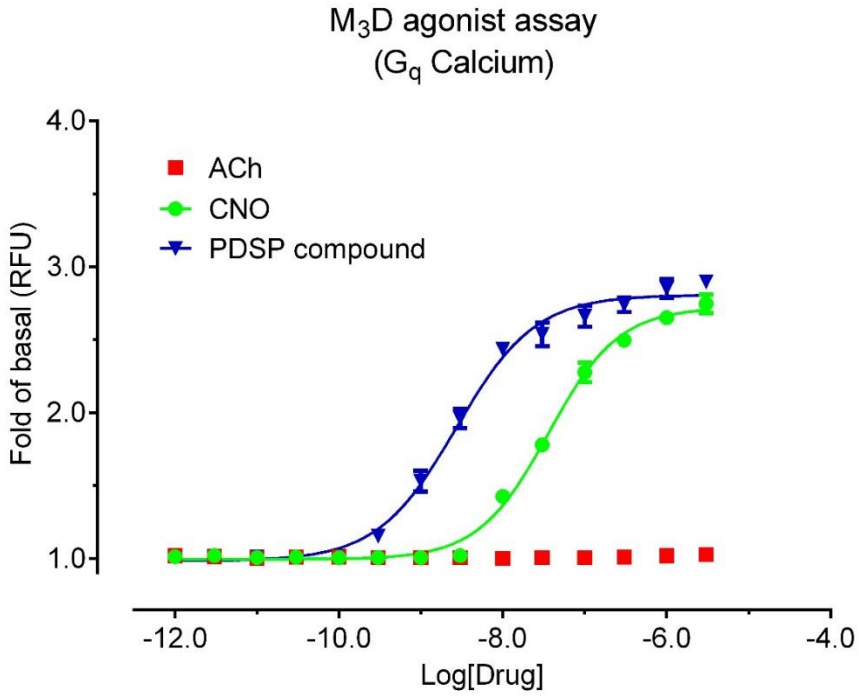

H1 agonist assay
(Gq Calcium)
Fold of basal (RFU)
3.0
2.0
1.0
PDSP compound
Histamine
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

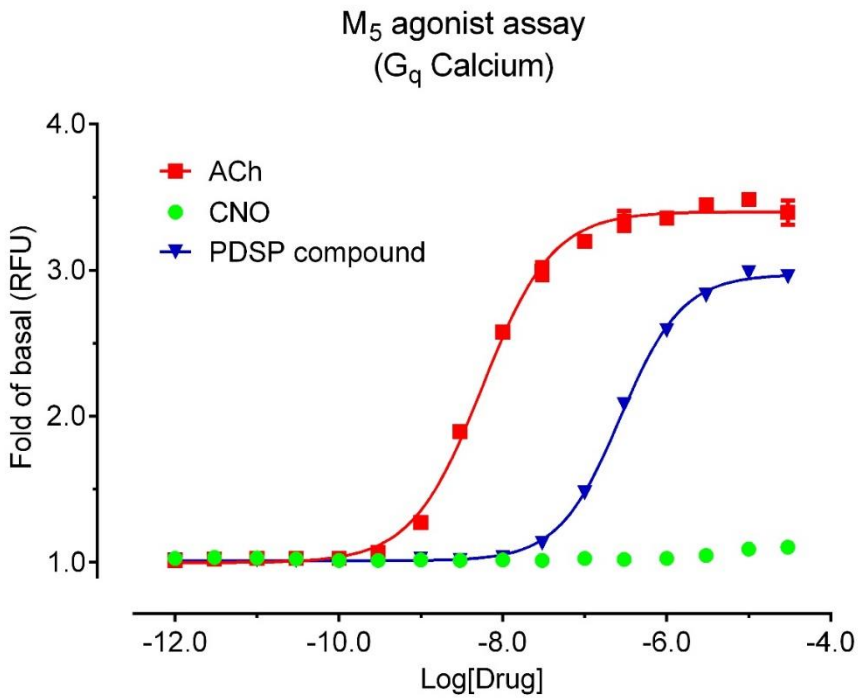

H2 agonist assay
(Gq Calcium)
Fold of basal (RFU)
2.0
1.5
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Histamine]

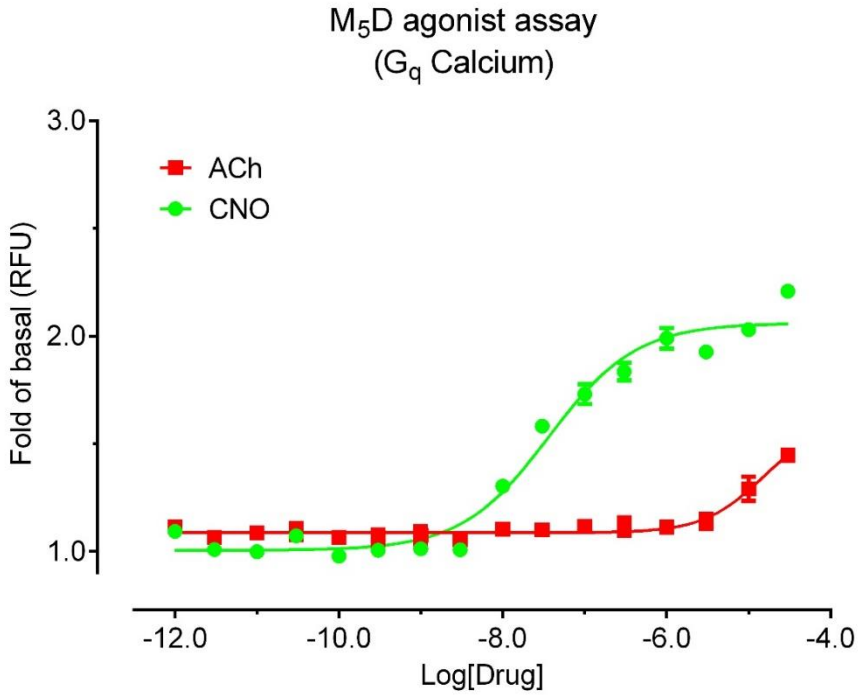

M1 agonist assay
(Gq Calcium)
Fold of basal (RFU)
4.0
3.0
2.0
1.0
ACh
CNO
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

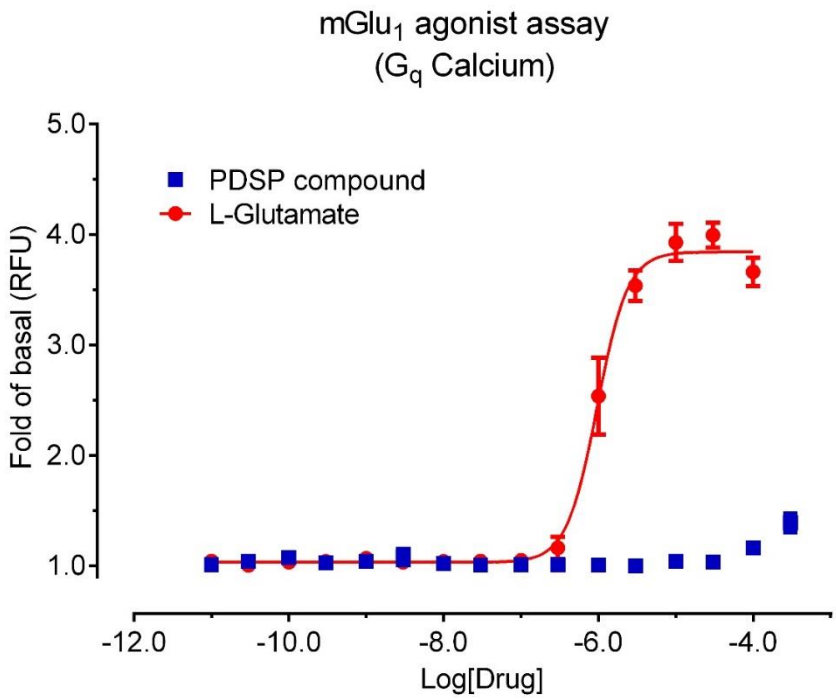

M1D agonist assay
(Gq Calcium)
2.0
1.5
1.0
Fold of basal (RFU)
CNO
ACh
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

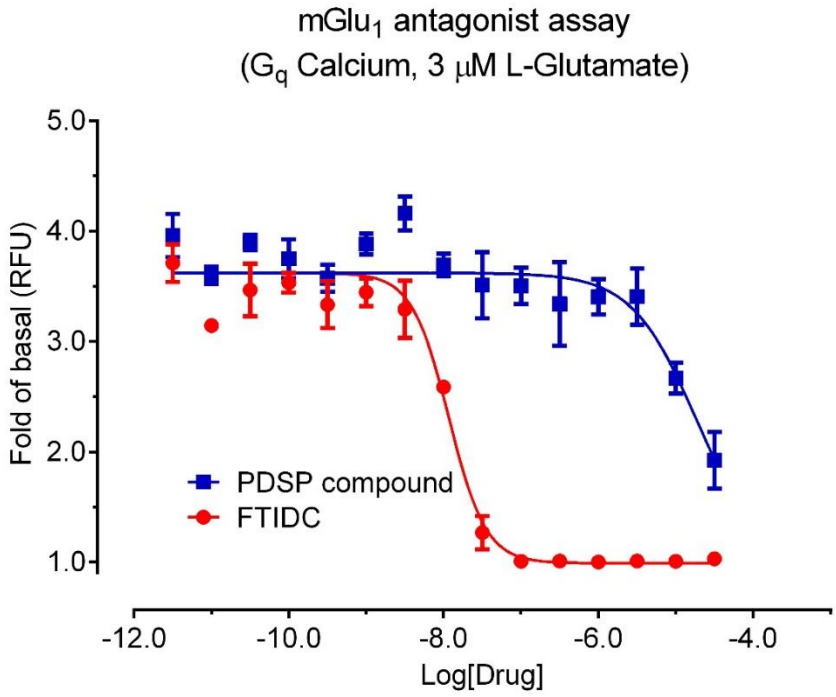

M3 agonist assay
(Gq Calcium)
4.0
3.0
2.0
1.0
Fold of basal (RFU)
ACh
CNO
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

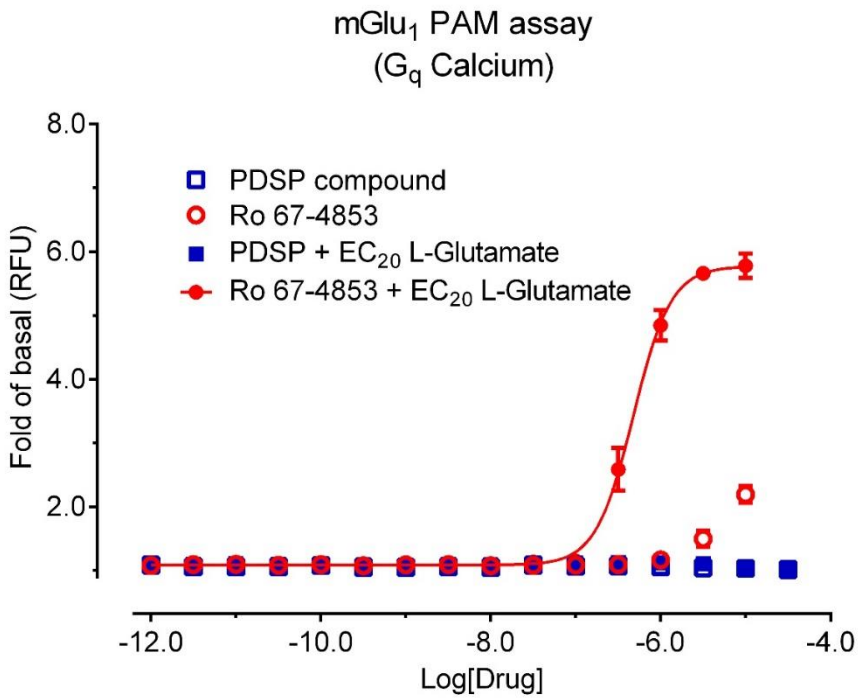

M3D agonist assay
(Gq Calcium)
ACh
CNO
PDSP compound
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

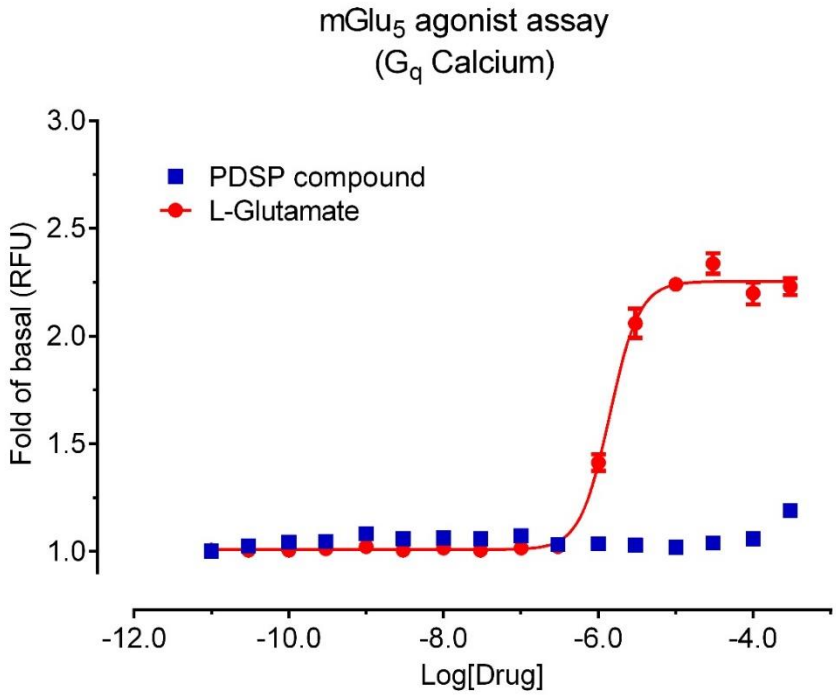

M5 agonist assay
(Gq Calcium)
ACh
CNO
PDSP compound
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

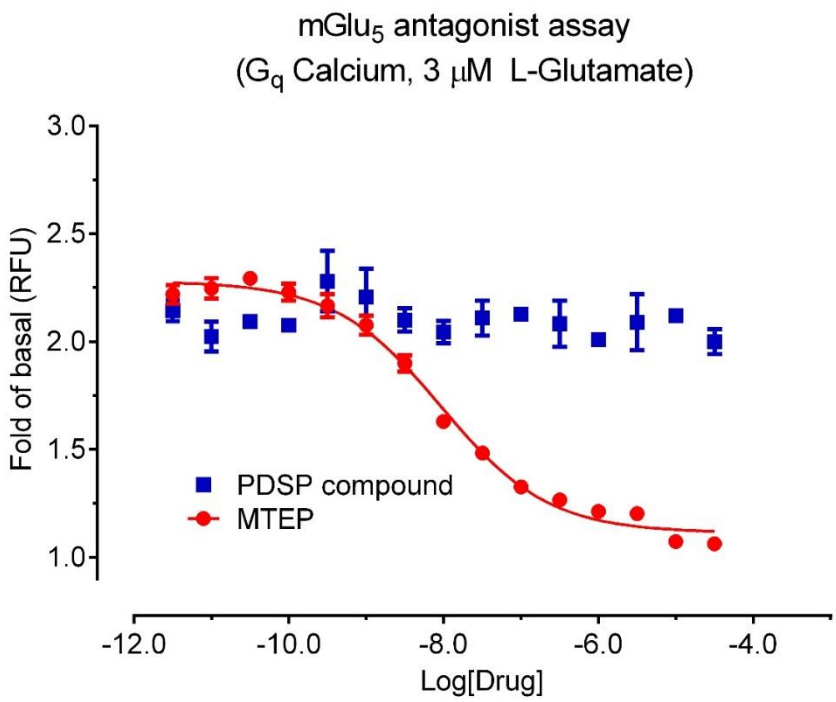

M5D agonist assay
(Gq Calcium)
ACh
CNO
Fold of basal (RFU)
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

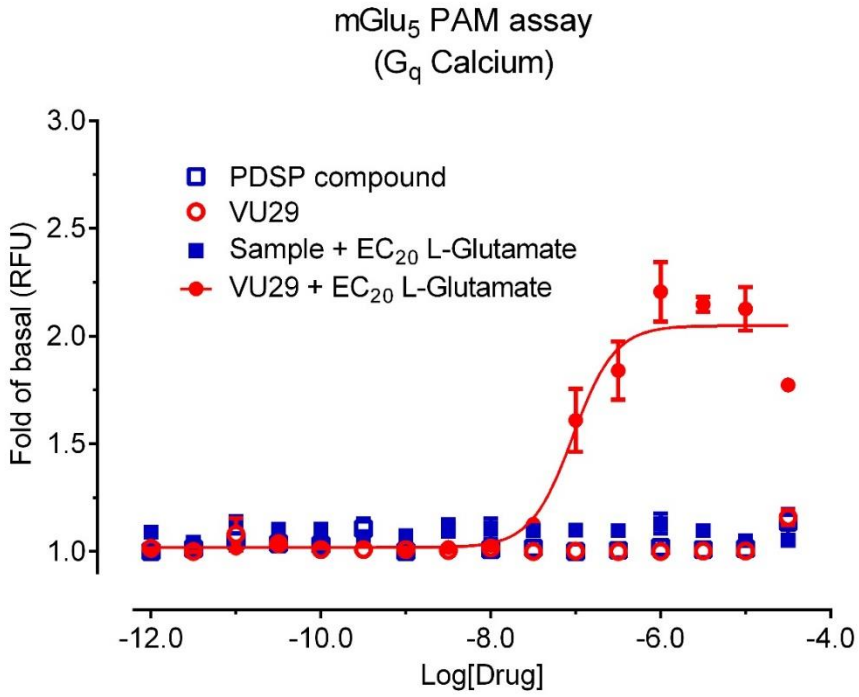

mGlu1 agonist assay
(Gq Calcium)
PDSP compound
L-Glutamate
Fold of basal (RFU)
5.0
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

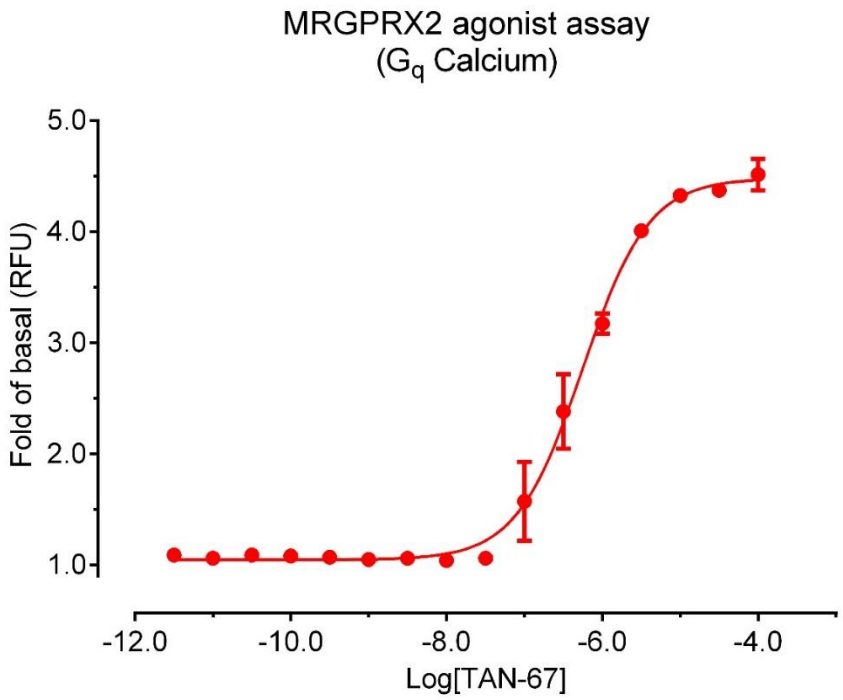

mGlu1 antagonist assay
(Gq Calcium, 3 μM L-Glutamate)
Fold of basal (RFU)
5.0
4.0
3.0
2.0
1.0
PDSP compound
FTIDC
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

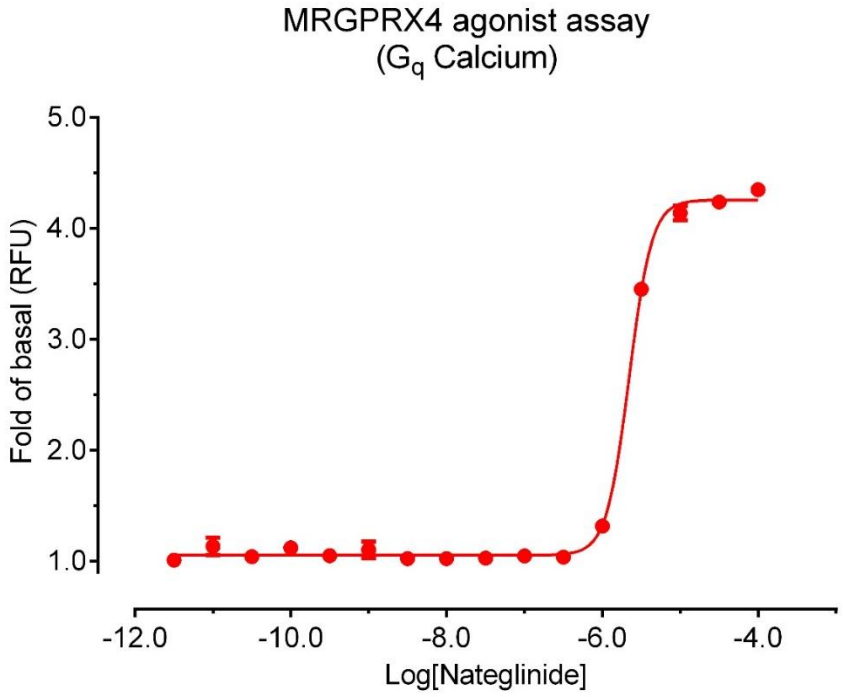

mGlu1 PAM assay
(Gq Calcium)
Fold of basal (RFU)
8.0
6.0
4.0
2.0
PDSP compound
Ro 67-4853
PDSP + EC20 L-Glutamate
Ro 67-4853 + EC20 L-Glutamate
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

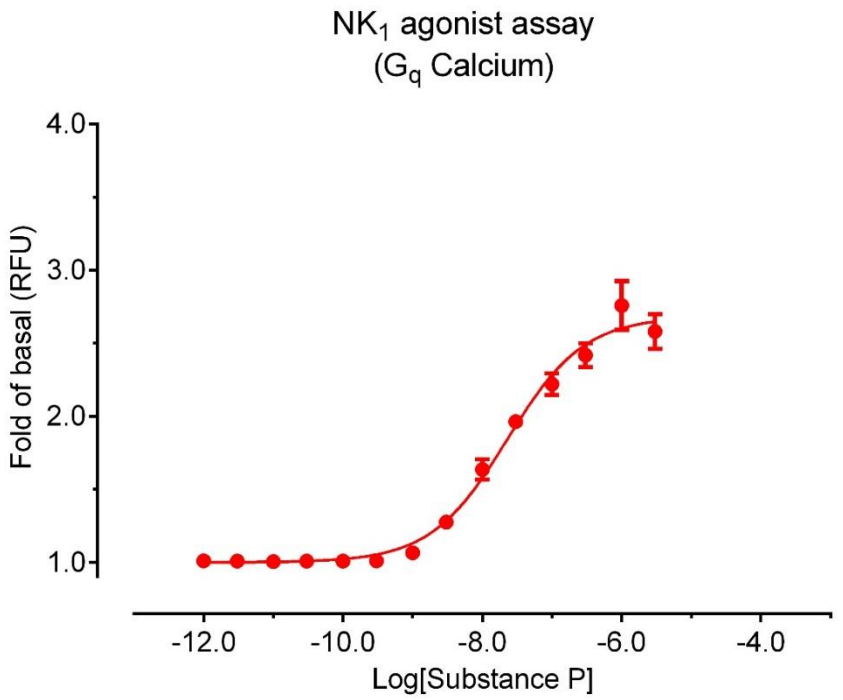

mGlu5 agonist assay
(Gq Calcium)
PDSP compound
L-Glutamate
Fold of basal (RFU)
3.0
2.5
2.0
1.5
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

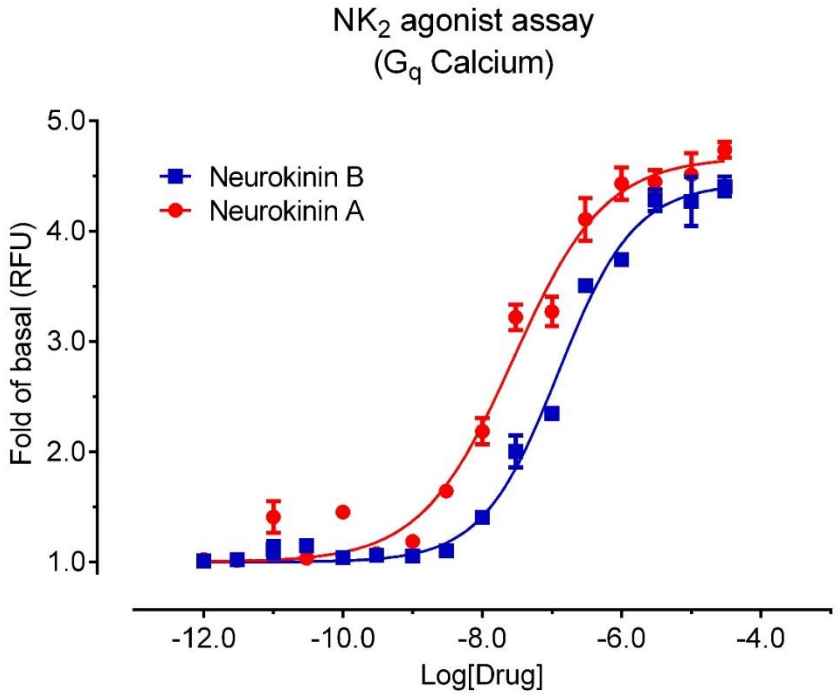

mGlu5 antagonist assay
(Gq Calcium, 3 μM L-Glutamate)
PDSP compound
MTEP
Fold of basal (RFU)
3.0
2.5
2.0
1.5
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

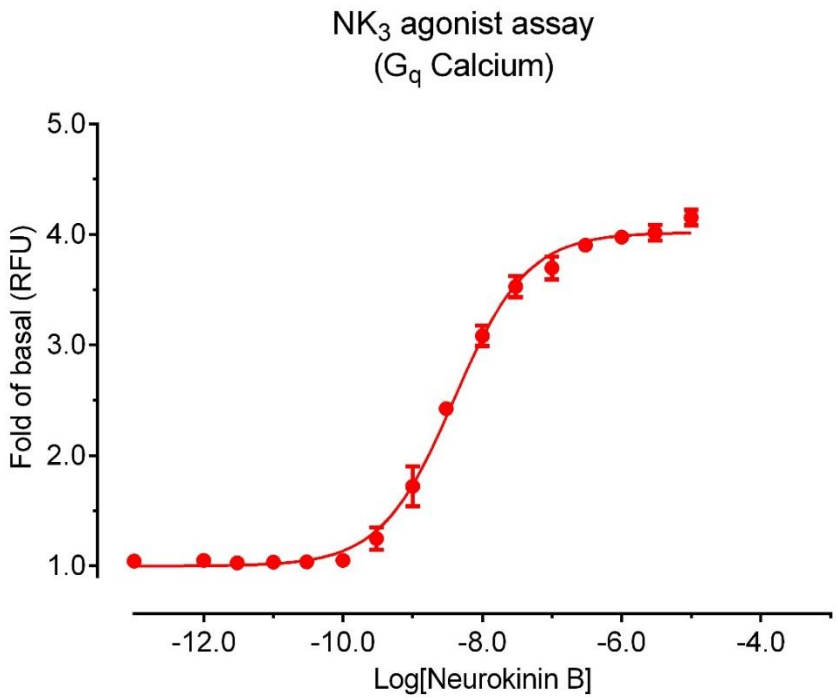

mGlu5 PAM assay
(Gq Calcium)
PDSP compound
VU29
Sample + EC20 L-Glutamate
VU29 + EC20 L-Glutamate
Fold of basal (RFU)
3.0
2.5
2.0
1.5
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

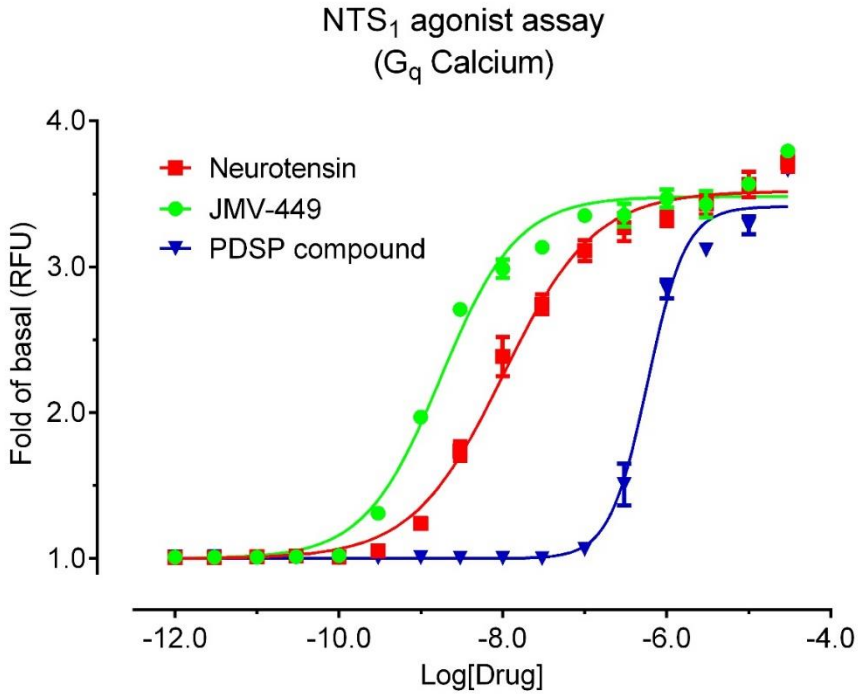

MRGPRX2 agonist assay
(Gq Calcium)
Fold of basal (RFU)
5.0
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[TAN-67]

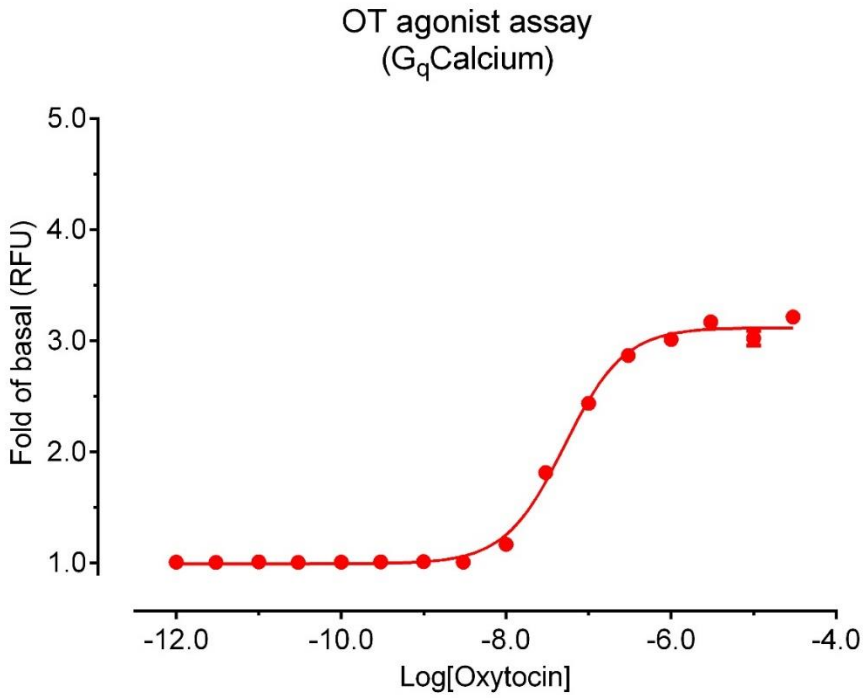

MRGPRX4 agonist assay
(G_q Calcium)
Fold of basal (RFU)
5.0
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Nateglinide]

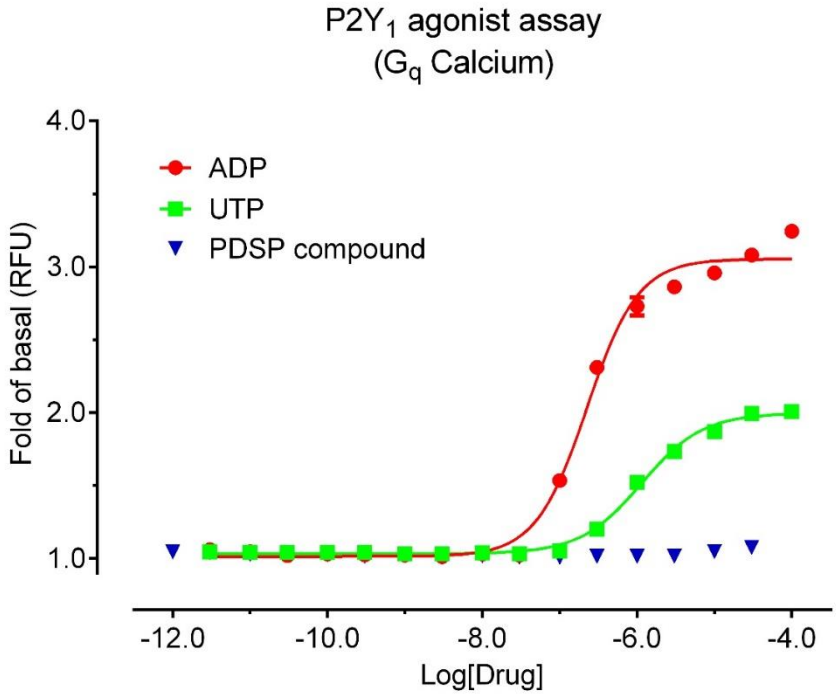

NK_1 agonist assay
(G_q Calcium)
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Substance P]

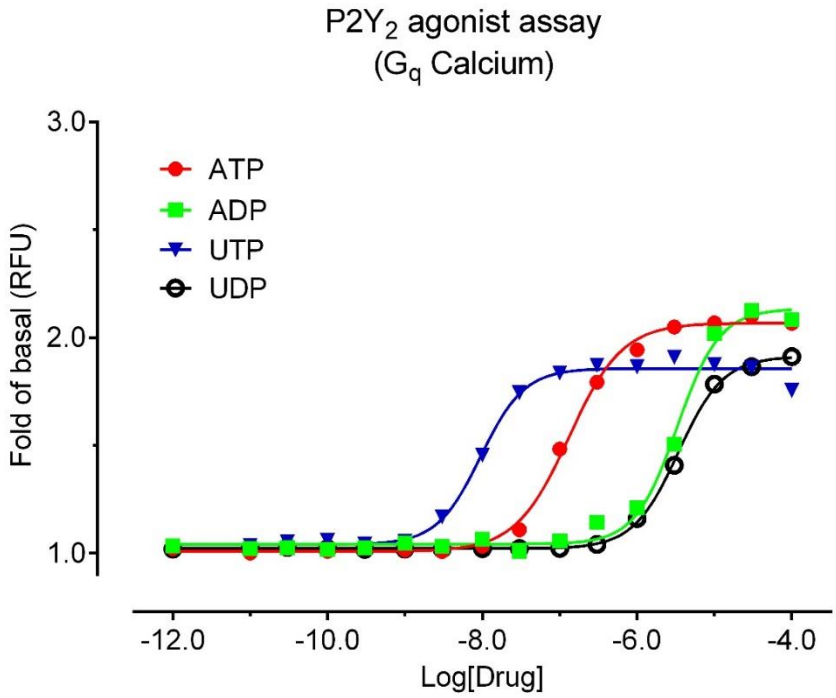

NK2 agonist assay
(Gq Calcium)
Neurokinin B
Neurokinin A
Fold of basal (RFU)
5.0
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

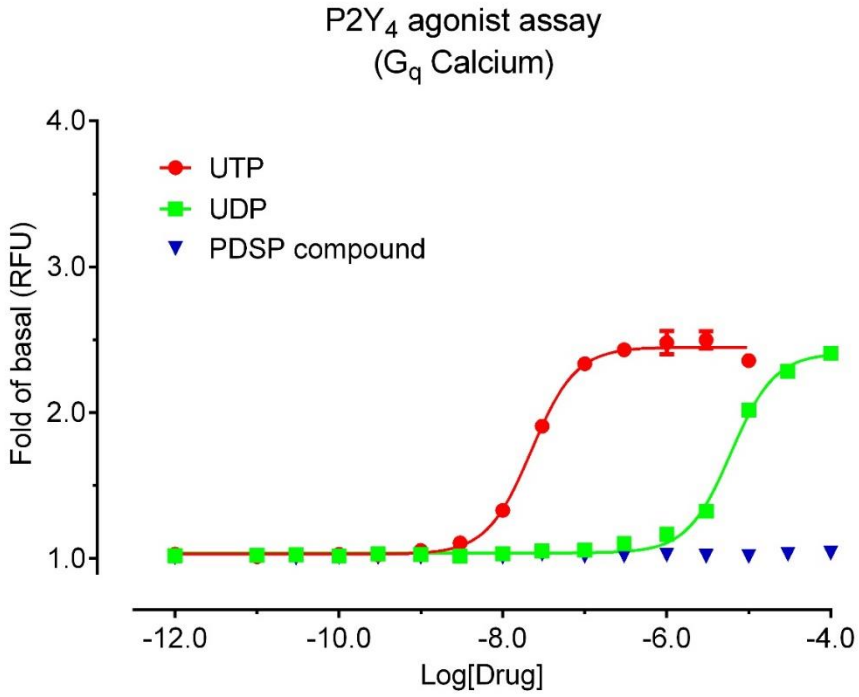

NK3 agonist assay
(Gq Calcium)
Fold of basal (RFU)
5.0
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Neurokinin B]

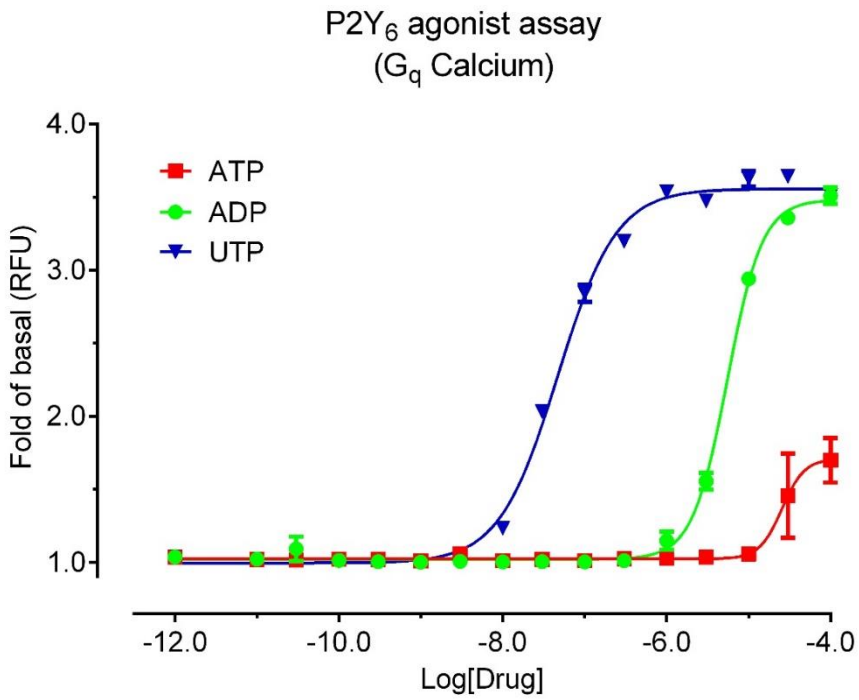

NTS1 agonist assay
(Gq Calcium)
Neurotensin
JMV-449
PDSP compound
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

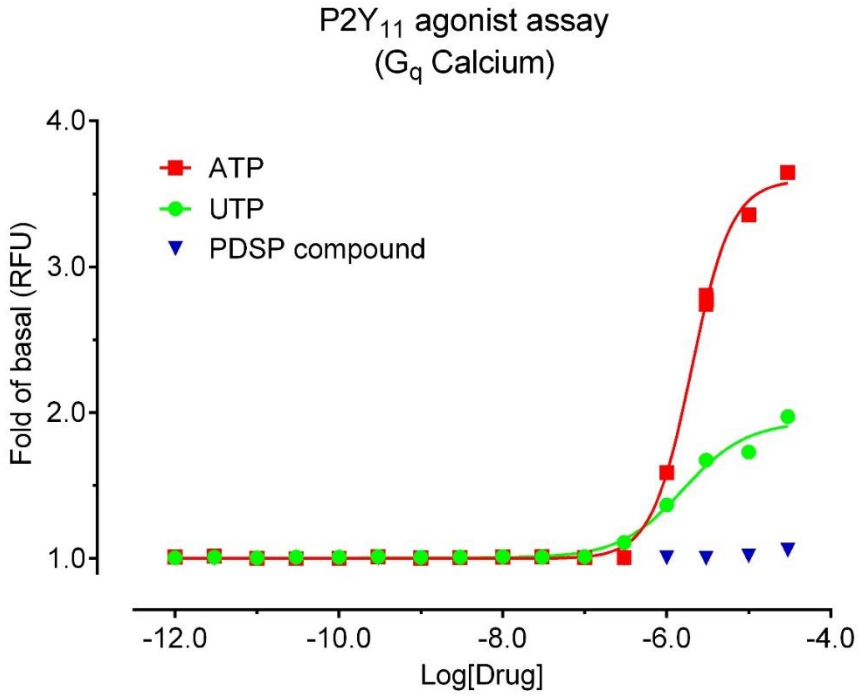

OT agonist assay
(GqCalcium)
Fold of basal (RFU)
5.0
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Oxytocin]

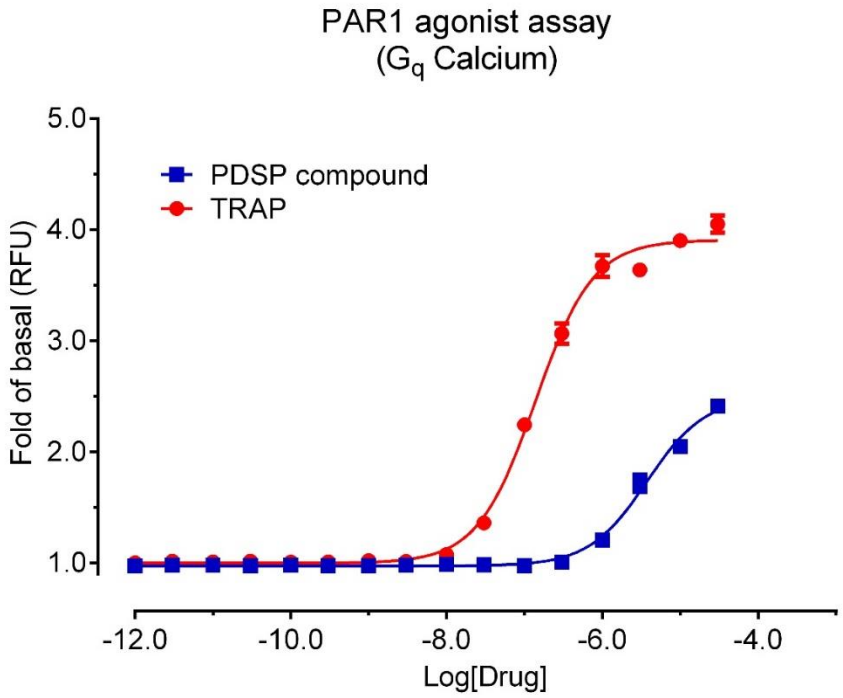

P2Y1 agonist assay
(Gq Calcium)
4.0
3.0
2.0
1.0
Fold of basal (RFU)
ADP
UTP
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

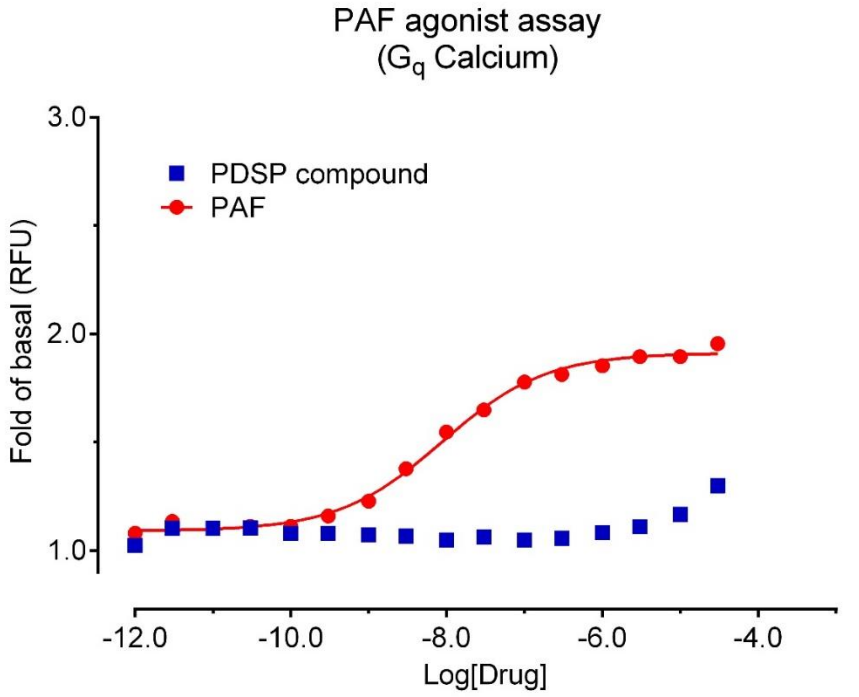

P2Y2 agonist assay
(Gq Calcium)
3.0
2.0
1.0
Fold of basal (RFU)
ATP
ADP
UTP
UDP
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

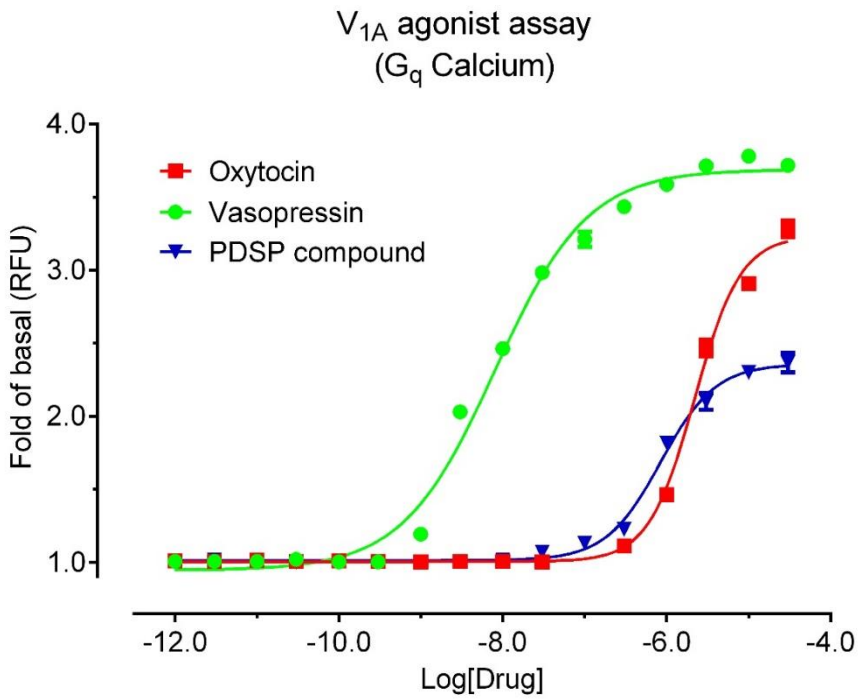

P2Y4 agonist assay
(Gq Calcium)
UTP
UDP
PDSP compound
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

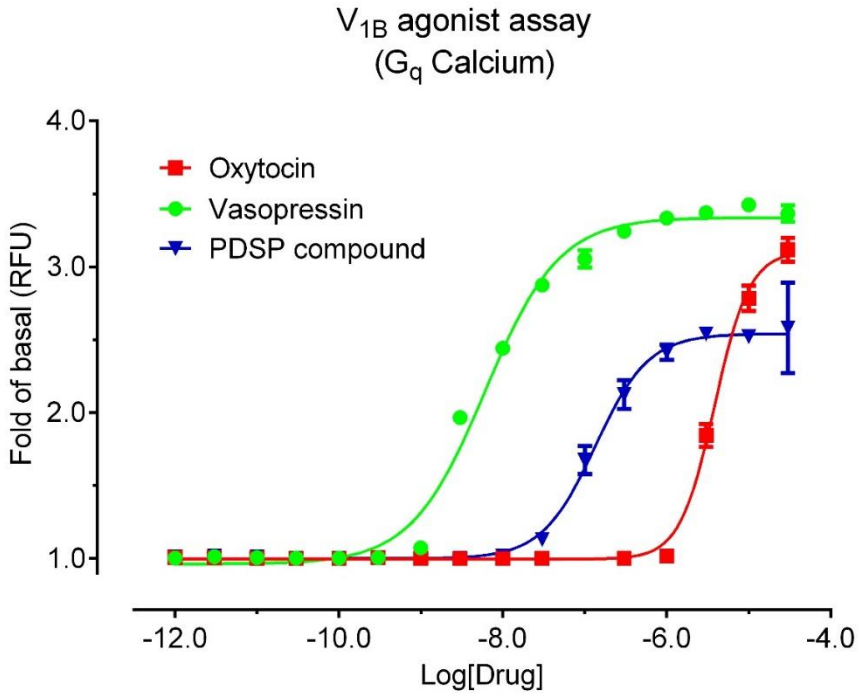

P2Y6 agonist assay
(Gq Calcium)
ATP
ADP
UTP
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


P2Y11 agonist assay
(Gq Calcium)
ATP
UTP
PDSP compound
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


PAR1 agonist assay
(Gq Calcium)
PDSP compound
TRAP
Fold of basal (RFU)
5.0
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


PAF agonist assay
(G_q Calcium)
PDSP compound
PAF
Fold of basal (RFU)
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


V_{1A} agonist assay
(G_q Calcium)
Oxytocin
Vasopressin
PDSP compound
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


V1B agonist assay
(Gq Calcium)
Oxytocin
Vasopressin
PDSP compound
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

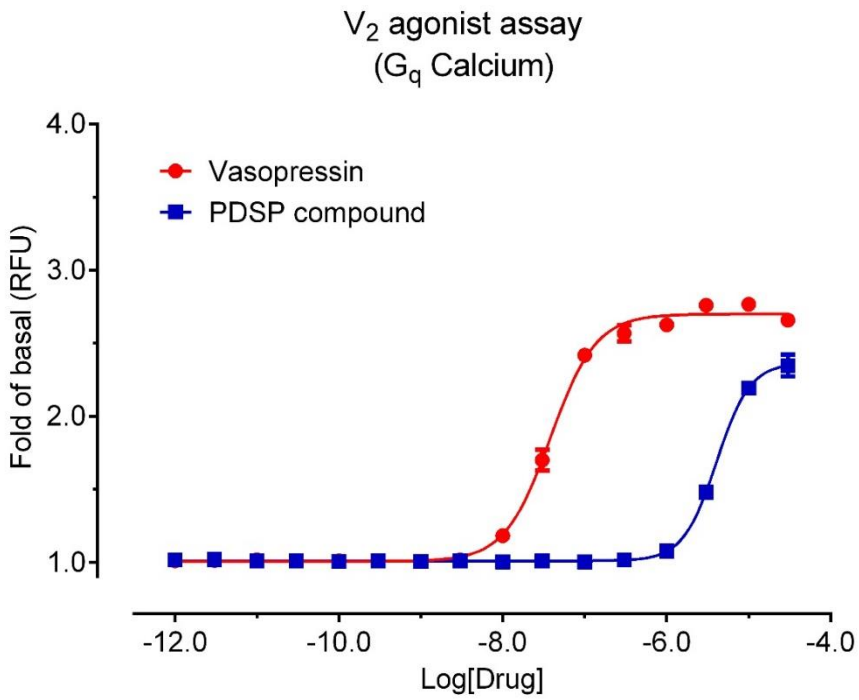

V2 agonist assay
(Gq Calcium)
Vasopressin
PDSP compound
Fold of basal (RFU)
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

Figure 40. Schild analysis with a PDSP compound at 5-HT_{2A} receptors (G_q Calcium, FLIPR). Results are analyzed according to published procedures (155–157).

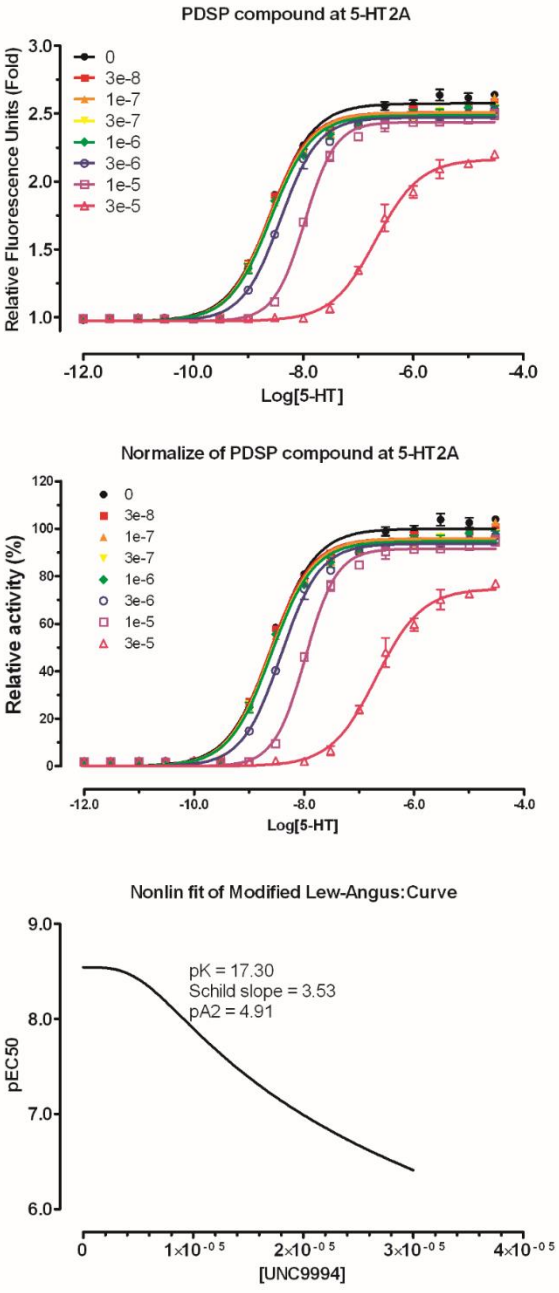


Figure 41. Schild analysis of a PDSP compound at 5-HT_{2B} receptors (G_q Calcium, FLIPR). Results are analyzed according to published procedures (155–157).

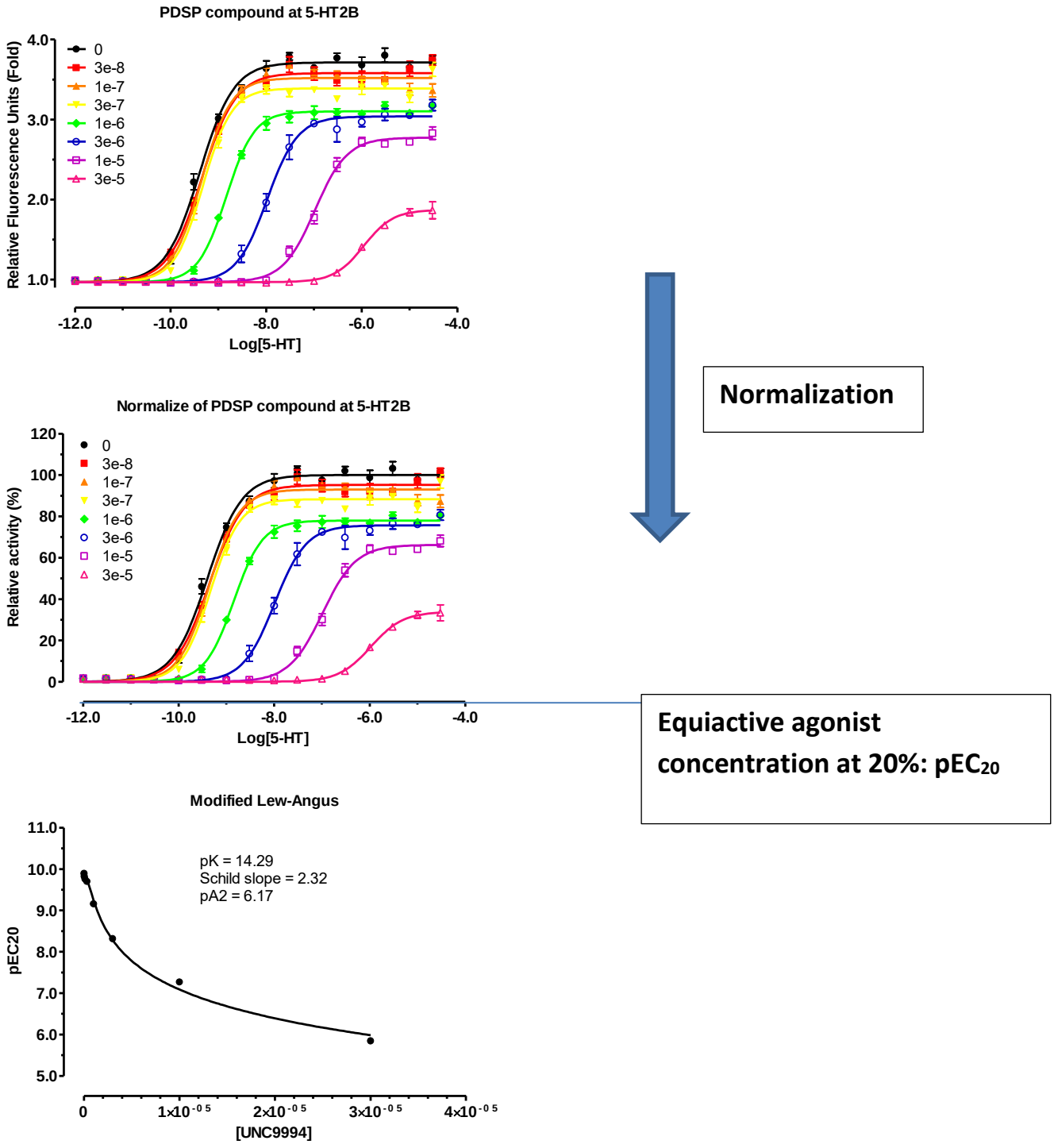


Figure 42. Schild analysis with a PDSP compound at 5-HT_{2C} receptors (G_q Calcium, FLIPR). Results are analyzed according to published procedures (155–157).

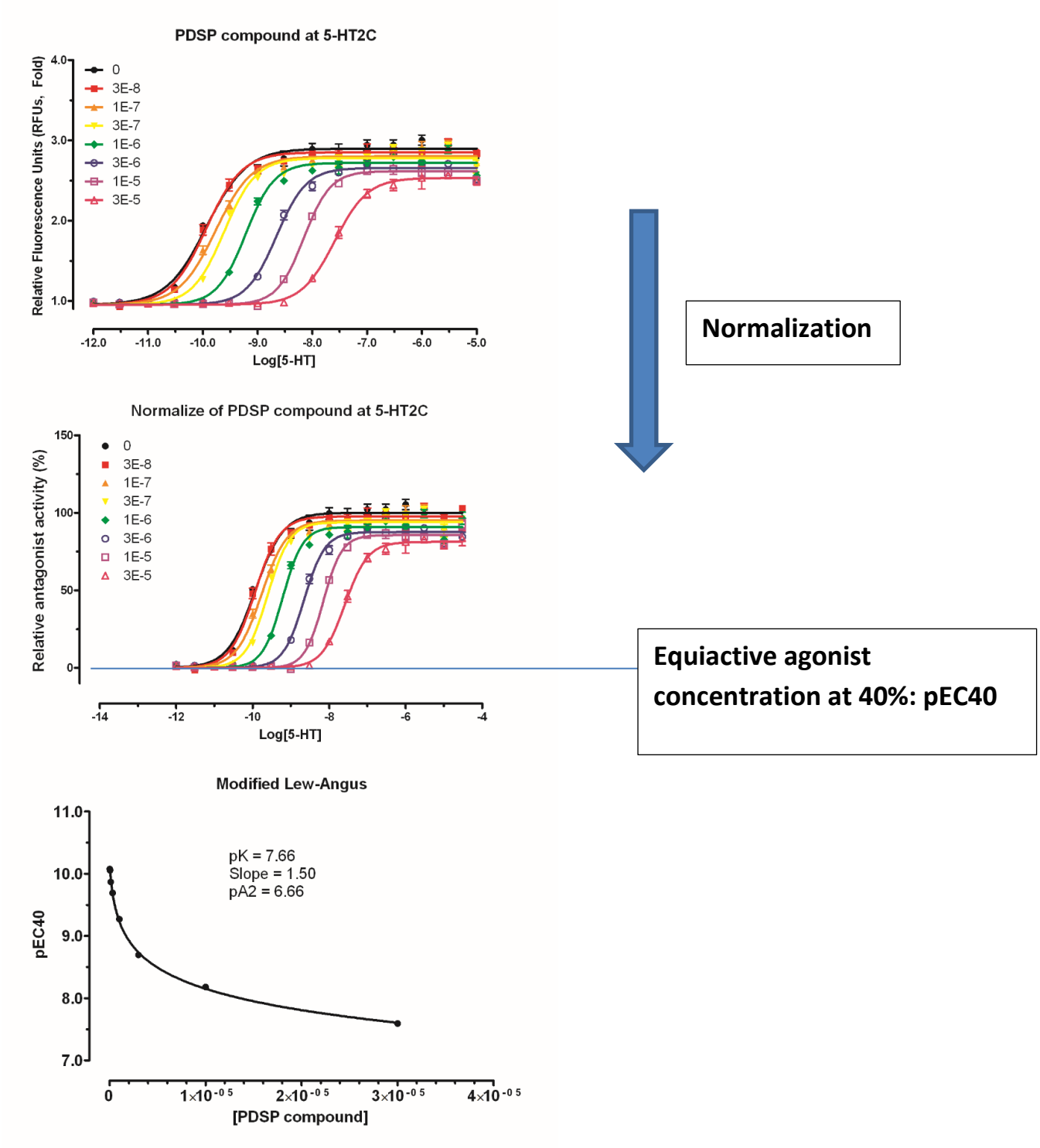
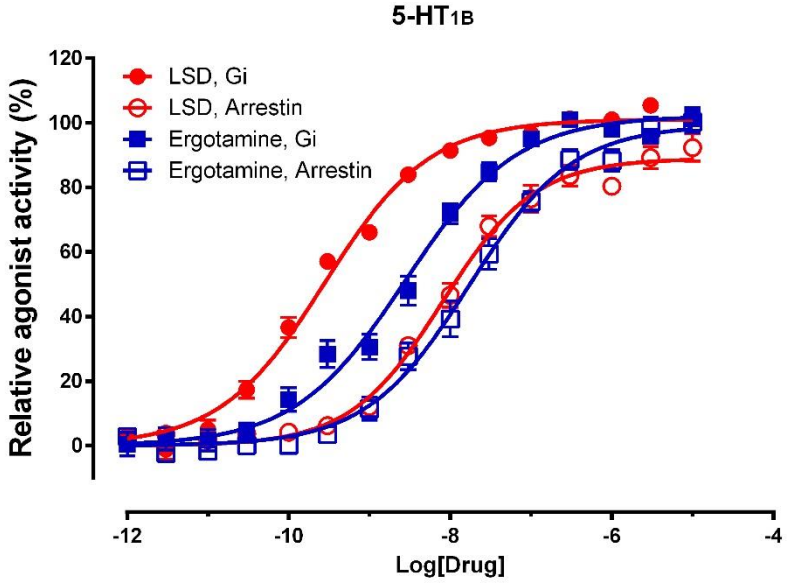


Figure 43. Representative figures for bias analysis. LSD and Ergotamine (ERG) agonist activity in G_i (for 5-HT_{1B}) or G_q (for 5-HT_{2B}) or β-arrestin signaling pathways were determined at 5-HT_{1B} and 5-HT_{2B} receptors as outlined in the functional assay section, and results were normalized to corresponding 5-HT activity and analyzed in Prism using Black and Leff operational model to estimate transduction coefficient, $Log(\tau/K_A)$, as listed in the Table on the next page. Results are from published papers (142, 158) and modified for presentation here.



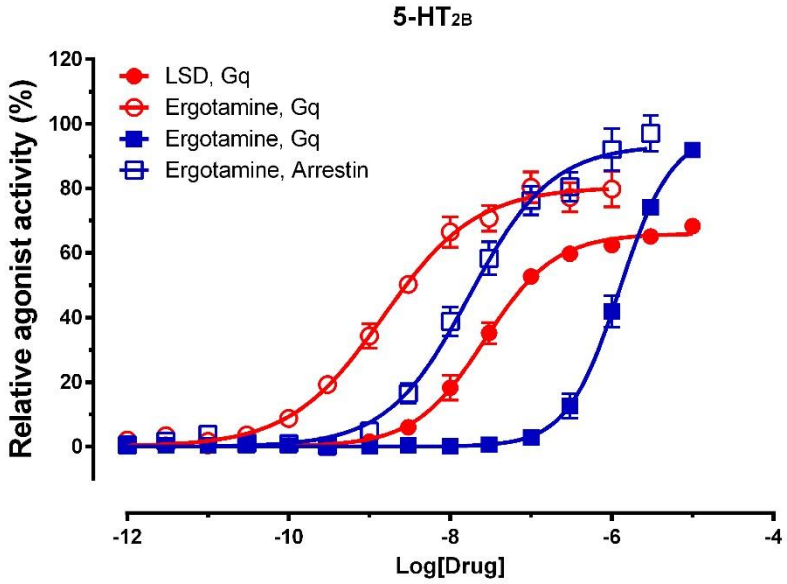


Table 28. Transduction coefficients, Log(τ/K_A), and bias factor calculations for indicated pathways of agonists at 5-HT_{1B}. See representative dose-response curves in **Figure 43** on previous page. Bias factor = $10^{\Delta\Delta Log(\tau/KA)}$. Results are from published papers (142, 158) and are modified for presentation here.

Ligands	Bias calculation at 5-HT_{1B} receptors					
	G_i pathway	$\Delta Log(\tau/K_A)$	β-arrestin pathway	$\Delta Log(\tau/K_A)$	$\Delta\Delta Log(\tau/K_A)$	Bias Factor
5-HT	9.54 ± 0.08	0	7.47 ± 0.13	0	0	1.0
LSD	9.53 ± 0.09	-0.01	8.08 ± 0.29	0.61	0.62	4.2
Ergotamine	8.53 ± 0.18	-1.01	7.82 ± 0.20	0.35	1.36	22.9
DHE	8.89 ± 0.21	-0.65	7.87 ± 0.19	0.40	1.05	11.2
MTE	9.41 ± 0.12	-0.13	7.72 ± 0.15	0.25	0.38	2.4
Pergolide	7.82 ± 0.25	-1.72	6.41 ± 0.11	-1.06	0.66	4.6
Cabergoline	6.58 ± 0.08	-2.96	6.32 ± 0.14	-1.15	1.81	64.6
Ro 60-0175	6.37 ± 0.35	-3.17	5.32 ± 0.53	-2.15	1.02	10.5
Sumatriptan	8.16 ± 0.19	-1.38	6.94 ± 0.11	-0.53	0.85	7.1
Donitriptan	9.51 ± 0.37	-0.03	8.13 ± 0.18	0.66	0.69	4.9
Eletriptan	8.13 ± 0.29	-1.41	7.07 ± 0.29	-0.40	1.01	10.2
Rizatriptan	7.80 ± 0.43	-1.74	6.63 ± 0.21	-0.84	0.90	7.9

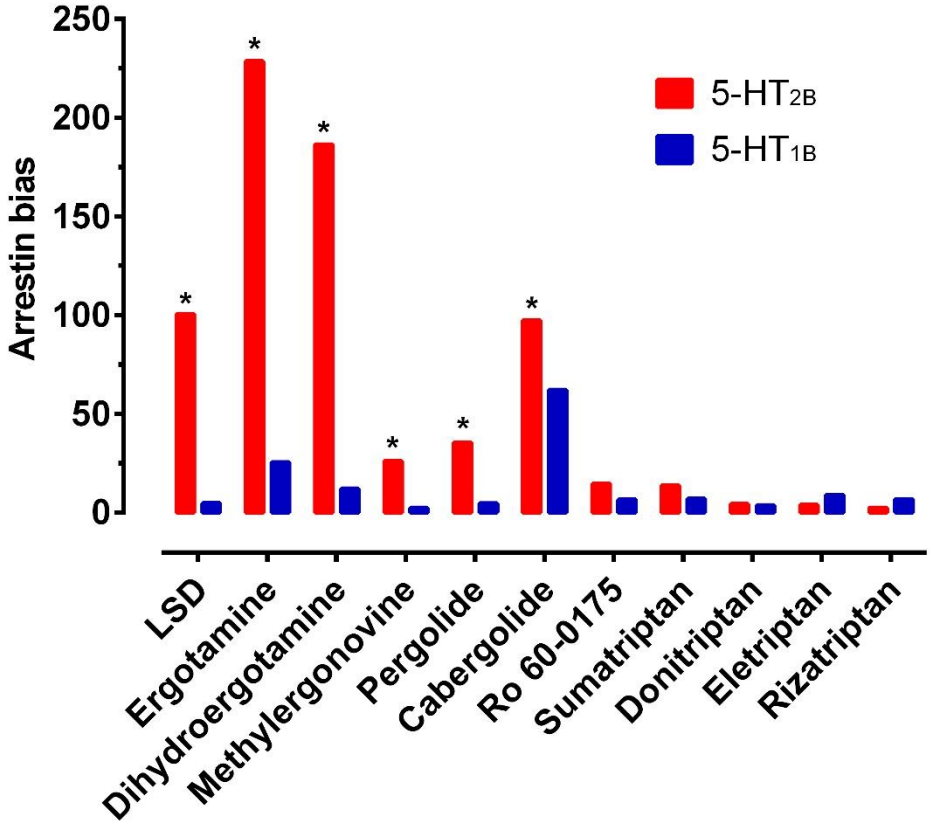
DHE = Dihydroergotamine; MTE = Methylergonovine

Table 29. Transduction coefficients, Log(τ/K_A), and bias factor calculation for indicated pathways of agonists at 5-HT_{2B}. See representative dose-response curves in **Figure 43** on previous page. Results are from published papers (142, 158) and are modified for presentation here.

Ligands	Bias calculation at 5-HT_{2B} receptors					
	G_q pathway	ΔLog(τ/K_A)	β-arrestin pathway	ΔLog(τ/K_A)	$\Delta\Delta$Log(τ/K_A)	Bias Factor
5-HT	9.61 ± 0.05	0	8.30 ± 0.09	0	0	1.0
LSD	7.63 ± 0.15	-1.98	8.38 ± 0.09	0.08	2.06	114.8
Ergotamine	5.95 ± 0.10	-3.66	7.05 ± 0.16	-1.25	2.41	257.0
DHE	6.03 ± 0.07	-3.58	7.13 ± 0.33	-1.17	2.41	257.0
MTE	7.93 ± 0.23	-1.68	8.05 ± 0.20	-0.25	1.43	26.9
Pergolide	7.50 ± 0.22	-2.11	7.96 ± 0.08	-0.34	1.77	58.9
Cabergoline	6.92 ± 0.18	-2.69	7.76 ±0.22	-0.54	2.15	141.3
Ro 60-0175	9.04 ± 0.15	-0.57	9.00 ± 0.10	0.7	1.27	18.6
Sumatriptan	5.24 ± 0.12	-4.37	5.17 ± 0.89	-3.13	1.24	17.4
Donitriptan	6.65 ± 0.12	-2.96	5.92 ± 0.04	-2.38	0.58	3.8
Eletriptan	6.17 ± 0.05	-3.44	5.41 ± 0.20	-2.89	0.55	3.5
Rizatriptan	5.86 ± 0.15	-3.75	5.03 ± 0.03	-3.27	0.48	3.0

DHE = Dihydroergotamine; MTE = Methylergonovine

Figure 44. Comparison of bias factors of various ligands at 5-HT_{1B} and 5-HT_{2B} receptors. Values are taken from **Tables 28 and 29** and **Figure 43**. * indicates significant difference $p < 0.0001$ (two-way ANOVA). Results are from published papers (142, 158) and are modified for presentation here.



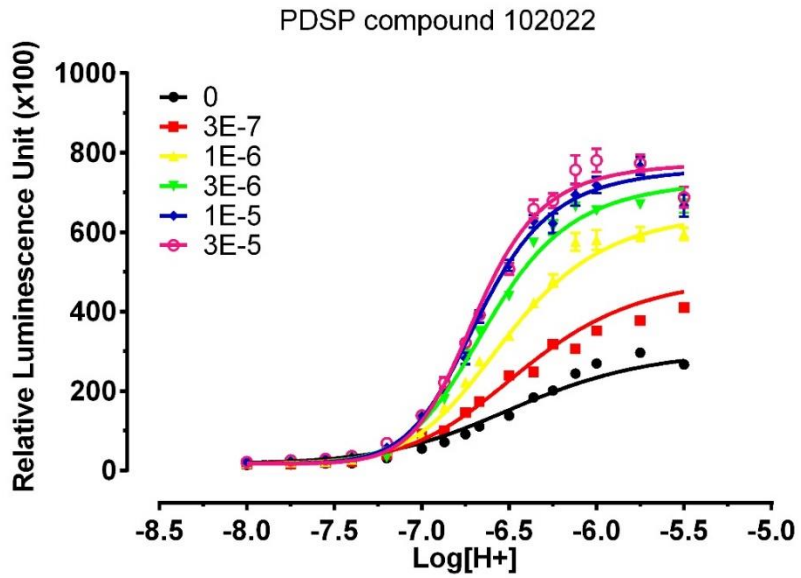


Figure 44. Representative curves for the allosteric operational model. GPR68-mediated cAMP production was determined in the absence and presence of increasing concentration of PDSP compound #102022. Results are analyzed using the allosteric operational model and best-fit values are listed in the following table. In the curve-fitting, Log(K_A) is set at -6.50 (equivalent to the pEC_{50} value of protons in the absence of #102022), while the E_{max} is constrained to 800 (which is the maximum activity of the system); τ_B is constrained to "0", since #102022 has no agonist activity by itself.

[102022] M	0	3.00E-7	1.00E-6	3.00E-6	1.00E-5	3.00E-5	Global (shared)
Best-fit values							
LogK_A	(= -6.50)	(= -6.50)	(= -6.50)	(= -6.50)	(= -6.50)	(= -6.50)	
LogK_B	-6.14	-6.14	-6.14	-6.14	-6.14	-6.14	-6.14
Basal	16.32	16.32	16.32	16.32	16.32	16.32	16.32
E_{max}	(=800)	(=800)	(=800)	(=800)	(=800)	(=800)	
τ_A	0.678	0.678	0.678	0.678	0.678	0.678	0.678
α	0.877	0.877	0.877	0.877	0.877	0.877	0.877
β	4.055	4.055	4.055	4.055	4.055	4.055	4.055
B	= 0.0	3.00E-07	1.00E-06	3.00E-06	1.00E-05	3.00E-05	
τ_B	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	
n	1.443	1.993	2.441	2.799	3.116	3.451	

2.5. Functional assays for G_i or G_s coupled GPCRs - Split luciferase cAMP assay

Main equipment: luminescence counter

Reagents: GloSensor cAMP construct from Promega and Luciferin

Assay buffer: 20 mM HEPES, 1x HBSS, pH 7.40

2.5.1. Cell culture and transfection: To determine G_i or G_s GPCR-mediated cAMP production, the PDSP uses Promega's split luciferase based GloSensor cAMP biosensor technology. With the cells stably expressing target receptors, we transfect with the GloSensor cAMP DNA construct overnight; otherwise, we co-transfect HEK 293T cells with target receptor DNA and GloSensor cAMP DNA construct overnight. For detailed transfection protocol, see above section "Calcium precipitation transfection". To prepare plates for assays, cells are seeded into PLL-coated 384-well white clear bottom cell culture plates in DMEM supplemented with 1% dFBS at a density of 15-20K cells in a volume of 40 µl per well. The plates can be used for assays after 6 hours or overnight.

2.5.2. Split luciferase biosensor cAMP assay – Luciferin first protocol: The GloSensor cAMP assays have been widely used in determining G_i- or G_s-GPCR mediated cAMP production in live cells (142, 147, 158–161). The PDSP uses these assays for cell-based functional assays with G_i- or G_s-coupled GPCRs. On the day of assay, cells are removed from culture medium and loaded with 20 µl of 4 mM luciferin prepared in assay buffer for 60 min at 37°C. All the following steps are carried out at room temperature. To measure agonist activity at G_s-coupled receptors, 10 µl of 3x drug solutions are added and the plate is counted for chemiluminescence after 15 minutes. To measure antagonist activity at G_s-coupled receptors, cells are preincubated with drugs for 15 minutes before addition of an EC_{80} concentration of a reference agonist, and chemiluminescence is counted after 15 minutes. To measure agonist activity at G_i-coupled receptors, 10 µl 3x drug solutions is added for 15 minutes before addition of 5 µl of isoproterenol at a final concentration of 200 nM, and the plate is counted for chemiluminescence after 15 minutes. An alternative way to directly activate adenylyl cyclases is by the use of 30 µM forskolin(162–164), especially when cells that do not have enough β_2-AR mediated adenylyl cyclase activity (such as CHO cells) are used for GloSensor cAMP assays. To

measure antagonist activity at G_i-coupled receptors, cells are preincubated with drugs for 15 minutes before the addition of an EC_{80} concentration of a reference agonist for another 15 minutes, followed by the addition of 5 µl isoproternol at a final concentration of 200 nM and counting after 15 minutes. Isoproterenol is used to activate the endogenous G_s protein through endogenous $ß_2$ adrenergic receptors. Different receptors and different cell lines might need different preincubation times; preliminary assays are done to determine the best count window before large-scale screening assays.

2.5.3. Split luciferase biosensor cAMP assay - Drug first protocol: On the day of assay, cells are removed from culture medium and receive 20 µl/well assay buffer, followed by addition of 10 µl of 3x drug solutions for 15 minutes at room temperature. To measure agonist activity for G_s-coupled receptors, 10 µl of 4 mM luciferin prepared in assay buffer is added, and counting is done after 15 minutes. To measure agonist activity for G_i-coupled receptors, 10 µl of 4 mM Luciferin supplemented with Isoproternol at final of 200 nM is added, and counting is done after 15 minutes. To measure antagonist activity at Gi-coupled receptors, cells are preincubated with drugs for 15 minutes before addition of an EC_{80} concentration of a reference agonist for another 15 minutes, followed by addition of 10 µl of 4 mM luciferin supplemented with isoproternol at a final concentration of 200 nM and counting after 15 minutes. Different receptors and different cell lines might need different preincubation times; therefore, a preliminary assay are performed to determine the best count window before large-scale screening assays.

2.5.3.1. Primary assays - Single concentration assays. Each new compound is tested on all receptors at a single concentration (10 µM) for activity as an agonist or an antagonist. Testing for antagonism is performed in presence of the EC_{50} concentration of a typical agonist (as described above). Each compound is tested in duplicate in two separate experiments performed on different lots of cells. In addition to the tested compounds, each 96-well plate contains wells for the determination of basal activity, maximal agonist stimulation, agonist EC_{50} concentrations (i.e., concentration-response isotherm), and the IC_{50} concentration of a known antagonist for purposes of positive control and for activity calculations. The reported results for each compound are calculated for agonists as the % of maximal activity (as obtained with maximal agonist concentrations), and for antagonists as the %

inhibition of receptor activity (in presence of an EC_{50} concentration of the agonist). Results are expressed as means ± SEM from four replicates.

2.5.3.2. Secondary assays - Dose-response assays. Compounds determined to be active as agonists or antagonists may be tested for their potency in dose-response experiments. Eight-point dose-response curves are performed in duplicate twice on two separate lots of cells (sometimes a third curve may be needed if in the first experiment the range of concentrations used is outside of the active range). For antagonists, these curves are performed in the presence of the EC_{50} concentration of the agonist. For each compound, the results from four replicates are averaged and then either EC_{50} or IC_{50} values are calculated by non-linear regression using the 4-parameter logistic equation. Results are reported as EC_{50} or IC_{50} values for each tested compound (and receptor) and include the EC_{50} or IC_{50} values of a known agonist or antagonist for comparison purposes.

2.5.4. Data processing and analysis: The luminescence counter records chemiluminescence in relative luminescence units (RLU) and saves files in 384-well format in Excel sheet for easy processing. Results in RLU are plotted and analyzed in GraphPad Prism v5.0 as outlined in **Section 2.3**.

2.5.5. Table and Figures. List of GPCRs for which the PDSP provides functional assays to determine G_i, G_s, or G_{olf} activity, and their corresponding dose-response curves. PDSP will also provide functional assays for other G_i or G_s coupled GPCRs upon request.

Table 30. List of GPCRs for which the PDSP provides cAMP measurements using GloSensor cAMP technology for G_i or G_s coupled receptors, and representative figures. PDSP will also design and develop functional assays for other G_i- or G_s-coupled GPCRs upon request and approval.

Receptor	G_i or G_s	Cell	Ligands (references)	Emax (fold)*	pEC_{50} (nM) or pIC_{50} (nM)	Hill slope
$5\text{-}HT_{1A}$	G_i	HEK T	5-HT	5.9	8.71 (1.9)	-1.02
$5\text{-}HT_{1B}$	G_i	HEK T	5-HT	5.1	9.71 (0.20)	-0.94
$5\text{-}HT_{1D}$	G_i	HEK T	5-HT	2.0	9.45 (0.36)	-1.77
$5\text{-}HT_{1E}$	G_i	HEK T	5-HT	3.2	9.12 (0.77)	-0.87
$5\text{-}HT_4$	G_s	HEK T	5-HT	2.3	10.17 (0.07)	0.97
$5\text{-}HT_{5A}$	G_s	CHO	5-HT	Being developed		
$5\text{-}HT_6$	G_s	HEK T	5-HT	14.6	9.29 (0.5)	0.78
$5\text{-}HT_{7A}$	G_s	HEK T	5-HT	9.1	7.75 (17.6)	0.81
			Clozapine (inverse agonist)	7.9	6.91 (123)	-0.77
M_2	G_i	HEK T	Acetylcholine	2.1	7.62 (24.1)	-0.74
M_2D	G_i	HEK T	CNO	3.4	6.30 (497)	-0.79
M_4	G_i	HEK T	Acetylcholine	2.7	8.23 (5.8)	-0.94
M_4D	G_i	HEK T	CNO	2.6	9.46 (0.35)	-1.25
G_s-DREADD	G_s	HEK T	CNO	13.3	7.89 (12.9)	1.44
			Acetylcholine	8.3	4.60 (25 μM)	1.91
A_1	G_i	HEK T	NECA	3.6	9.11 (0.78)	-0.99
			DPCPX (antagonist)	11.0	5.18 (6.6 μM)	0.81
A_{2A}	G_s	HEK T	NECA	2.4	9.58 (0.26)	0.74
			CCPA	1.9	7.80 (15.8)	1.42
			CGS21680	2.2	9.67 (0.22)	0.72
			CGS15943 (inverse agonist)	10.4	7.65 (22.5)	-1.27
A_{2B}	G_s	HEK T	NECA	30.4	8.03 (9.4)	1.09
			CGS15943 (antagonist)	24.3	6.44 (366)	-0.74
CRF-1	G_s	HEK T	CRF	95.8	8.55 (2.8)	0.78
CRF-2	G_s	HEK T	CRF	125.0	7.26 (54.7)	0.95
D_1	G_s	HEK T	Dopamine	153.6	8.18 (6.7)	0.94
D_2	G_i	HEK T	Dopamine	2.9	8.98 (1.1)	-0.97
D_3	G_i	HEK T	Dopamine	Being developed		
D_4	G_i	HEK T	Dopamine	2.0	9.19 (0.6)	-1.67
D_5	G_s	HEK T	Dopamine	37.6	9.24 (0.6)	0.80
H_2	G_s	HEK T	Histamine	40.0	8.07 (84.7)	1.08
			Cimetidine (antagonist)	16.6	5.98 (1039)	-1.45
H_3	G_i	HEK T	Histamine	4.6	8.90 (1.3)	-0.70

Receptor	G_i or G_s	Cell	Ligands (references)	Emax (fold)*	pEC_{50} (nM) or pIC_{50} (nM)	Hill slope
β_1	G_s	HEK T	Isoproterenol	20.8	11.10 (7.9 pM)	1.32
ß$_2$	G_s	HEK T	Isoproterenol	54.3	10.71 (19.3 pM)	0.94
			Salemeterol	42.7	9.78 (0.17)	0.86
β_3	G_s	HEK T	Isoprotenerol	30.5	8.22 (6.0)	0.62
Control HEK T (endogenous β receptors)	G_s	HEK T	Isopreternol	16.3	7.89 (12.9)	1.19
			Norepinephrine	7.8	6.28 (520)	0.89
			Cimaterol	10.7	7.98 (11.8)	0.98
			Dobutamine	7.5	5.51 (3090)	0.98
			Fenoterol	19.5	7.37 (44.0)	0.63
			Metaproterenol	8.0	5.94 (1159)	1.55
			Salbuterol	6.4	6.94 (116)	1.34
			Terbutaline	7.8	6.16 (691)	0.87
HCA_1	G_i	CHO	Niacin	1.5	4.94 (11.6 μM)	0.43
			Acifran	1.5	4.25 (56.7 μM)	0.55
HCA_2	G_i	CHO	Acifran	1.5	4.25 (56.7 μM)	0.55
			Acifran	2.8	6.36 (438)	0.69
HCA_3	G_i	CHO	Niacin	2.0	3.43 (376 μM)	0.56
			Acifran	3.6	4.31 (49.3 μM)	0.67
GLP-1	G_s	HEKT	GLP-1	173.6	7.24 (58.1)	0.81
			Glucagon	175.4	8.19 (6.5)	0.95
DOR	G_i	HEK T	DADLE	2.7	7.36 (43.6)	-0.94
			Naltrindole (antagonist)	1.6	7.73 (18.7)	1.46
KOR	G_i	HEK T	Salvinorin A	2.4	9.43 (0.37)	-1.33
			GNTI (antagonist)	3.2	8.13 (7.4)	1.54
MOR	G_i	HEK T	DAMGO	3.2	8.94 (1.2)	-0.75
			Naltrexone (antagonist)	2.6	7.70 (20.2)	0.67
NOP	G_i	HEK T	Nociceptin	3.6	8.64 (2.3)	-1.06
			SB612111 (antagonist)	1.8	7.66 (22.0)	1.64
Oxoglutarate	G_i	HEK T	α-Ketoglutaric Acid	2.2	3.49 (327 μM)	-1.52
MC_1	G_s	HEK T	α-MSH	19.1	7.45 (35.5)	1.14
			Melanotan II	21.2	7.89 (13.0)	0.87
MC_3	G_s	HEK T	α-MSH	91.0	7.06 (87.4)	1.26
			Melanotan II	86.1	7.62 (23.9)	0.93
MC_4	G_s	HEK T	α-MSH	80.6	9.19 (64.0)	0.98
			Melanotan II	89.1	7.72 (18.9)	0.76
MC_5	G_s	HEK T	α-MSH	35.9	6.49 (327)	1.65
			Melanotan II	29.8	6.81 (156)	1.54
MT_1	G_i	HEK T	Melatonin	2.5	10.51 (0.031)	-1.84

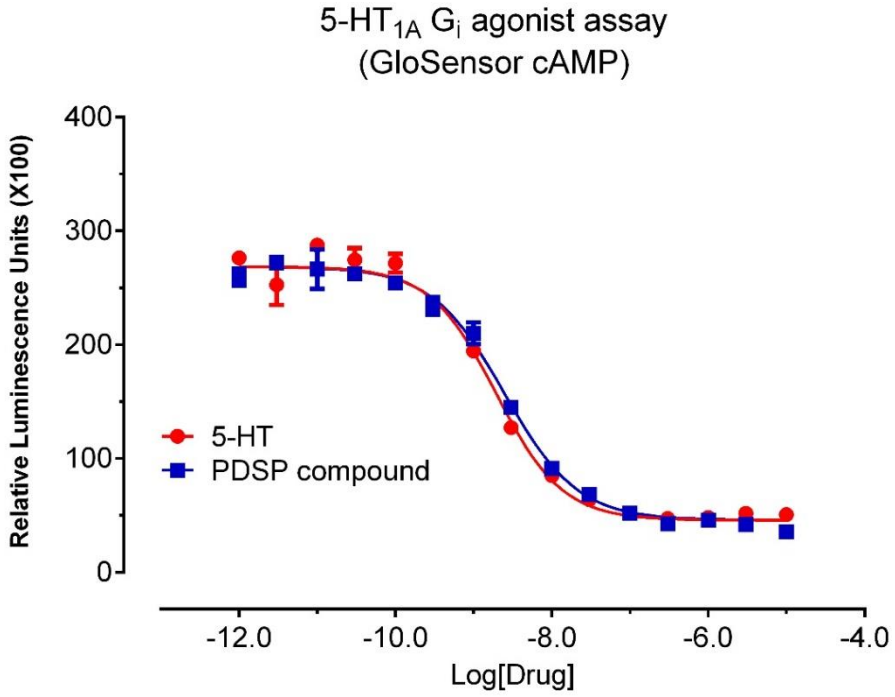
Receptor	G_i or G_s	Cell	Ligands (references)	Emax (fold)*	pEC_{50} (nM) or pIC_{50} (nM)	Hill slope
MT_2	G_i	HEK T	Melatonin	1.9	10.85 (0.014)	-1.18
$mGlu_2$	G_i	HEK	L-Glutamate	5.8	5.14 (7287)	-1.13
			LY379268	5.8	8.39 (4.1)	-0.89
			LY341495 (antagonist)	4.1	7.27 (53.5)	1.52
$mGlu_3$	G_i	HEK	L-Glutamate	2.3	6.30 (505)	-1.05
			LY341495 (antagonist)	3.2	7.27 (53.2)	2.13
$mGlu_4$	G_i	HEK	L-SOP	4.8	5.80 (1590)	-1.02
			LY341495 (antagonist)	2.3	5.27 (5381)	3.38
$mGlu_6$	G_i	HEK	L-Glutamate	1.9	4.84 (14.5 μM)	-1.25
			L-SOP	1.9	6.17 (684)	-1.01
			LY341495 (antagonist)	2.8	5.90 (1264)	1.33
$mGlu_7$	G_i	Not available yet, in development				
$mGlu_8$	G_i	HEK	L-Glutamate	3.6	5.87 (1336)	-0.68
			LY341495 (antagonist)	2.6	6.79 (163)	1.28
$NPBW_1$	G_i	HEK T	Neuropeptide W-23 (165)	2.1	8.05 (8.8)	-0.88
$NPBW_2$	G_i	HEK T		1.6	9.56 (0.27)	-0.38
RXFP1	G_s	HEK T	Relaxin-2	95.7	9.15 (0.7)	0.92
RXFP2	G_s	HEK T	Relaxin-2	21.1	7.48 (33.4)	0.96
RXFP3	G_i	HEK T	Relaxin-3	2.4	9.85 (0.14)	-1.40
RXFP4	G_i	HEK T	Relaxin-3	2.4	9.19 (0.06)	-0.78
SSTR5	G_i	HEK T	Somastotatin	7.7	8.63 (2.4)	-1.66
TP	G_s	HEK T	U46619(166)	27	5.98 (1055)	0.98
			S18886 (antagonist)(167)	26	8.29 (5.2)	-1.21
GPR88	G_i	HEK T	PDSP reference#	6.1	5.99 (1030)	-0.74
GPR4	G_s	HEK T	H^+ (161, 168)	5.9	7.99 (10.1)	5.53
GPR65	G_s	HEK T		9.1	7.44 (36.4)	3.56
GPR68	G_s	HEK T		13.6	6.80 (157)	3.36
GPR39	G_s	HEK T	GPR39-C3 (146–148)	16.2	6.28 (527)	1.13

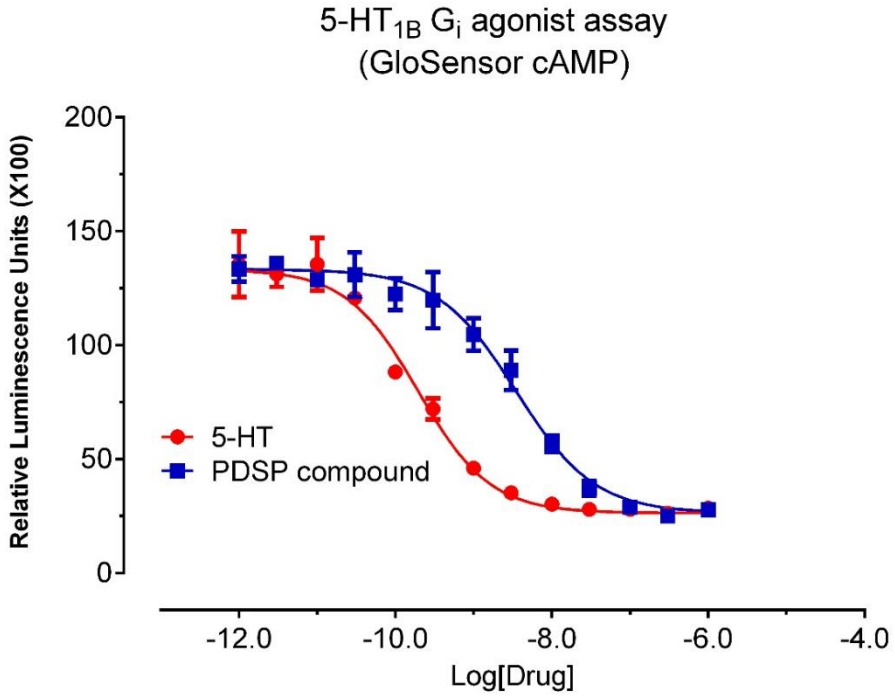
Notes:

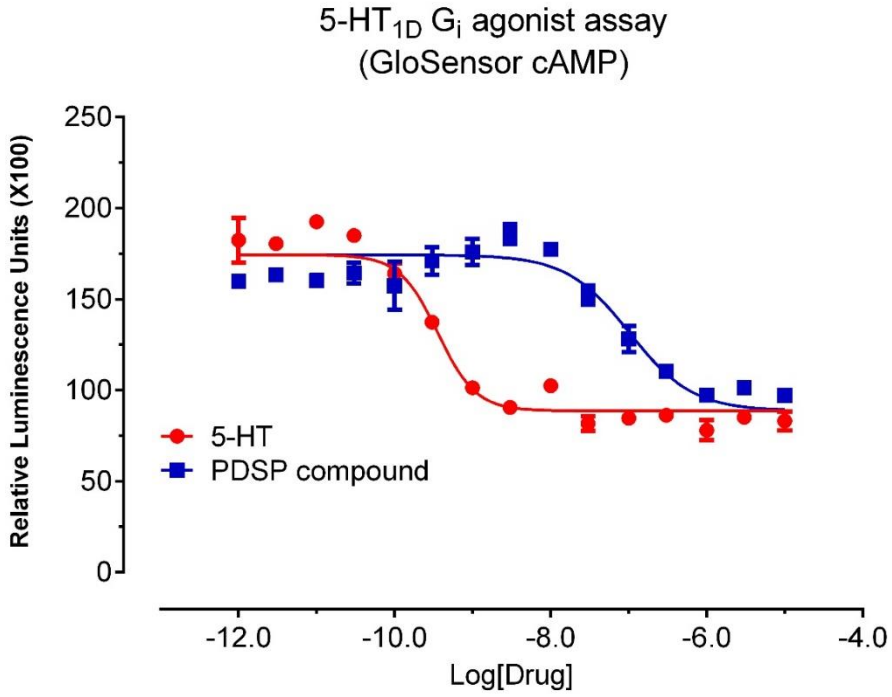
#: Roth lab unpublished results

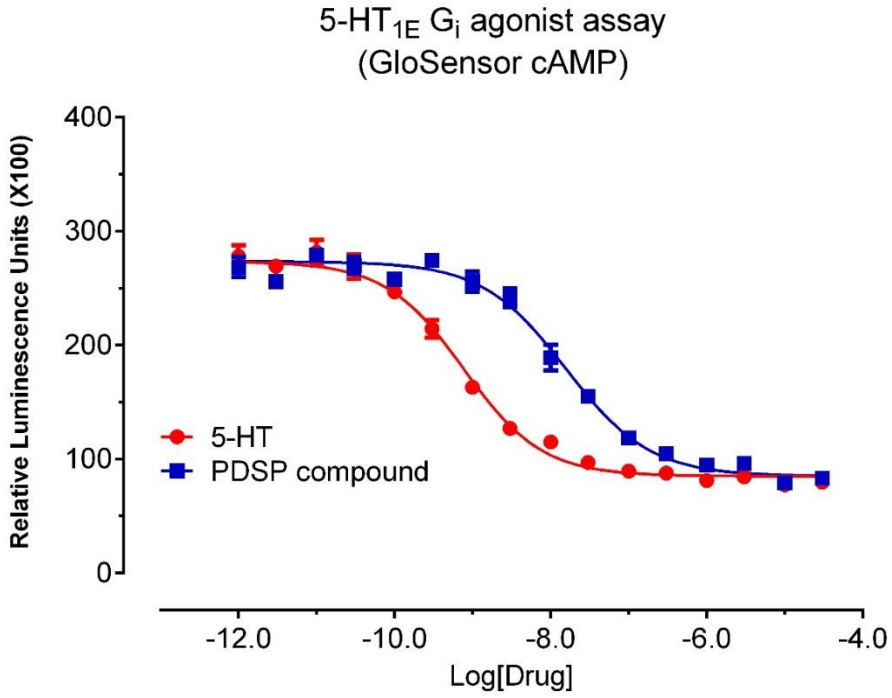
*: Emax in fold for G_i pathway represents the ratio of basal vs maximal inhibition; Emax in fold for G_s pathway represents the ratio of maximal activation vs basal.

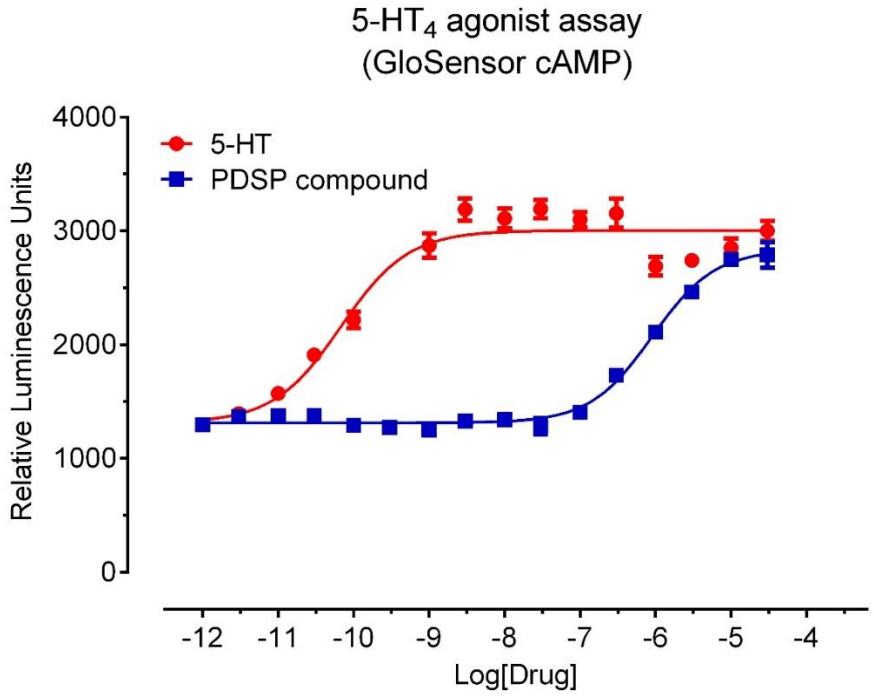
Figure 45. Representative curves for the GloSensor cAMP assay. The assays were conducted according to above procedures and analyzed in GraphPad Prism 6.0.

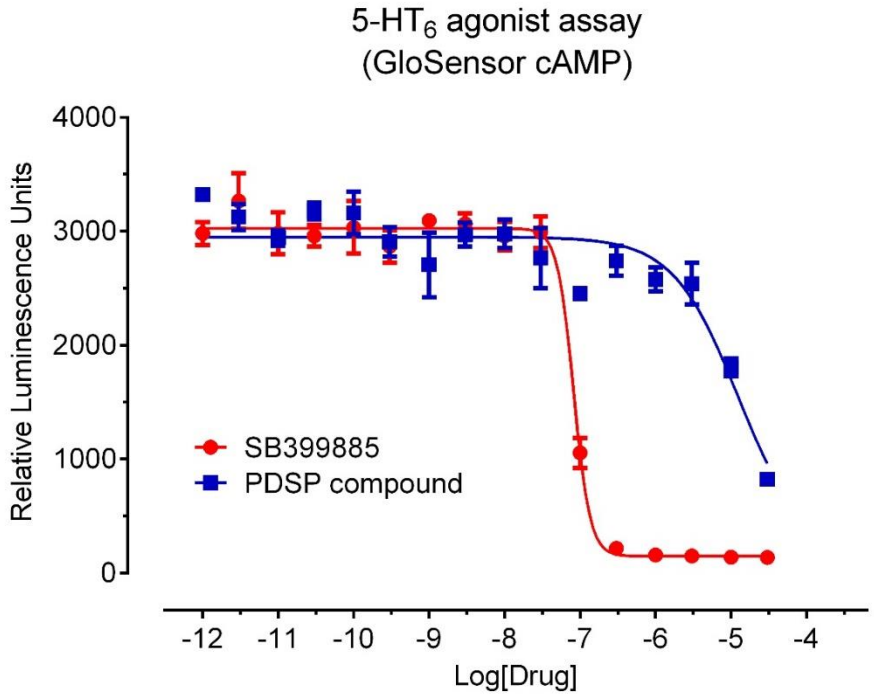

5-HT1A Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units (X100)
400
300
200
100
0
5-HT
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

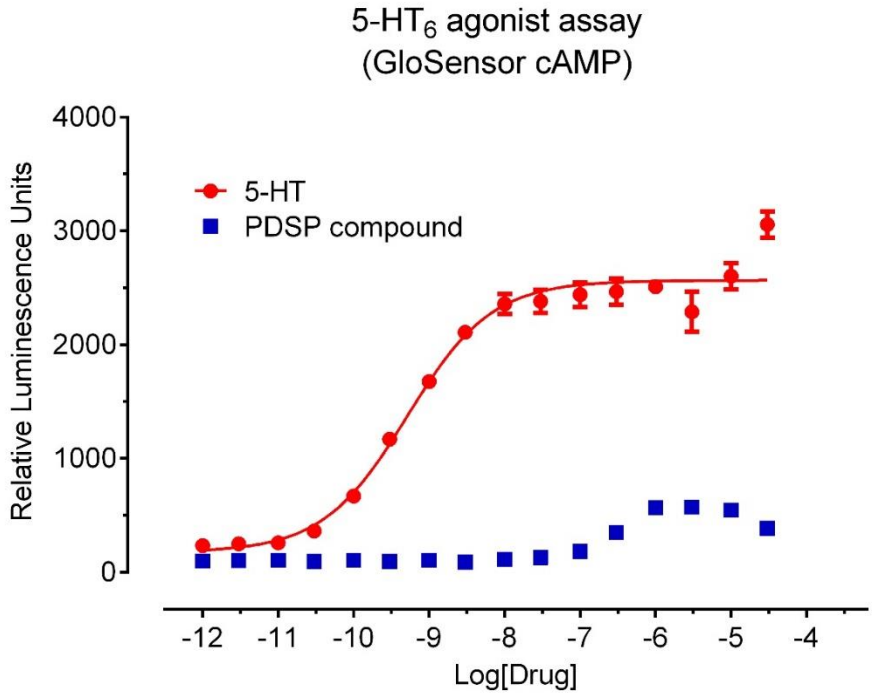

5-HT1B Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units (X100)
200
150
100
50
0
5-HT
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

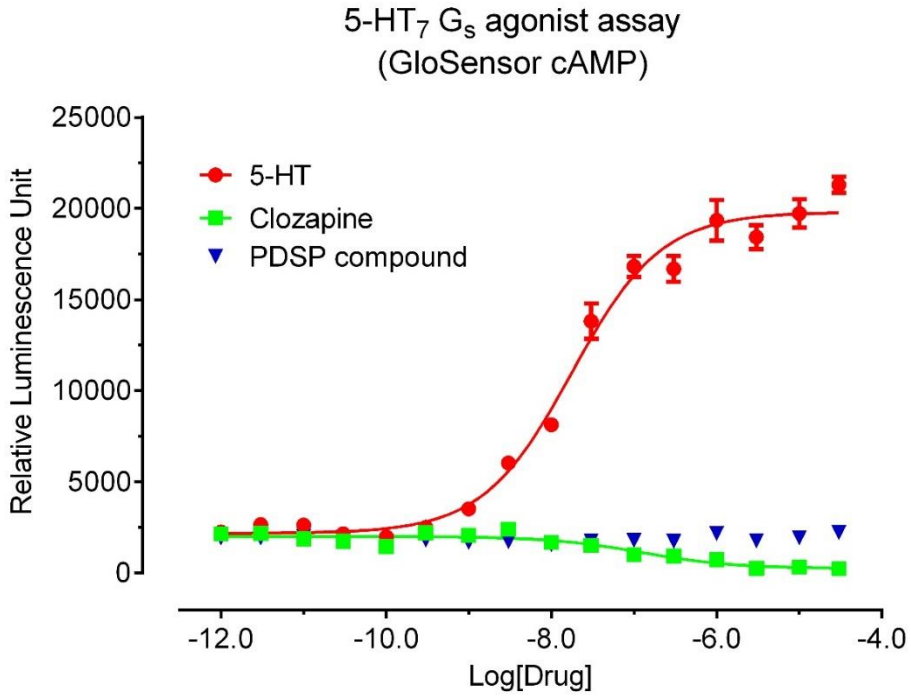

5-HT1D Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units (X100)
250
200
150
100
50
0
5-HT
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

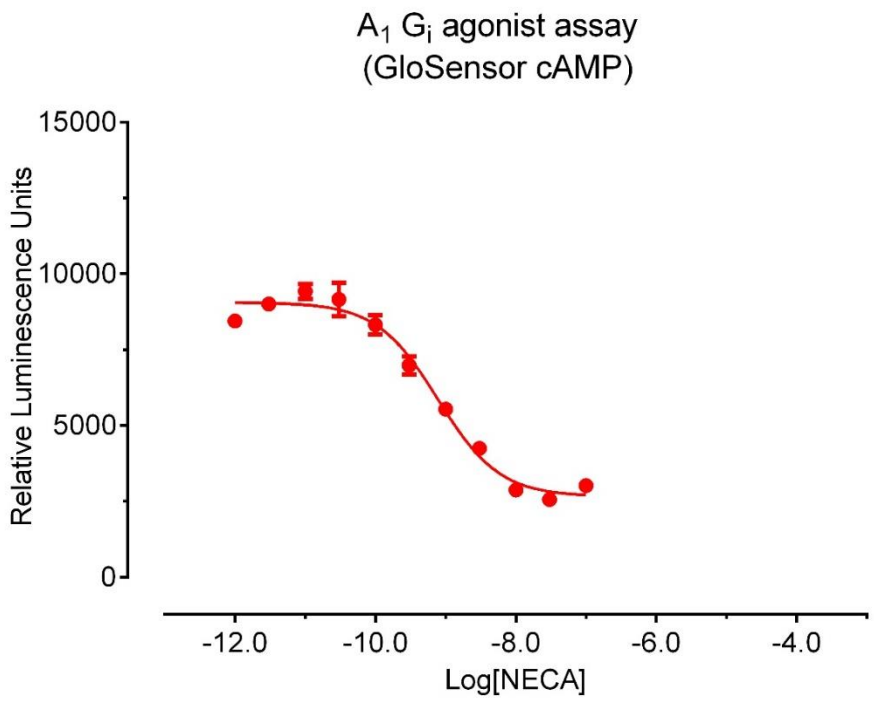

5-HT1E Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units (X100)
400
300
200
100
0
5-HT
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

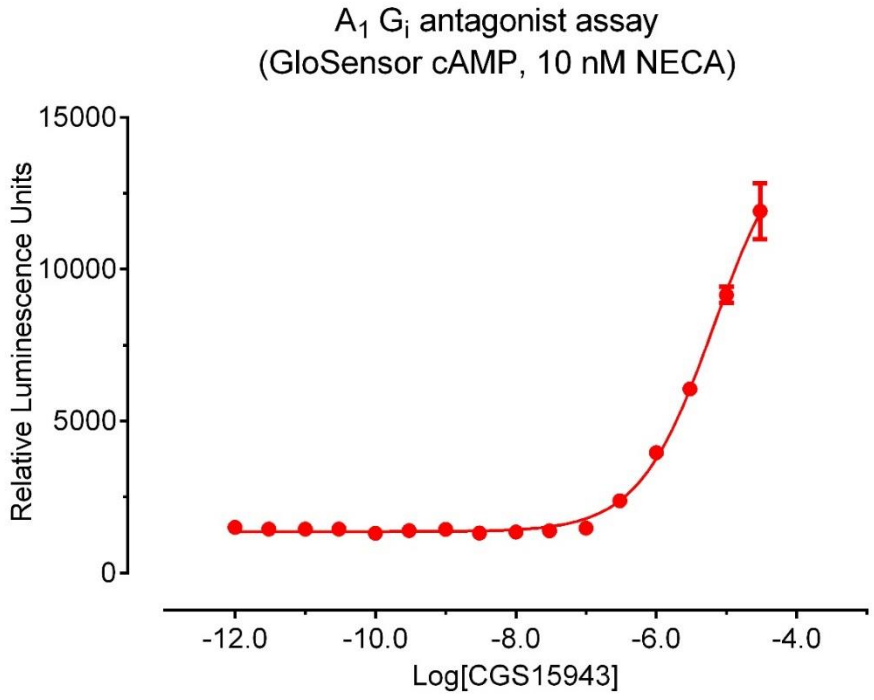

5-HT4 agonist assay
(GloSensor cAMP)
5-HT
PDSP compound
Relative Luminescence Units
4000
3000
2000
1000
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

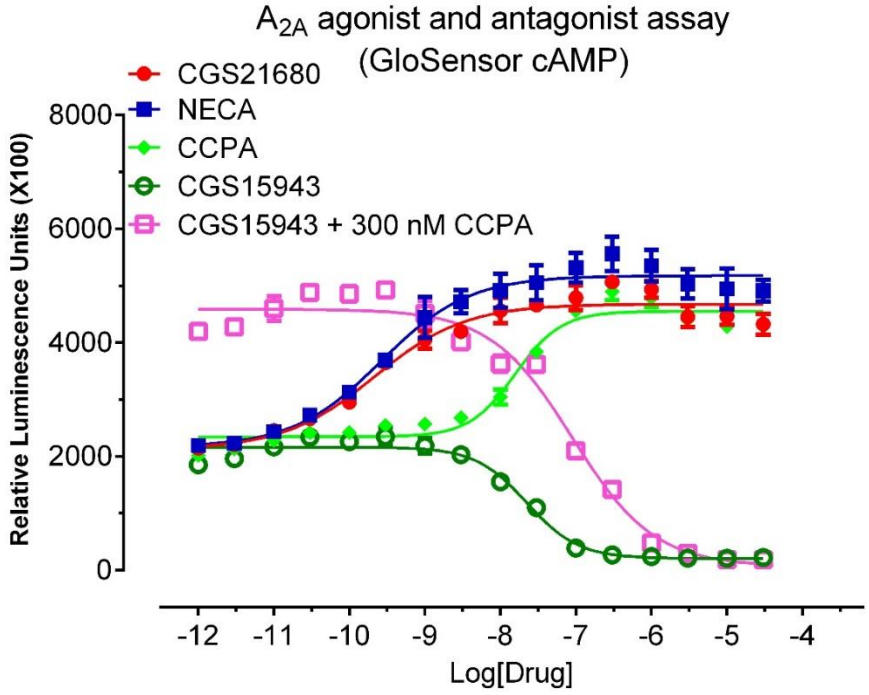

5-HT6 agonist assay
(GloSensor cAMP)
SB399885
PDSP compound
Relative Luminescence Units
4000
3000
2000
1000
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

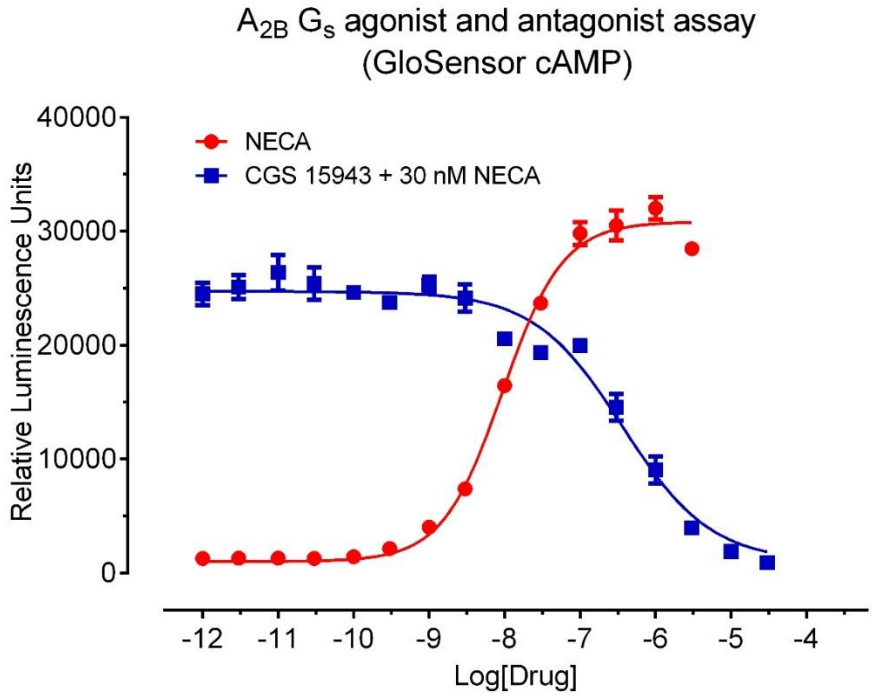

5-HT6 agonist assay
(GloSensor cAMP)
Relative Luminescence Units
4000
3000
2000
1000
0
5-HT
PDSP compound
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

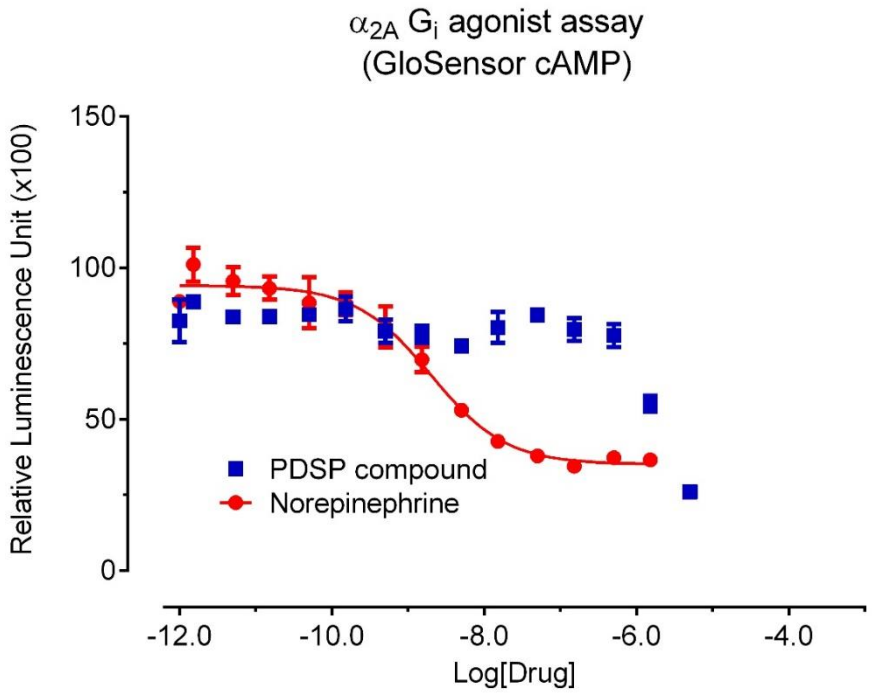

5-HT7 Gs agonist assay
(GloSensor cAMP)
Relative Luminescence Unit
25000
20000
15000
10000
5000
0
5-HT
Clozapine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

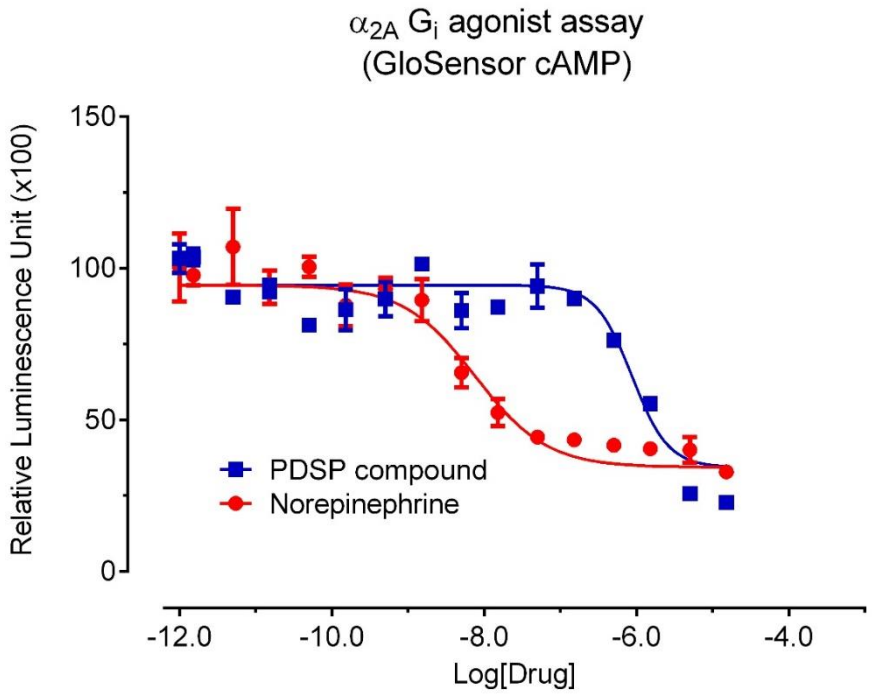

A1 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
15000
10000
5000
0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[NECA]

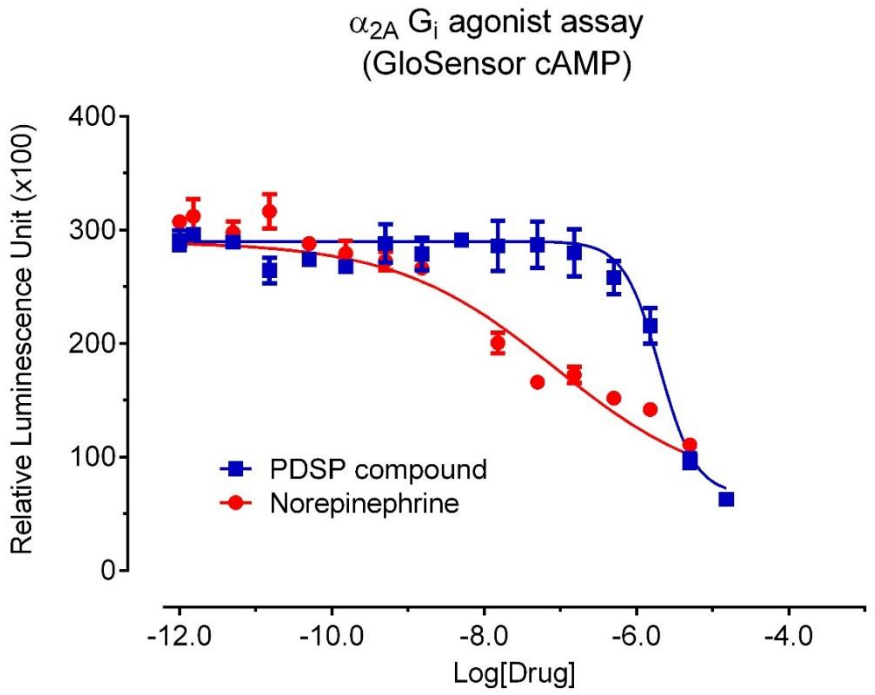

A1 Gi antagonist assay
(GloSensor cAMP, 10 nM NECA)
Relative Luminescence Units
15000
10000
5000
0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[CGS15943]

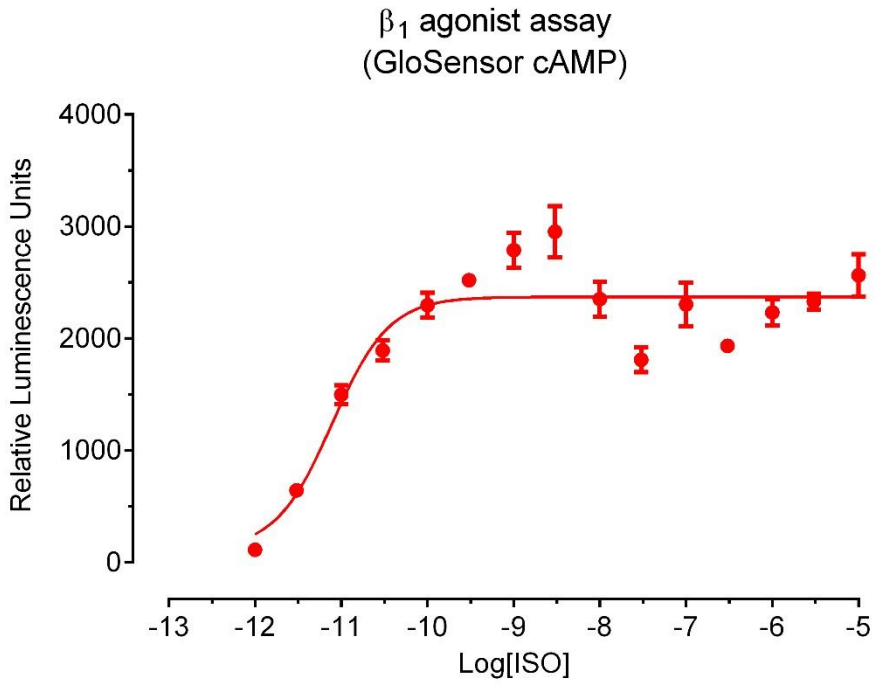

A2A agonist and antagonist assay
(GloSensor cAMP)
CGS21680
NECA
CCPA
CGS15943
CGS15943 + 300 nM CCPA
Relative Luminescence Units (X100)
8000
6000
4000
2000
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

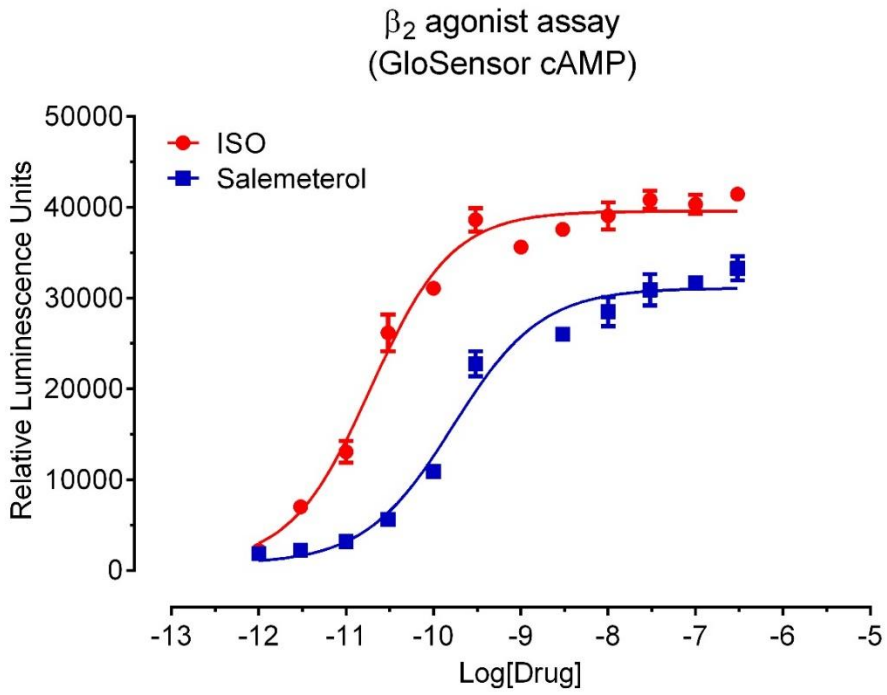

A2B Gs agonist and antagonist assay
(GloSensor cAMP)
NECA
CGS 15943 + 30 nM NECA
Relative Luminescence Units
40000
30000
20000
10000
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

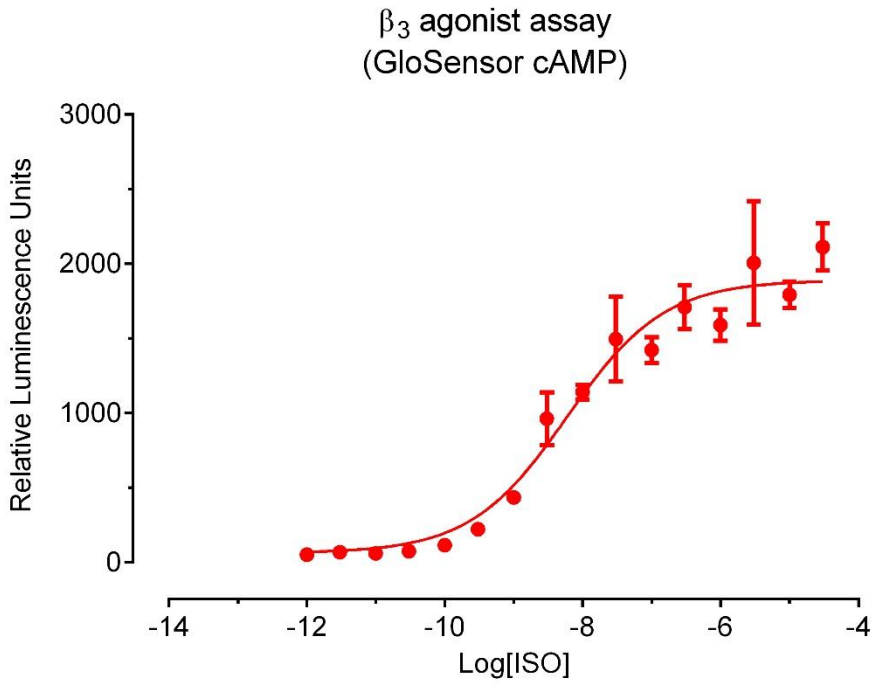

α_{2A} G_i agonist assay
(GloSensor cAMP)
Relative Luminescence Unit (x100)
150
100
50
0
PDSP compound
Norepinephrine
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

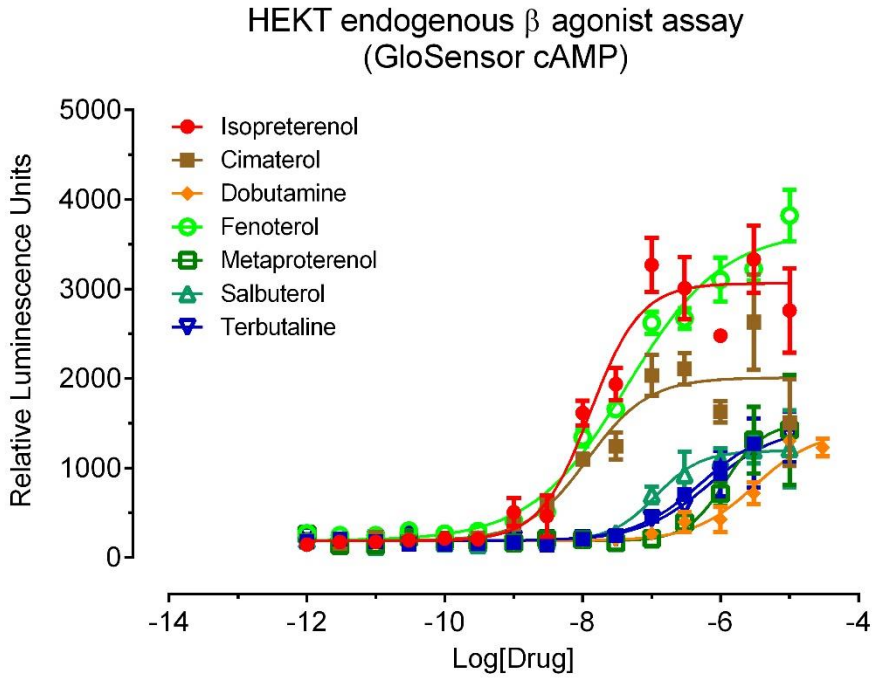

α_{2A} G_i agonist assay
(GloSensor cAMP)
Relative Luminescence Unit (x100)
150
100
50
0
PDSP compound
Norepinephrine
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

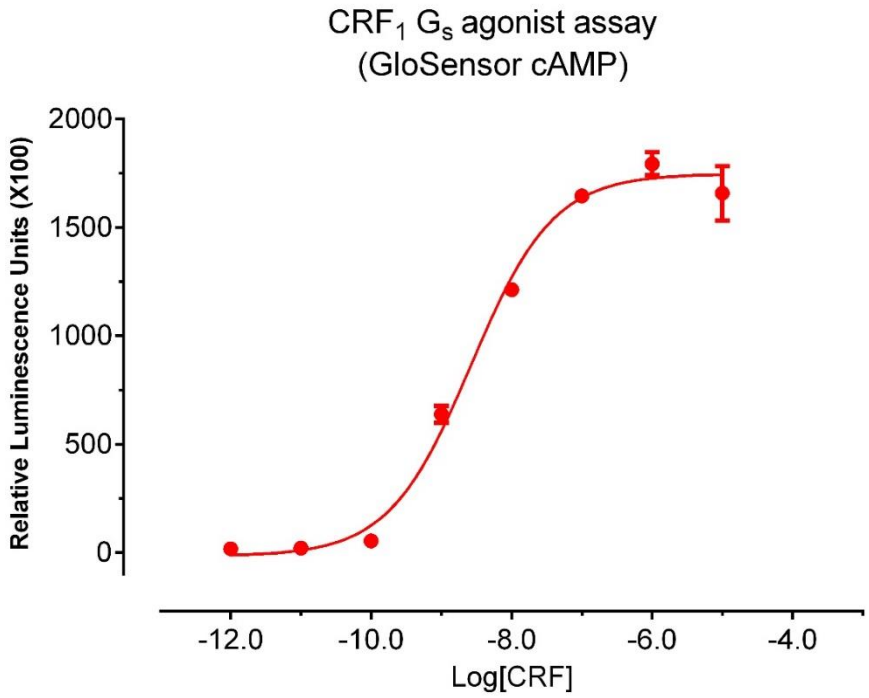

α_{2A} G_i agonist assay
(GloSensor cAMP)
Relative Luminescence Unit (x100)
400
300
200
100
0
PDSP compound
Norepinephrine
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

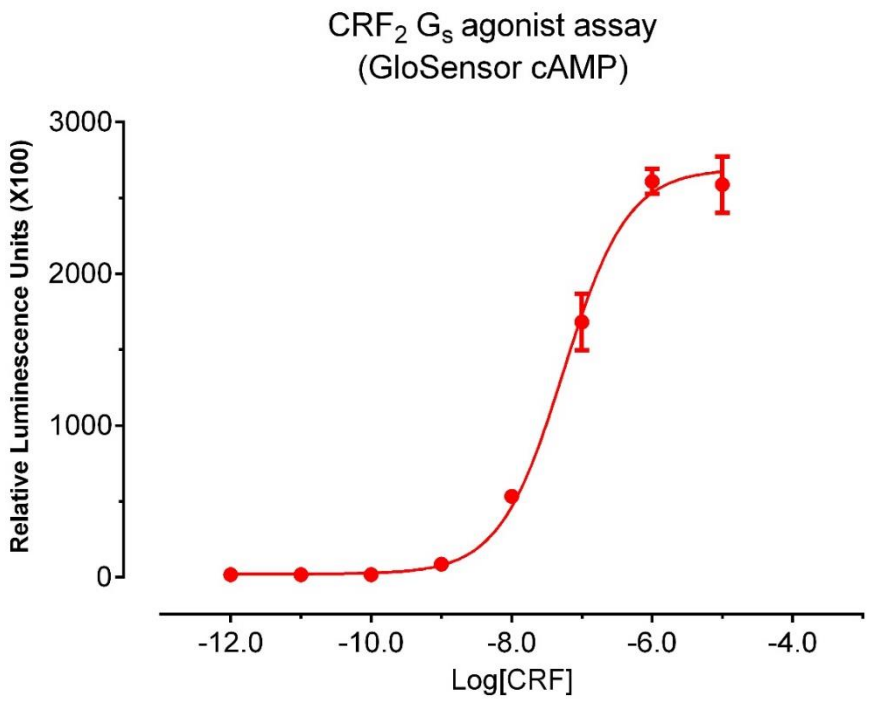

β_1 agonist assay
(GloSensor cAMP)
Relative Luminescence Units
4000
3000
2000
1000
0
-13
-12
-11
-10
-9
-8
-7
-6
-5
Log[ISO]

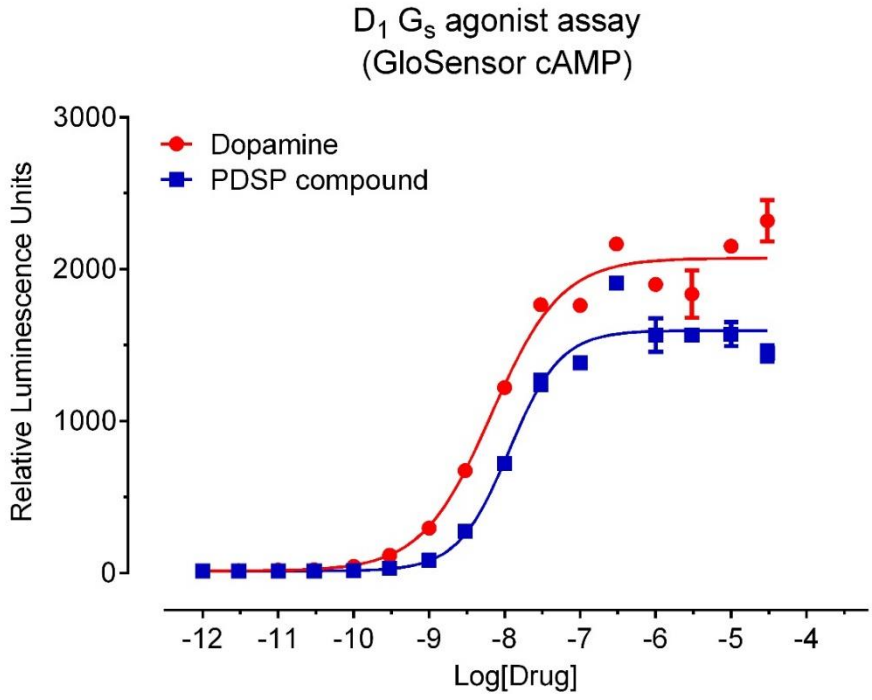

β2 agonist assay
(GloSensor cAMP)
ISO
Salemeterol
Relative Luminescence Units
50000
40000
30000
20000
10000
0
-13
-12
-11
-10
-9
-8
-7
-6
-5
Log[Drug]

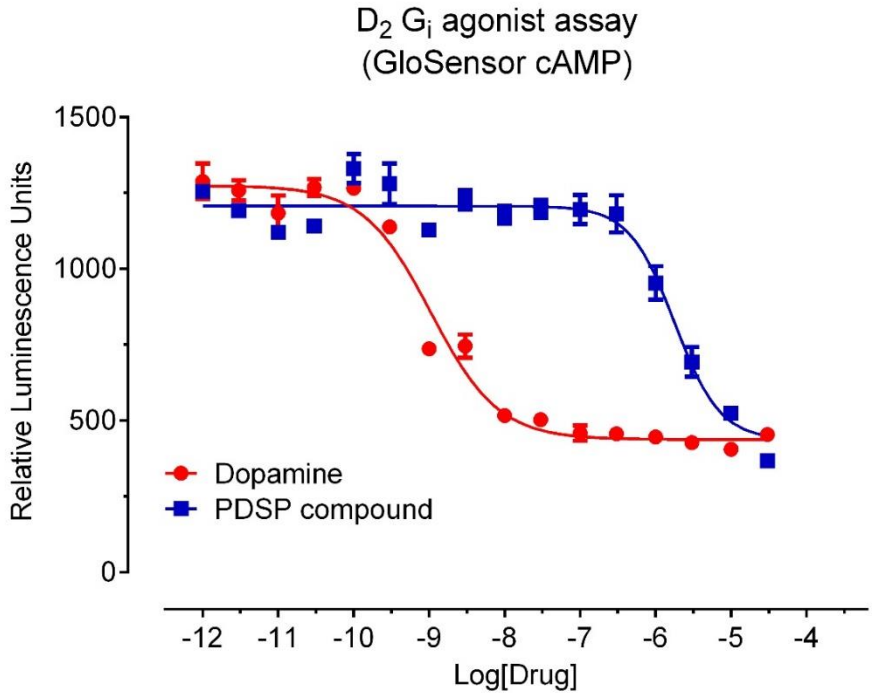

β3 agonist assay
(GloSensor cAMP)
Relative Luminescence Units
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[ISO]

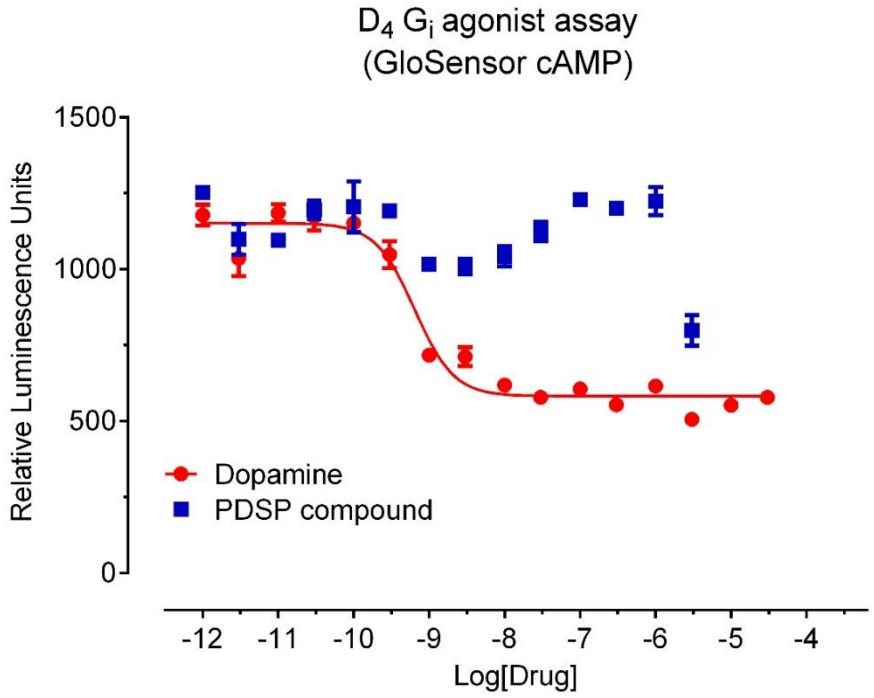

HEKT endogenous β agonist assay
(GloSensor cAMP)
Relative Luminescence Units
5000
4000
3000
2000
1000
0
Isopreterenol
Cimaterol
Dobutamine
Fenoterol
Metaproterenol
Salbuterol
Terbutaline
-14
-12
-10
-8
-6
-4
Log[Drug]

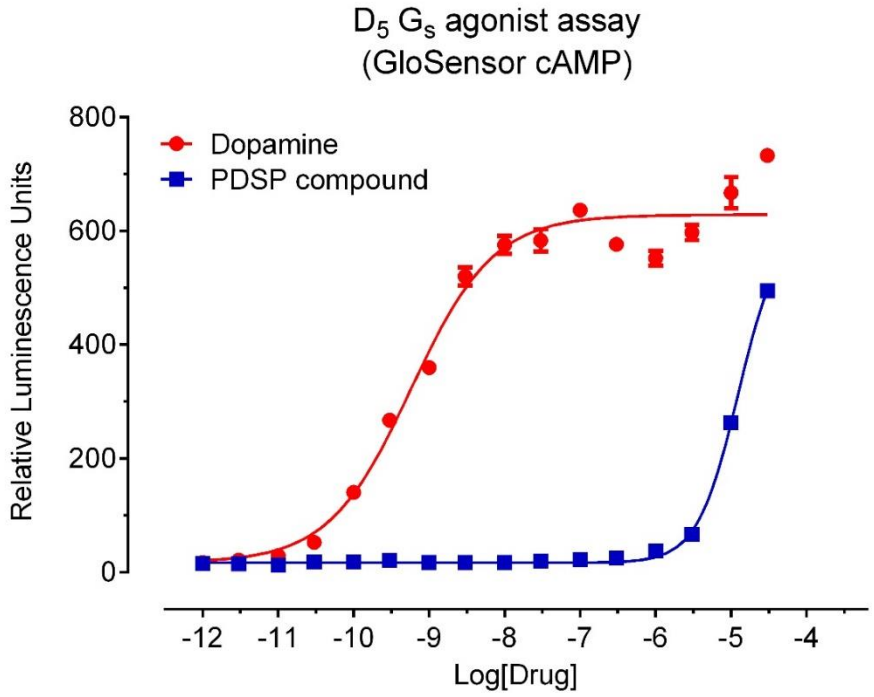

CRF1 Gs agonist assay
(GloSensor cAMP)
Relative Luminescence Units (X100)
2000
1500
1000
500
0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[CRF]

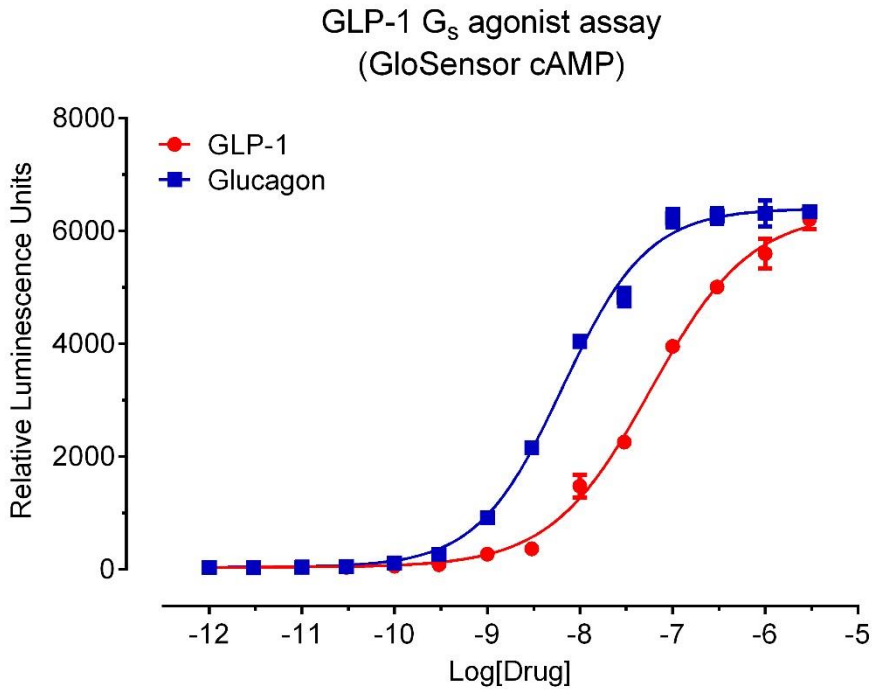

CRF2 Gs agonist assay
(GloSensor cAMP)
Relative Luminescence Units (X100)
3000
2000
1000
0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[CRF]

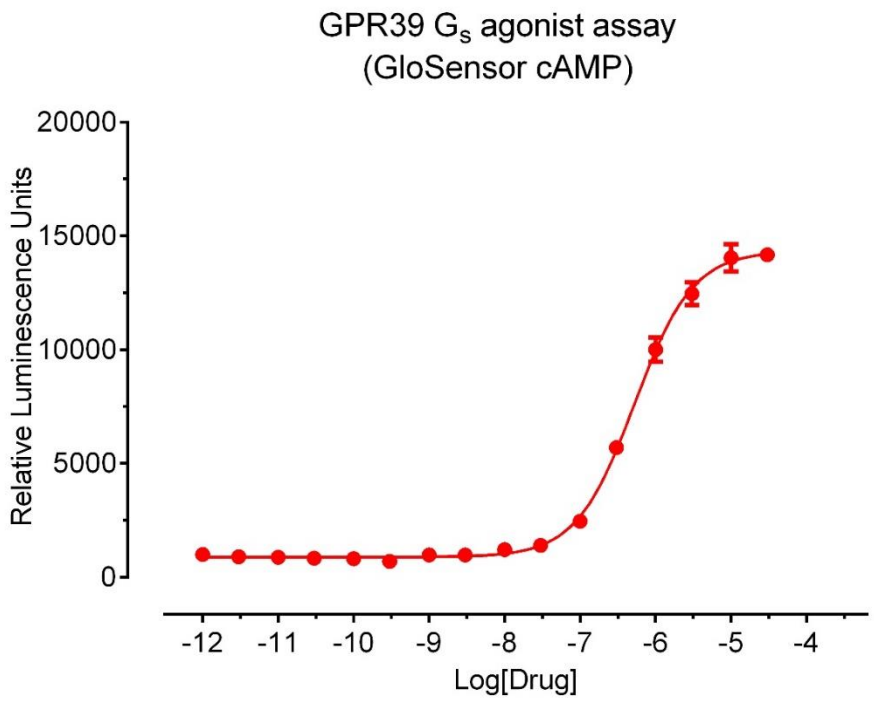

D1 Gs agonist assay
(GloSensor cAMP)
Relative Luminescence Units
3000
2000
1000
0
Dopamine
PDSP compound
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

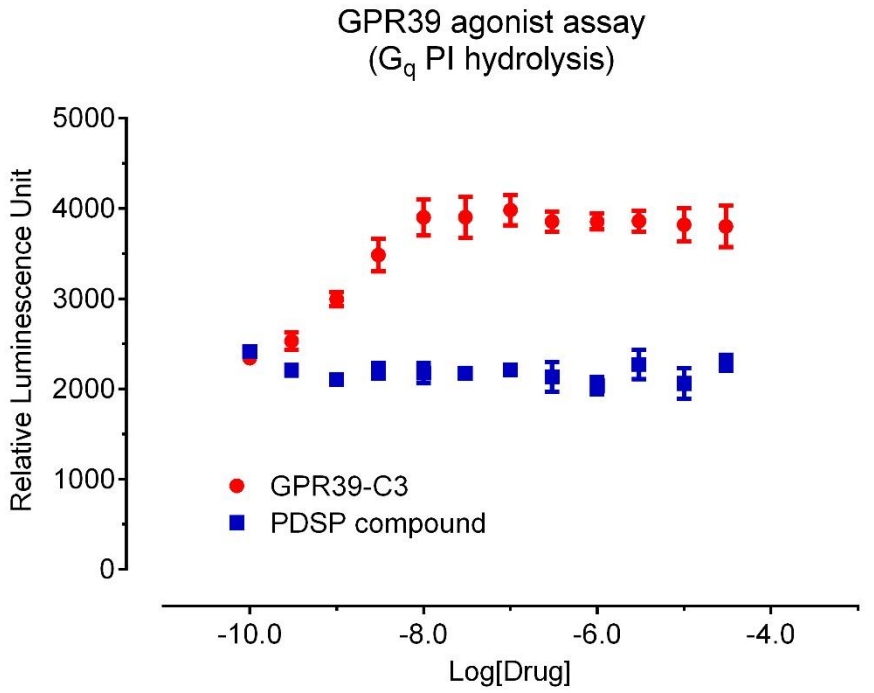

D2 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
1500
1000
500
0
Dopamine
PDSP compound
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

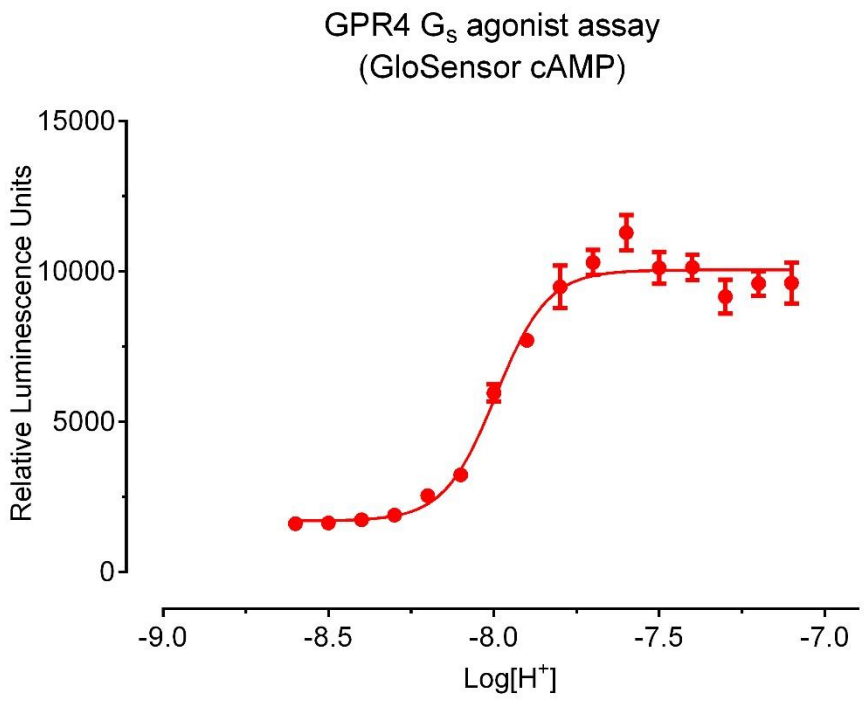

D4 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
1500
1000
500
0
Dopamine
PDSP compound
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

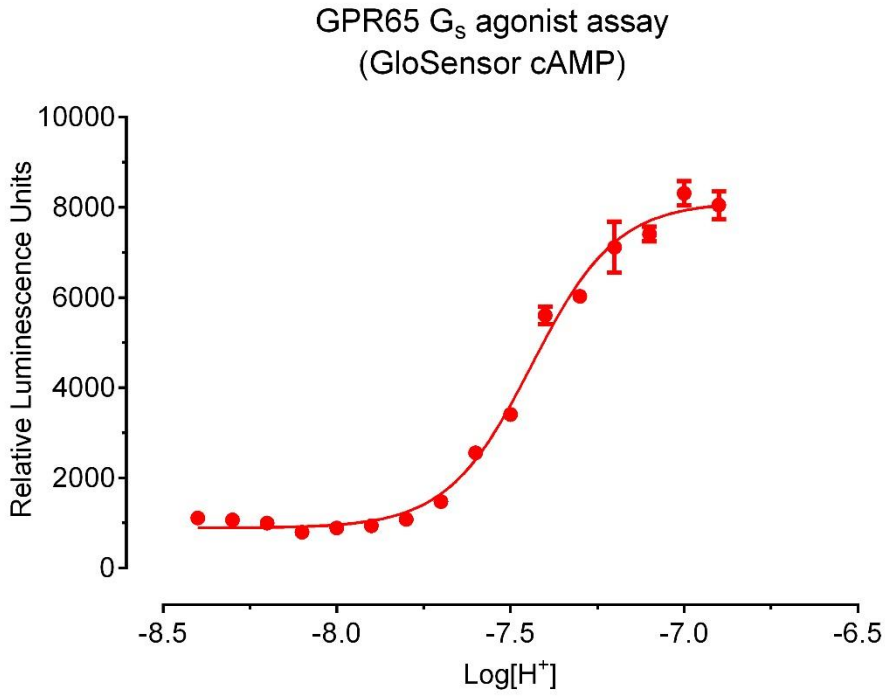

D5 Gs agonist assay
(GloSensor cAMP)
Dopamine
PDSP compound
Relative Luminescence Units
800
600
400
200
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

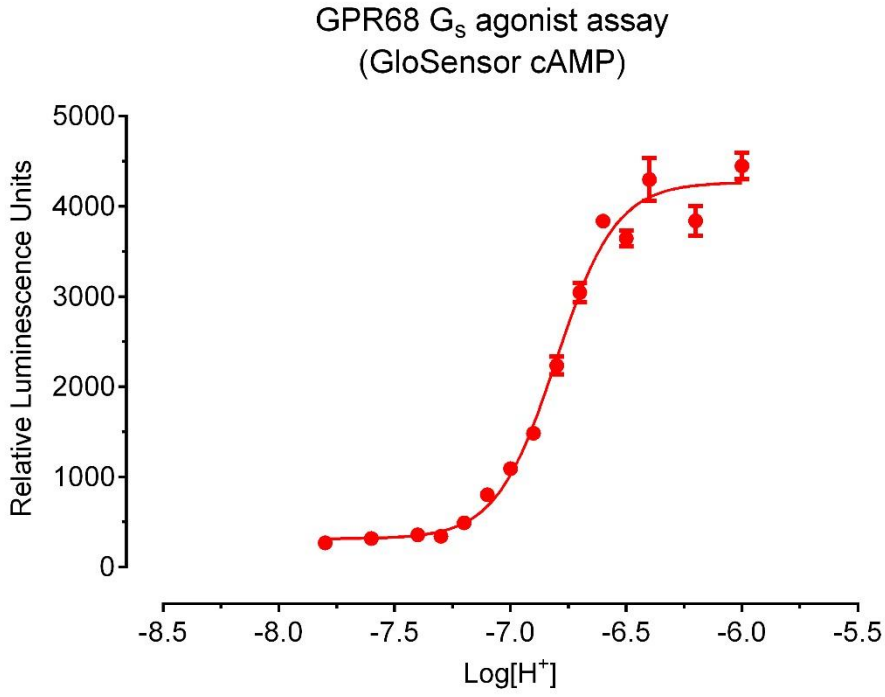

GLP-1 Gs agonist assay
(GloSensor cAMP)
GLP-1
Glucagon
Relative Luminescence Units
8000
6000
4000
2000
0
-12
-11
-10
-9
-8
-7
-6
-5
Log[Drug]

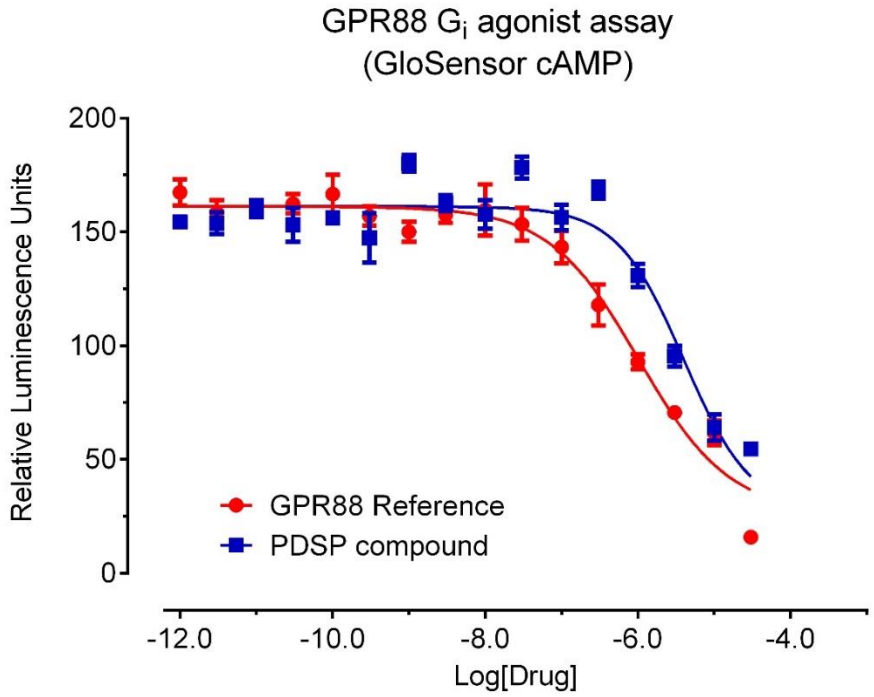

GPR39 G_s agonist assay
(GloSensor cAMP)
Relative Luminescence Units
20000
15000
10000
5000
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

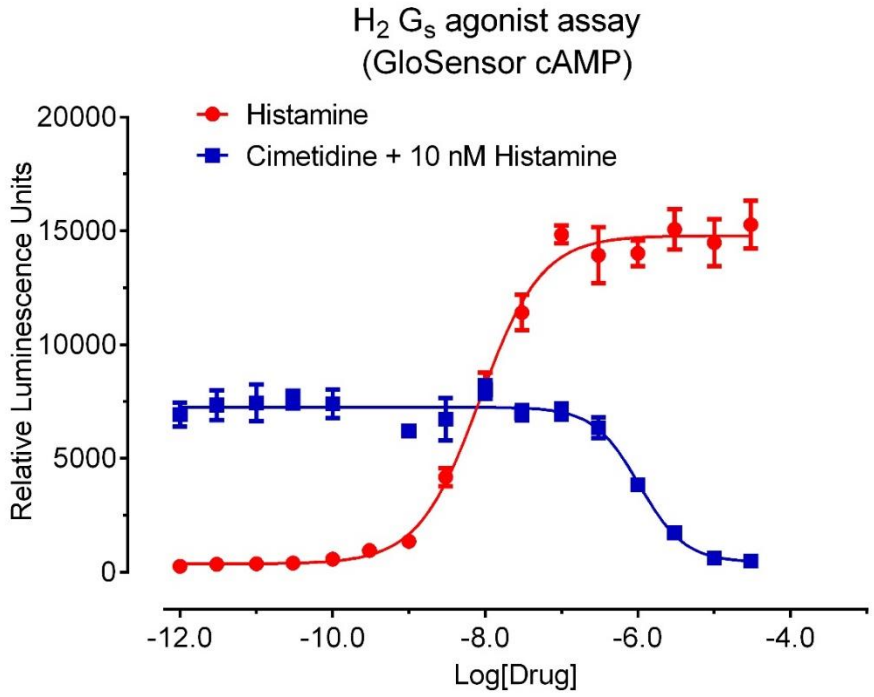

GPR39 agonist assay
(G_q PI hydrolysis)
Relative Luminescence Unit
5000
4000
3000
2000
1000
0
GPR39-C3
PDSP compound
-10.0
-8.0
-6.0
-4.0
Log[Drug]

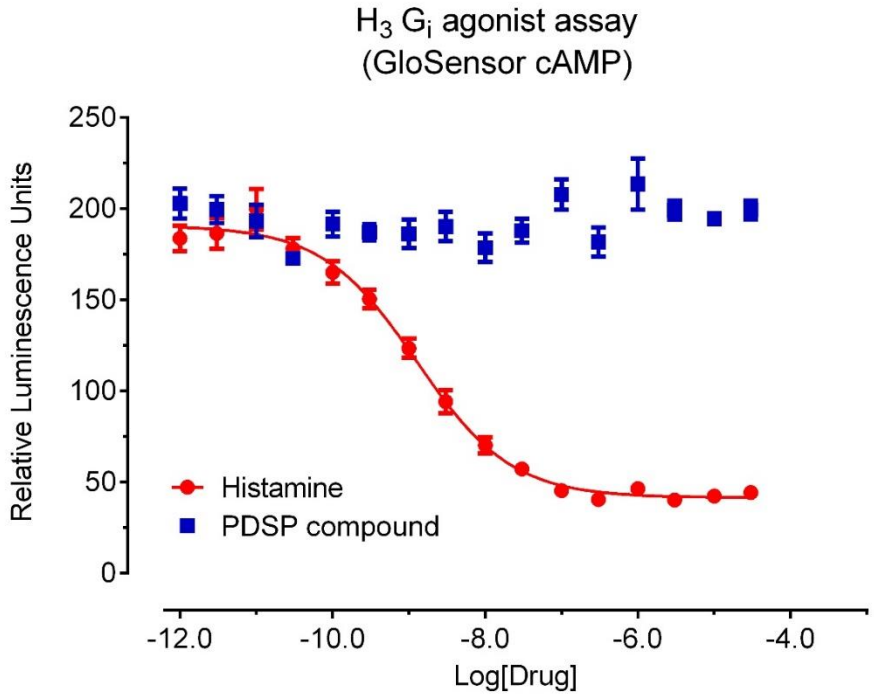

GPR4 G_s agonist assay
(GloSensor cAMP)
Relative Luminescence Units
15000
10000
5000
0
-9.0
-8.5
-8.0
-7.5
-7.0
Log[H+]

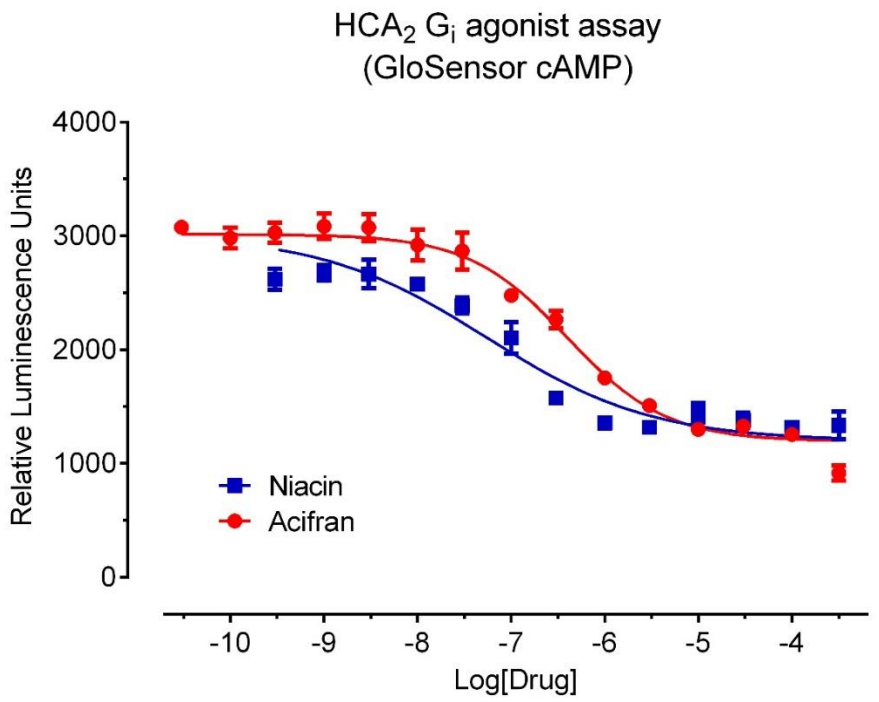

GPR65 G_s agonist assay
(GloSensor cAMP)
Relative Luminescence Units
10000
8000
6000
4000
2000
0
-8.5
-8.0
-7.5
-7.0
-6.5
Log[H+]

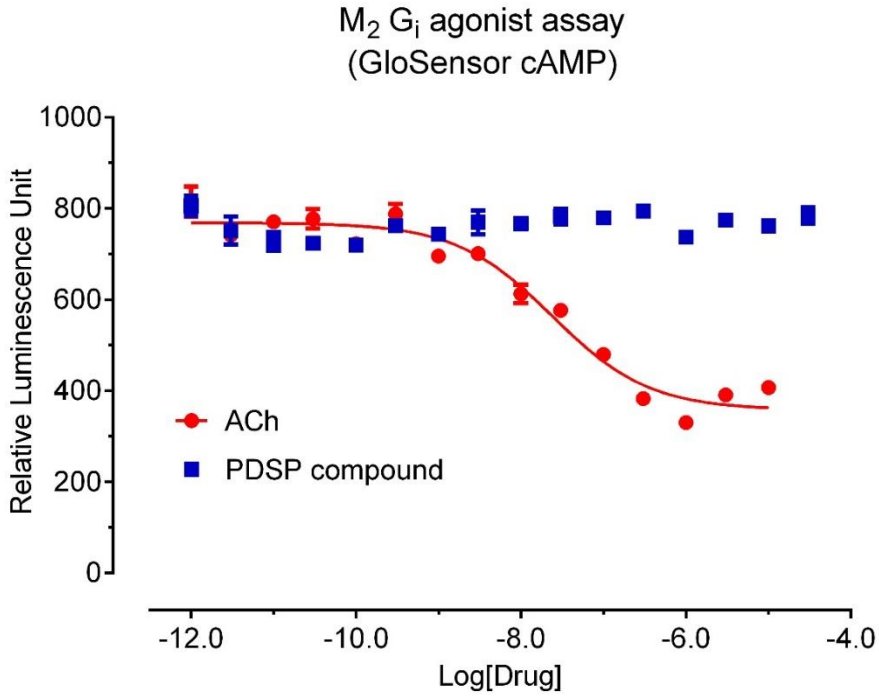

GPR68 G_s agonist assay
(GloSensor cAMP)
Relative Luminescence Units
5000
4000
3000
2000
1000
0
-8.5
-8.0
-7.5
-7.0
-6.5
-6.0
-5.5
Log[H^+]

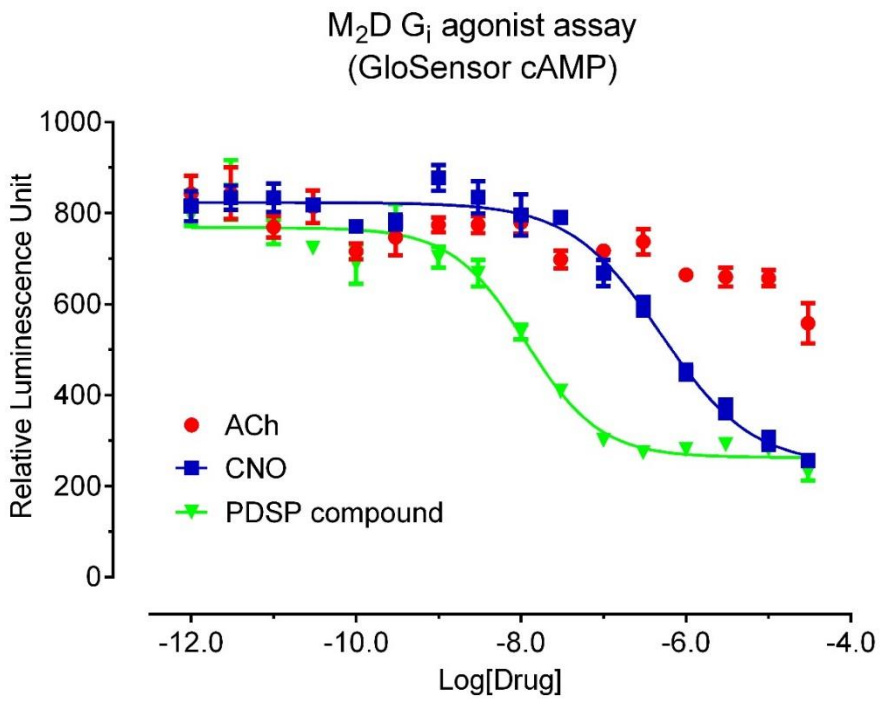

GPR88 G_i agonist assay
(GloSensor cAMP)
Relative Luminescence Units
200
150
100
50
0
GPR88 Reference
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

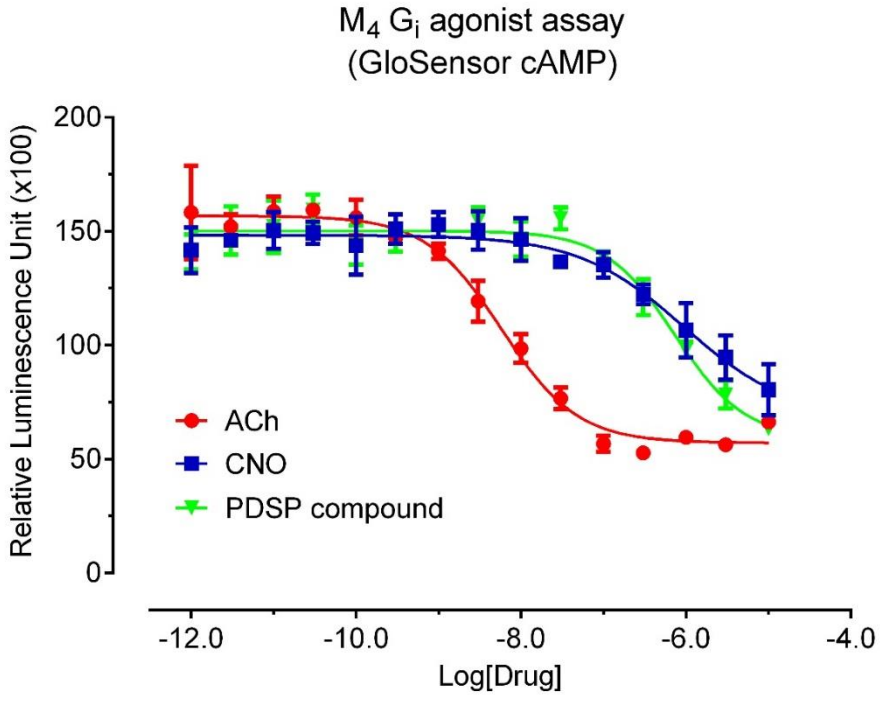

H2 Gs agonist assay
(GloSensor cAMP)
Histamine
Cimetidine + 10 nM Histamine
Relative Luminescence Units
20000
15000
10000
5000
0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

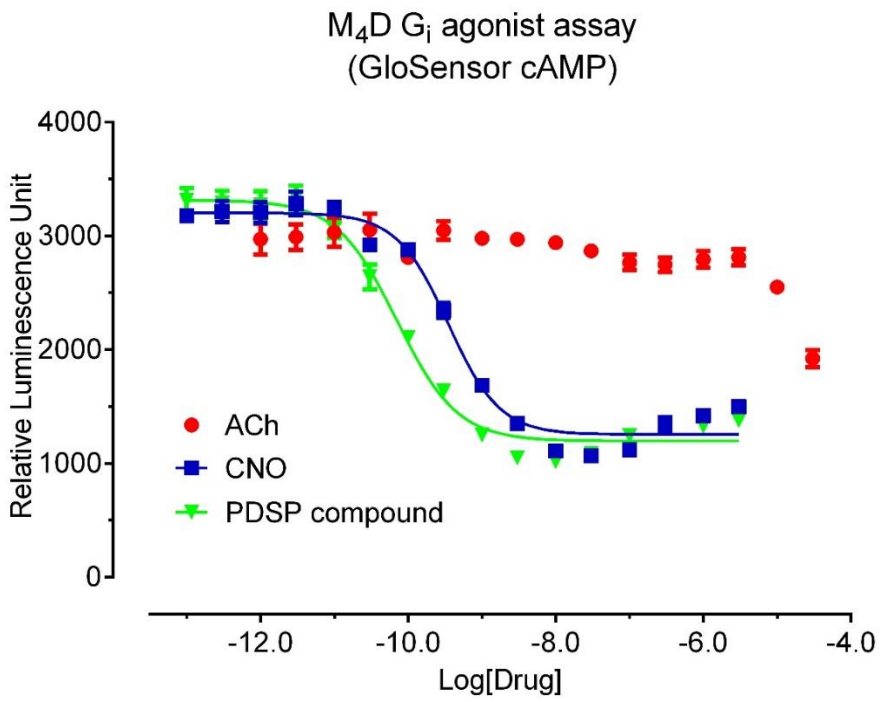

H3 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
250
200
150
100
50
0
Histamine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

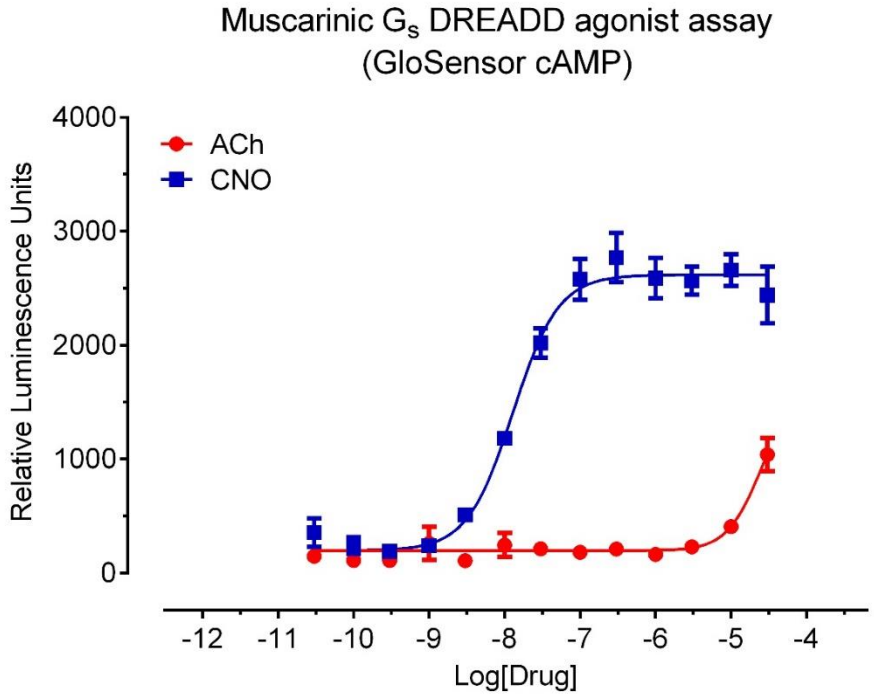

HCA2 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
4000
3000
2000
1000
0
Niacin
Acifran
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

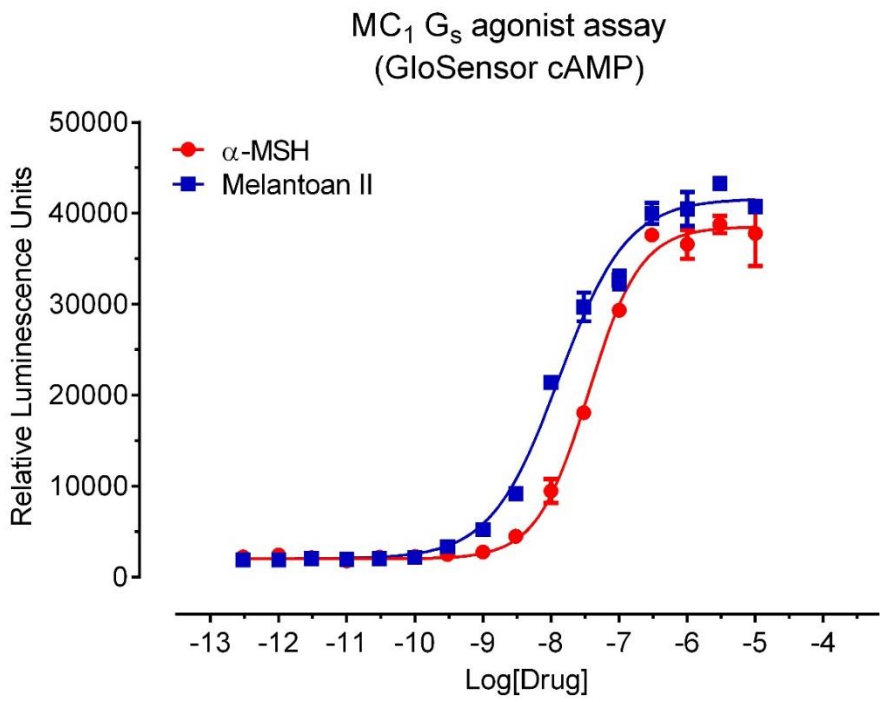

M2 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Unit
1000
800
600
400
200
0
ACh
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

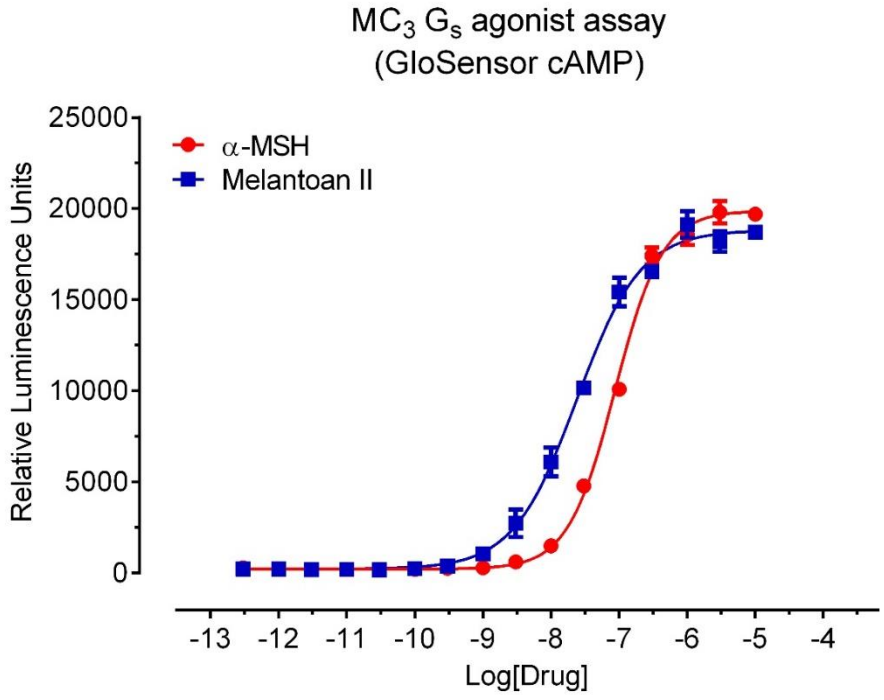

M2D Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Unit
1000
800
600
400
200
0
ACh
CNO
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

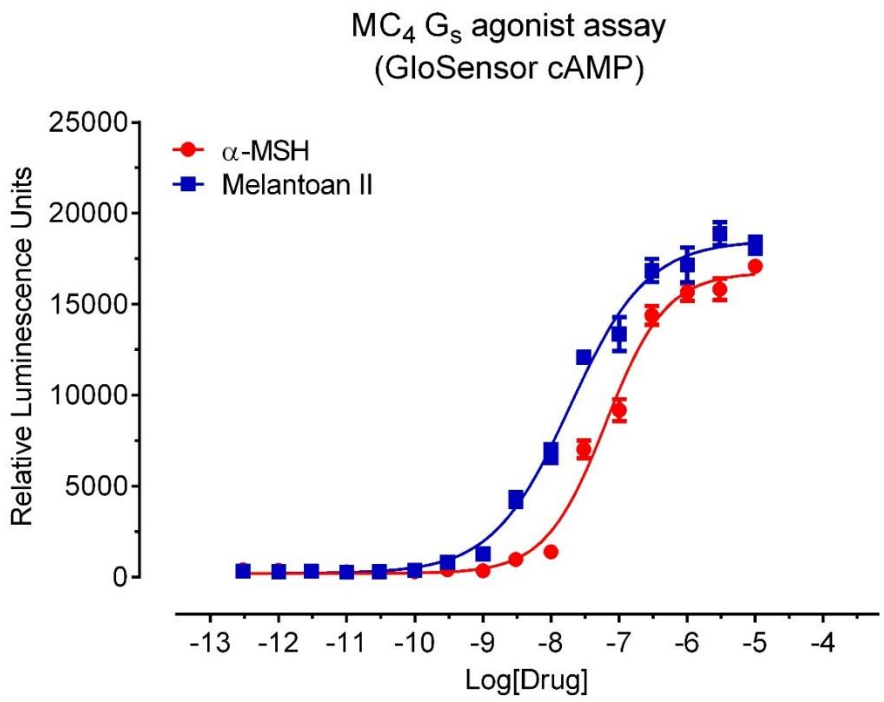

M4 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Unit (x100)
200
150
100
50
0
ACh
CNO
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

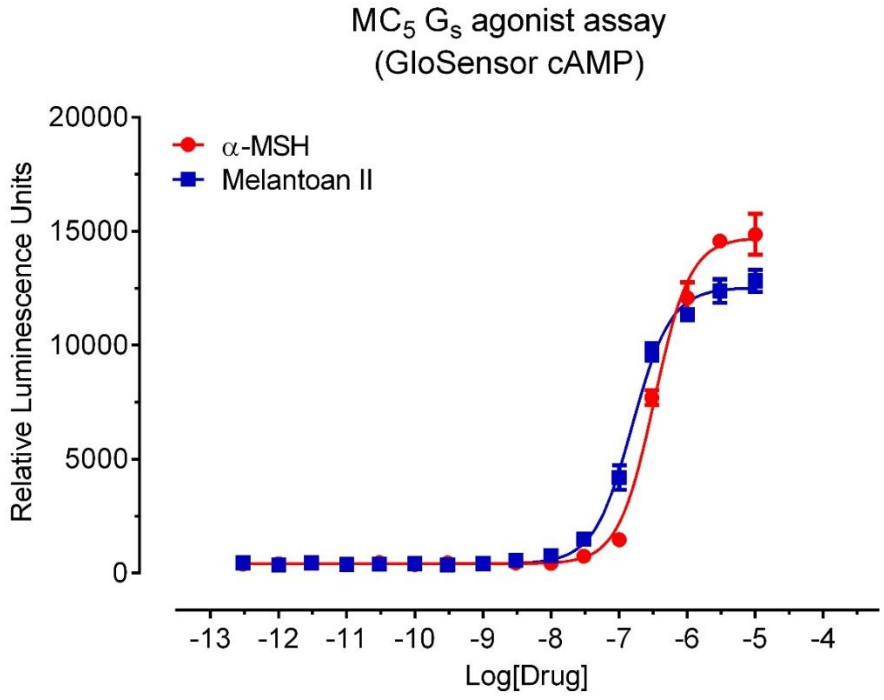

M4D Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Unit
4000
3000
2000
1000
0
ACh
CNO
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

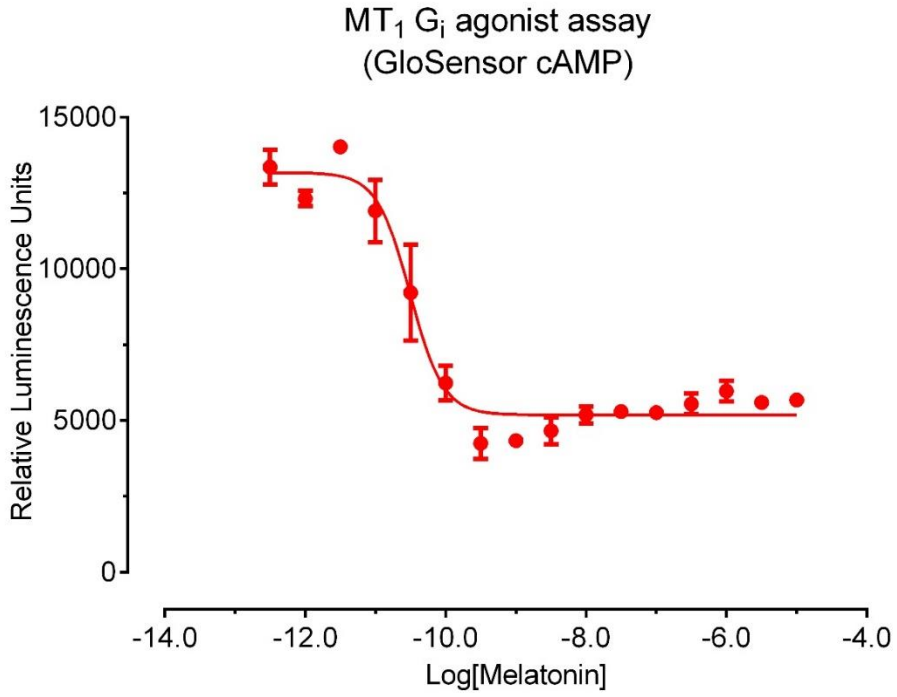

Muscarinic Gs DREADD agonist assay
(GloSensor cAMP)
Relative Luminescence Units
4000
3000
2000
1000
0
ACh
CNO
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

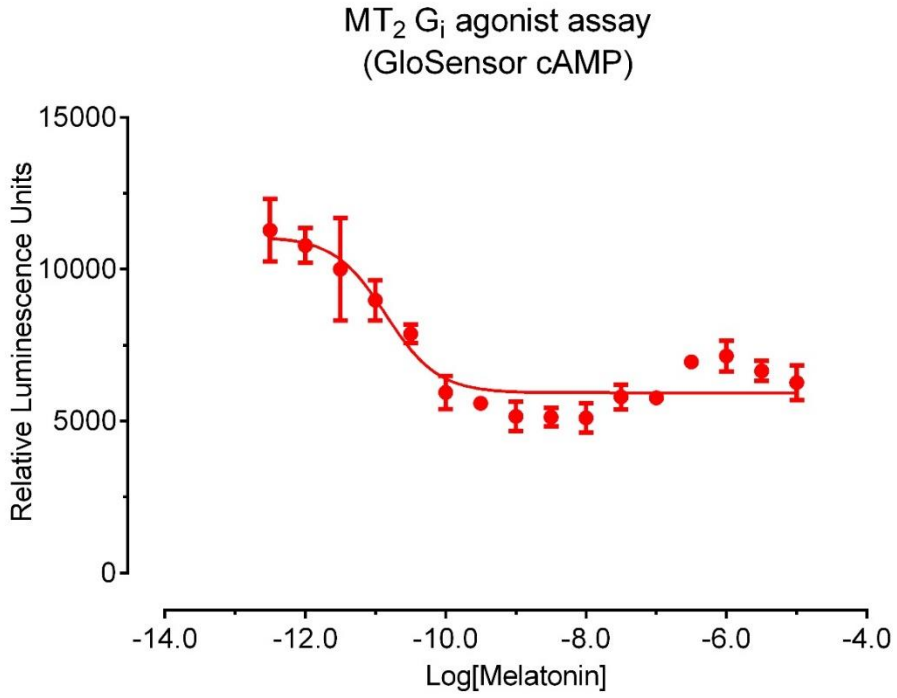

MC1 Gs agonist assay
(GloSensor cAMP)
α-MSH
Melantoan II
Relative Luminescence Units
50000
40000
30000
20000
10000
0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
Log[Drug]

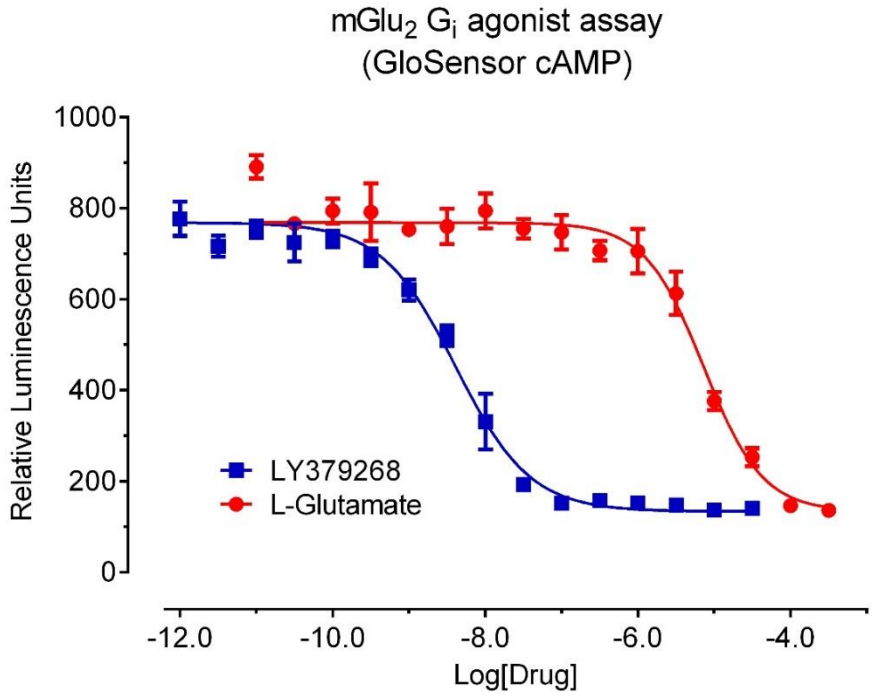

MC3 Gs agonist assay
(GloSensor cAMP)
α-MSH
Melantoan II
Relative Luminescence Units
25000
20000
15000
10000
5000
0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
Log[Drug]

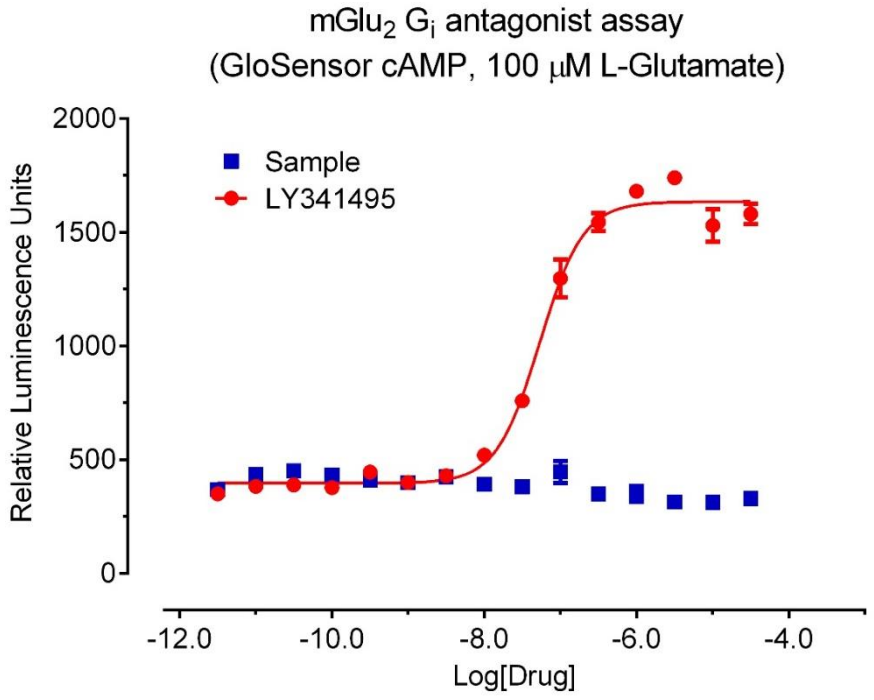

MC4 Gs agonist assay
(GloSensor cAMP)
α-MSH
Melantoan II
Relative Luminescence Units
25000
20000
15000
10000
5000
0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
Log[Drug]

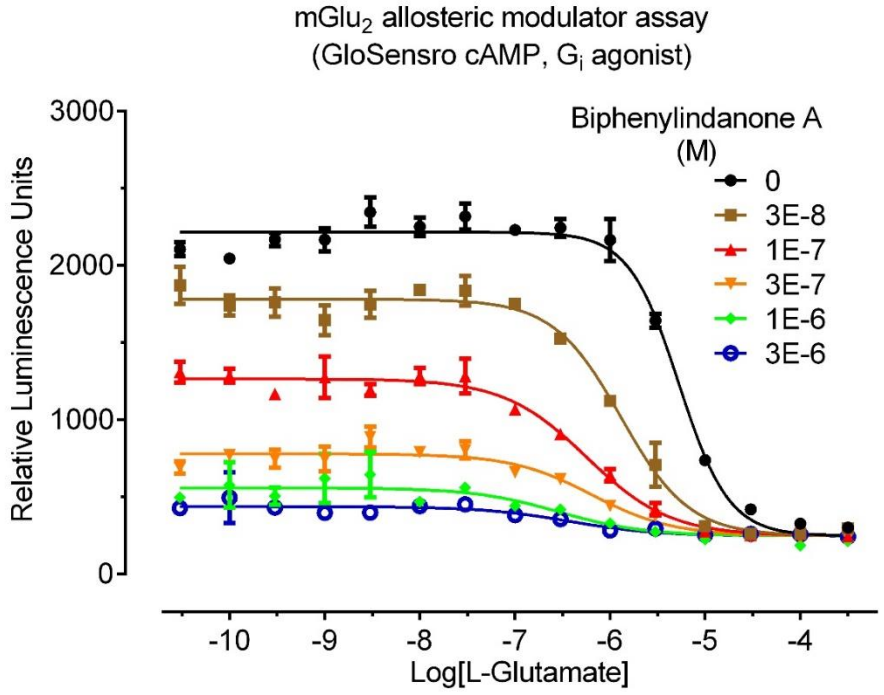

MC5 Gs agonist assay
(GloSensor cAMP)
α-MSH
Melantoan II
Relative Luminescence Units
20000
15000
10000
5000
0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
Log[Drug]

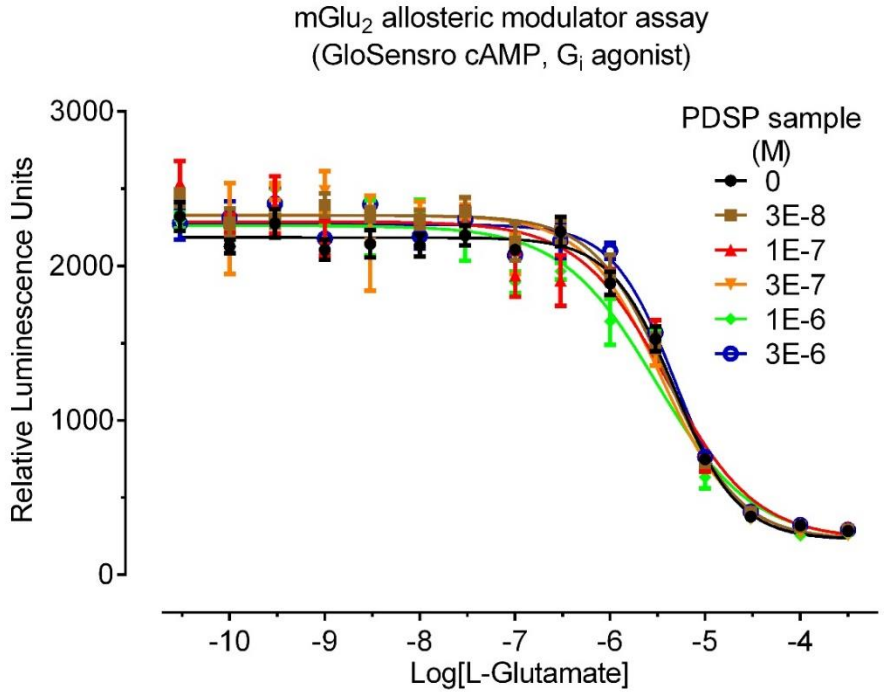

MT1 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
15000
10000
5000
0
-14.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Melatonin]

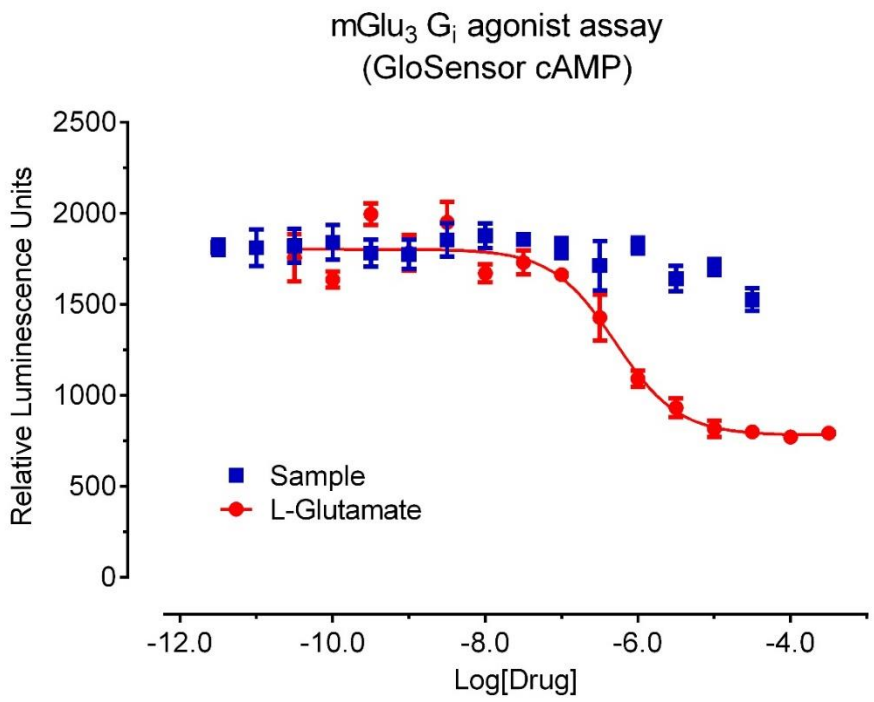

MT2 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
15000
10000
5000
0
-14.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Melatonin]

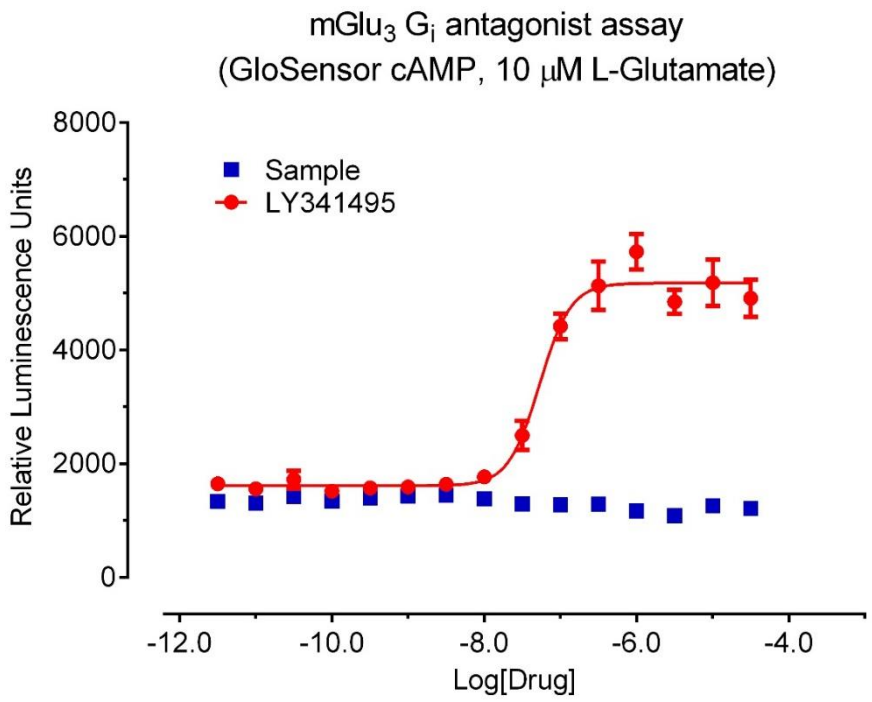

mGlu2 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
1000
800
600
400
200
0
LY379268
L-Glutamate
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

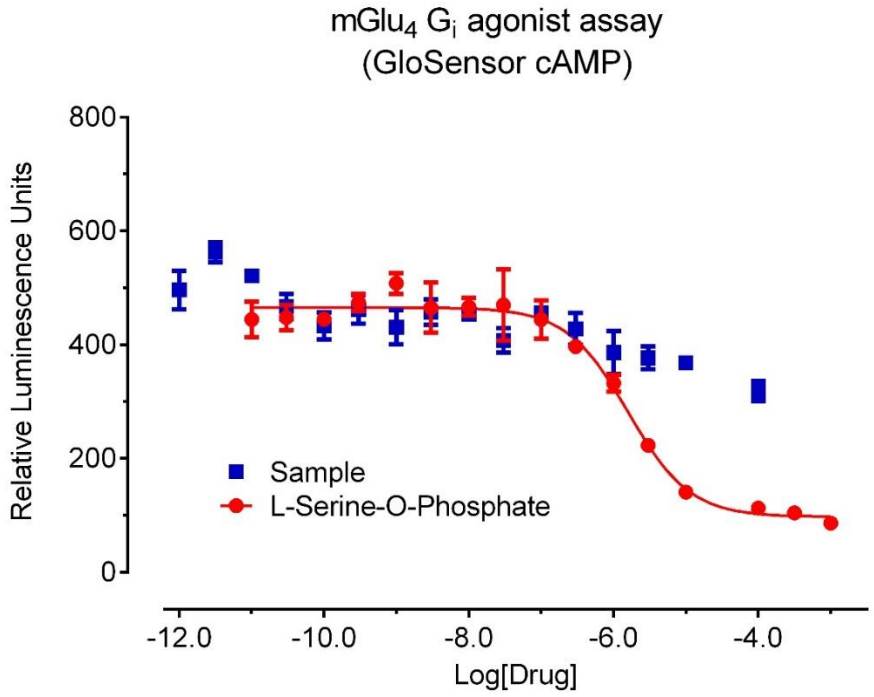

mGlu2 Gi antagonist assay
(GloSensor cAMP, 100 μM L-Glutamate)
Relative Luminescence Units
2000
1500
1000
500
0
Sample
LY341495
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

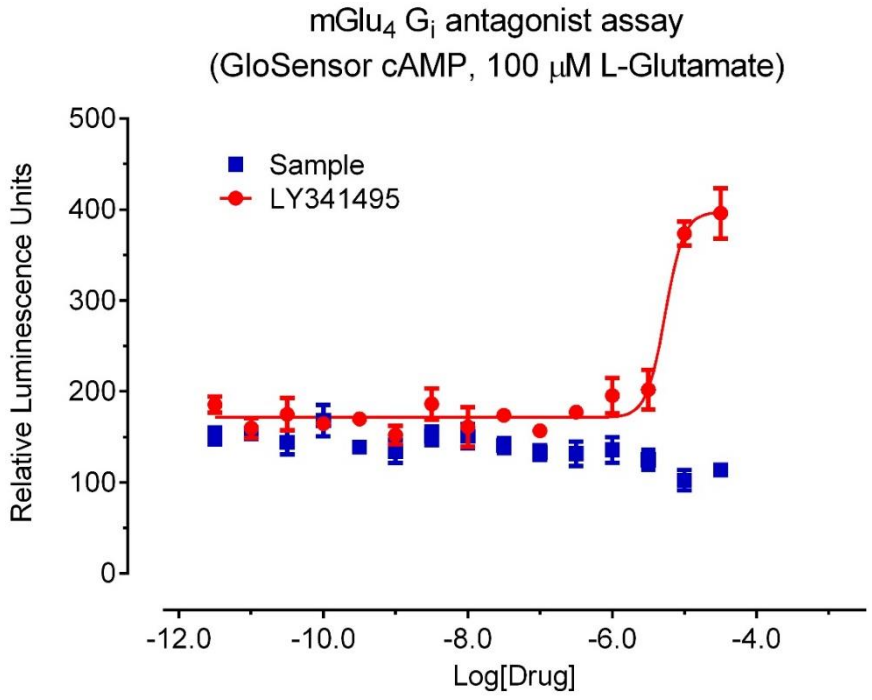

mGlu2 allosteric modulator assay
(GloSensro cAMP, Gi agonist)
Biphenylindanone A
(M)
0
3E-8
1E-7
3E-7
1E-6
3E-6
Relative Luminescence Units
3000
2000
1000
0
-10
-9
-8
-7
-6
-5
-4
Log[L-Glutamate]

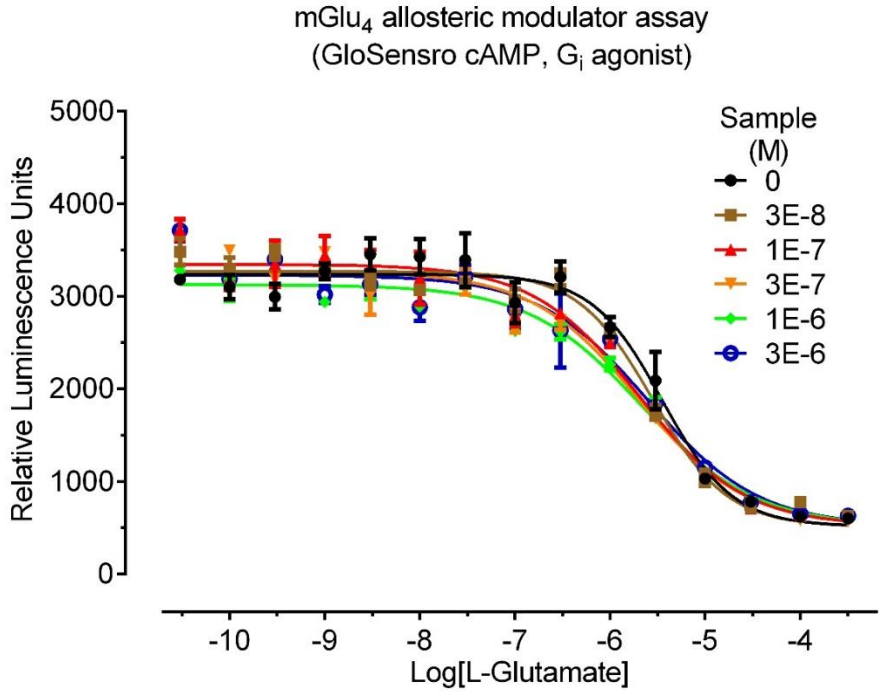

mGlu2 allosteric modulator assay
(GloSensro cAMP, Gi agonist)
PDSP sample
(M)
0
3E-8
1E-7
3E-7
1E-6
3E-6
Relative Luminescence Units
3000
2000
1000
0
-10
-9
-8
-7
-6
-5
-4
Log[L-Glutamate]

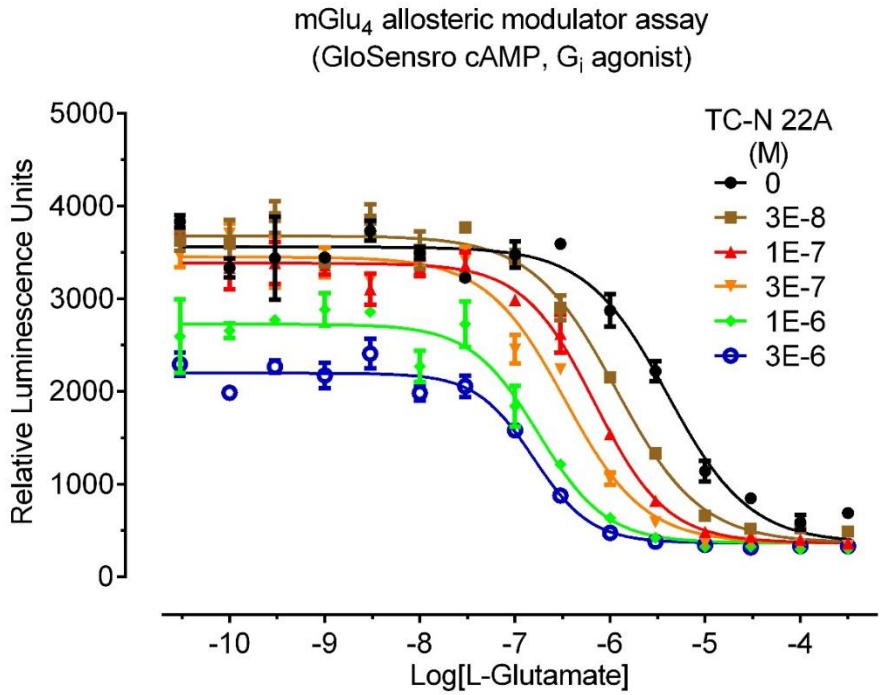

mGlu3 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
2500
2000
1500
1000
500
0
Sample
L-Glutamate
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

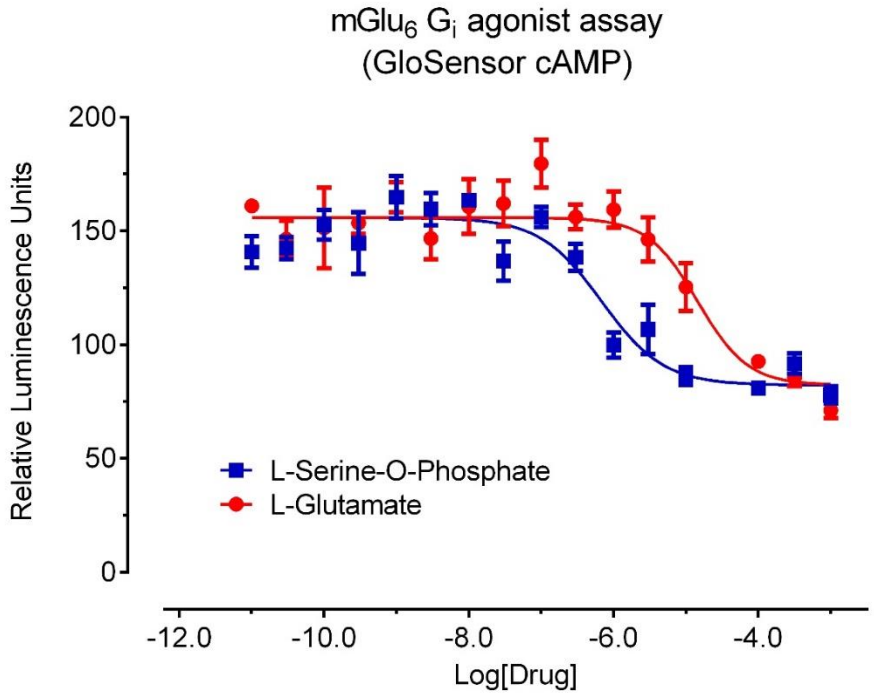

mGlu3 Gi antagonist assay
(GloSensor cAMP, 10 μM L-Glutamate)
Relative Luminescence Units
8000
6000
4000
2000
0
Sample
LY341495
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

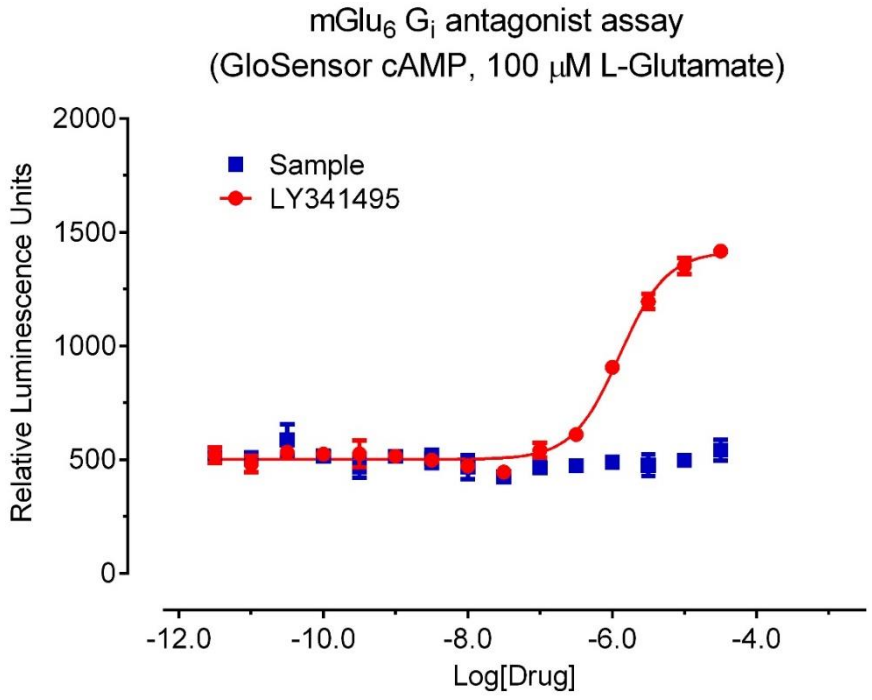

mGlu4 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
800
600
400
200
0
Sample
L-Serine-O-Phosphate
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

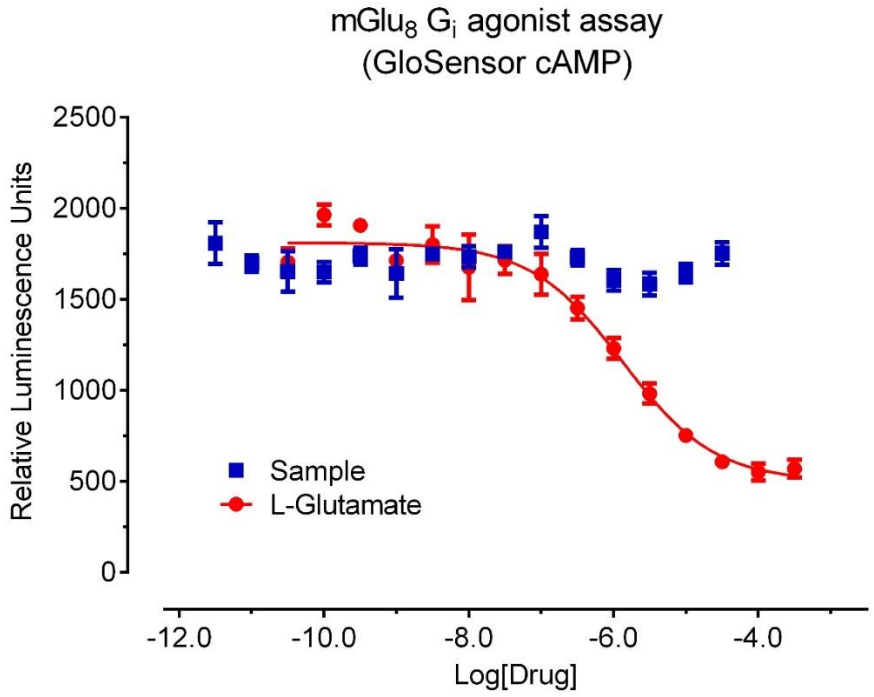

mGlu4 Gi antagonist assay
(GloSensor cAMP, 100 μM L-Glutamate)
Relative Luminescence Units
500
400
300
200
100
0
Sample
LY341495
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

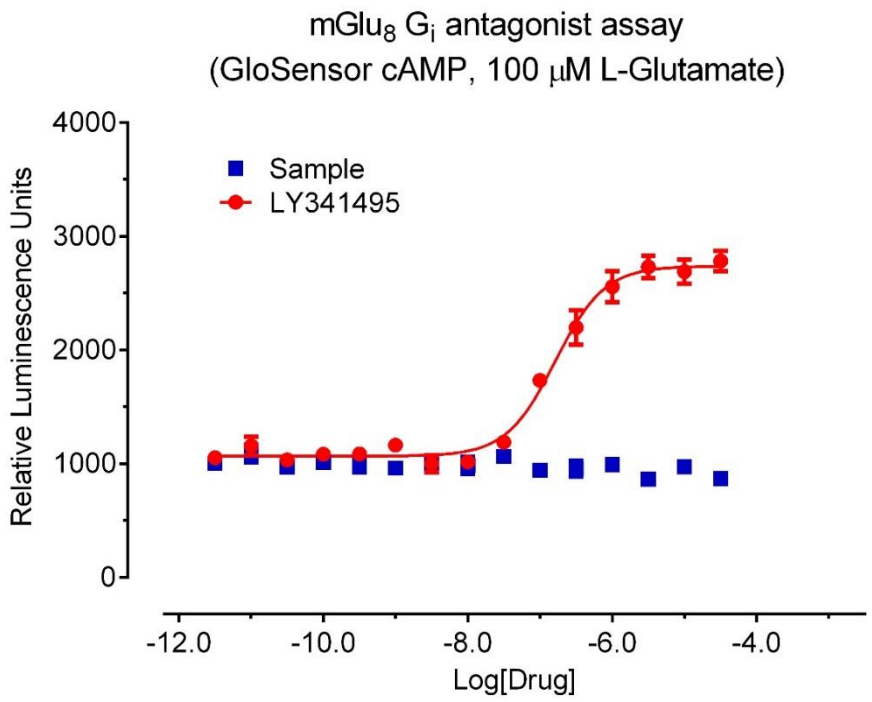

mGlu4 allosteric modulator assay
(GloSensro cAMP, Gi agonist)
Relative Luminescence Units
5000
4000
3000
2000
1000
0
Sample
(M)
0
3E-8
1E-7
3E-7
1E-6
3E-6
-10
-9
-8
-7
-6
-5
-4
Log[L-Glutamate]

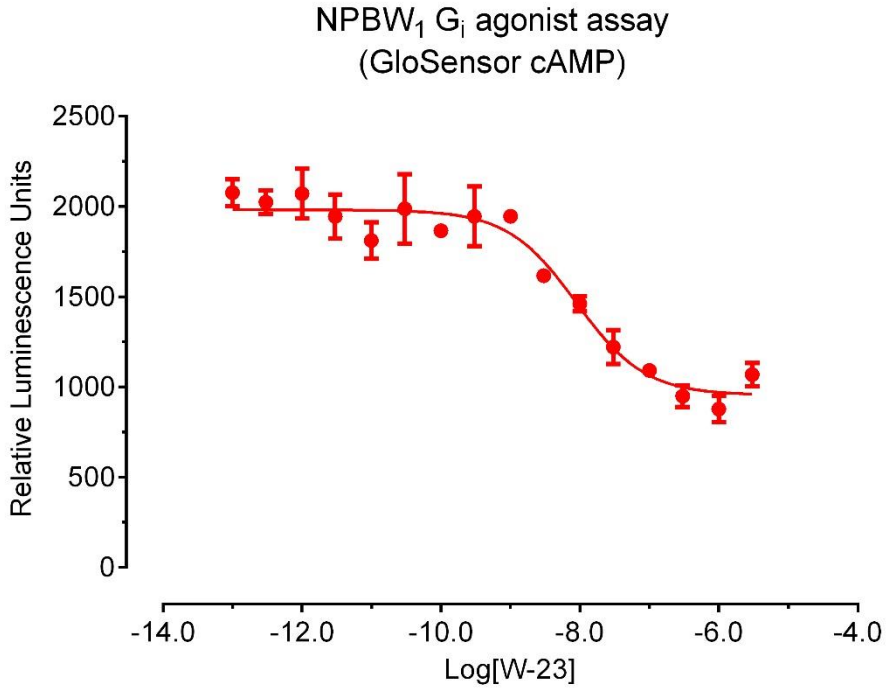

mGlu4 allosteric modulator assay
(GloSensro cAMP, Gi agonist)
Relative Luminescence Units
5000
4000
3000
2000
1000
0
TC-N 22A
(M)
0
3E-8
1E-7
3E-7
1E-6
3E-6
-10
-9
-8
-7
-6
-5
-4
Log[L-Glutamate]

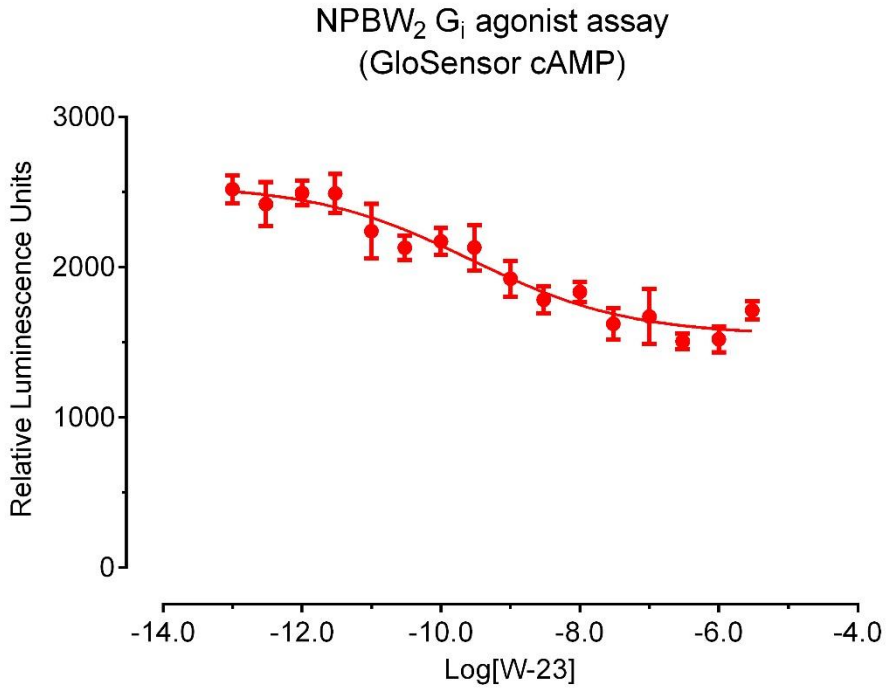

mGlu6 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
200
150
100
50
0
L-Serine-O-Phosphate
L-Glutamate
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

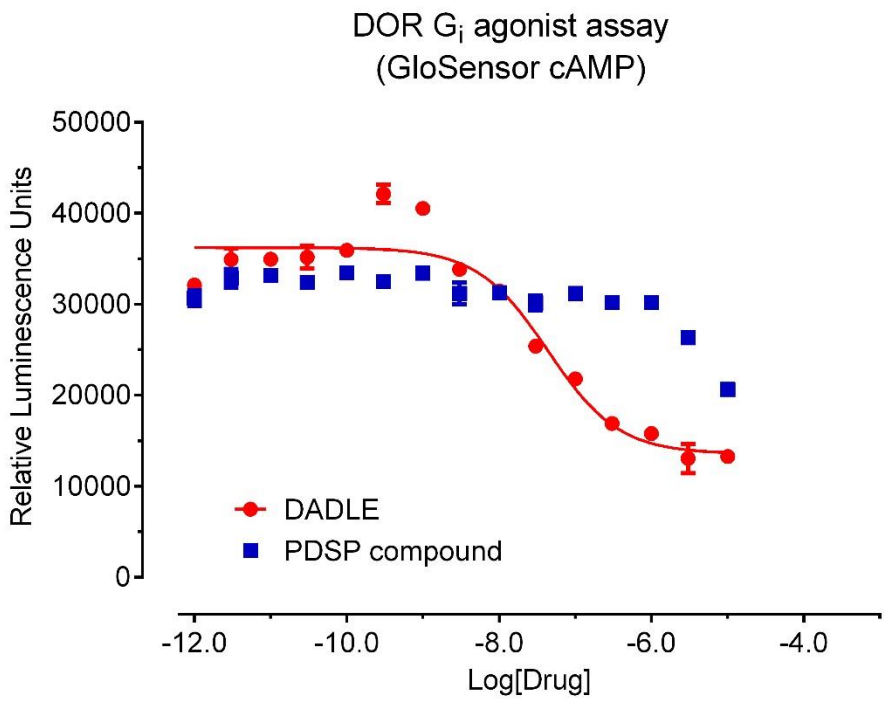

mGlu6 Gi antagonist assay
(GloSensor cAMP, 100 μM L-Glutamate)
Relative Luminescence Units
2000
1500
1000
500
0
Sample
LY341495
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

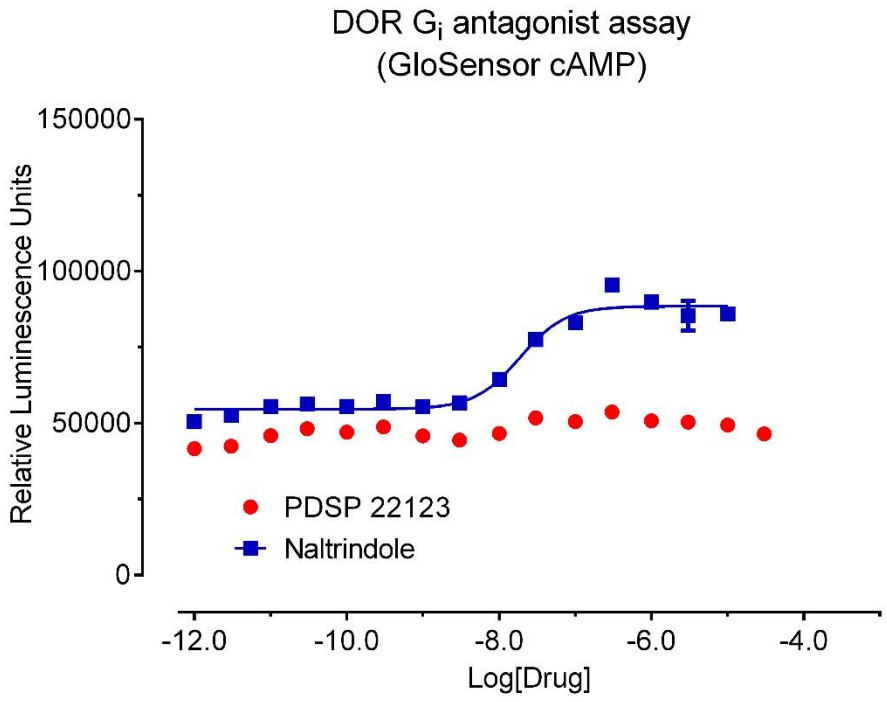

mGlu8 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
2500
2000
1500
1000
500
0
Sample
L-Glutamate
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

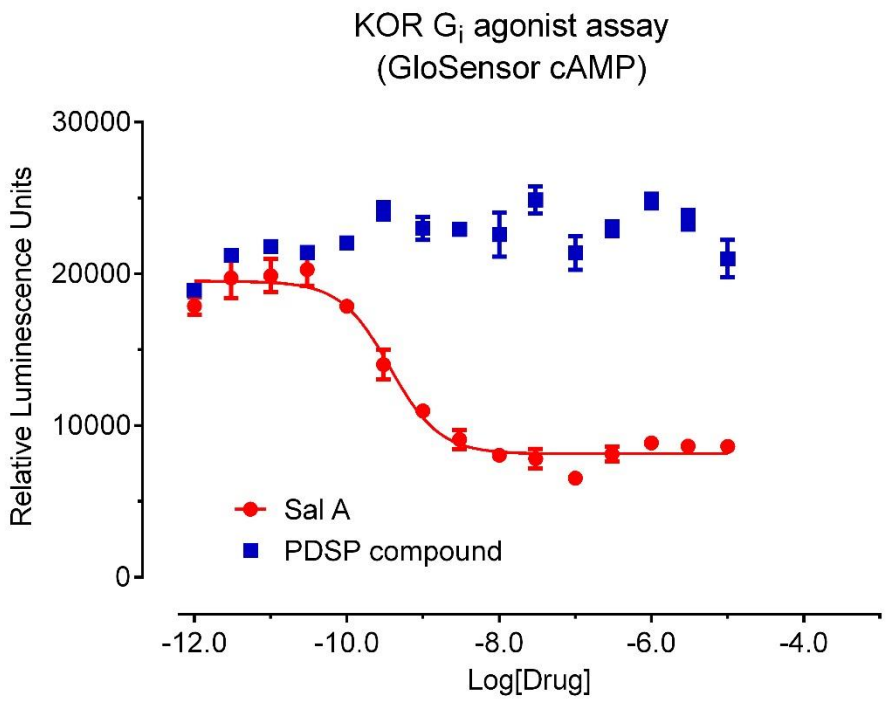

mGlu8 Gi antagonist assay
(GloSensor cAMP, 100 μM L-Glutamate)
Relative Luminescence Units
4000
3000
2000
1000
0
Sample
LY341495
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

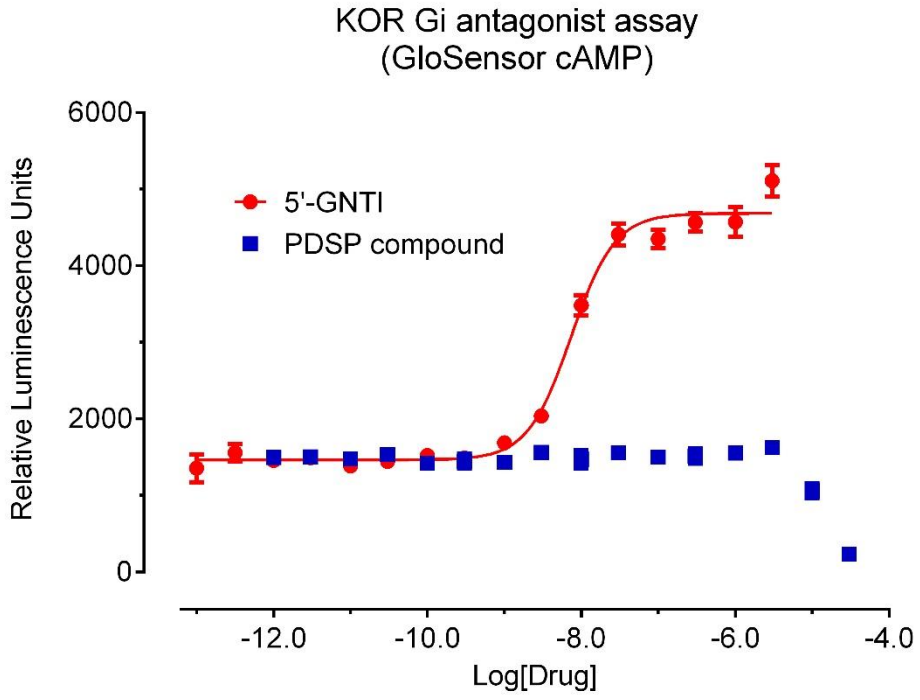

NPBW1 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
2500
2000
1500
1000
500
0
-14.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[W-23]

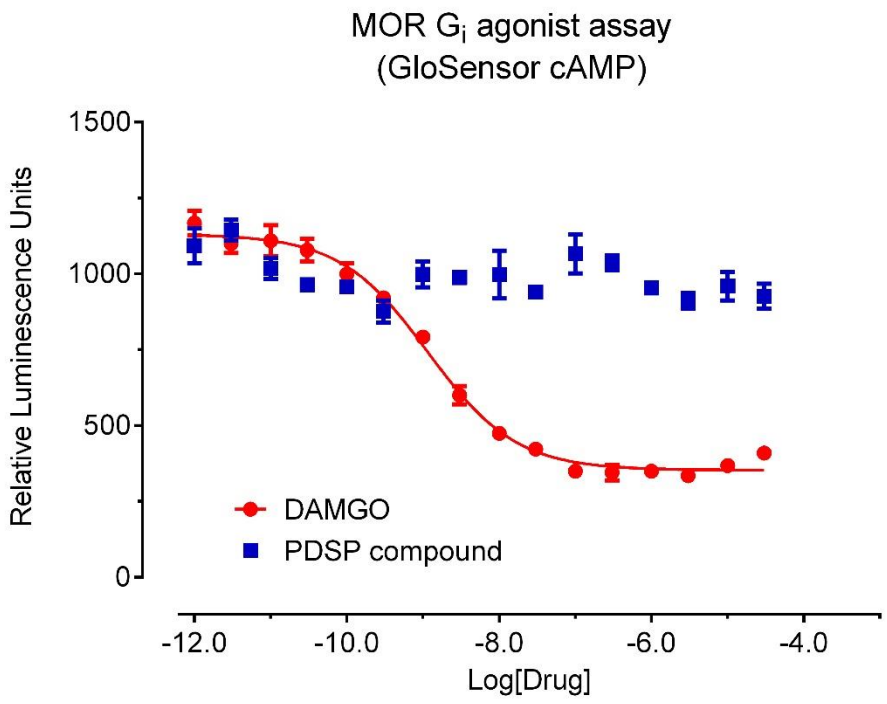

NPBW2 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units
3000
2000
1000
0
-14.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[W-23]

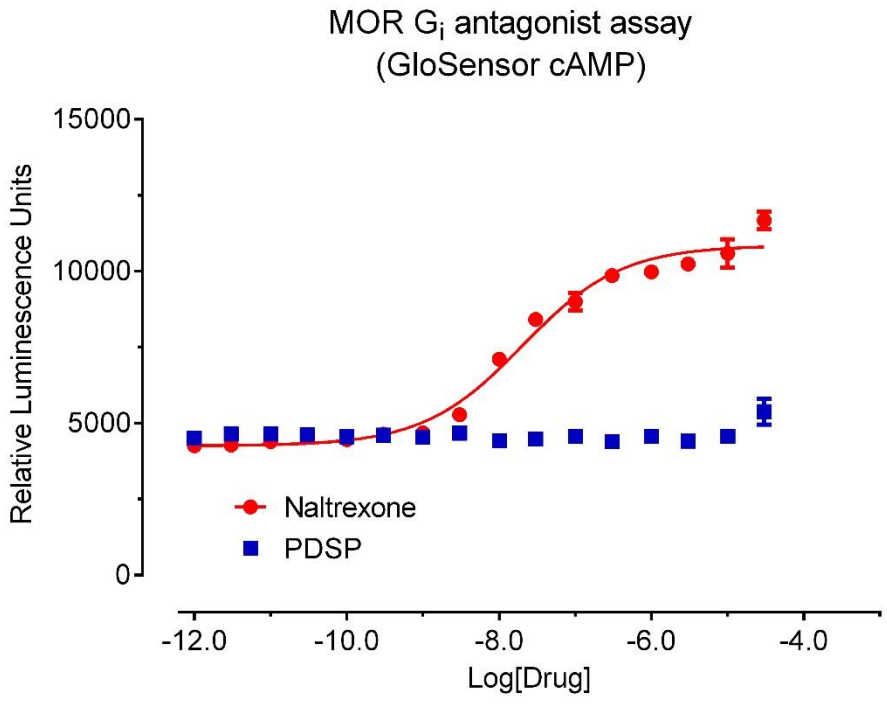

DOR G_i agonist assay
(GloSensor cAMP)
Relative Luminescence Units
50000
40000
30000
20000
10000
0
DADLE
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

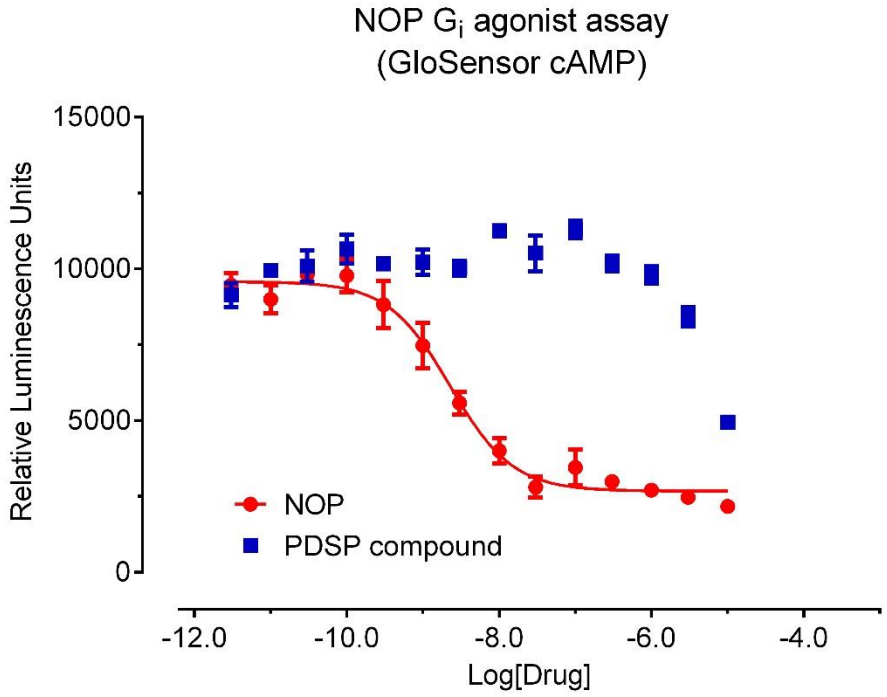

DOR G_i antagonist assay
(GloSensor cAMP)
Relative Luminescence Units
150000
100000
50000
0
PDSP 22123
Naltrindole
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

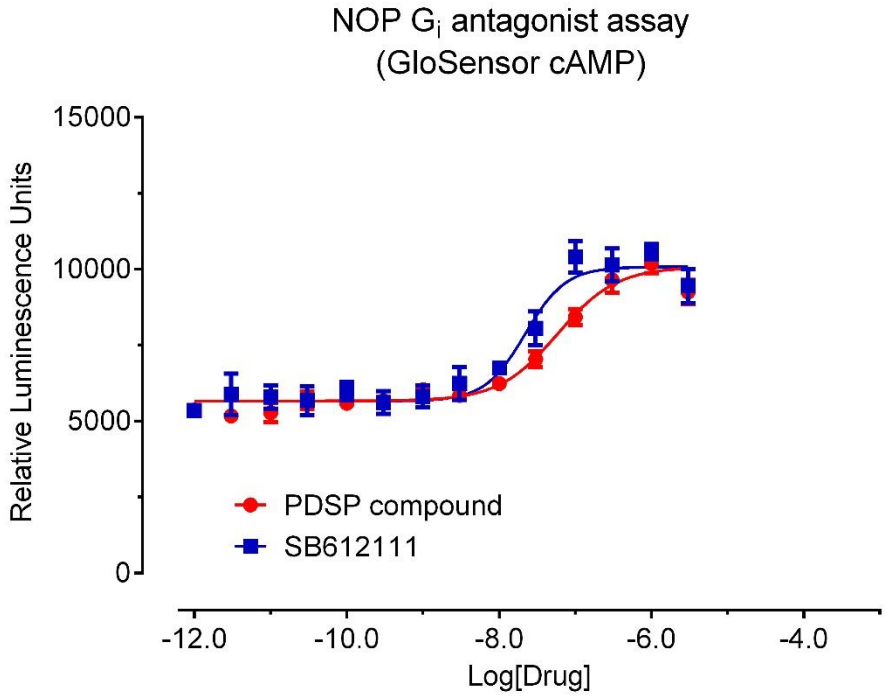

KOR G_i agonist assay
(GloSensor cAMP)
Relative Luminescence Units
30000
20000
10000
0
Sal A
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

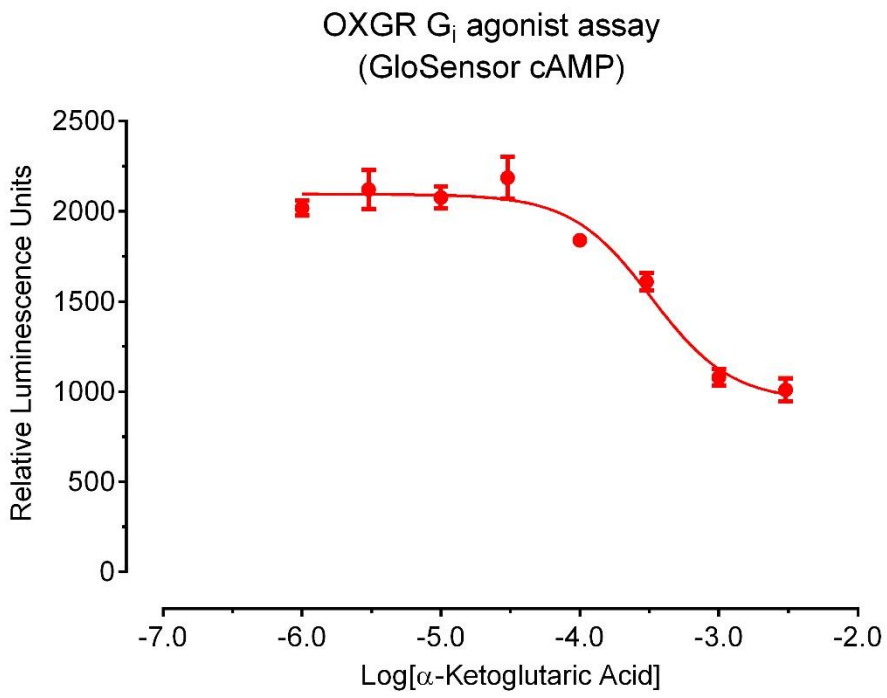

KOR Gi antagonist assay
(GloSensor cAMP)
Relative Luminescence Units
6000
4000
2000
0
5'-GNTI
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

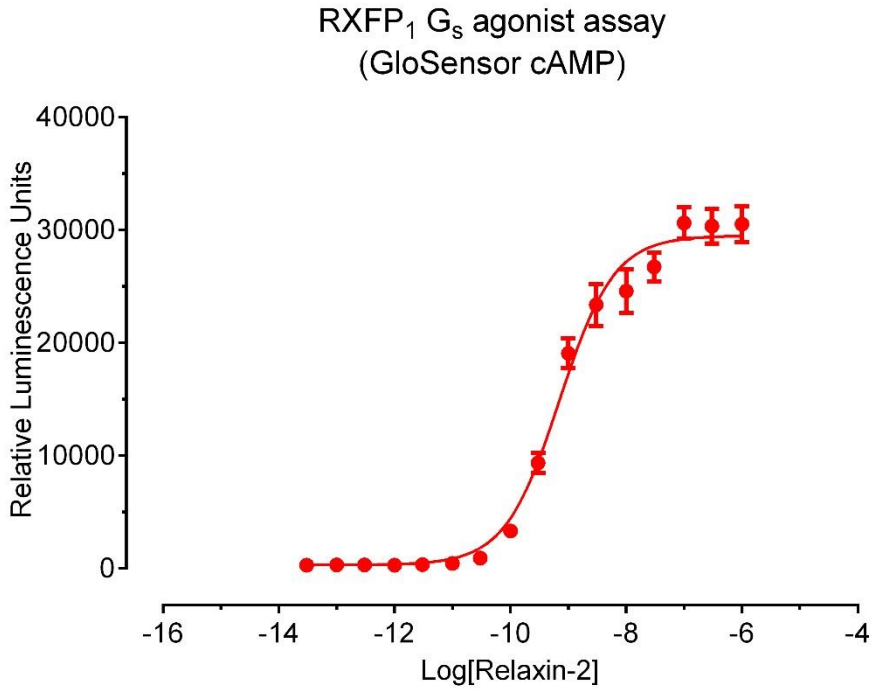

MOR G_i agonist assay
(GloSensor cAMP)
Relative Luminescence Units
1500
1000
500
0
DAMGO
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

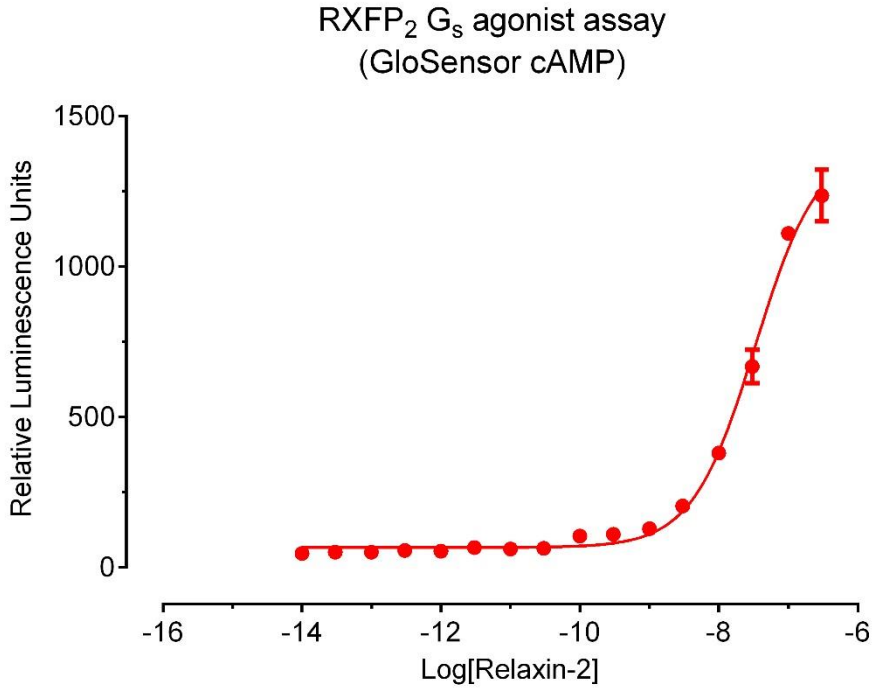

MOR G_i antagonist assay
(GloSensor cAMP)
Relative Luminescence Units
15000
10000
5000
0
Naltrexone
PDSP
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

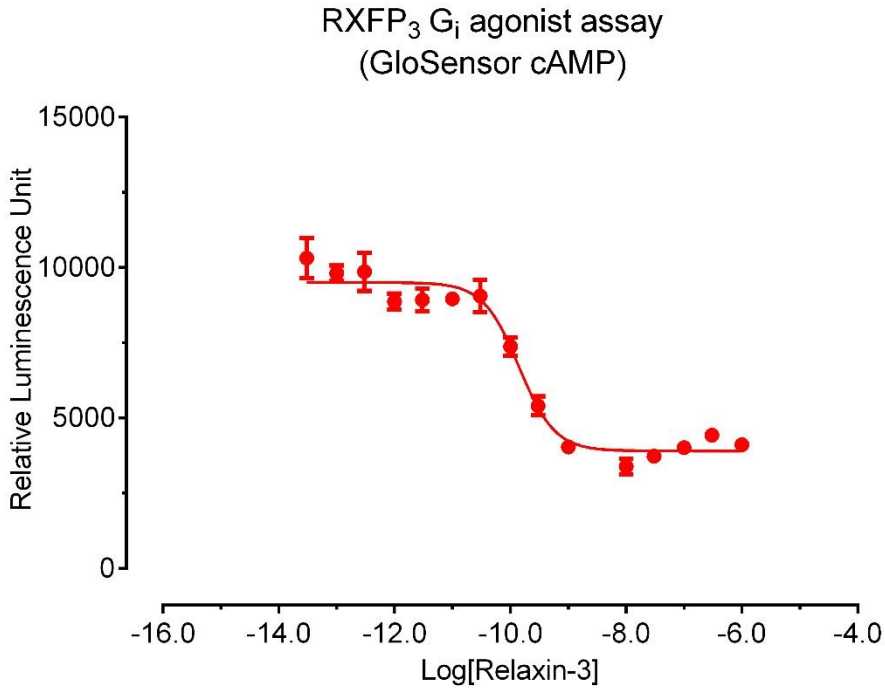

NOP G_i agonist assay
(GloSensor cAMP)
Relative Luminescence Units
15000
10000
5000
0
NOP
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

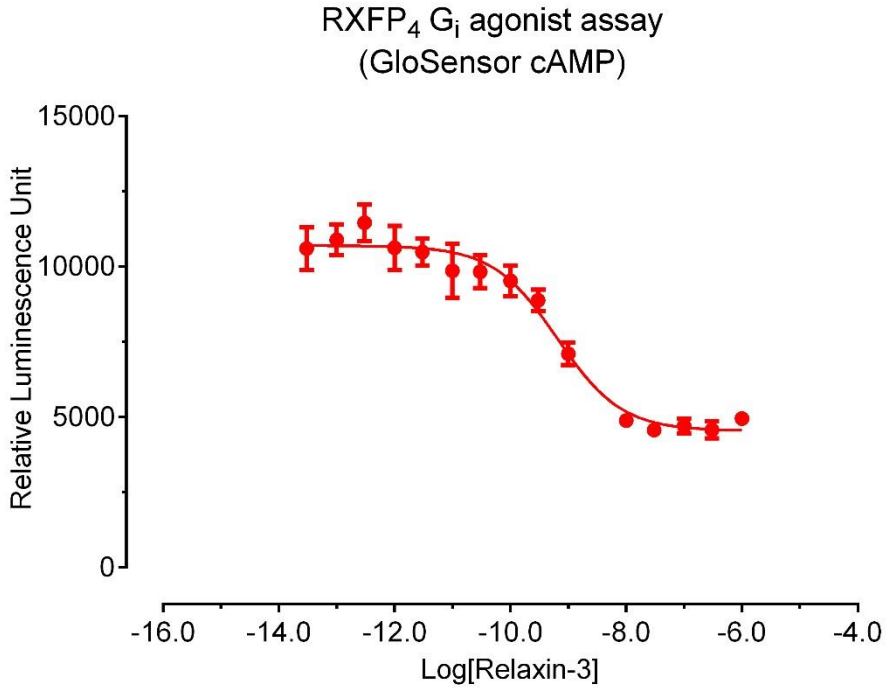

NOP G_i antagonist assay
(GloSensor cAMP)
Relative Luminescence Units
15000
10000
5000
0
PDSP compound
SB612111
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

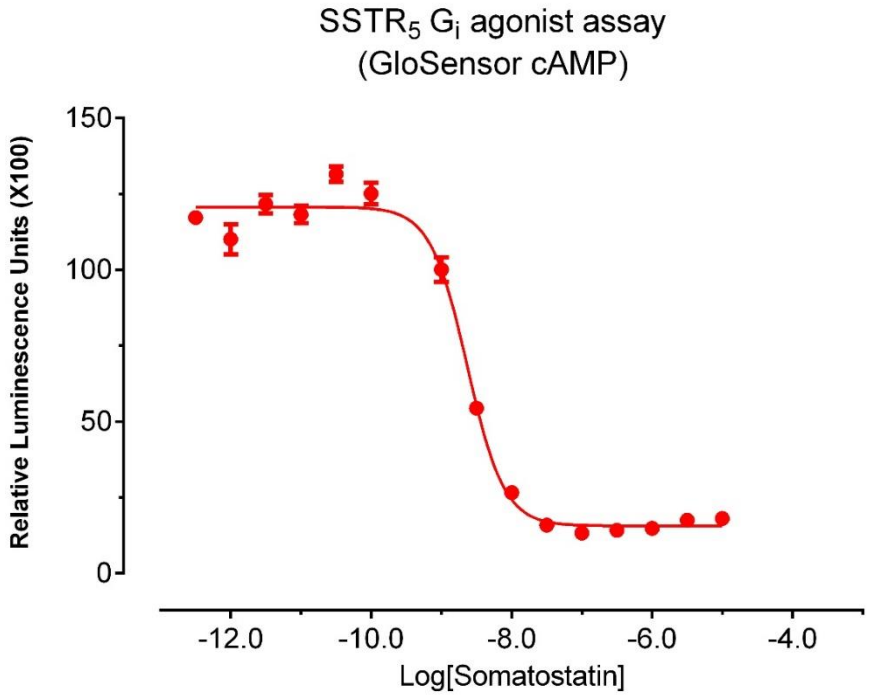

OXGR G_i agonist assay
(GloSensor cAMP)
Relative Luminescence Units
2500
2000
1500
1000
500
0
-7.0
-6.0
-5.0
-4.0
-3.0
-2.0
Log[α-Ketoglutaric Acid]

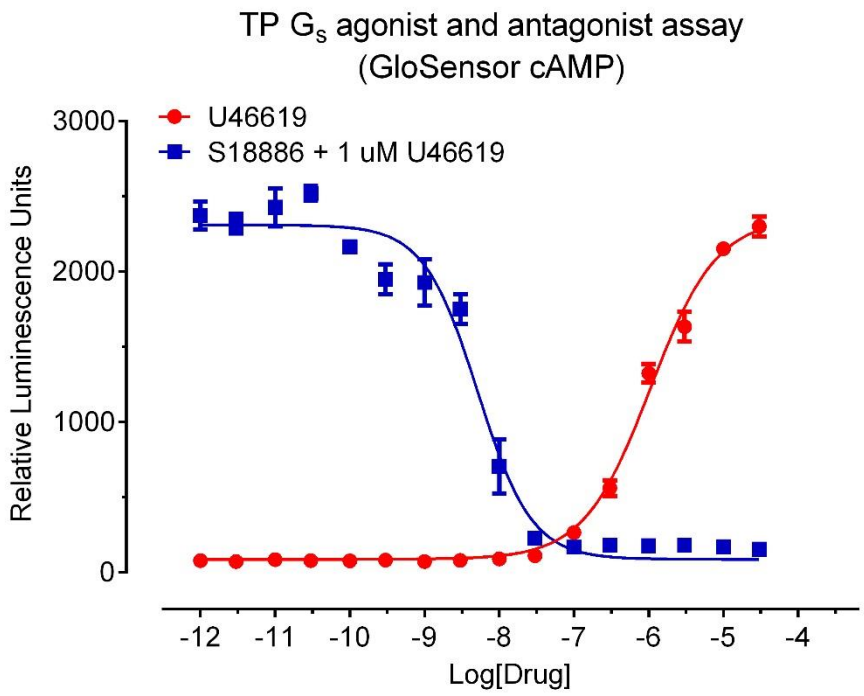

$RXFP_1$ G_s agonist assay
(GloSensor cAMP)
Relative Luminescence Units
40000
30000
20000
10000
0
-16
-14
-12
-10
-8
-6
-4
Log[Relaxin-2]


RXFP2 Gs agonist assay
(GloSensor cAMP)
Relative Luminescence Units
1500
1000
500
0
-16
-14
-12
-10
-8
-6
Log[Relaxin-2]


RXFP3 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Unit
15000
10000
5000
0
-16.0
-14.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Relaxin-3]


RXFP4 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Unit
15000
10000
5000
0
-16.0
-14.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Relaxin-3]


SSTR5 Gi agonist assay
(GloSensor cAMP)
Relative Luminescence Units (X100)
150
100
50
0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Somatostatin]


TP G_s agonist and antagonist assay
(GloSensor cAMP)
U46619
S18886 + 1 uM U46619
Relative Luminescence Units
3000
2000
1000
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

2.6. GPCR Tango assays: G-protein independent β-arrestin recruitment

Main equipment: Luminescence counter

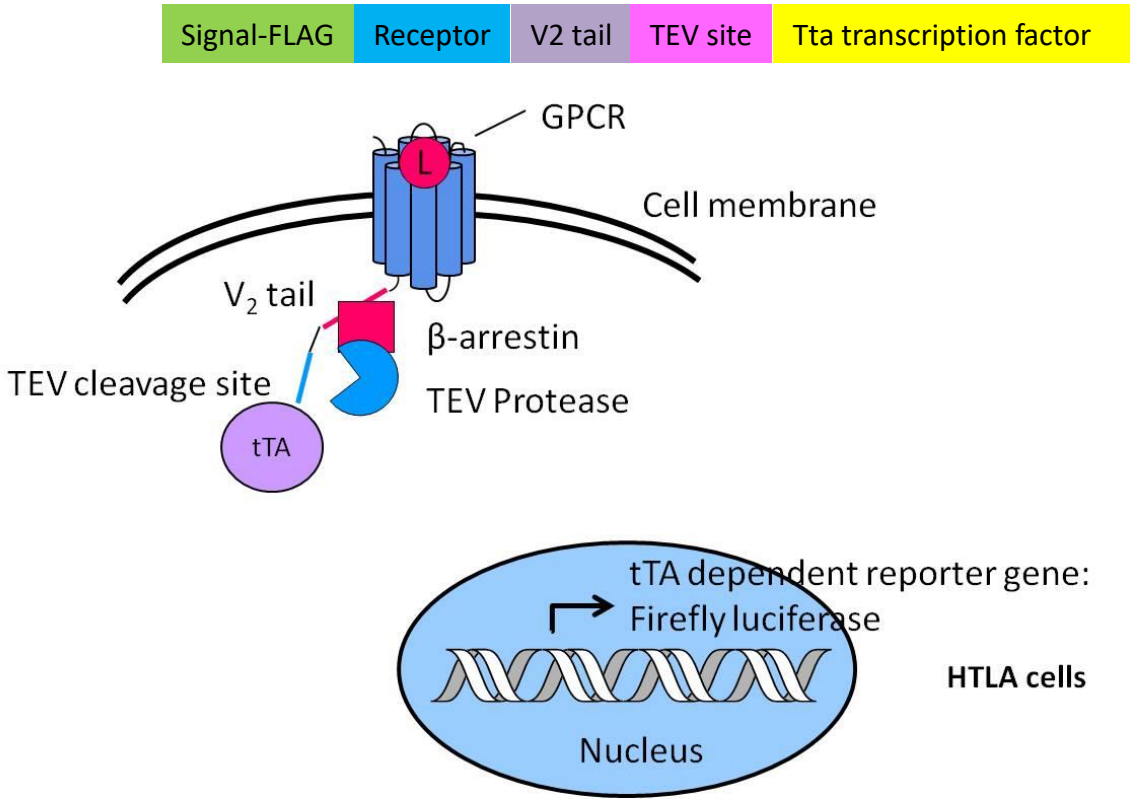
Main reagents: BrightGlo® reagents from Promega

Tango assay buffer: 20 mM HEPES, 1x HBSS, pH 7.40

2.6.1. Tango construct design and cell culture: To measure GPCR mediated ß-arrestin translocation activity, we adopted the Tango assay system originally developed by Richard Axel and his colleagues (169). GPCR Tango constructs (**Figure 42**, below) were codon optimized for better expression in mammalian cell lines and total synthesis was by Blue Heron Biotech (Bothell, WA), with independent sequencing confirmation (154). HTLA cells (an HEK293 cell line stably expressing a tTA-dependent luciferase reporter and a ß-arrestin2-TEV fusion gene, **Figure 34**, bottom) was gifted from Richard Axel's lab, and are maintained in DMEM supplemented with 10% FBS and 2 µg/ml Puromycin and 100 µg/ml Hygromycin. The FLAG tag was designed into the GPCR Tango constructs for confirmation of surface expression (**Figure 43**) and comparison of expression levels.

2.6.2. GPCR Tango assay: HTLA cells are transfected with GPCR tango constructs overnight (see calcium mobilization section for detailed transfection protocol) and are plated in DMEM supplemented with 1% dialyzed FBS in Poly-L-Lys (PLL)-coated 384-well white clear bottom cell culture plates at a density of 15,000 cells in a volume of 40 µl per well. Cells are incubated for at least 6 hours (or overnight) to allow them to recover before receiving drug stimulation. Drug stimulation solutions are prepared in sterile-filtered Tango assay buffer at 5x concentration and added to cells (10 µl per well) overnight. To measure antagonist activity, drug solutions are made at 6x of the final concentration and are preincubated with cells for 30 min before addition of 10 µl of a final EC_{80} concentration of reference agonist. The EC_{80} concentration is determined in separate preliminary dose-response assays. On the day of measurement, medium and drug solutions are removed and 20 µl per well of BrightGlo reagent (diluted 20-fold with Tango assay buffer) are added. Plates are incubated for 20 min at room temperature in the dark before being counted on a luminescence counter.

Figure 46. Tango construct design (**top**) and GPCR tango assay principle (**bottom**). GPCR Tango constructs were designed as indicated (top). Each construct contains these elements in the following order (1) signal/FLAG tag; (2) gene of interest; (3) Vasopressin 2 C-tail; (4) TEV protease site; (5) tTA transcription factor. The GPCR Tango assay is carried out in transiently transfected HEK T cells that are genetically modified to express β-arrestin2 fused with a TEV protease and a Tta mediated luciferase reporter gene. Activation of the transfected GPCR leads to β-arrestin translocation, which guides TEV protease to cleave the Tta transcription factor from the GPCR tail. The free Tta transcription factor then activates the luciferase reporter gene in the nucleus**.**



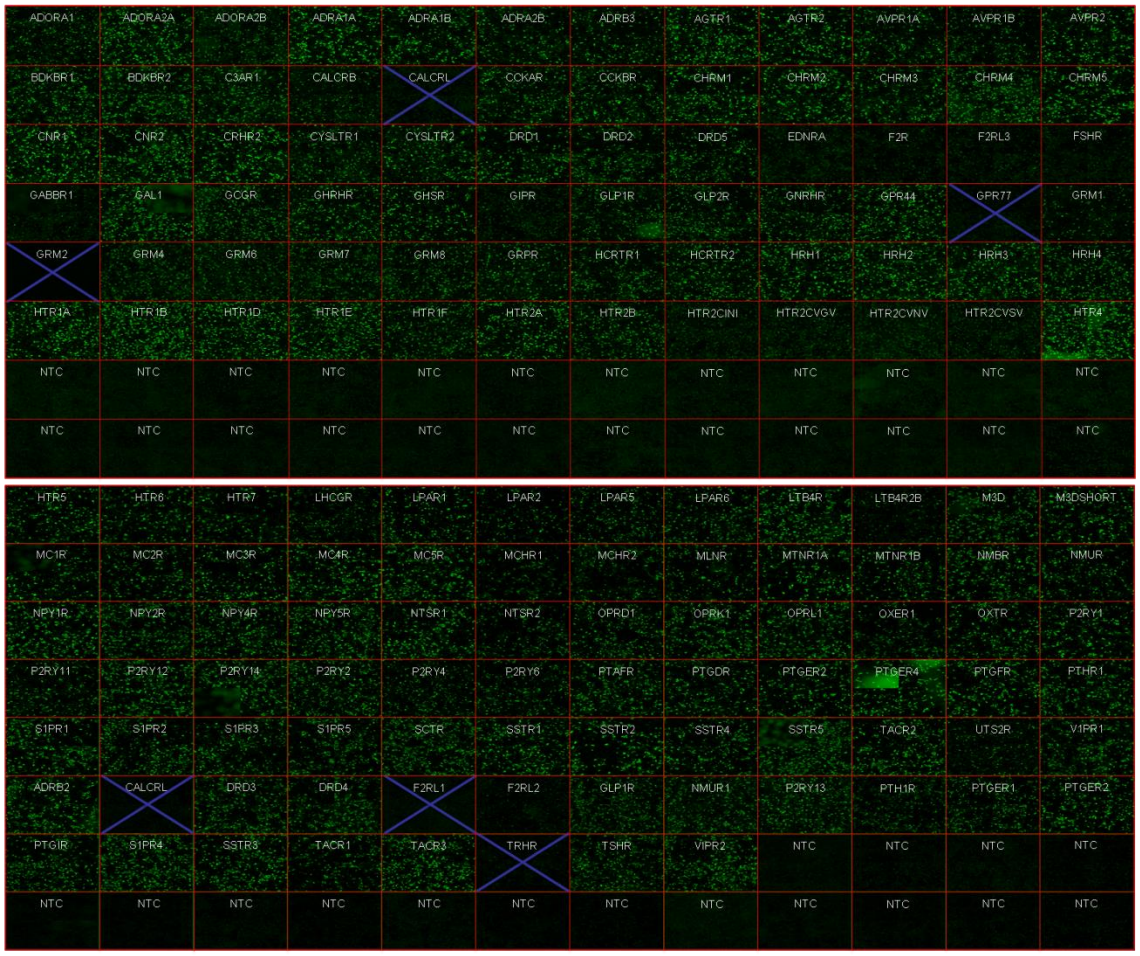


Figure 47. Confirmation of surface expression of FLAG-tagged non-orphan non-olfactory GPCRs used for the β-arrestin recruitment assay. Cells were pre-fixed with paraformaldehyde (PFA) for 30 min, blocked using rabbit anti-FLAG antibody (primary), and incubated for 1h at room temperature and then overnight at 4°C. On day 2, the plate was incubated with Alexa Fluor 594 goat anti-rabbit antibody (secondary) and Hoechst 33342 dye for 1 h. After thorough washing, the plate was post-fixed with PFA and stored at 4°C in the dark. Images were taken using the B-D Pathway High Throughput Bioimager. Blue crosses indicated constructs that were not expressed.

2.6.3. Data processing and analysis: Luminescence counter records chemiluminescence in relative luminescence units (RLU) and saves files in 384-well format in Excel sheet for easy processing. Results in RLU are plotted against concentrations and analyzed in GraphPad Prism v5.0 as outlined in **Section 2.3**.

Table 31. List of GPCR Tango constructs with representative agonist activity curves. All DNA constructs are human clones, designed according to the original design by Barnea et al., (2008) (169), codon optimized, synthesized by Blue Heron Biotech, and sequence-confirmed (154). Most representative curves were recently published (154) and modified for this protocol book. Some receptors have high variations in E_{max} for different reference agonists (such as 5-HT vs LSD at 5-HT receptors) because they were not tested from the same batch of transfected cells, and different assays/transfections may have different efficiency. In addition, some constructs were not optimized when the results were obtained, such as the HTR2C constructs – the ones without N-terminal Signal/FLAG peptide work better than the ones with the Signal/FLAG peptide (Roth lab, unpublished results).

Gene Name	IUPHAR Receptor Name	Agonists (references)	E_{max} (fold of basal)	pEC_{50} (EC_{50} nM)	Hill slope
HTR1A	5-HT_{1A}	5-HT	15	6.35 (448)	0.67
		LSD	139	7.22 (59.0)	1.16
HTR1B	5-HT_{1B}	5-HT	3.2	7.49 (32.7)	0.66
		LSD	1.6	8.62 (2.4)	0.99
HTR1D	5-HT_{1D}	5-HT	3.4	7.16 (698)	0.70
		LSD	3.4	8.39 (4.0)	0.56
HTR1E	5-HT_{1E}	5-HT	3.6	8.39 (4.0)	0.94
		LSD	1.4	8.61 (2.4)	0.98
HTR1F	5-HT_{1F}	5-HT	77	7.27 (54.2)	0.54
		LSD	13	7.75 (17.9)	0.62
HTR2A	5-HT_{2A}	5-HT	17	6.60 (254)	0.79
		LSD	123	8.97 (1.1)	0.66
HTR2B	5-HT_{2B}	5-HT	2.7	8.65 (2.2)	1.20
		LSD	1.7	8.95 (1.1)	0.39
HTR2C	5-HT_{2C} INI	5-HT	2.0	7.42 (38.4)	0.92
		LSD	4.3	7.09 (81.4)	0.43
	5-HT_{2C} VGV	5-HT		<4.50 (>30 μM)	0.49
	5-HT_{2C} VNV	5-HT	37	<4.50 (>30 μM)	0.64
		LSD	1.9	8.30 (5.1)	0.79
	5-HT_{2C} VSV	5-HT		<4.50 (>30 μM)	0.69
		LSD	1.6	8.89 (1.3)	0.76
HTR4	5-HT_4	5-HT	59	7.59 (25.7)	1.18

Gene Name	IUPHAR Receptor Name	Agonists (references)	E_{max} (fold of basal)	pEC_{50} (EC_{50} nM)	Hill slope
HTR4	5-HT_4	LSD	2.0	5.49 (3265)	3.14
HTR5A	5-HT_{5A}	5-HT	11	8.06 (8.7)	0.66
		LSD	16	9.74 (0.18)	0.71
HTR6	5-HT_6	5-HT	40	6.52 (301)	0.93
		LSD	16.1	8.65 (2.2)	0.76
HTR7A	5-HT_{7A}	5-HT	1.9	7.13 (73.4)	2.23
CHRM1	M_1	Acetylcholine	2.1	4.58	
		Carbachol	1.6	6.66 (218)	0.74
		Arecoline	3.7	<5	
CHRM2	M_2	Acetylcholine	8.1	6.41	
		Carbachol	3.8	6.62 (243)	0.96
		Arecoline	23	5.67	
CHRM3	M_3	Acetylcholine	>10	<5	
		Carbachol	18	5.36 (4388)	0.90
		Arecoline	>10	<5	
	M_3D	CNO	27	7.68 (21.1)	1.14
	M_4D	CNO	6.1	8.60 (2.5)	1.01
CHRM4	M_4	Acetylcholine	11.5	5.61	
		Carbachol	35	5.62 (2428)	1.06
		Arecoline	33.8	5.62	
CHRM5	M_5	Acetylcholine	<2	<5	
		Carbachol	1.6	6.88 (131)	1.36
		Arecoline	8.9	<5	
ADORA1	A_1	NECA	4.8	7.93 (11.7)	1.10
ADRA1A	α_{1A}	Oxymetazoline	3.6	7.97 (10.6)	0.82
ADRA1B	α_{1B}	Epinephrine	11	6.61 (244)	0.90
ADRA1D	α_{1D}	Norepinephrine	7.6	5.95 (1118)	1.49
ADRA2A	α_{2A}	Clonidine	5.4	8.36 (4.4)	0.99
ADRA2B	α_{2B}	Norepinephrine	8.9	7.20 (63.1)	0.89
ADRA2C	α_{2C}	Clonidine	61	7.20 (63.3)	1.30
ADRB1	ß$_1$	Epinephrine	22	5.74 (1838)	2.06
ADRB2	ß$_2$	Epinephrine	17	6.21 (613)	1.70
ADRB3	ß$_3$	Carvedilol	44	5.53 (2934)	2.51
AGTR1	AT_1	Angiotensin II	13	7.95 (11.1)	0.57
APLNR	Apelin	Apelin-17	6.5	8.80 (1.6)	0.68
GPBAR1	GPBA	Taurodeoxycholate	>2.3	<5	~0.85
NMBR	BB_1	Bombesin	4.6	7.06 (86.9)	1.75

Gene Name	IUPHAR Receptor Name	Agonists (references)	E_{max} (fold of basal)	pEC_{50} (EC_{50} nM)	Hill slope
		Gastrin-releasing peptide (GRP)	4.6	6.98 (105)	2.00
		Neuromedin B	6.0	8.68 (2.1)	0.87
GRPR	BB_2	Bombesin	6.2	8.50 (3.2)	1.39
		Gastrin-releasing peptide (GRP)	6.1	8.32 (4.8)	1.21
		Neuromedin B	5.3	7.49 (32.6)	1.16
BRS3	BB_3	Saqvinavir (154)	7.5	7.22 (61.0)	0.97
BDKRB1	B_1	Bradykinin	14	4.60 (25220)	0.53
BDKRB2	B_2	Bradykinin	6.8	9.00 (1.0)	0.65
CNR1	CB_1	WIN 55212-2	3.2	6.24 (583)	3.04
		CP55940	19	7.07 (84.5)	1.14
CNR2	CB_2	CP55940	53	8.30 (5.0)	1.44
CCR6	CCR6	CCL20 (Exodus-1)	2.3	7.84 (14.6)	1.07
CXCR1	CXCR1	IL-8	46	8.44 (3.7)	0.72
		CXCL6	3.5	6.60 (252)	0.99
		CXCL8 (IL-8)	4.1	6.95 (111)	1.05
CXCR2	CXCR2	IL-8	1.9	8.59	1.55
		CXCL6	2.6	6.49 (322)	0.29
		CXCL8 (IL-8)	2.3	6.35 (451)	0.43
CXCR4	CXCR4	CXCL12 (SDF1-α)	2.0	8.65 (2.2)	1.14
CXCR6	CXCR6	CXCL16 (SRPOX)	>10	<5	
ACKR3	ACKR3	CXCL12 (SDF1-α)	49	8.85 (1.4)	1.66
CX3CR1	CX_3CR1	CX3CL1	5.8	9.52 (0.3)	2.87
CCKAR	CCK_1	[Thr28, Nle31]CCK(25-33)	3.4	7.50 (31.4)	1.81
C3AR1	C3a	C3a (70-77)	22	6.24 (582)	2.60
DRD1	D_1	Cabergoline	17	4.69 (2033)	0.73
DRD2	D_2	LSD	68	9.29 (0.5)	1.64
DRD3	D_3	Quinpirole	35	7.33 (46.3)	0.75
DRD4	D_4	Lisuride	5.5	7.05 (88.5)	0.39
		Quinpirole	7.0	6.61 (243)	0.70
DRD5	D_5	LSD	28	6.62 (238)	1.39
FPR1	FPR1	fMLP	84	8.61 (2.5)	0.88
FPR2	FPR2/ALX	fMLP	>18	<5	
FPR3	FPR3	fMLP	1.2	6.61 (247)	0.99
GAL1	GAL_1	Galanin	16	5.71 (1940)	0.70
GAL2	GAL_2	Galnon	3.6	8.29	
GAL3	GAL_3	Galanin	3.4	9.92 (1.2)	1.63

Gene Name	IUPHAR Receptor Name	Agonists (references)	E_{max} (fold of basal)	pEC_{50} (EC_{50} nM)	Hill slope
GHSR	Ghrelin	Ghrelin	1.8	7.10 (8.0)	0.70
GLP-1R	GLP-1	Glucagon	8.8	<5	
SCTR	Secretin	Secretin	5.5	5.39 (4116)	1.10
GNRHR	GnRH	Leuprolide	2.8	8.43 (3.8)	1.96
CYSLTR1	CysLT1	Leukotriene D4	4.0	7.61 (24.8)	1.82
EDNRA	ET_A	Endothelin-1	70	9.21 (0.6)	1.03
HRH1	H_1	N-Methylhistaprodifen	1.3	7.19 (65.2)	1.37
HRH2	H_2	Histamine	6.9	5.40 (3982)	2.94
HRH3	H_3	N-Methylhistamine	21	7.71 (19.6)	0.76
HRH4	H_4	Histamine	3.2	6.81 (155)	0.94
HCAR2	HCA_2	Niacin	1.9	5.11 (7729)	0.71
HCRTR1	OX_1	Orexin-A	86	7.82 (15.3)	1.53
HCRTR2	OX_2	Orexin-A	60	8.14 (7.2)	1.94
LTB4R	BLT_1	Leukotriene D4	96	6.71 (196)	0.61
	BLT_1*	Leukotriene D4	28	7.55 (28.2)	0.69
LPAR1	LPA_1	1-oleoyl LPA	6.0	5.36 (4380)	1.35
LPAR2	LPA_2	1-oleoyl LPA	69	<5	
LPAR5	LPA_5	1-oleoyl LPA	14	<5	
S1PR1	$S1P_1$	Sphingosine-1-phosphate	9.3	5.46 (3435)	0.67
S1PR2	$S1P_2$	Sphingosine-1-phosphate	>8	<5	
S1PR3	$S1P_3$	Sphingosine-1-phosphate	17	<5	
MCHR1	MCH_1	[Ala-17]-MCH	6.9	7.67 (21)	1.01
MCHR2	MCH_2	[Ala-17]-MCH	20	8.39 (4.1)	0.90
MC1R	MC_1	α-MSH	2.4	7.41 (38.5)	1.64
MC3R	MC_3	Melanotan II	11	8.15 (7.1)	0.87
MC4R	MC_4	ACTH	4.6	6.14 (734)	1.15
MC5R	MC_5	α-MSH	>1.7	<5	
MTNR1A	MT_1	Melatonin	15	10.81 (0.02)	0.52
MTNR1B	MT_2	Melatonin	8.1	8.87 (1.3)	0.61
MLNR	Motilin	Motilin	159	8.04 (9.2)	0.97
NMUR1	NMU1	Neuromedin S	34	8.04 (9.1)	1.33
NMUR2	NMU2	Neuromedin S	8.1	7.83 (14.9)	1.05
NPS	NPS	Neuropeptide S	44	6.25 (566)	1.33
NPBWR2	NPBW2	Neuropeptide W-23	35	<5	
NPY1R	Y_1	Pancreatic polypeptide	3.9	9.27 (0.5)	0.85
NPY2R	Y_2	Pancreatic polypeptide	23	8.73 (1.9)	0.80
NPY4R	Y_4	Neuropeptide Y	1.9	7.44 (36.0)	7.05
NTSR2	NTS_2	SR48692(154)	11	6.23 (589)	1.04

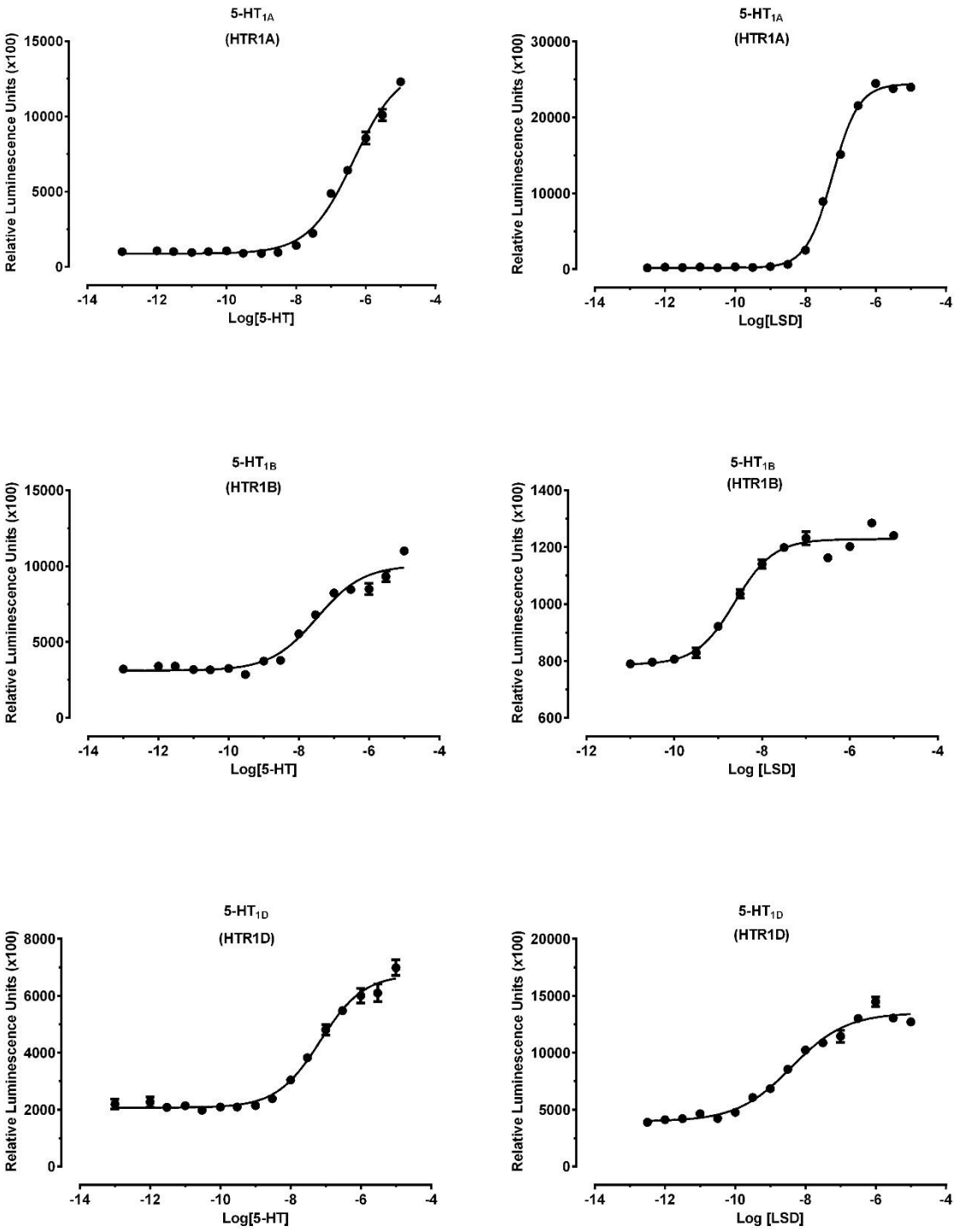
Gene Name	IUPHAR Receptor Name	Agonists (references)	E_{max} (fold of basal)	pEC_{50} (EC_{50} nM)	Hill slope
OPRD1	δ (DOR)	DADLE	7.8	9.14 (0.7)	0.84
KOR	κ (KOR)	Salvinorin A	9.3	7.49 (32.5)	1.29
OPRM1	μ (MOR)	Morphine	10		
OPRL1	NOP	Orphanin	110	7.92 (12.1)	1.04
P2RY1	$P2Y_1$	2-MeS-ADP	46	5.31 (4957)	0.69
P2RY2	$P2Y_2$	UTP	44	<5	
P2RY4	$P2Y_4$	UTP	5.9	5.13 (735)	1.04
P2RY6	$P2Y_6$	UTP	2.5	5.74 (1824)	1.03
P2YR11	$P2Y_{11}$	ATP	4.7	<5	
P2RY12	$P2Y_{12}$	2-MeS-ADP	46	7.34 (46.1)	0.86
P2RY13	$P2Y_{13}$	2-MeS-ADP	2.3	8.20 (6.3)	0.48
P2RY14	$P2Y_{14}$	UDP-Glucose	1.6	6.16 (696)	0.65
PTHR1	PTH1	PTH (1-42)	9.0	6.63 (234)	1.05
PTAFR	PAF	PAF (C16)	1.3	9.32 (0.5)	1.18
PTGDR2	DP_2	Prostaglandin D2	2.8	7.19 (64.9)	1.21
PTGER1	EP_1	Prostaglandin E2	9.6	7.23 (59.5)	0.73
PTGER2	EP_2	Prostaglandin E2	49	5.78 (1652)	0.58
PTGER3	EP_3	Prostaglandin E2	3.7	8.14 (7.3)	0.82
PTGER4	EP_4	Prostaglandin E2	29	8.92 (1.2)	1.16
PTGFR	FP	Prostaglandin F2α	26	6.98 (104)	0.86
PTGIR	IP	Iloprost	19	<5	
SSTR1	SST_1	Somatostatin	9.6	5.47 (3372)	0.64
SSTR2	SST_2	Somatostatin	129	5.59 (2583)	0.89
SSTR3	SST_3	Somatostatin	124	5.31 (4948)	0.99
SSTR4	SST_4	Somatostatin	72	5.93 (1175)	0.87
SSTR5	SST_5	Somatostatin	177	5.86 (1388)	1.09
TACR1	NK_1	Substance P	3.0	6.74 (180)	2.36
TACR2	NK_2	Substance P	15	8.72 (1.9)	2.09
TACR3	NK_3	Substance P	3.0	6.73 (187)	2.28
TA1	TA_1	β-Phenylethylamine	4.5	3.96 (110 μM)	1.63
UTSR	UT	Urotensin II	8.9	8.72 (1.9)	1.47
AVPR1A	V_{1A}	Vasopressin	145	8.58 (2.6)	0.97
AVPR1B	V_{1B}	Vasopressin	8.9	8.65 (2.2)	0.81
AVPR2	V_2	Vasopressin	13	8.18 (6.6)	0.72
OXTR	Oxytocin	Oxytocin	35	7.88 (13.2)	0.92
VIPR1	$VPAC_1$	Vasoactive Intestinal Peptide (VIP)	2.8	5.00 (10 μM)	2.66

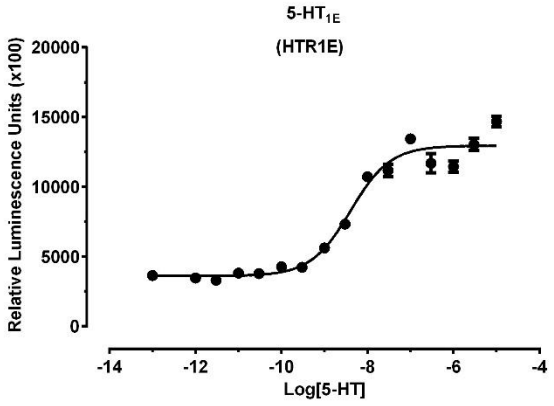
Gene Name	IUPHAR Receptor Name	Agonists (references)	E_{max} (fold of basal)	pEC_{50} (EC_{50} nM)	Hill slope
VIPR2	$VPAC_2$	Vasoactive Intestinal Peptide (VIP)	>5	<5	
GPR35	GPR35	PDSP reference#	4.5	7.59 (25.5)	1.05
GPR39	GPR39	GPR39-C3 (146–148)	209	5.98 (1052)	0.57
GPR55	GPR55	Rimonabant (SR141716)	>10	<5	
GPR183	GPR183	7α,25-dihydroxy Cholesterol (170, 171)	60	7.32 (48.1)	0.88
MAS1	MAS1	PDSP reference#	2.4	5.85 (1425)	1.01
MRGRPX1	MRGPRX1	PDSP reference#	1.5	<5	
MRGRPX2	MRGPRX2	TAN-67 (153)	3.1	5.73 (1880)	1.11
MRGRPX4	MRGPRX4	Nateglinide (154)	38	6.37 (423)	1.78

Notes

1. * Modified Tango construct without V2 tail
2. # Roth lab unpublished results

Figure 48. Representative agonist dose-response curves for GPCR mediated ß-arrestin translocation.

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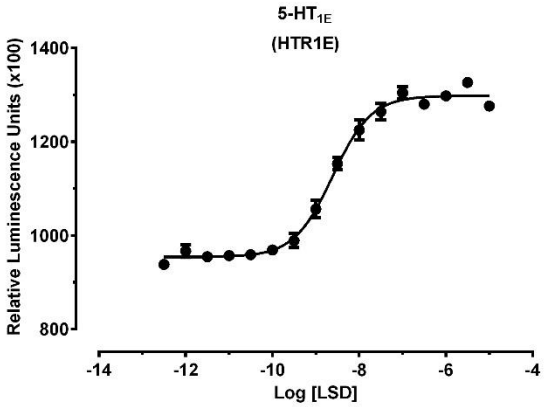
-12
-10
-8
-6
-4
Log[5-HT]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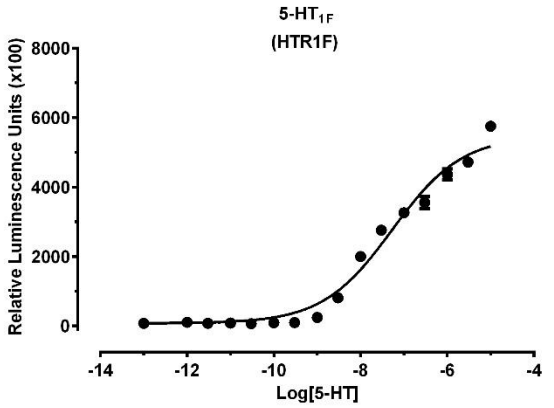
-10
-8
-6
-4
Log [LSD]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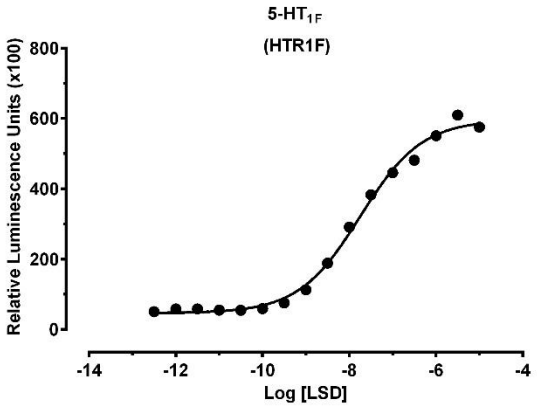
-12
-10
-8
-6
-4
Log[5-HT]


5-HT1F
(HTR1F)
Relative Luminescence Units (x100)
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-10
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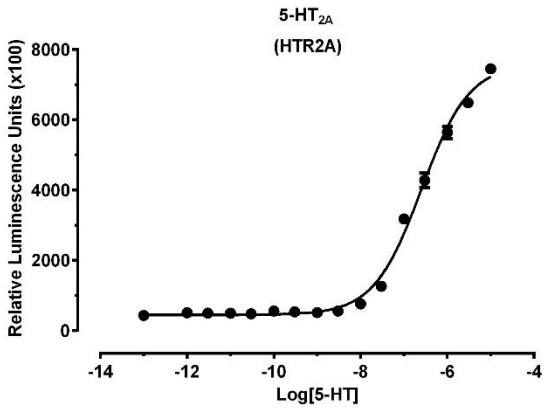
-4
Log [LSD]


5-HT2A
(HTR2A)
Relative Luminescence Units (x100)
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4000
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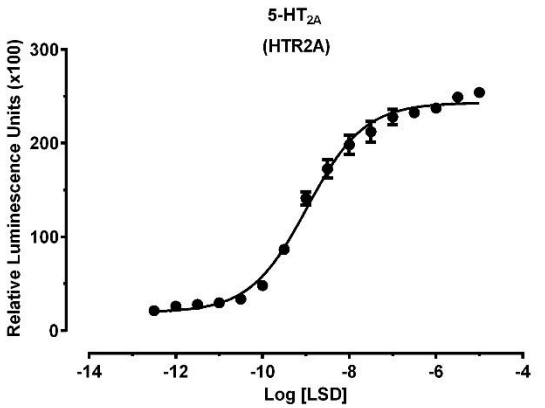
-12
-10
-8
-6
-4
Log[5-HT]


5-HT2A
(HTR2A)
Relative Luminescence Units (x100)
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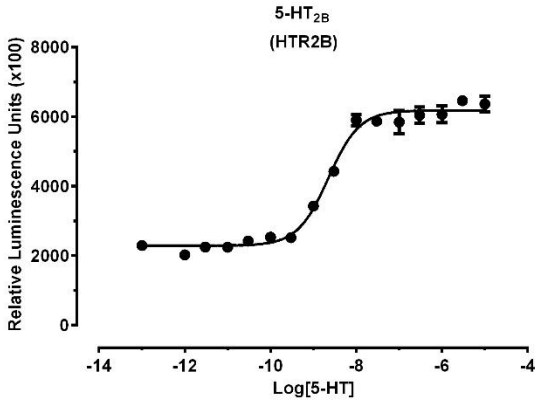
200
100
0
-14
-12
-10
-8
-6
-4
Log [LSD]


5-HT2B
(HTR2B)
Relative Luminescence Units (x100)
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4000
2000
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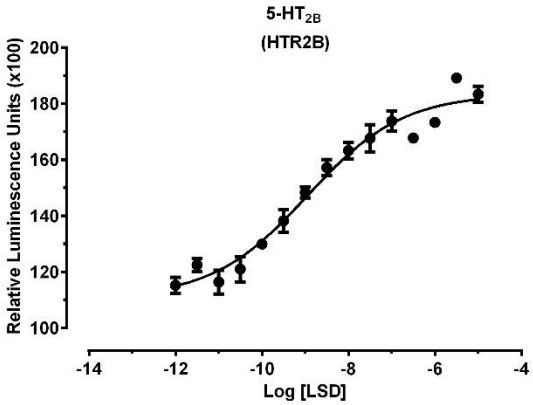
-14
-12
-10
-8
-6
-4
Log[5-HT]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140
120
100
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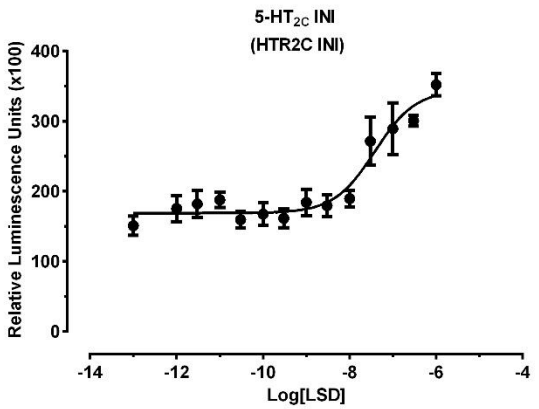
-12
-10
-8
-6
-4
Log [LSD]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Relative Luminescence Units (x100)
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200
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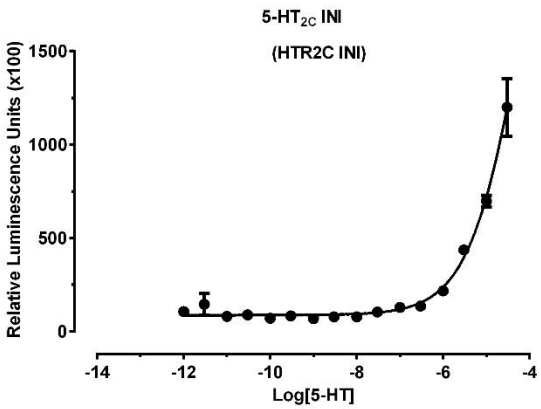
-12
-10
-8
-6
-4
Log[LSD]


5-HT2C INI
(HTR2C INI)
Relative Luminescence Units (x100)
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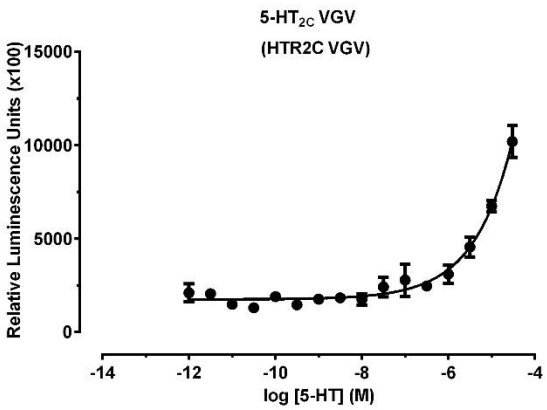
1000
500
0
-14
-12
-10
-8
-6
-4
Log[5-HT]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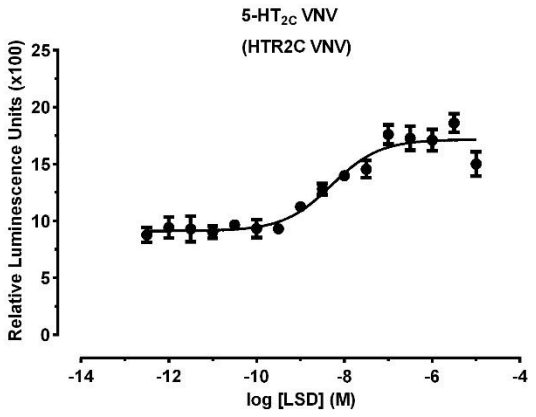
-12
-10
-8
-6
-4
log [5-HT] (M)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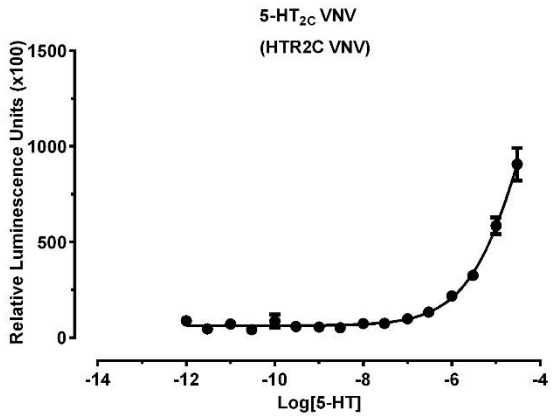
Relative Luminescence Units (x100)
log [LSD] (M)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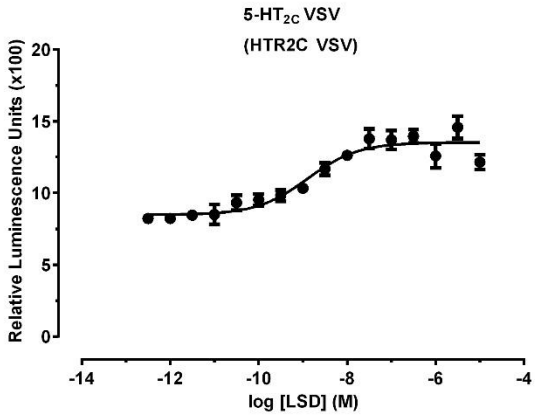
Relative Luminescence Units (x100)
Log[5-HT]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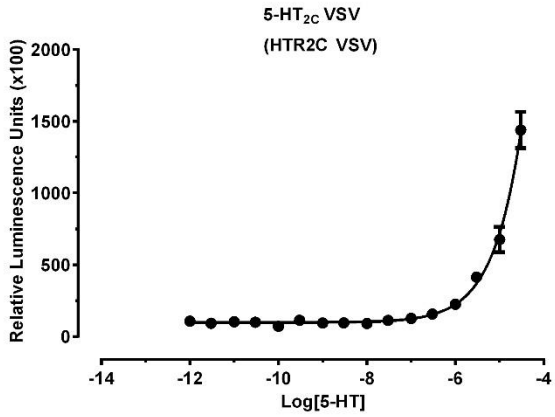
log [LSD] (M)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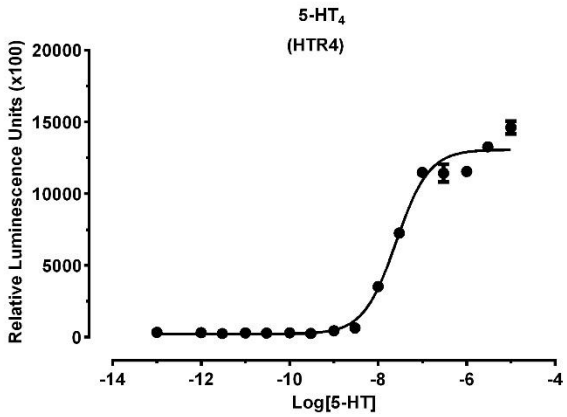
Log[5-HT]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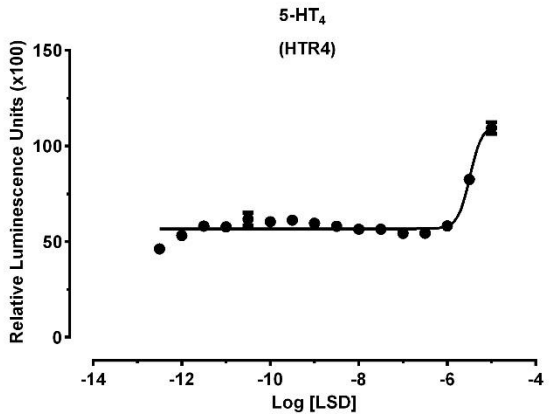
Log[5-HT]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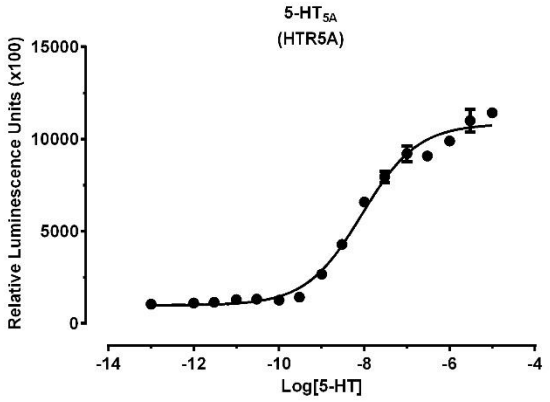
Log [LSD]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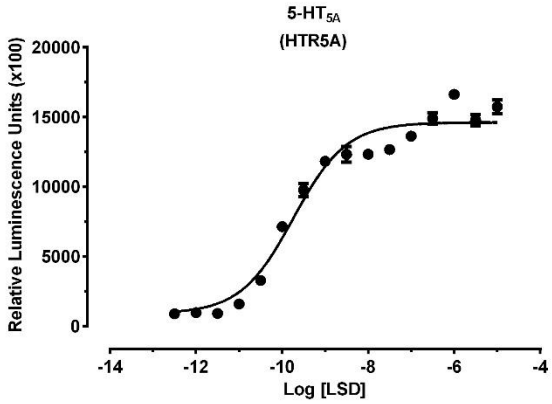
Log[5-HT]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10000
5000
0
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-12
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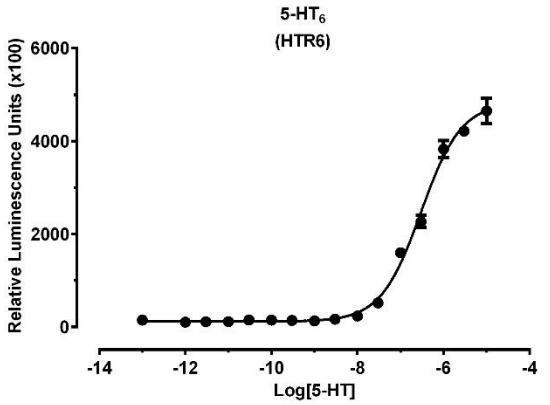
-6
-4
Log [LSD]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2000
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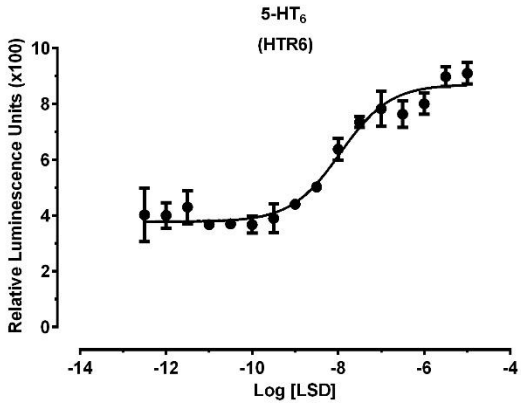
-14
-12
-10
-8
-6
-4
Log[5-HT]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-10
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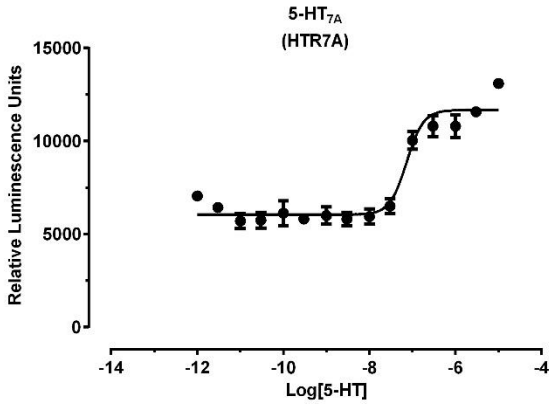
-6
-4
Log [LSD]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(HTR7A)
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-8
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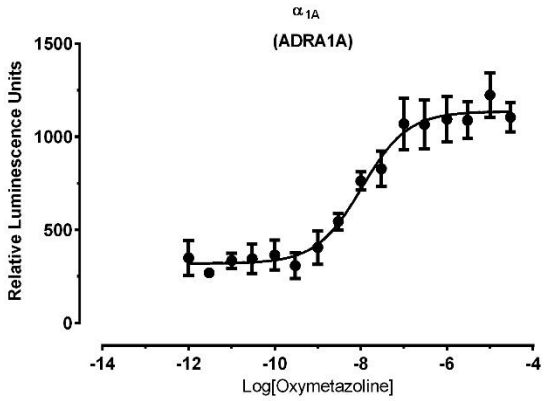
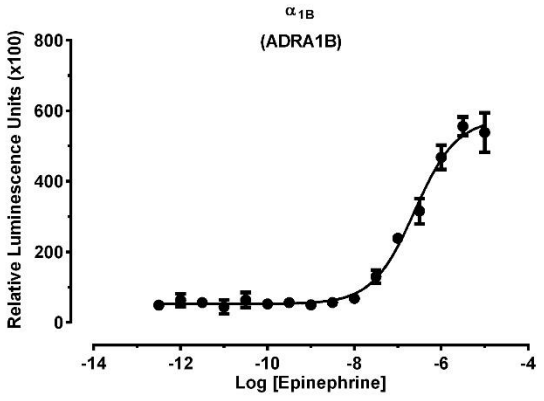
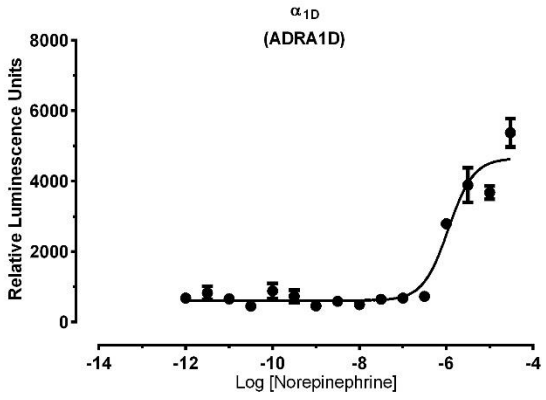
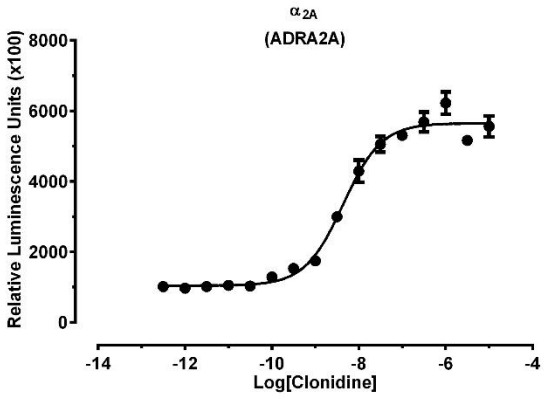
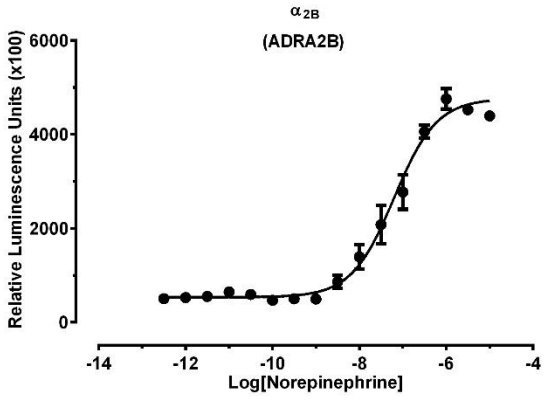
-4
Log[5-HT]

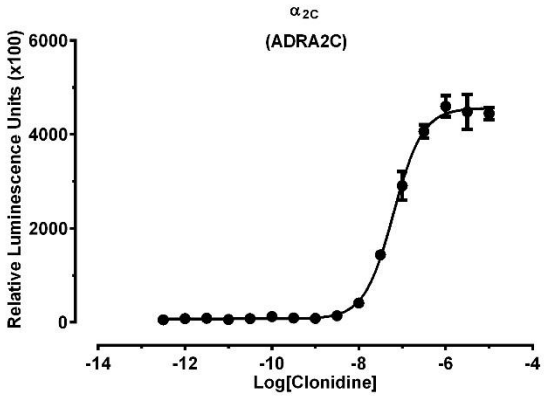

α1A
(ADRA1A)
Relative Luminescence Units
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Oxymetazoline]

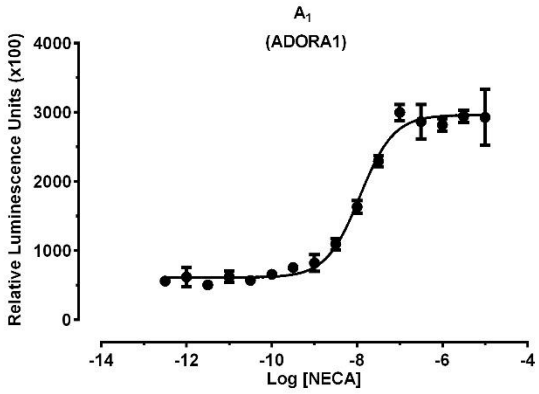

α1B
(ADRA1B)
Relative Luminescence Units (x100)
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log [Epinephrine]

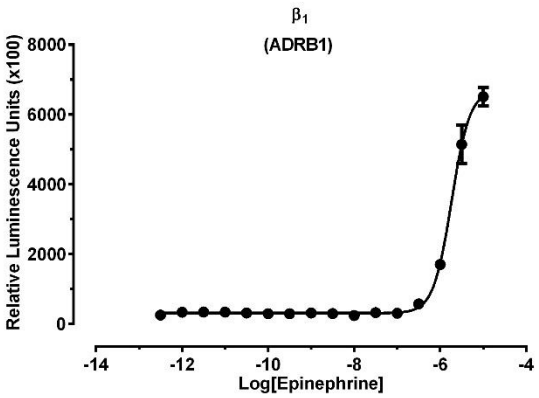

α1D
(ADRA1D)
Relative Luminescence Units
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log [Norepinephrine]

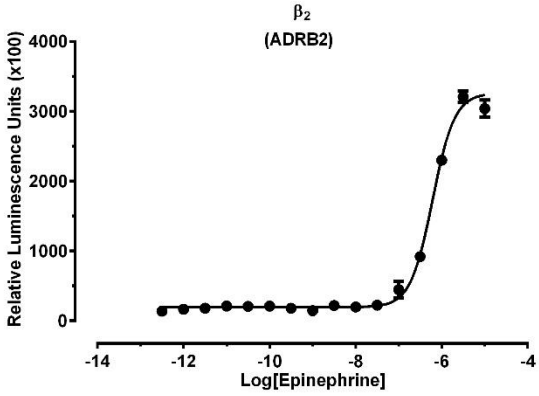

α2A
(ADRA2A)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Clonidine]

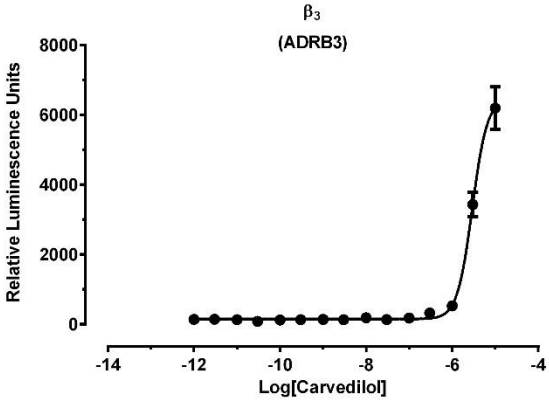

α2B
(ADRA2B)
Relative Luminescence Units (x100)
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Norepinephrine]

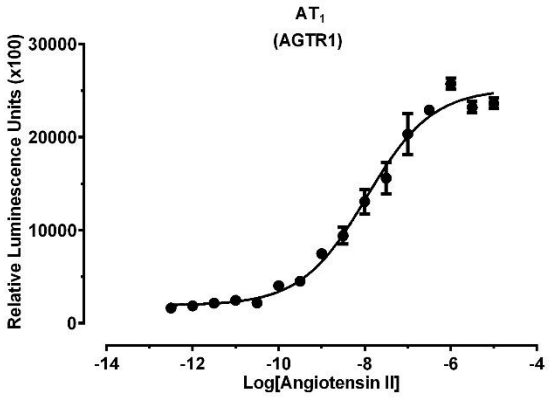

α2C
(ADRA2C)
Relative Luminescence Units (x100)
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Clonidine]

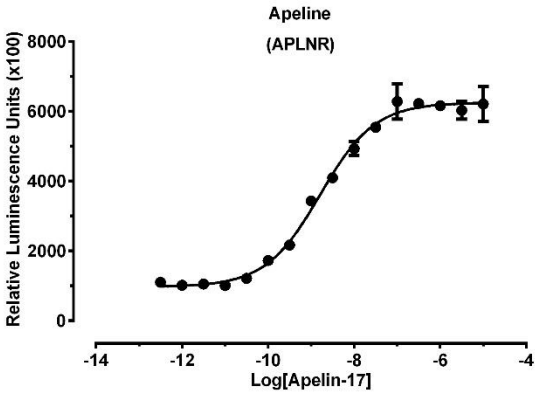

A1
(ADORA1)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log [NECA]

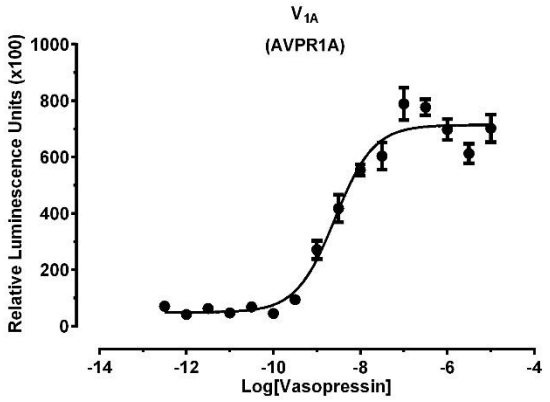

β1
(ADRB1)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Epinephrine]

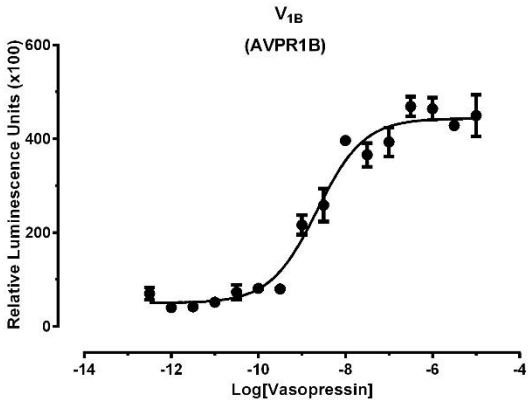

β2
(ADRB2)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Epinephrine]

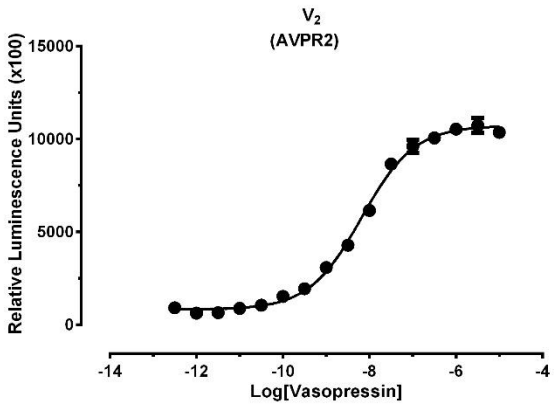

β3
(ADRB3)
Relative Luminescence Units
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Carvedilol]

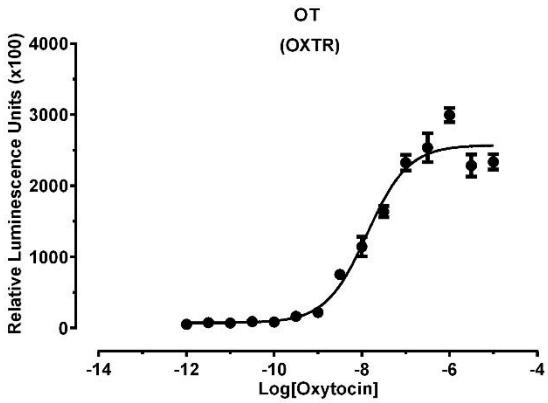

AT1
(AGTR1)
Relative Luminescence Units (x100)
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[Angiotensin II]

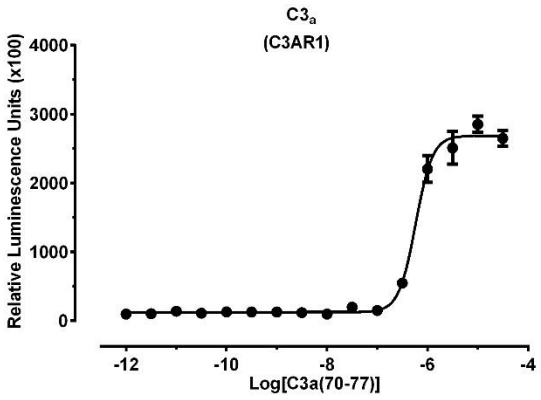

Apeline
(APLNR)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Apelin-17]

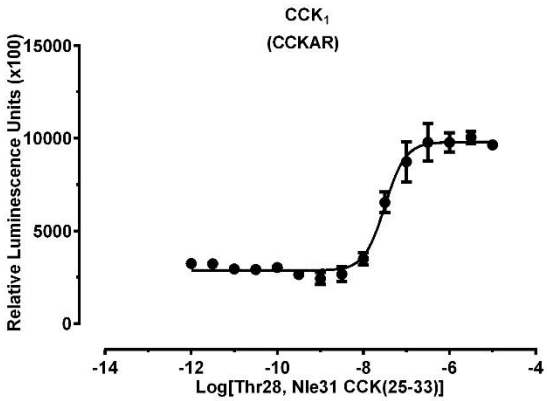

V1A
(AVPR1A)
Relative Luminescence Units (x100)
1000
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Vasopressin]

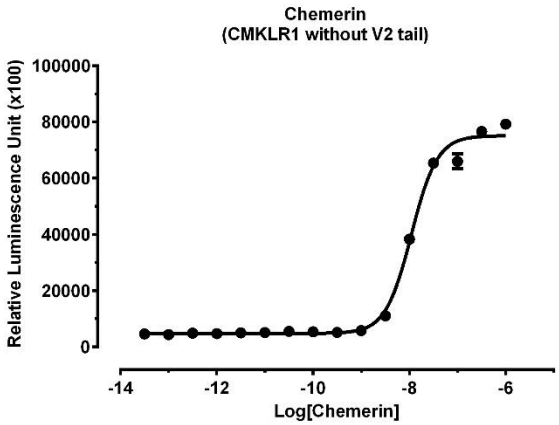

V1B
(AVPR1B)
Relative Luminescence Units (x100)
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Vasopressin]

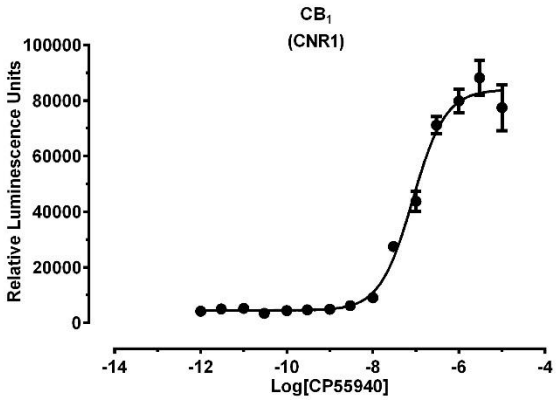

V2
(AVPR2)
Relative Luminescence Units (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Vasopressin]

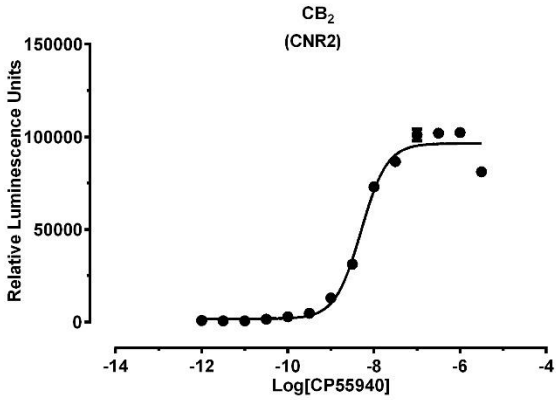

OT
(OXTR)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Oxytocin]

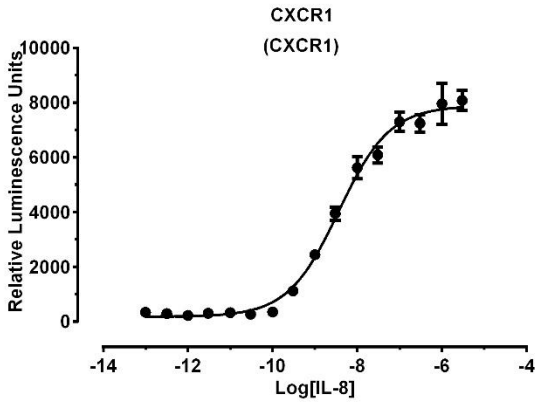

C3a
(C3AR1)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-12
-10
-8
-6
-4
Log[C3a(70-77)]

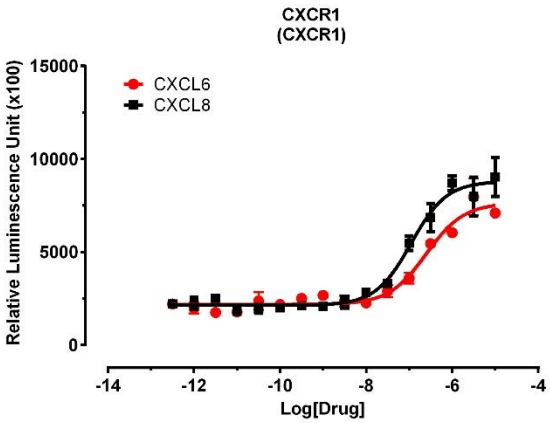

CCK1
(CCKAR)
Relative Luminescence Units (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Thr28, Nle31 CCK(25-33)]

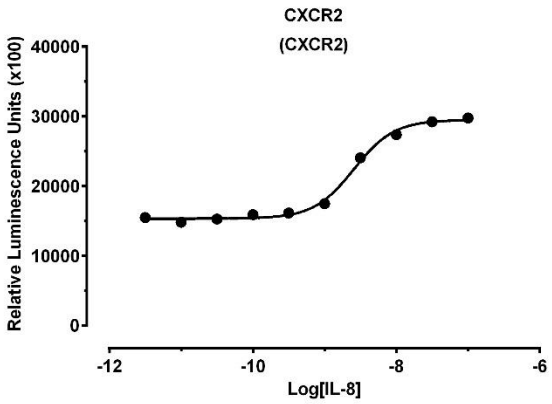

Chemerin
(CMKLR1 without V2 tail)
Relative Luminescence Unit (x100)
100000
80000
60000
40000
20000
0
-14
-12
-10
-8
-6
Log[Chemerin]

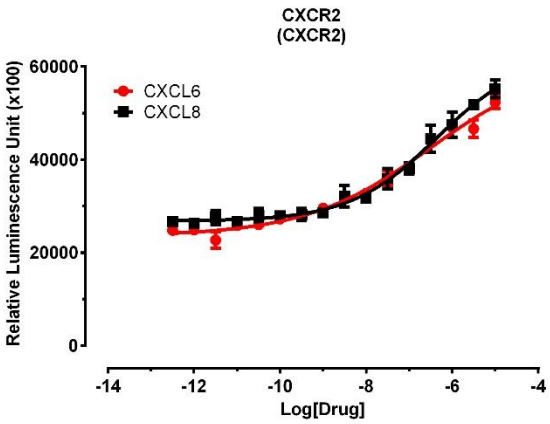

CB1
(CNR1)
Relative Luminescence Units
100000
80000
60000
40000
20000
0
-14
-12
-10
-8
-6
-4
Log[CP55940]

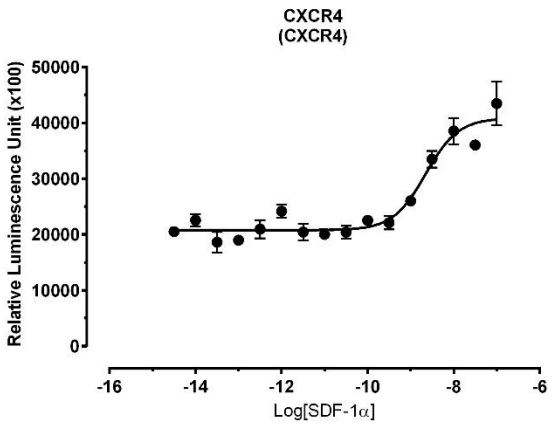

CB2
(CNR2)
Relative Luminescence Units
150000
100000
50000
0
-14
-12
-10
-8
-6
-4
Log[CP55940]

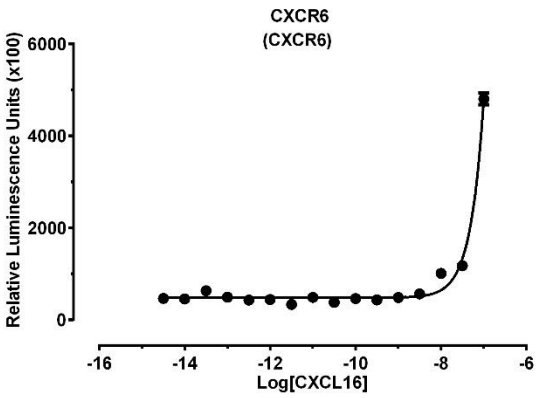

CXCR1
(CXCR1)
Relative Luminescence Units
10000
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[IL-8]

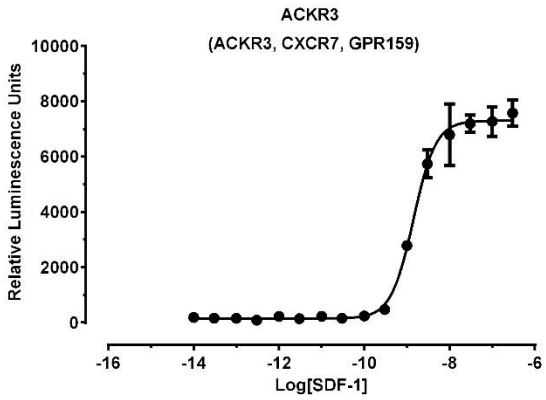

CXCR1
(CXCR1)
CXCL6
CXCL8
Relative Luminescence Unit (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Drug]

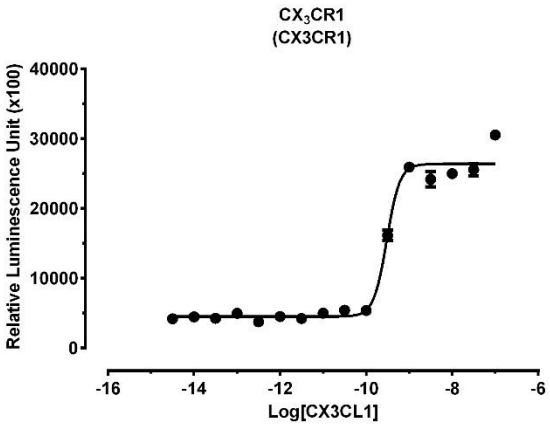

CXCR2
(CXCR2)
Relative Luminescence Units (x100)
40000
30000
20000
10000
0
-12
-10
-8
-6
Log[IL-8]

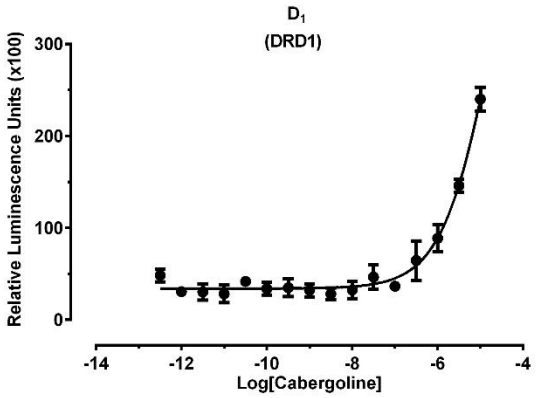

CXCR2
(CXCR2)
CXCL6
CXCL8
Relative Luminescence Unit (x100)
60000
40000
20000
0
-14
-12
-10
-8
-6
-4
Log[Drug]

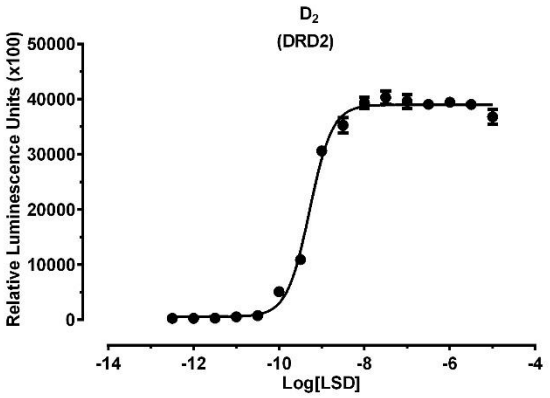

CXCR4
(CXCR4)
Relative Luminescence Unit (x100)
50000
40000
30000
20000
10000
0
-16
-14
-12
-10
-8
-6
Log[SDF-1α]

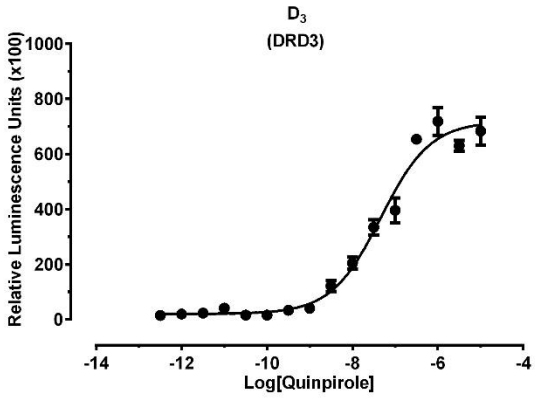

CXCR6
(CXCR6)
Relative Luminescence Units (x100)
6000
4000
2000
0
-16
-14
-12
-10
-8
-6
Log[CXCL16]

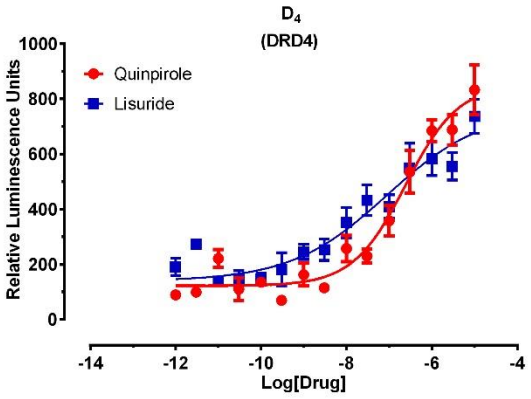

ACKR3
(ACKR3, CXCR7, GPR159)
Relative Luminescence Units
10000
8000
6000
4000
2000
0
-16
-14
-12
-10
-8
-6
Log[SDF-1]

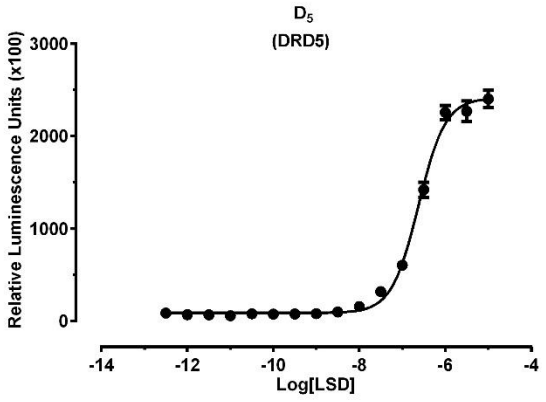

CX3CR1
(CX3CR1)
Relative Luminescence Unit (x100)
40000
30000
20000
10000
0
-16
-14
-12
-10
-8
-6
Log[CX3CL1]

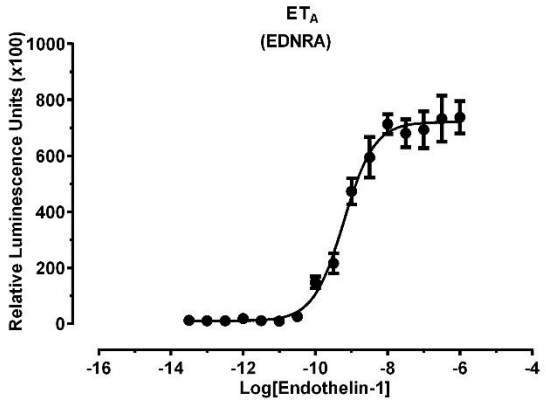

D1
(DRD1)
Relative Luminescence Units (x100)
300
200
100
0
-14
-12
-10
-8
-6
-4
Log[Cabergoline]

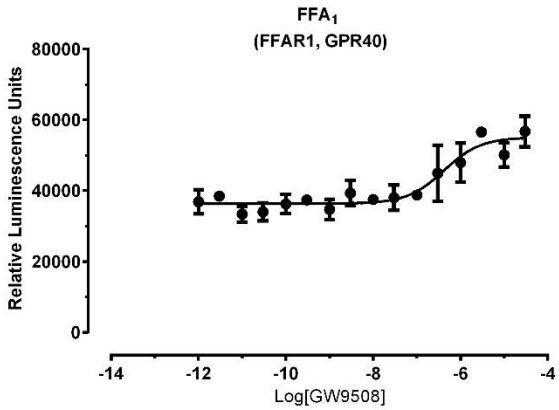

D2
(DRD2)
Relative Luminescence Units (x100)
50000
40000
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[LSD]

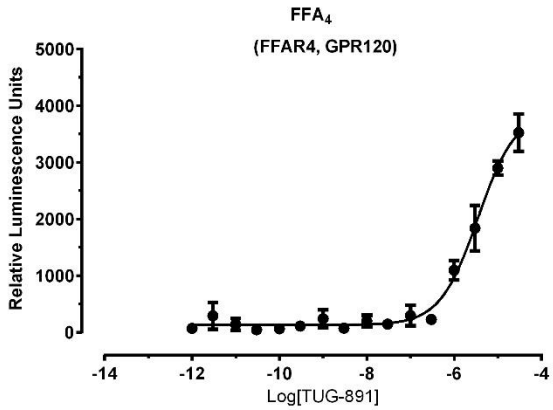

D3
(DRD3)
Relative Luminescence Units (x100)
1000
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Quinpirole]

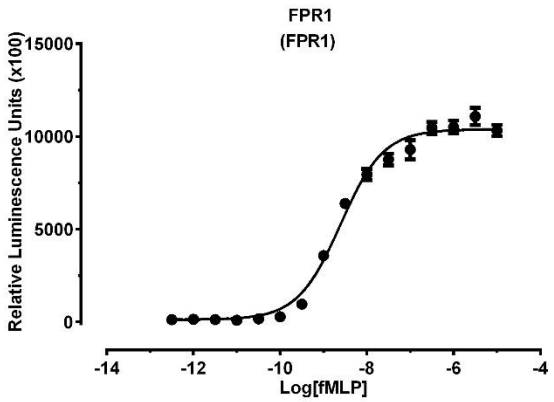

D4
(DRD4)
Quinpirole
Lisuride
Relative Luminescence Units
1000
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Drug]

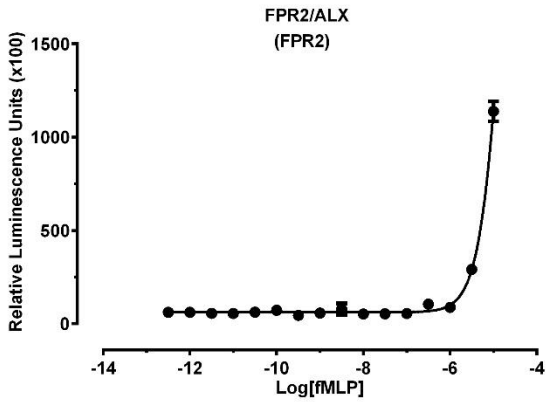

D5
(DRD5)
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[LSD]

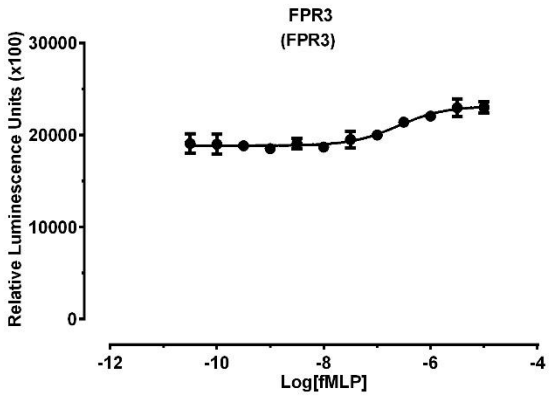

ETA
(EDNRA)
Relative Luminescence Units (x100)
1000
800
600
400
200
0
-16
-14
-12
-10
-8
-6
-4
Log[Endothelin-1]

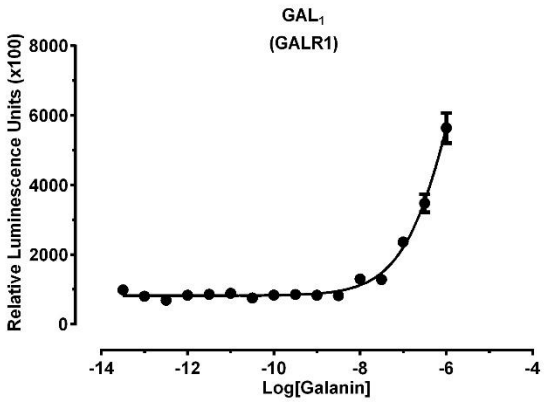

FFA1
(FFAR1, GPR40)
Relative Luminescence Units
80000
60000
40000
20000
0
-14
-12
-10
-8
-6
-4
Log[GW9508]

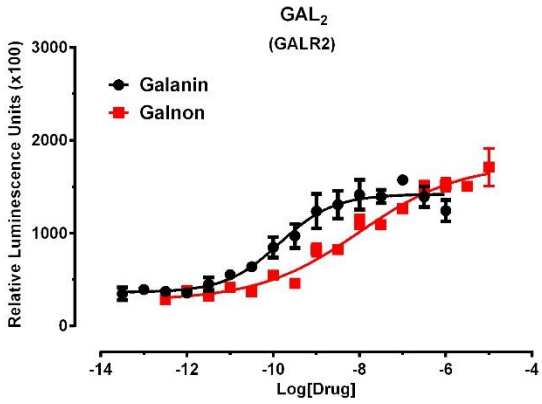

FFA4
(FFAR4, GPR120)
Relative Luminescence Units
5000
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[TUG-891]

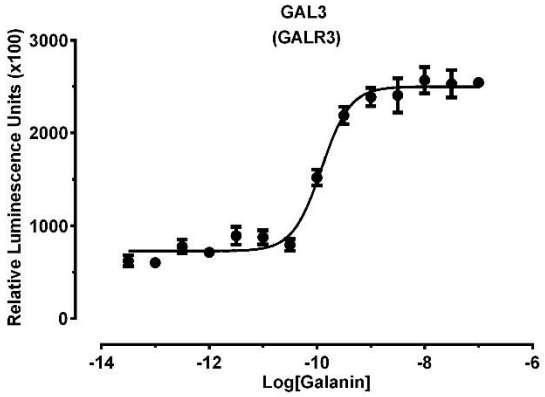

FPR1
(FPR1)
Relative Luminescence Units (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[fMLP]

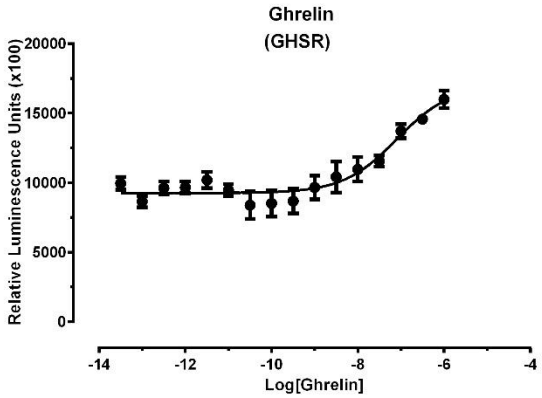

FPR2/ALX
(FPR2)
Relative Luminescence Units (x100)
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[fMLP]

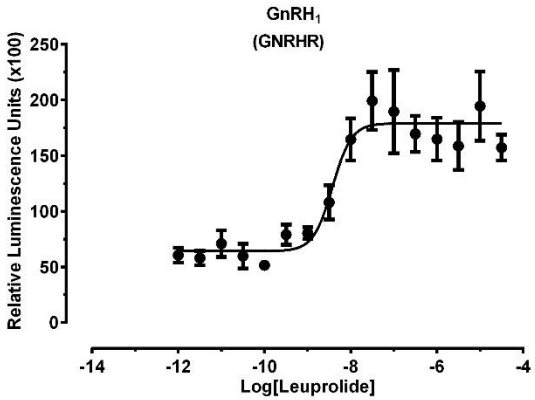

FPR3
(FPR3)
Relative Luminescence Units (x100)
30000
20000
10000
0
-12
-10
-8
-6
-4
Log[fMLP]

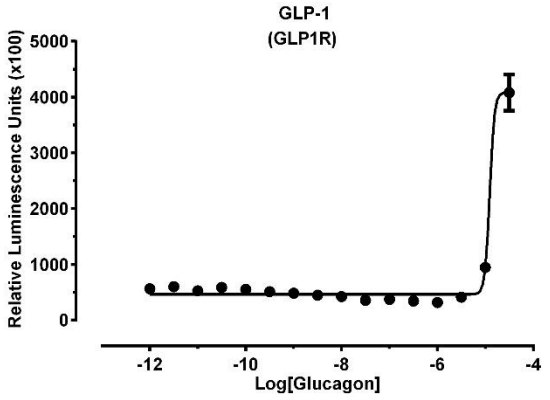

GAL1
(GALR1)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Galanin]

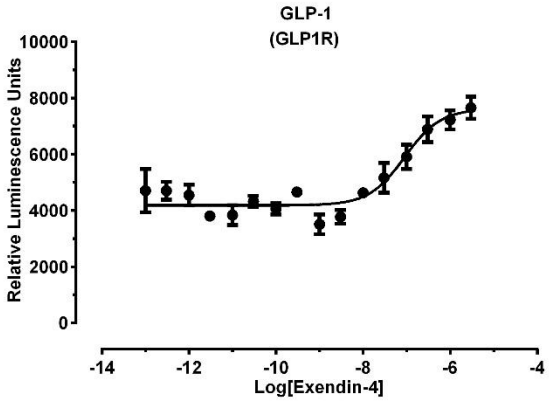

GAL2
(GALR2)
Galanin
Galnon
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Drug]

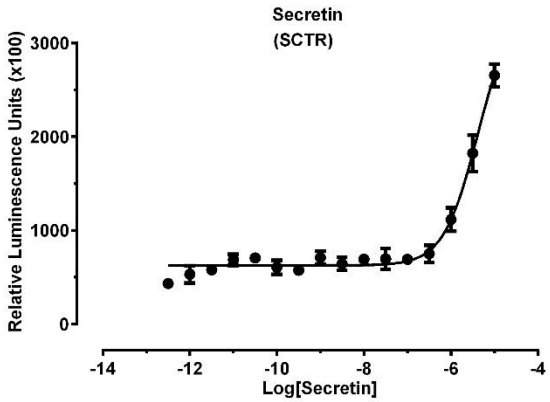

GAL3
(GALR3)
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
Log[Galanin]

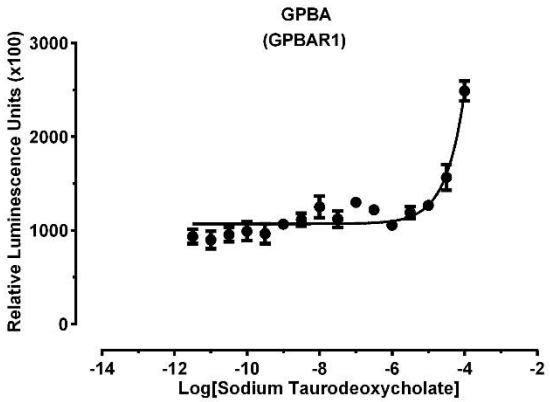

Ghrelin
(GHSR)
Relative Luminescence Units (x100)
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Ghrelin]

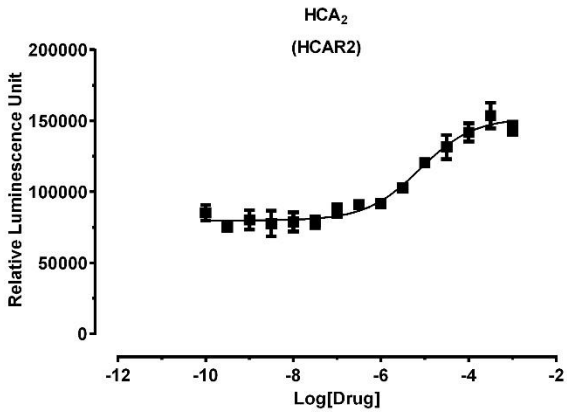

GnRH1
(GNRHR)
Relative Luminescence Units (x100)
250
200
150
100
50
0
-14
-12
-10
-8
-6
-4
Log[Leuprolide]

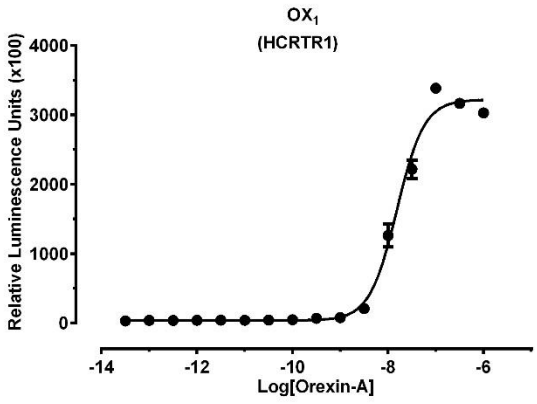

GLP-1
(GLP1R)
Relative Luminescence Units (x100)
5000
4000
3000
2000
1000
0
-12
-10
-8
-6
-4
Log[Glucagon]

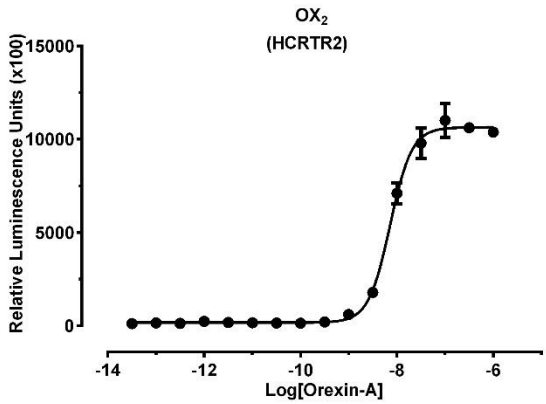

GLP-1
(GLP1R)
Relative Luminescence Units
10000
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Exendin-4]

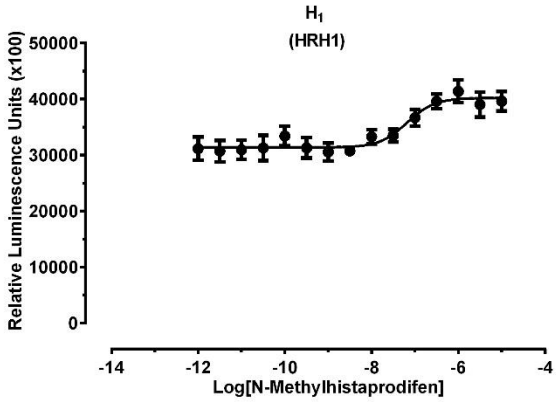

Secretin
(SCTR)
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Secretin]

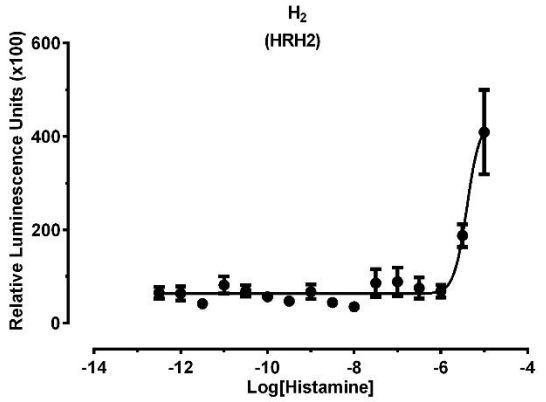

GPBA
(GPBAR1)
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
-2
Log[Sodium Taurodeoxycholate]

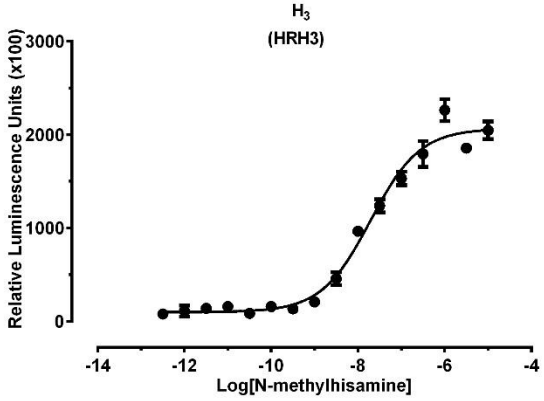

HCA2
(HCAR2)
Relative Luminescence Unit
200000
150000
100000
50000
0
-12
-10
-8
-6
-4
-2
Log[Drug]

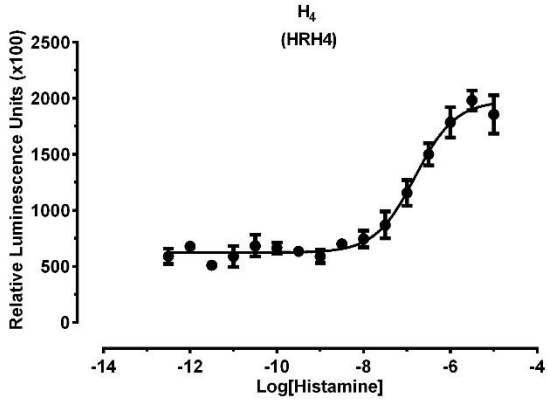

OX1
(HCRTR1)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
Log[Orexin-A]

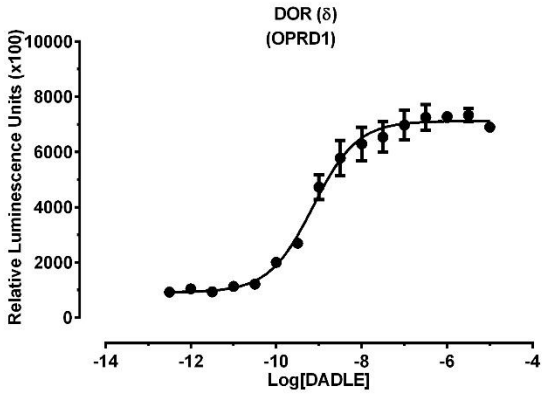

OX2
(HCRTR2)
Relative Luminescence Units (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
Log[Orexin-A]

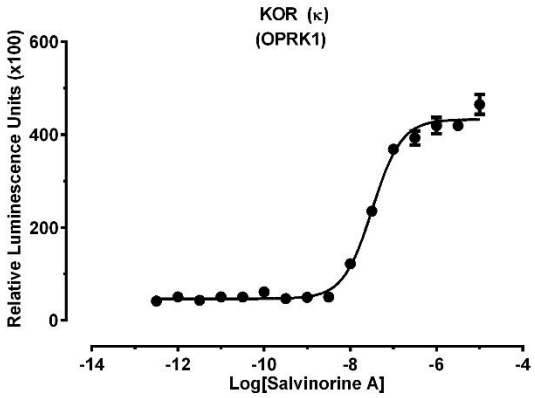

H1
(HRH1)
Relative Luminescence Units (x100)
50000
40000
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[N-Methylhistaprodifen]

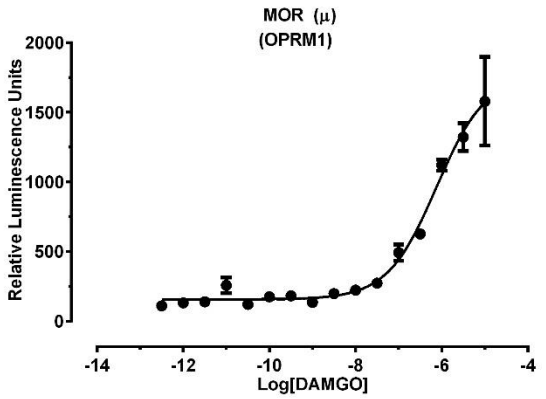

H2
(HRH2)
Relative Luminescence Units (x100)
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Histamine]

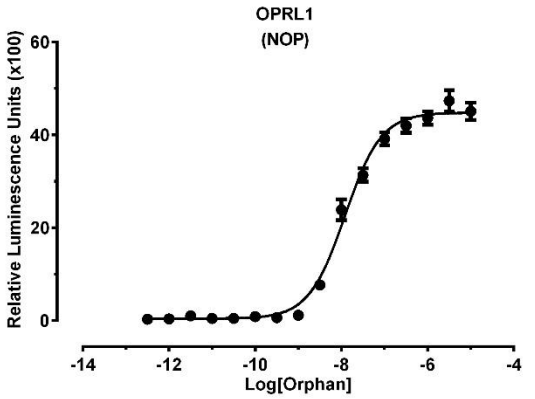

H3
(HRH3)
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[N-methylhisamine]

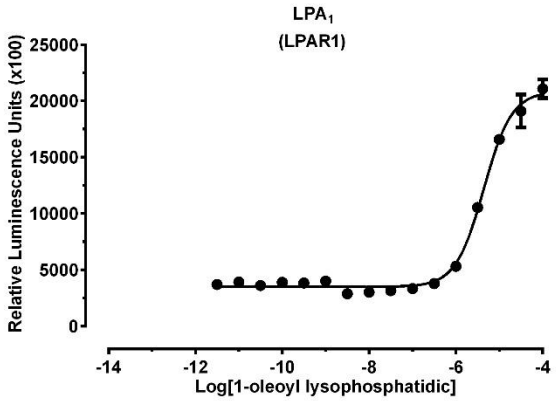

H4
(HRH4)
Relative Luminescence Units (x100)
2500
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Histamine]

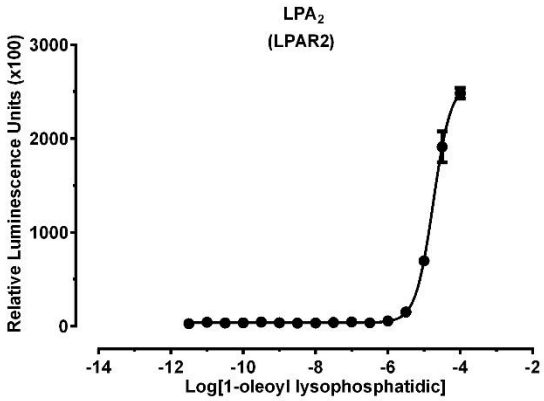

DOR (δ)
(OPRD1)
Relative Luminescence Units (x100)
10000
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[DADLE]

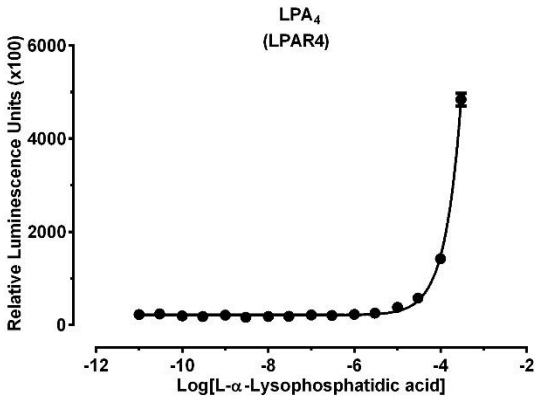

KOR (κ)
(OPRK1)
Relative Luminescence Units (x100)
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Salvinorine A]

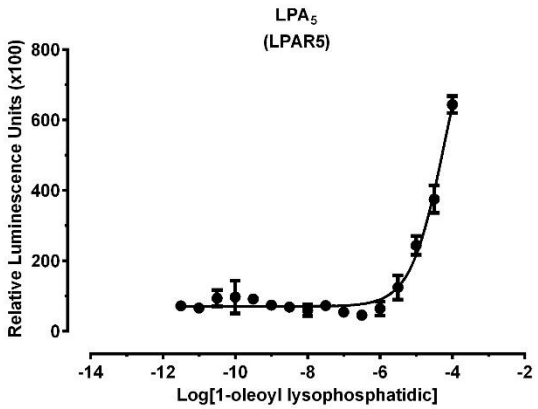

MOR (μ)
(OPRM1)
Relative Luminescence Units
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[DAMGO]

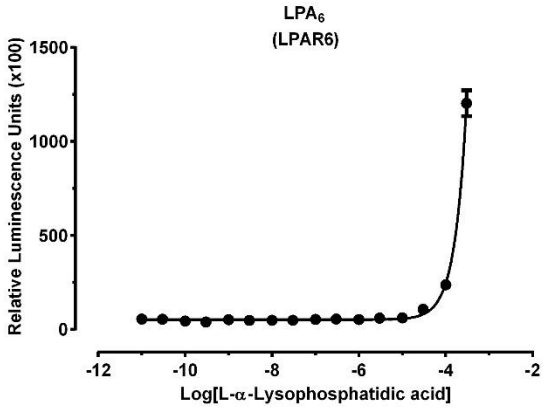

OPRL1
(NOP)
Relative Luminescence Units (x100)
60
40
20
0
-14
-12
-10
-8
-6
-4
Log[Orphan]

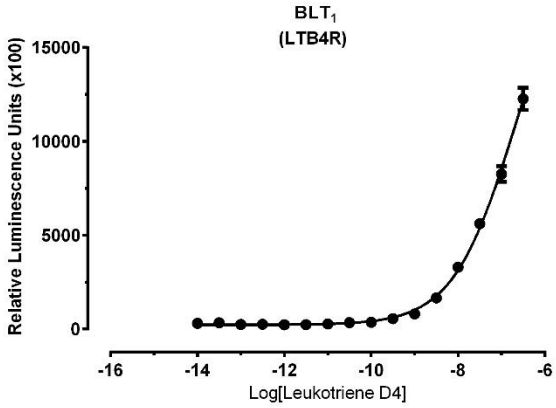

LPA1
(LPAR1)
Relative Luminescence Units (x100)
25000
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[1-oleoyl lysophosphatidic]

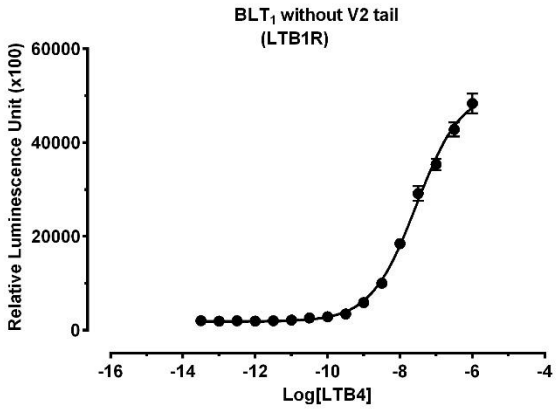

LPA2
(LPAR2)
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
-2
Log[1-oleoyl lysophosphatidic]

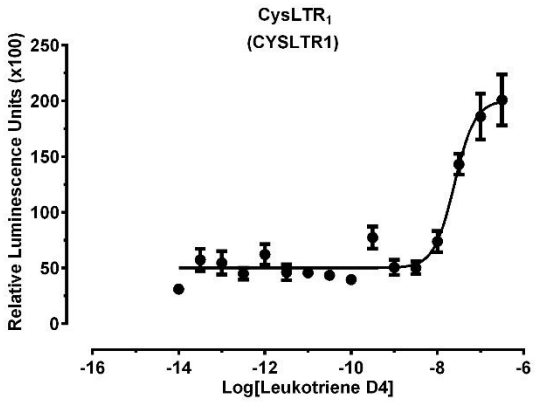

LPA4
(LPAR4)
Relative Luminescence Units (x100)
6000
4000
2000
0
-12
-10
-8
-6
-4
-2
Log[L-α-Lysophosphatidic acid]

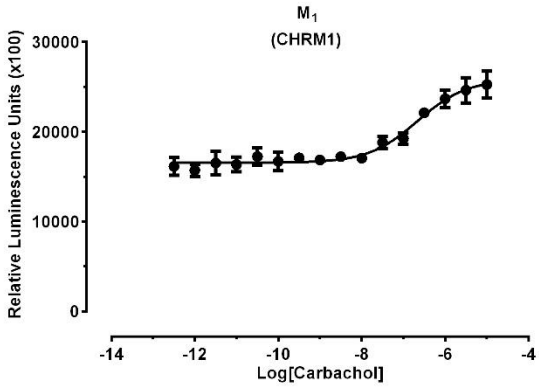

LPA5
(LPAR5)
Relative Luminescence Units (x100)
800
600
400
200
0
-14
-12
-10
-8
-6
-4
-2
Log[1-oleoyl lysophosphatidic]

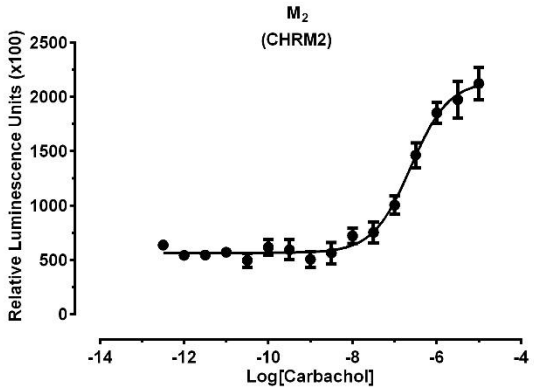

LPA6
(LPAR6)
Relative Luminescence Units (x100)
1500
1000
500
0
-12
-10
-8
-6
-4
-2
Log[L-α-Lysophosphatidic acid]

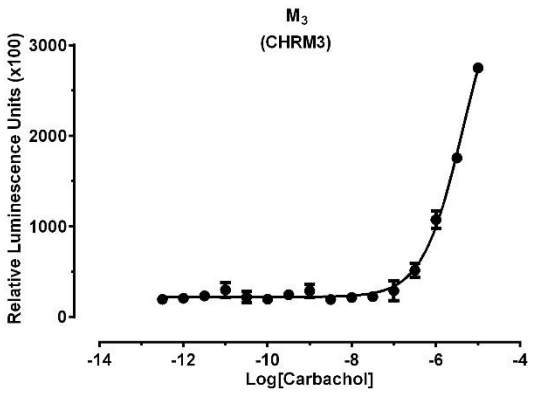

BLT1
(LTB4R)
Relative Luminescence Units (x100)
15000
10000
5000
0
-16
-14
-12
-10
-8
-6
Log[Leukotriene D4]

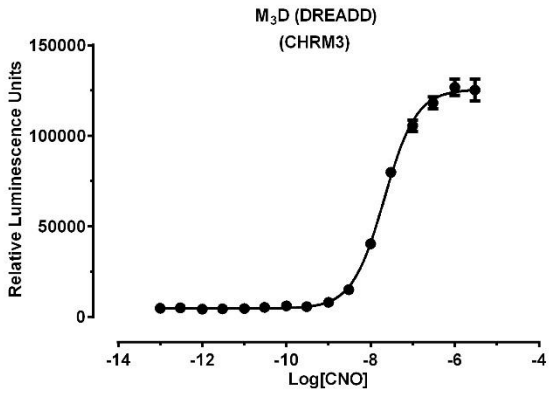

BLT1 without V2 tail
(LTB1R)
Relative Luminescence Unit (x100)
60000
40000
20000
0
-16
-14
-12
-10
-8
-6
-4
Log[LTB4]

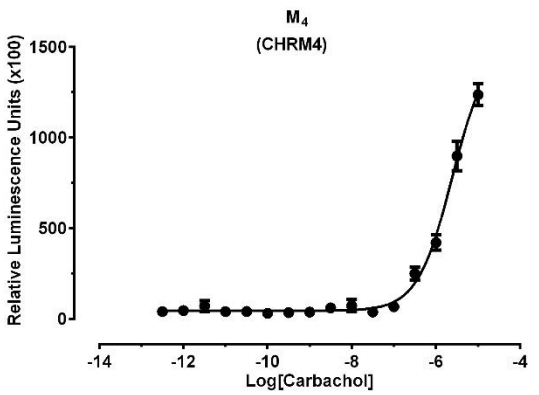

CysLTR1
(CYSLTR1)
Relative Luminescence Units (x100)
250
200
150
100
50
0
-16
-14
-12
-10
-8
-6
Log[Leukotriene D4]

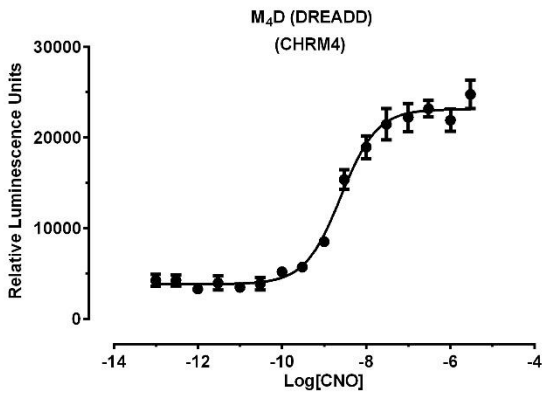

M1
(CHRM1)
Relative Luminescence Units (x100)
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[Carbachol]

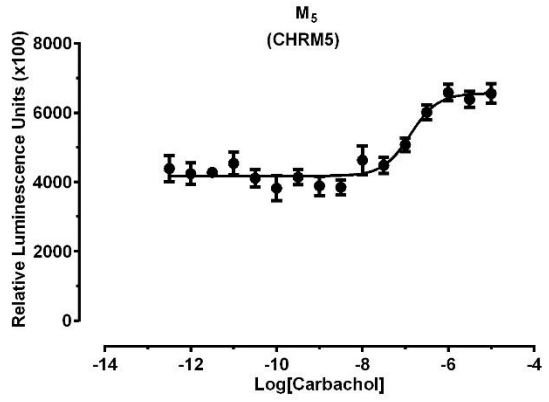

M2
(CHRM2)
Relative Luminescence Units (x100)
2500
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Carbachol]

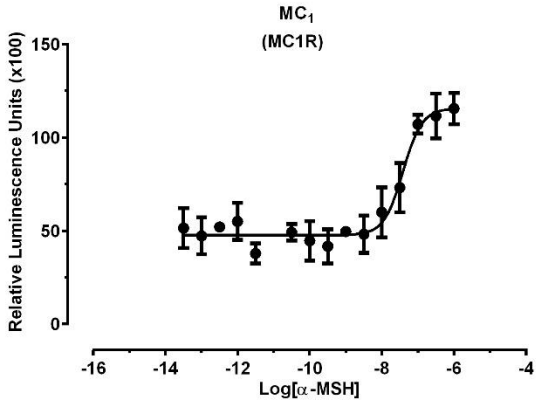

M3
(CHRM3)
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Carbachol]

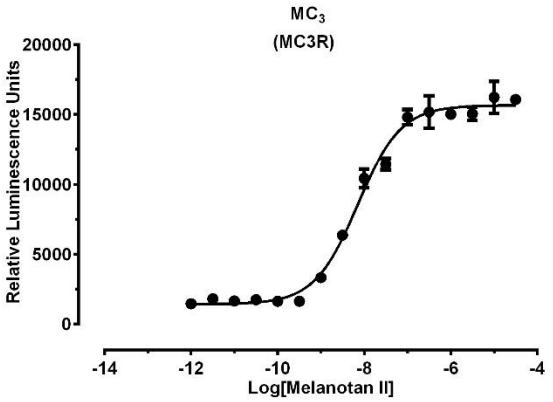

M3D (DREADD)
(CHRM3)
Relative Luminescence Units
150000
100000
50000
0
-14
-12
-10
-8
-6
-4
Log[CNO]

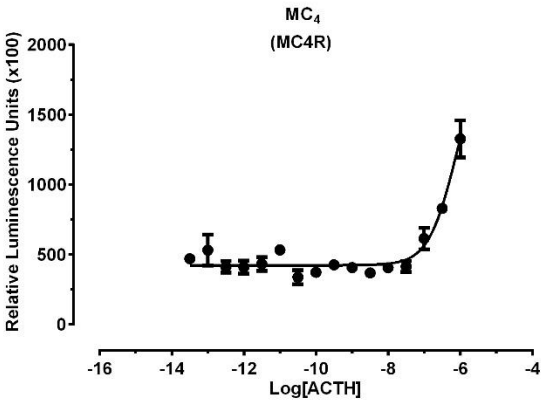

M4
(CHRM4)
Relative Luminescence Units (x100)
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Carbachol]

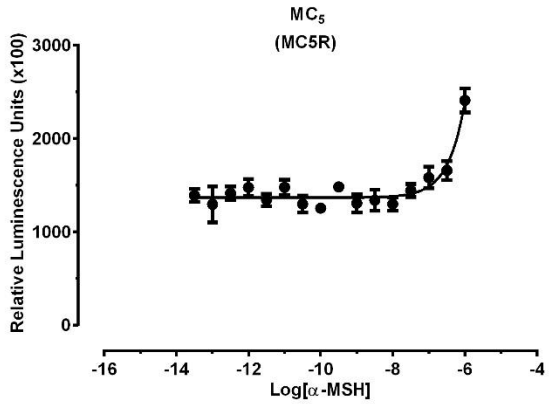

M4D (DREADD)
(CHRM4)
Relative Luminescence Units
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[CNO]

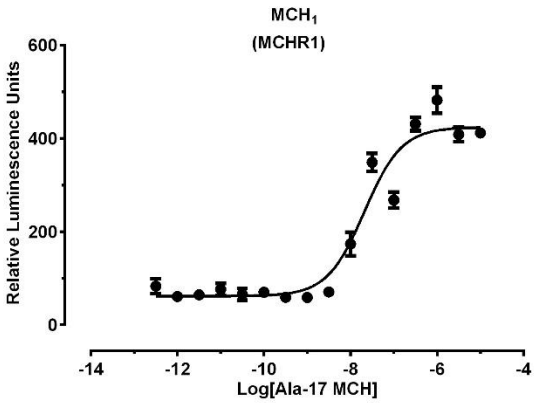

M5
(CHRM5)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Carbachol]

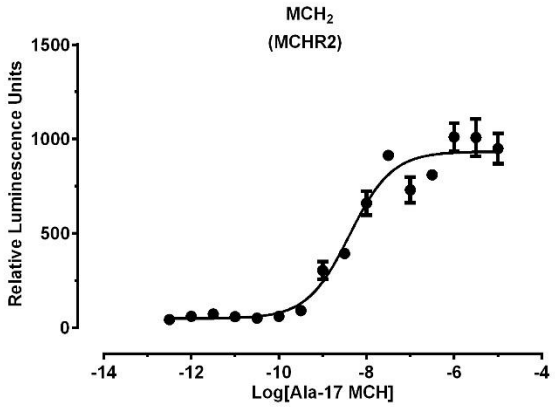

MC1
(MC1R)
Relative Luminescence Units (x100)
150
100
50
0
-16
-14
-12
-10
-8
-6
-4
Log[α-MSH]

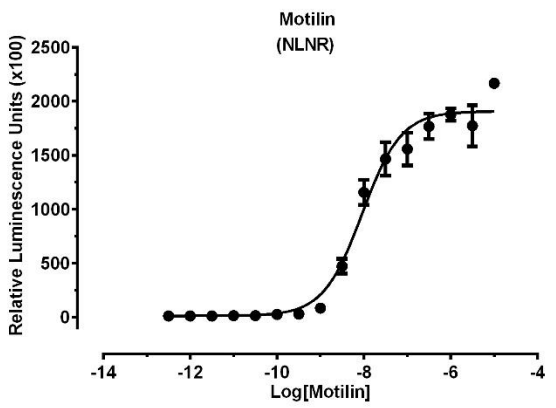

MC3
(MC3R)
Relative Luminescence Units
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Melanotan II]

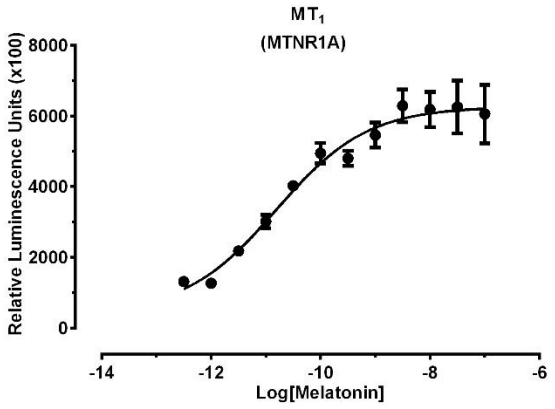

MC4
(MC4R)
Relative Luminescence Units (x100)
2000
1500
1000
500
0
-16
-14
-12
-10
-8
-6
-4
Log[ACTH]

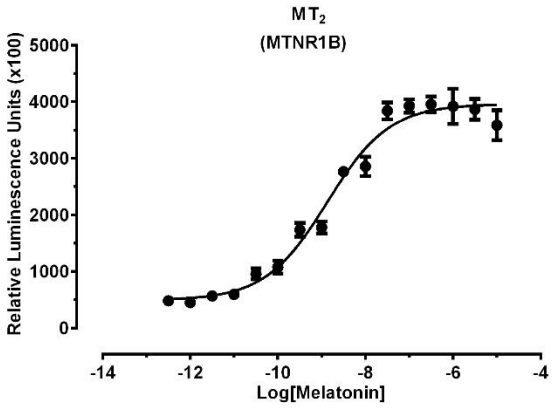

MC5
(MC5R)
Relative Luminescence Units (x100)
3000
2000
1000
0
-16
-14
-12
-10
-8
-6
-4
Log[α-MSH]

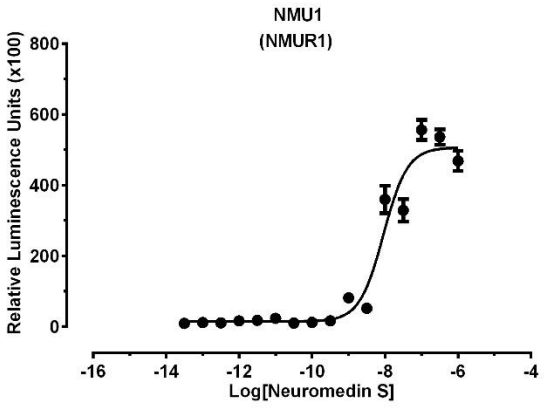

MCH1
(MCHR1)
Relative Luminescence Units
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Ala-17 MCH]

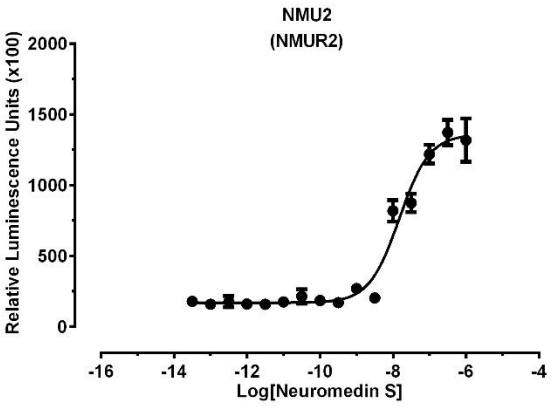

MCH2
(MCHR2)
Relative Luminescence Units
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Ala-17 MCH]

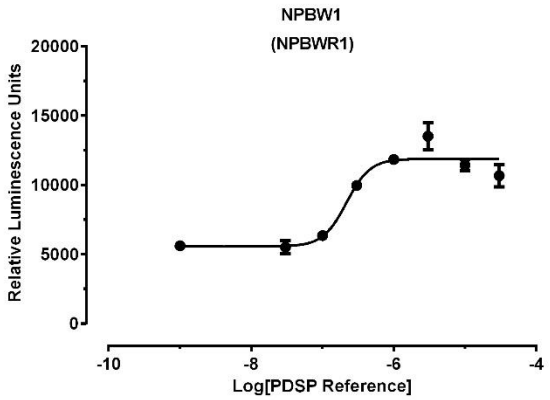

Motilin
(NLNR)
Relative Luminescence Units (x100)
2500
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Motilin]

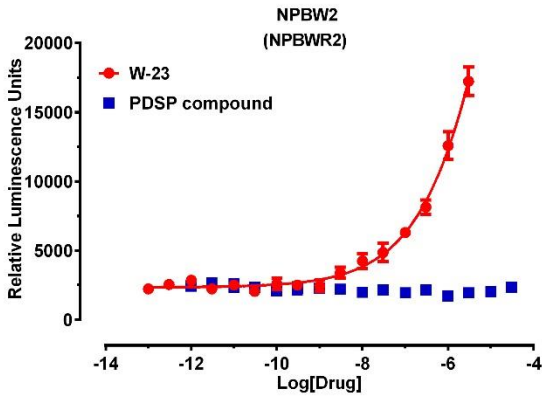

MT1
(MTNR1A)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
Log[Melatonin]

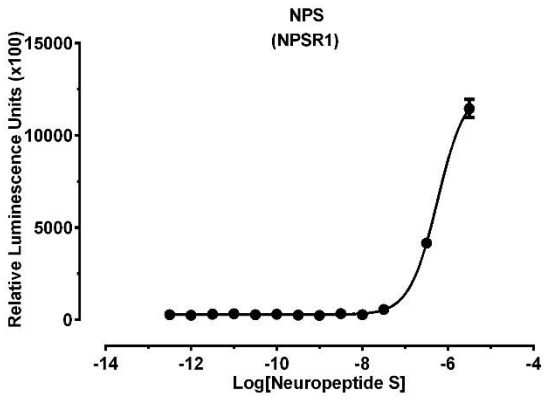

MT2
(MTNR1B)
Relative Luminescence Units (x100)
5000
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Melatonin]

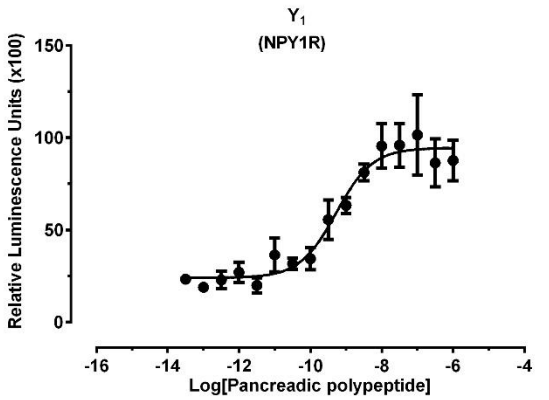

NMU1
(NMUR1)
Relative Luminescence Units (x100)
800
600
400
200
0
-16
-14
-12
-10
-8
-6
-4
Log[Neuromedin S]

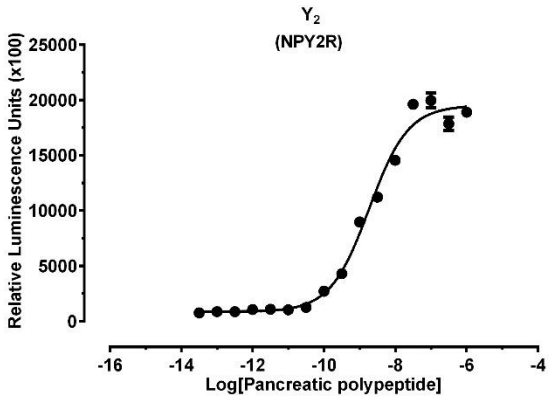

NMU2
(NMUR2)
Relative Luminescence Units (x100)
2000
1500
1000
500
0
-16
-14
-12
-10
-8
-6
-4
Log[Neuromedin S]

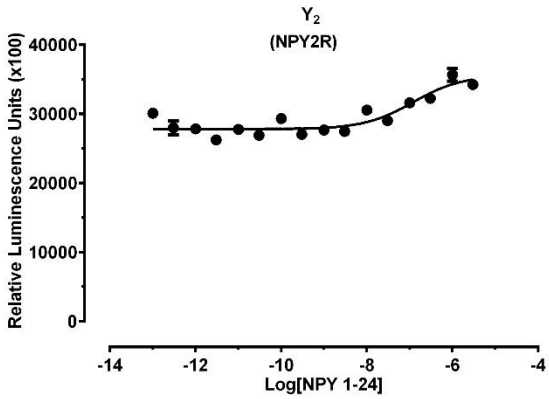

NPBW1
(NPBWR1)
Relative Luminescence Units
20000
15000
10000
5000
0
-10
-8
-6
-4
Log[PDSP Reference]

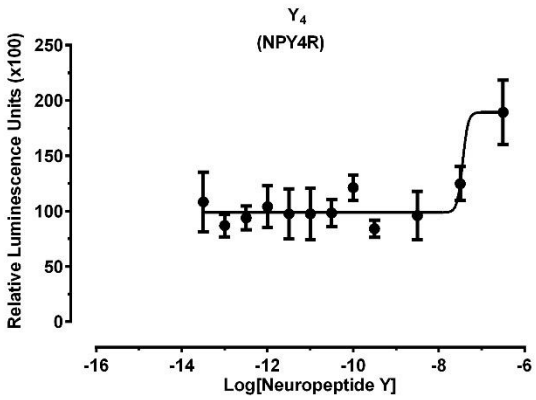

NPBW2
(NPBWR2)
W-23
PDSP compound
Relative Luminescence Units
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Drug]

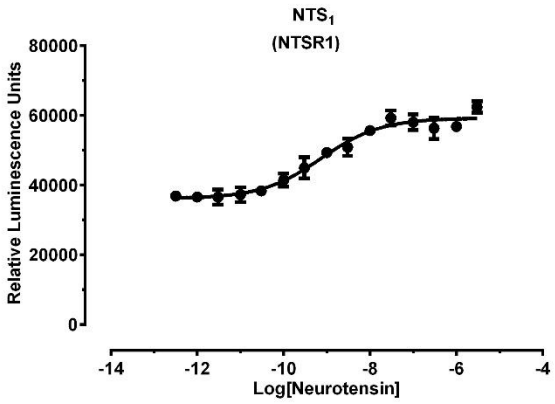

NPS
(NPSR1)
Relative Luminescence Units (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Neuropeptide S]

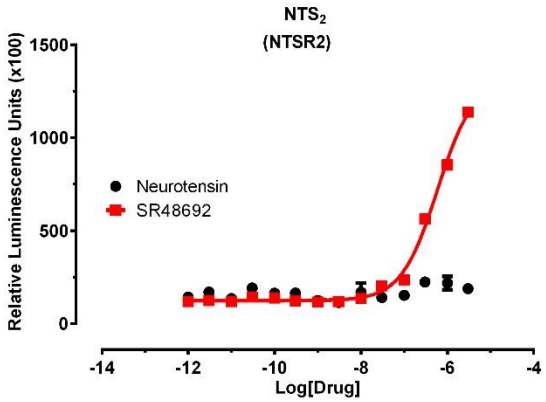

Y1
(NPY1R)
Relative Luminescence Units (x100)
150
100
50
0
-16
-14
-12
-10
-8
-6
-4
Log[Pancreadic polypeptide]

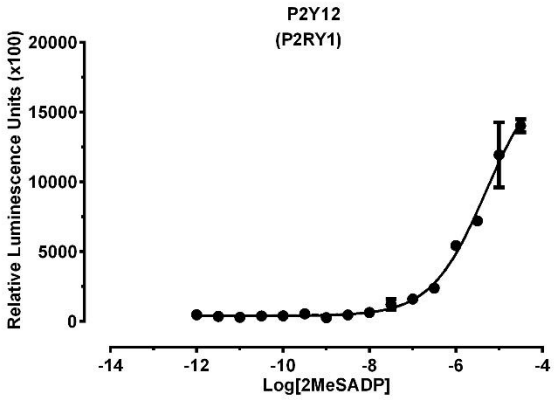

Y2
(NPY2R)
Relative Luminescence Units (x100)
25000
20000
15000
10000
5000
0
-16
-14
-12
-10
-8
-6
-4
Log[Pancreatic polypeptide]

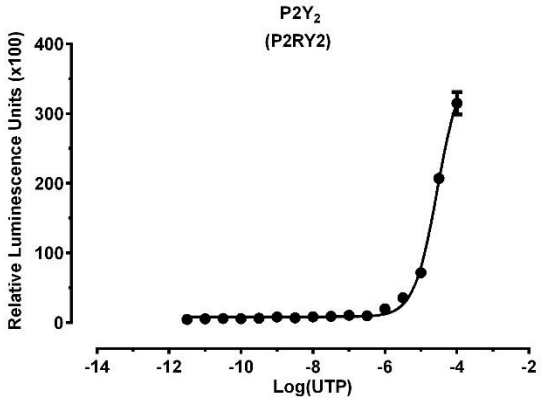

Y2
(NPY2R)
Relative Luminescence Units (x100)
40000
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[NPY 1-24]

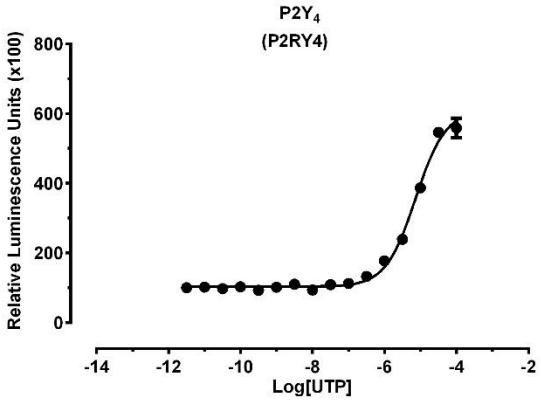

Y4
(NPY4R)
Relative Luminescence Units (x100)
250
200
150
100
50
0
-16
-14
-12
-10
-8
-6
Log[Neuropeptide Y]

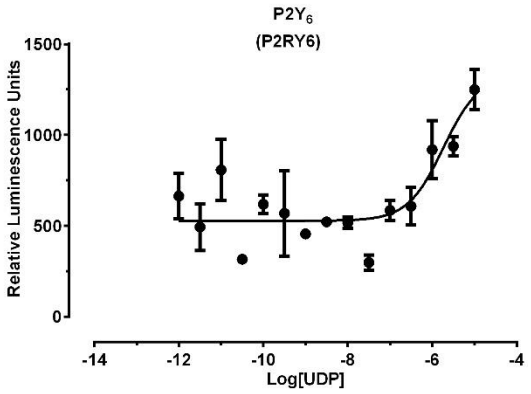

NTS1
(NTSR1)
Relative Luminescence Units
80000
60000
40000
20000
0
-14
-12
-10
-8
-6
-4
Log[Neurotensin]

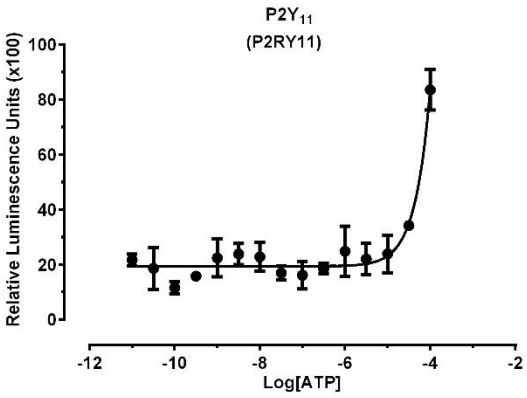

NTS2
(NTSR2)
Relative Luminescence Units (x100)
1500
1000
500
0
Neurotensin
SR48692
-14
-12
-10
-8
-6
-4
Log[Drug]

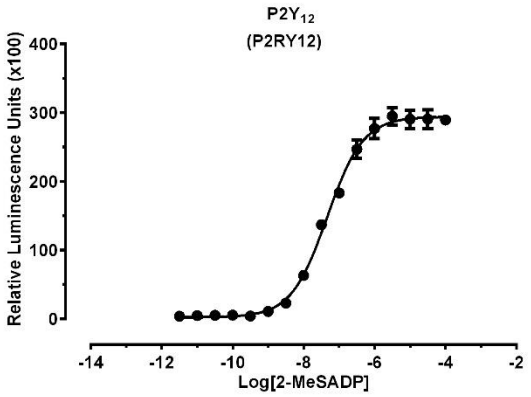

P2Y12
(P2RY1)
Relative Luminescence Units (x100)
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[2MeSADP]

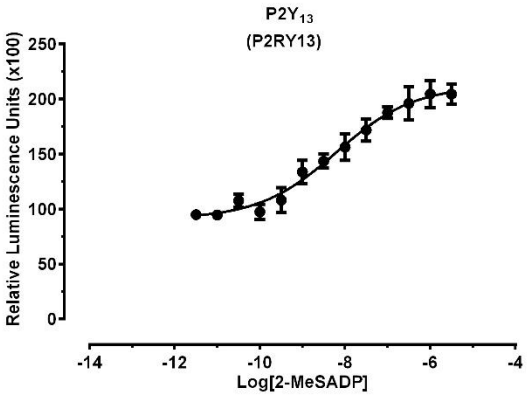

P2Y2
(P2RY2)
Relative Luminescence Units (x100)
400
300
200
100
0
-14
-12
-10
-8
-6
-4
-2
Log(UTP)

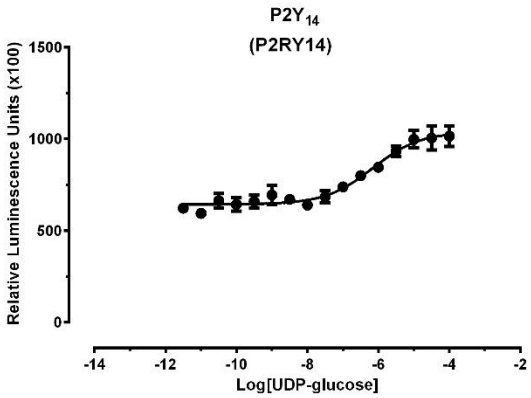

P2Y4
(P2RY4)
Relative Luminescence Units (x100)
800
600
400
200
0
-14
-12
-10
-8
-6
-4
-2
Log[UTP]

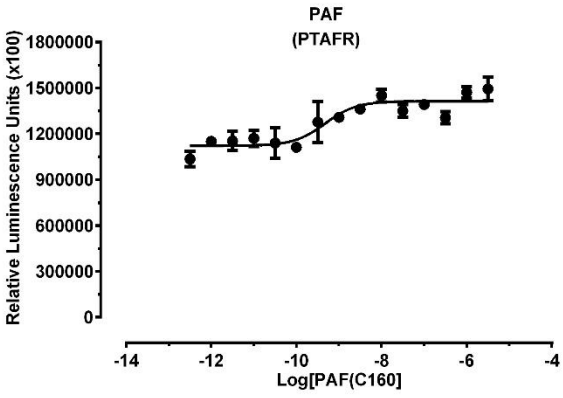

P2Y6
(P2RY6)
Relative Luminescence Units
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[UDP]

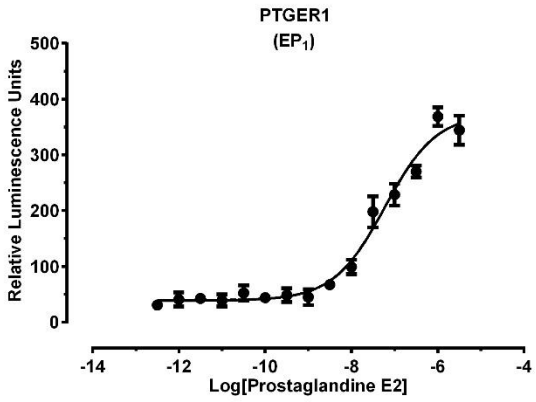

P2Y11
(P2RY11)
Relative Luminescence Units (x100)
100
80
60
40
20
0
-12
-10
-8
-6
-4
-2
Log[ATP]

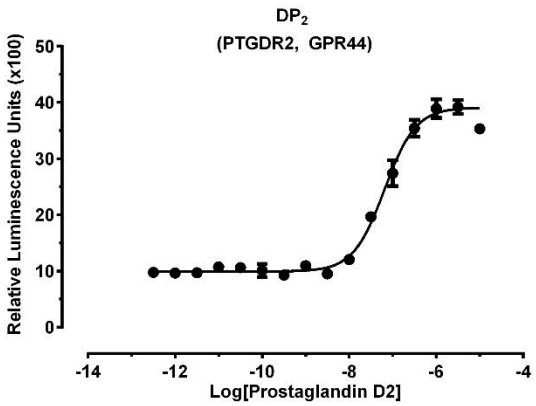

P2Y12
(P2RY12)
Relative Luminescence Units (x100)
400
300
200
100
0
-14
-12
-10
-8
-6
-4
-2
Log[2-MeSADP]

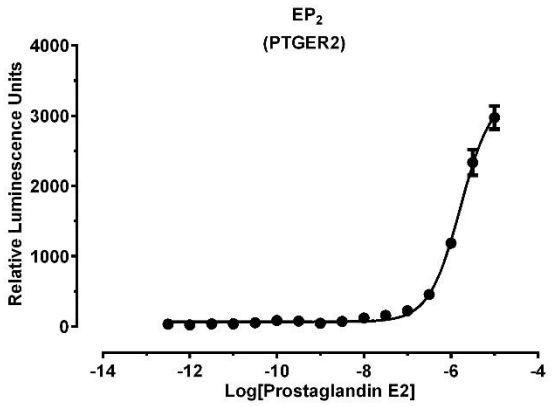

P2Y13
(P2RY13)
Relative Luminescence Units (x100)
250
200
150
100
50
0
-14
-12
-10
-8
-6
-4
Log[2-MeSADP]

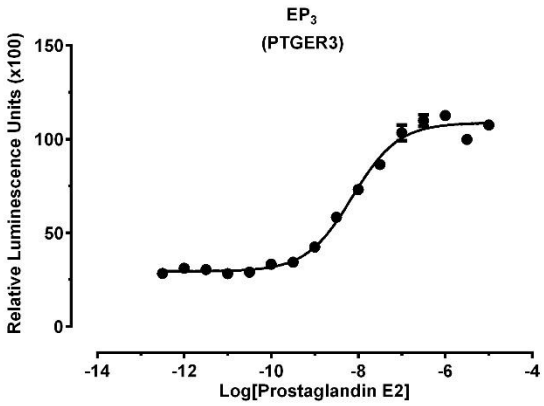

P2Y14
(P2RY14)
Relative Luminescence Units (x100)
1500
1000
500
0
-14
-12
-10
-8
-6
-4
-2
Log[UDP-glucose]

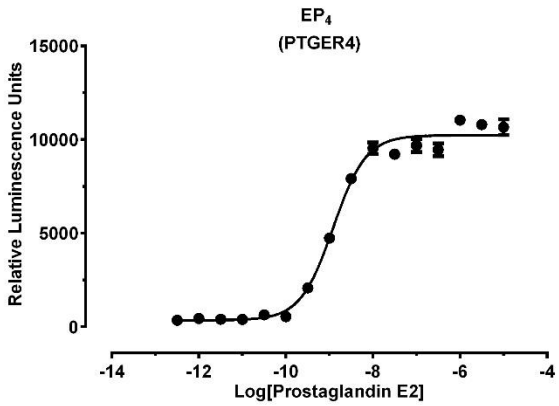

PAF
(PTAFR)
Relative Luminescence Units (x100)
1800000
1500000
1200000
900000
600000
300000
0
-14
-12
-10
-8
-6
-4
Log[PAF(C160]

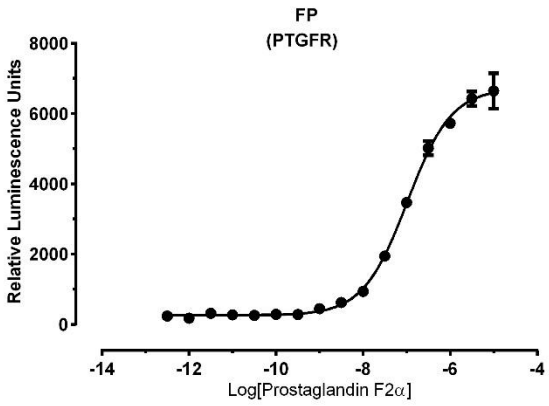

PTGER1
(EP1)
Relative Luminescence Units
500
400
300
200
100
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandine E2]

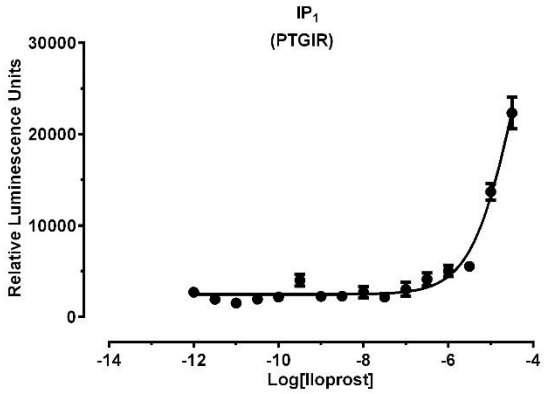

DP2
(PTGDR2, GPR44)
Relative Luminescence Units (x100)
50
40
30
20
10
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandin D2]

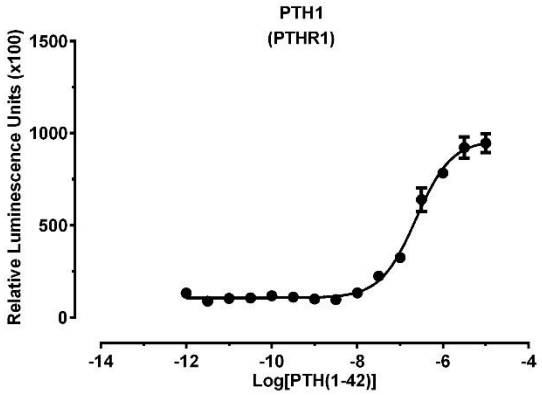

EP2
(PTGER2)
Relative Luminescence Units
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandin E2]

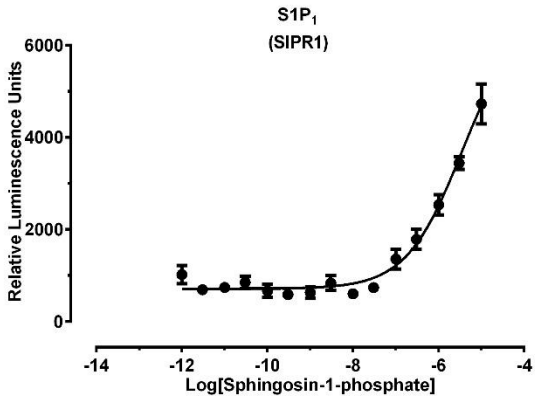

EP3
(PTGER3)
Relative Luminescence Units (x100)
150
100
50
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandin E2]

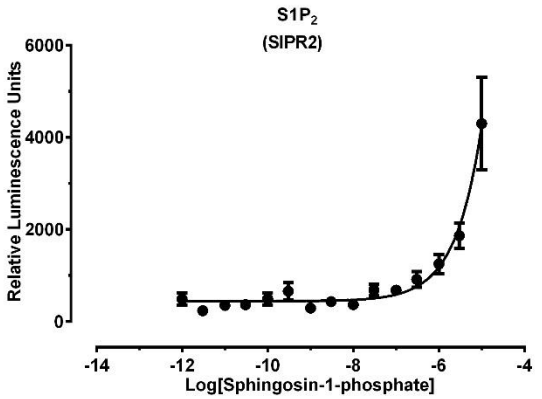

EP4
(PTGER4)
Relative Luminescence Units
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandin E2]

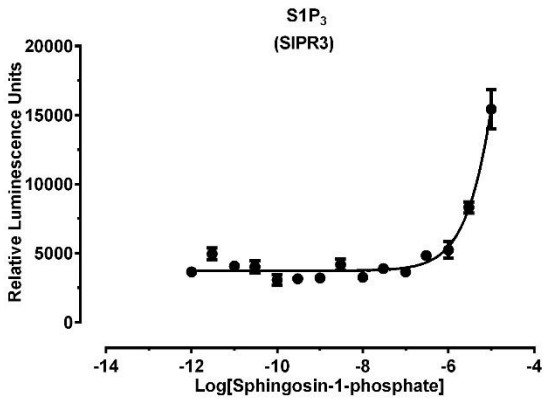

FP
(PTGFR)
Relative Luminescence Units
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandin F2α]

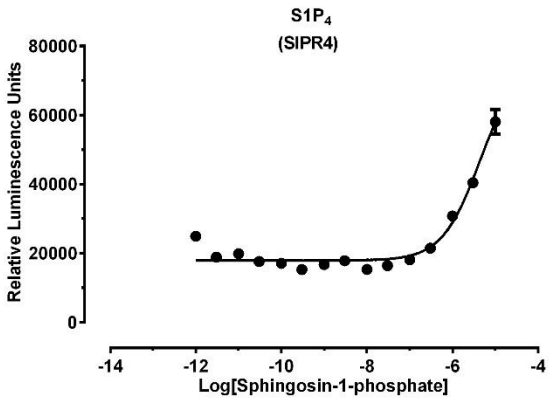

IP1
(PTGIR)
Relative Luminescence Units
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[Iloprost]

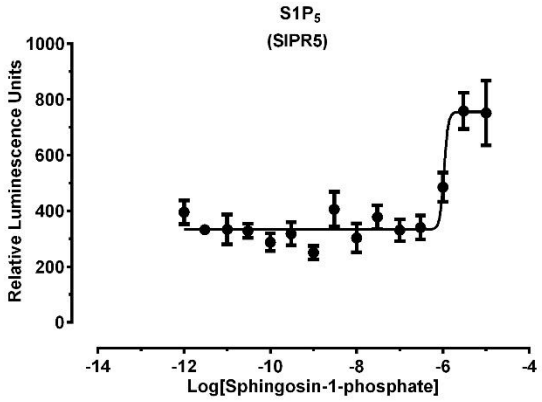

PTH1
(PTHR1)
Relative Luminescence Units (x100)
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[PTH(1-42)]

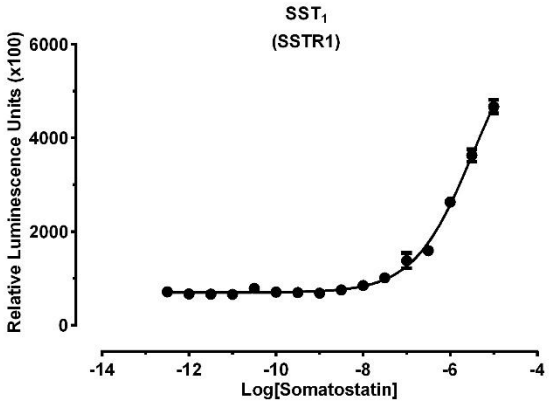

S1P1
(SIPR1)
Relative Luminescence Units
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Sphingosin-1-phosphate]

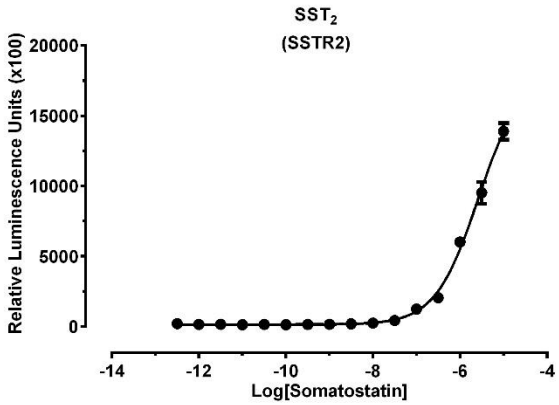

S1P2
(SIPR2)
Relative Luminescence Units
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Sphingosin-1-phosphate]

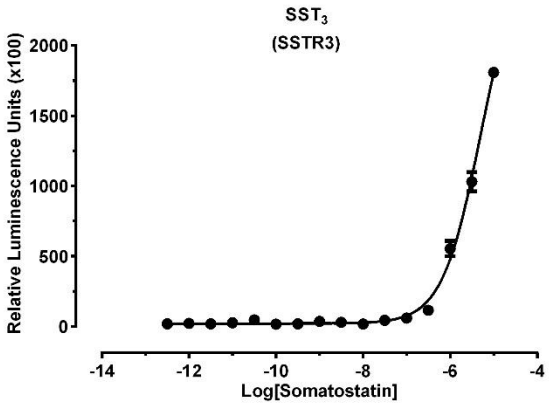

S1P3
(SIPR3)
Relative Luminescence Units
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Sphingosin-1-phosphate]

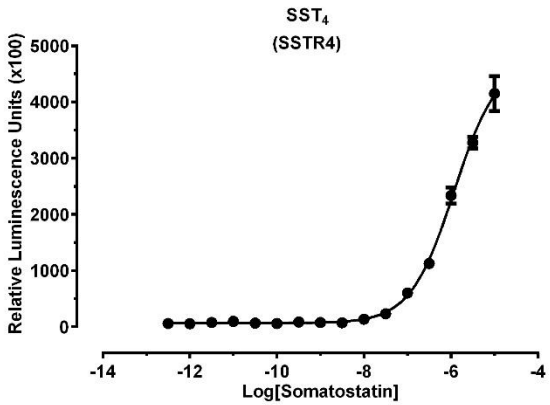

S1P4
(SIPR4)
Relative Luminescence Units
80000
60000
40000
20000
0
-14
-12
-10
-8
-6
-4
Log[Sphingosin-1-phosphate]

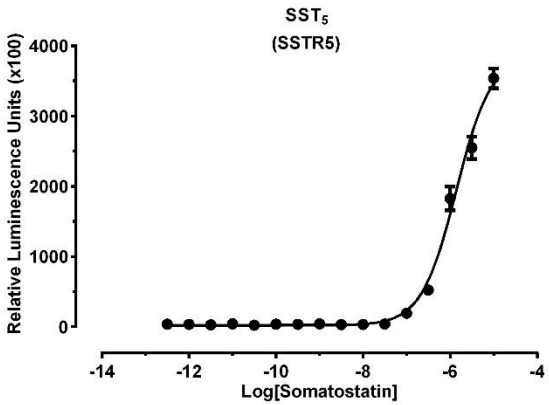

S1P5
(SIPR5)
Relative Luminescence Units
1000
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Sphingosin-1-phosphate]

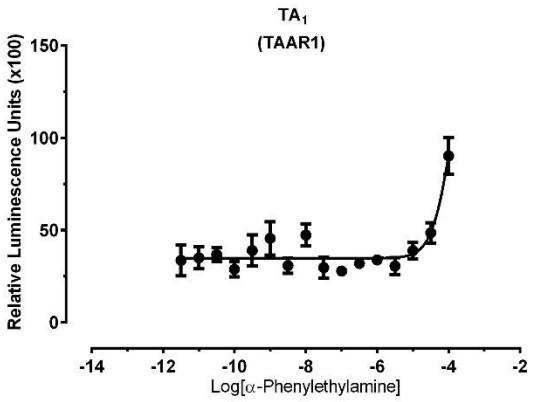

SST1
(SSTR1)
Relative Luminescence Units (x100)
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Somatostatin]

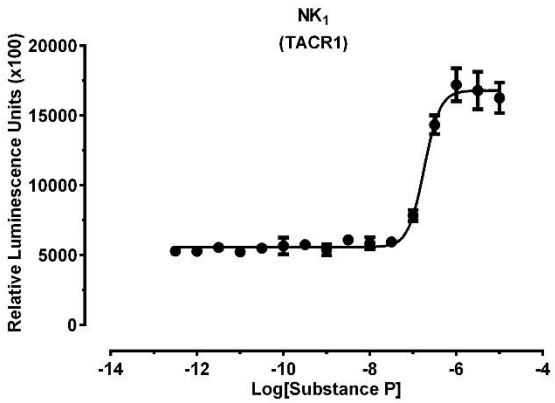

SST2
(SSTR2)
Relative Luminescence Units (x100)
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Somatostatin]

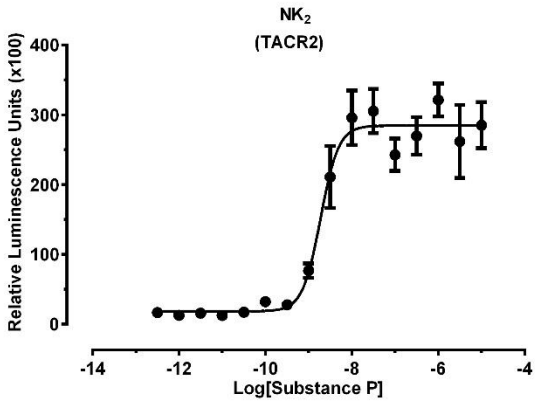

SST3
(SSTR3)
Relative Luminescence Units (x100)
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Somatostatin]

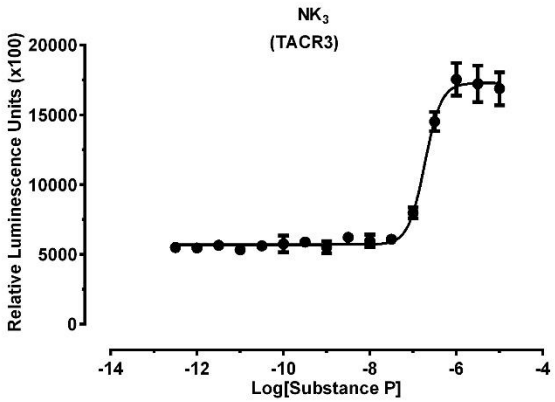

SST4
(SSTR4)
Relative Luminescence Units (x100)
5000
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Somatostatin]

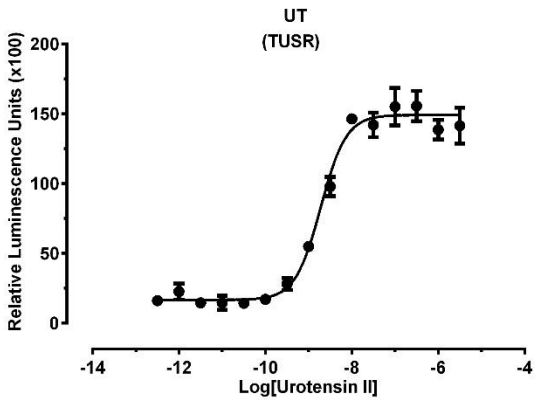

SST5
(SSTR5)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Somatostatin]

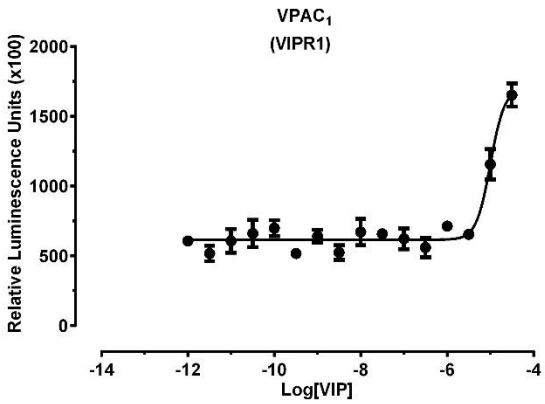

TA1
(TAAR1)
Relative Luminescence Units (x100)
150
100
50
0
-14
-12
-10
-8
-6
-4
-2
Log[α-Phenylethylamine]

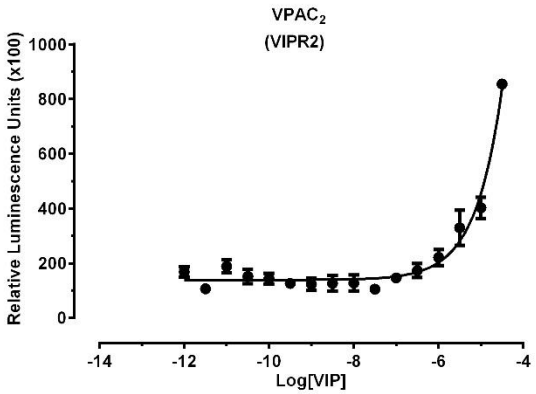

NK1
(TACR1)
Relative Luminescence Units (x100)
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Substance P]

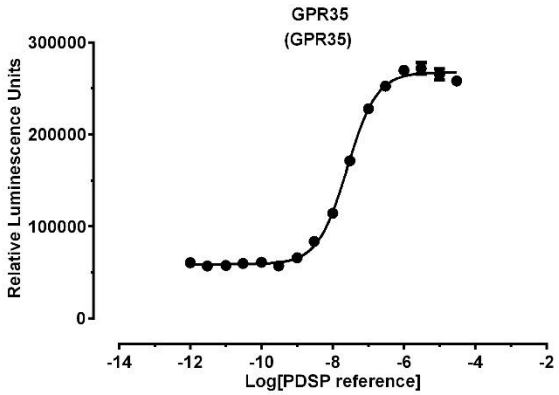

NK2
(TACR2)
Relative Luminescence Units (x100)
400
300
200
100
0
-14
-12
-10
-8
-6
-4
Log[Substance P]

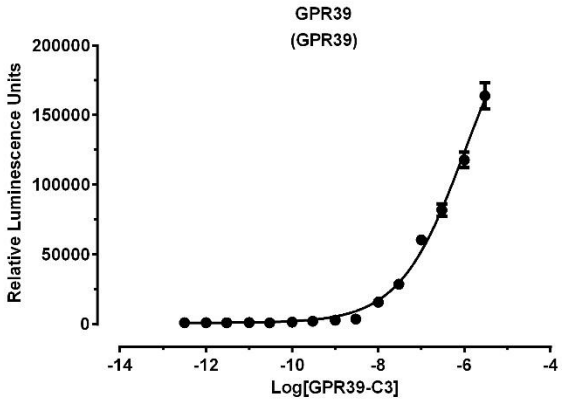

NK3
(TACR3)
Relative Luminescence Units (x100)
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Substance P]

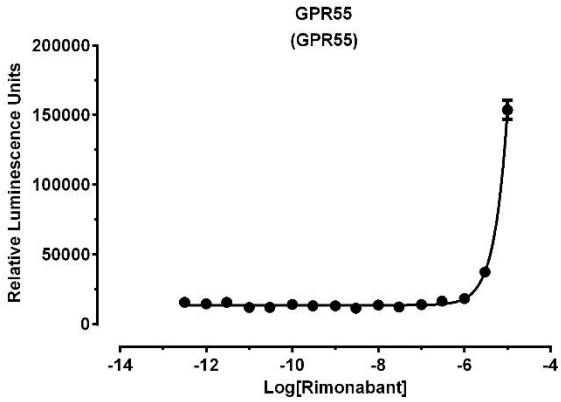

UT
(TUSR)
Relative Luminescence Units (x100)
200
150
100
50
0
-14
-12
-10
-8
-6
-4
Log[Urotensin II]

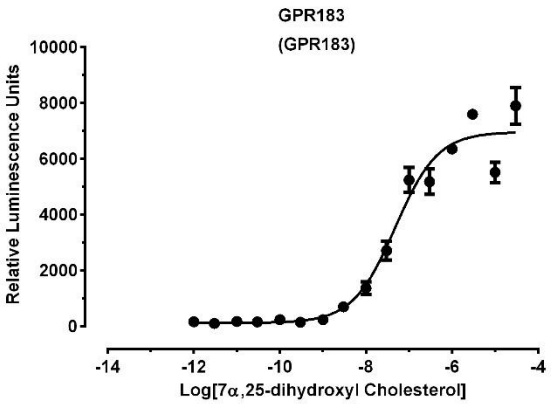

VPAC1
(VIPR1)
Relative Luminescence Units (x100)
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[VIP]

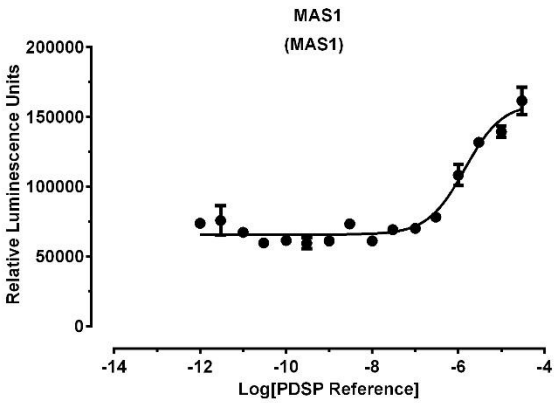

VPAC2
(VIPR2)
Relative Luminescence Units (x100)
1000
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[VIP]

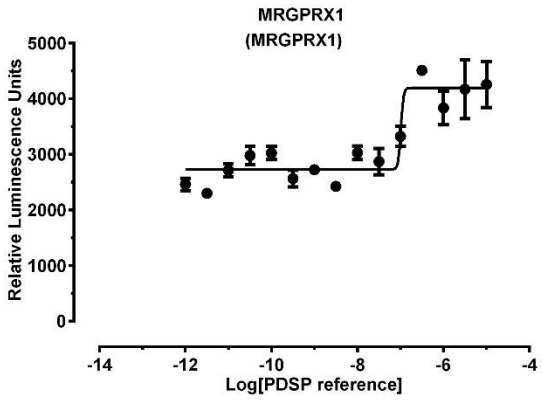

GPR35
(GPR35)
Relative Luminescence Units
300000
200000
100000
0
-14
-12
-10
-8
-6
-4
-2
Log[PDSP reference]

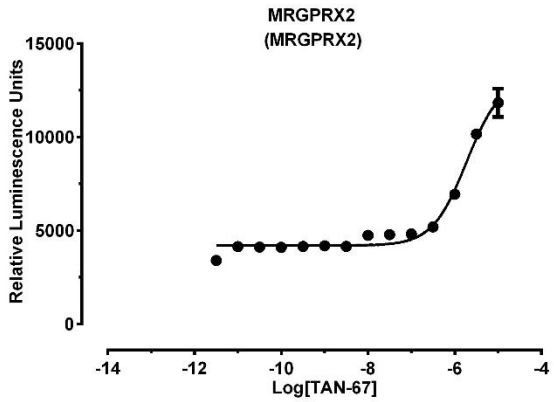

GPR39
(GPR39)
Relative Luminescence Units
200000
150000
100000
50000
0
-14
-12
-10
-8
-6
-4
Log[GPR39-C3]


GPR55
(GPR55)
Relative Luminescence Units
200000
150000
100000
50000
0
-14
-12
-10
-8
-6
-4
Log[Rimonabant]


GPR183
(GPR183)
Relative Luminescence Units
10000
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[7α,25-dihydroxyl Cholesterol]


MAS1
(MAS1)
Relative Luminescence Units
200000
150000
100000
50000
0
-14
-12
-10
-8
-6
-4
Log[PDSP Reference]


MRGPRX1
(MRGPRX1)
Relative Luminescence Units
5000
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[PDSP reference]


MRGPRX2
(MRGPRX2)
Relative Luminescence Units
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[TAN-67]

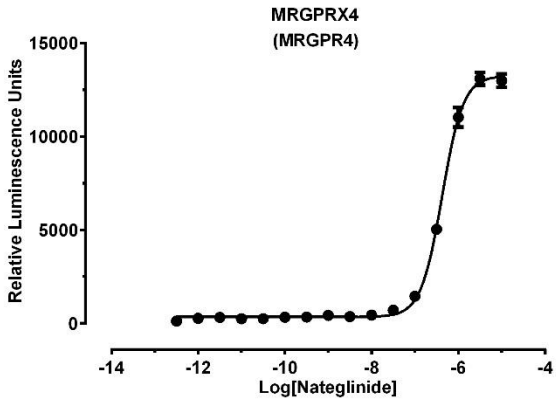

MRGPRX4
(MRGPR4)
Relative Luminescence Units
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Nateglinide]

Table 31. List of GPCR Tango constructs with representative antagonist activity assay conditions. Results are analyzed in GraphPad Prism 6.0. Representative curves are attached below in **Figure 49**. PDSP will develop Tango antagonist assays for other GPCRs upon request and approval, as long as reference agonist(s) and antagonist(s) are provided or commercially available. E_{max} lists the ratio of the top over bottom from the corresponding curves as an indication of signal windows, which is dependent on the concentration of reference agonist for antagonist assay.

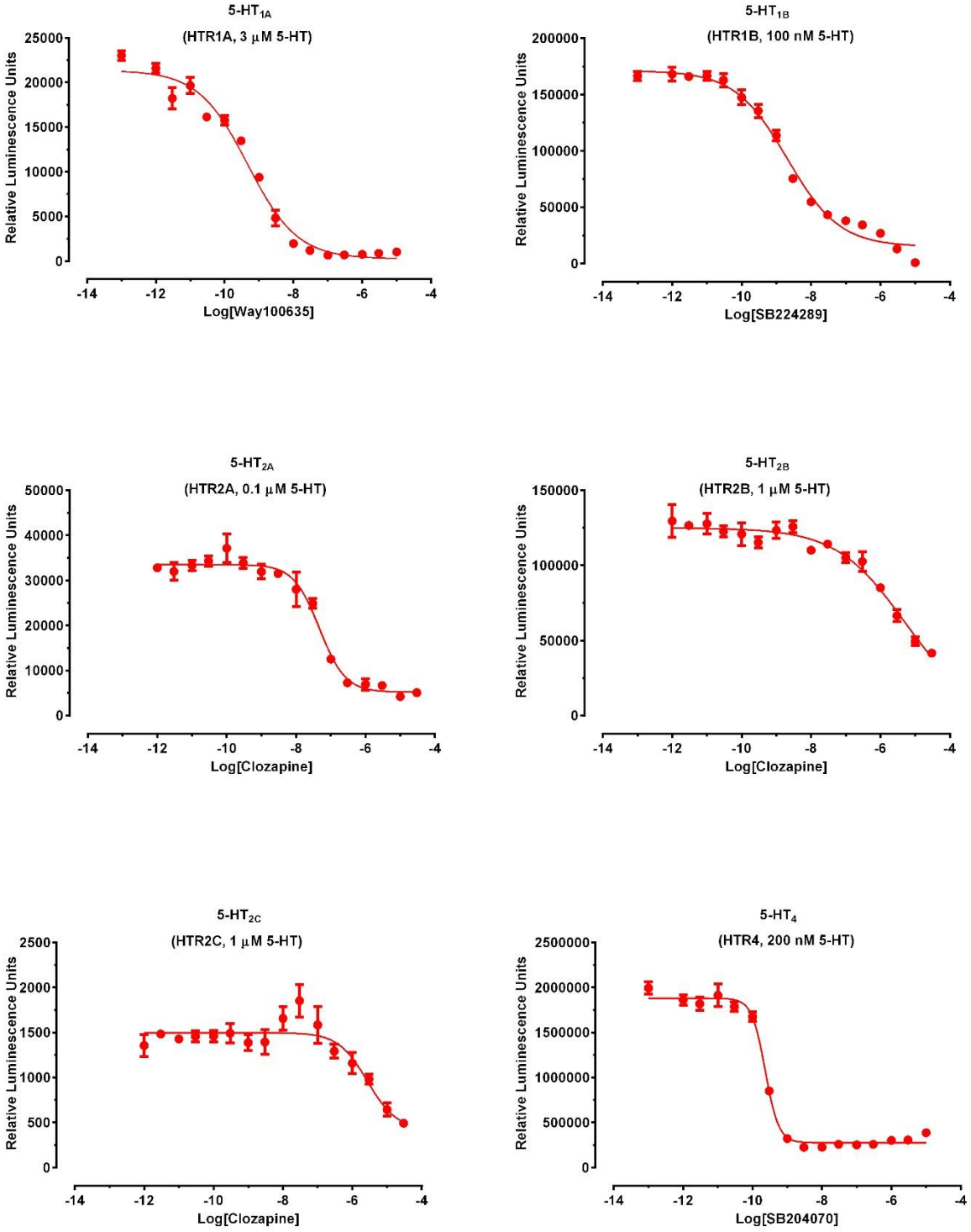
Note: # = Roth lab unpublished results.

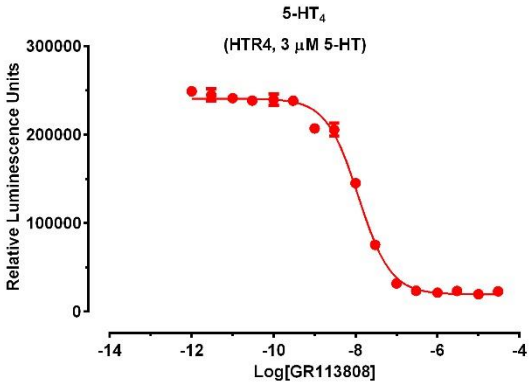
Gene Name	IUPHAR Receptor Name	Reference agonist Reference antagonist	E_{max} (fold of basal)	pIC_{50} (IC_{50} nM)	Hill slope
HTR1A	5-HT_{1A}	5-HT (3 μM) Way100635	83	9.33 (0.47)	-0.60
HTR1B	5-HT_{1B}	5-HT (10 nM) SB224289	114	8.68 (2.1)	-0.58
HTR2A	5-HT_{2A}	5-HT (0.1 μM) Clozapine	6.5	7.33 (47.0)	-1.16
HTR2B	5-HT_{2B}	5-HT (1 μM) Clozapine	242	5.31 (4853)	-0.44
HTR2C	5-HT_{2C}	5-HT (1 μM) Clozapine	3.5	5.57 (2709)	-1.03
HTR4	5-HT_4	5-HT (200 nM) SB204070	6.8	9.63 (2.3)	-2.24
		5-HT (3 μM) GR113808	12	7.93 (11.8)	-1.11
HTR5A	5-HT_{5A}	5-HT (1 μM) SB699551	98	6.04 (91.3)	-0.77
HTR6	5-HT6	5-HT (1 μM) Clozapine	16	7.11 (77.9)	-072
		5-HT (1 μM) SB399885	3.7	8.24 (5.8)	-1.65
HTR7A	5-HT_{7A}	5-HT (1 μM) SB269970	1.4	7.52 (30.2)	-1.73
CHRM1	M_1	Carbachol (10 μM) Atropine	72	7.03 (93.0)	-0.85
CHRM2	M_2	Carbachol (1 μM) NMS	11	8,87 (1.3)	-1.13
CHRM3	M_3	Carbachol (3 μM) NMS	47	9.58 (0.3)	-0.93
CHRM4	M_4	Carbachol (1 μM)	413	10.55 (0.03)	-1.40

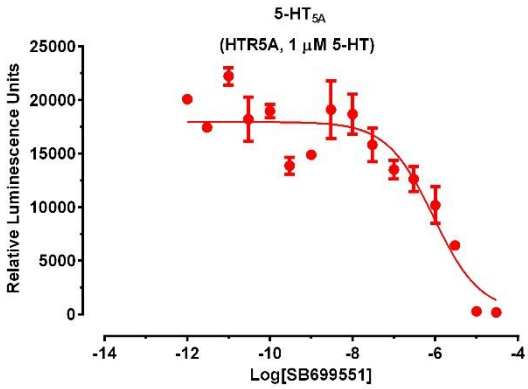
Gene Name	IUPHAR Receptor Name	Reference agonist Reference antagonist	E_{max} (fold of basal)	pIC_{50} (IC_{50} nM)	Hill slope
		Atropine			
CHRM5	M_5	Carbachol (10 μM) NMS	2.8	8.97 (1.1)	-0.88
DRD1	D_1	SKF81297 (3 μM) SCH23390	23	7.28 (52.4)	-1.27
DRD2	D_2	Quinpirole (10 nM) Haloperidol	24	8.90 (1.3)	-2.31
DRD3	D_3	Quinpirole (100 nM) Haloperidol	58	7.38 (41.5)	-1.16
DRD4	D_4	Lisuride (100 nM) Nemonapride	3.6	6.80 (160)	-2.91
DRD5	D_5	SKF81297 (3 μM) SCH23390	22	6.99 (103)	-1.07
ADRA1A	α_{1A}	Norepinephrine (1 μM) Prazosin	2.0	7.63 (23.3)	-0.88
ADRA1B	α_{1B}	Norepinephrine (1 μM) Prazosin	5.8	8.99 (1.0)	-1.19
ADRA1D	α_{1D}	Norepinephrine (1 μM) Prazosin	5.6	9.60 (0.3)	-1.17
ADRA2A	α_{2A}	Norepinephrine (300 nM) Rauwolscine	10	8.58 (2.6)	-0.97
ADRA2B	α_{2B}	Norepinephrine (300 nM) Rauwolscine	7.3	7.29 (51.9)	-0.94
ADRA2C	α_{2C}	Clonidine (300 nM) Rauwolscine	42	7.94 (11.4)	-1.06
ADRB1	β_1	Norepinephrine (10 μM) Carvedilol	28	8.56 (2.8)	-1.02
ADRB2	β_1	Norepinephrine (10 μM) Carvedilol	63	9.15 (0.7)	-0.70
ADORA1	A_1	NECA (100 nM) CGS15943	44	7.46 (34.6)	-0.88
CNR1	CB_1	CP55940 (300 nM) Rimonabant	59	7.03 (92.8)	-0.87
CNR2	CB_2	JWH-018 (300 nM) SR144528	18	6.19 (651)	-1.09
HRH1	H_1	Histamine (1 μM) Pyrilamine	2.2	6.53 (294)	-0.98
MLNR	Motilin	Motilin (1 μM)	44	8.47 (3.4)	-1.17

Gene Name	IUPHAR Receptor Name	Reference agonist Reference antagonist	E_{max} (fold of basal)	pIC_{50} (IC_{50} nM)	Hill slope
		MS 2029			
NMUR2	NMU2	Neuromedin S (1 μM) PDSP reference#	2.2	7.23 (59.6)	-0.75
OPRD1	DOR (δ)	DADLE (20 nM) Naltrindole	5.5	8.79 (1.6)	-1.12
OPRK1	KOR (κ)	Salvinorin A (60 nM) GNTI	4.4	8.49 (3.2)	-1.58
OPRM1	MOR (μ)	DAMGO (1 μM) Naltrexone	5.8	8.20 (6.3)	-2.04
OPRL1	NOP	Nociceptin (100 nM) SB612111	6.6	8.62 (2.4)	-0.85
OXTR	OT	Oxytocin (3 μM) PDSP reference#	15	5.89 (1299)	-0.92
P2RY1	$P2Y_1$	2MeS-ADP (3 μM) MRS 2179	4.6	6.26 (546)	-0.61
P2RY2	$P2Y_2$	UTP (10 μM) AR-C 118925XX	4.5	6.39 (408)	-0.82
PTGER4	EP_4	Prostaglandin E2 (10 nM) L-161982	1.9	7.49 (32.1)	-1.25

Figure 49. Representative curves for GPCR Tango antagonist assays.




5-HT4
(HTR4, 3 μM 5-HT)
Relative Luminescence Units
300000
200000
100000
0
-14
-12
-10
-8
-6
-4
Log[GR113808]


5-HT5A
(HTR5A, 1 μM 5-HT)
Relative Luminescence Units
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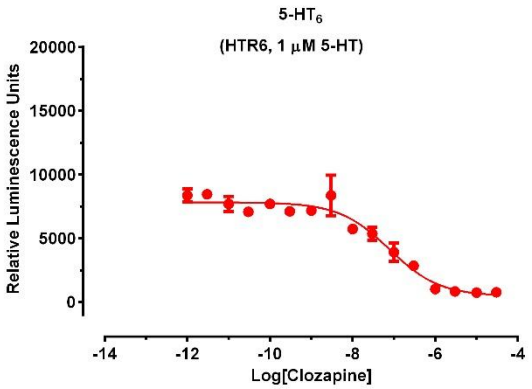
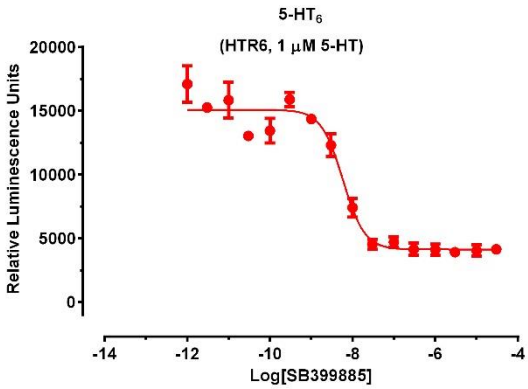
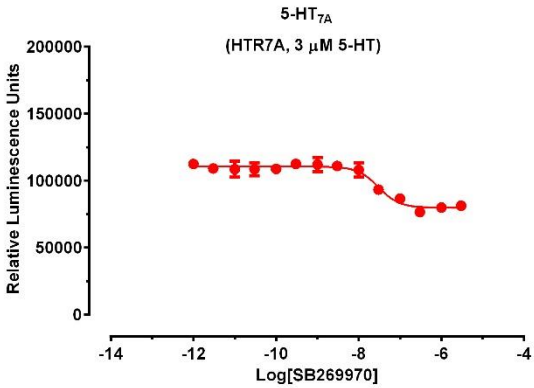
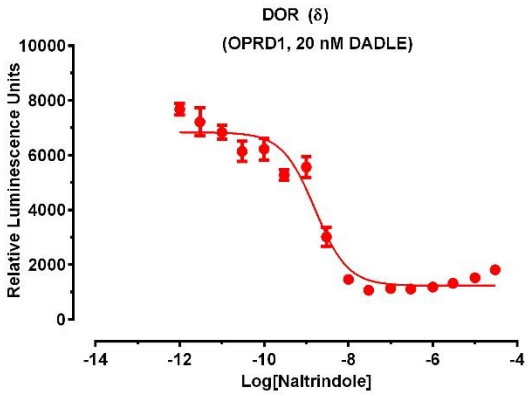
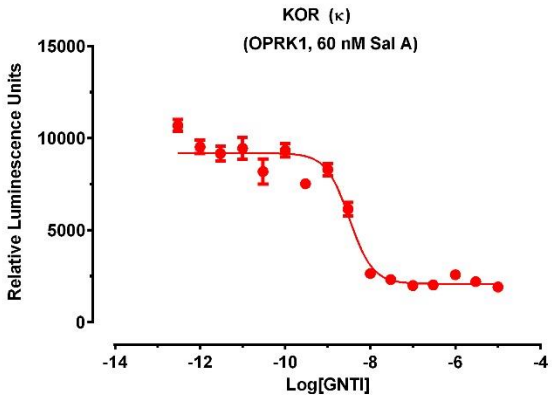
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[SB699551]

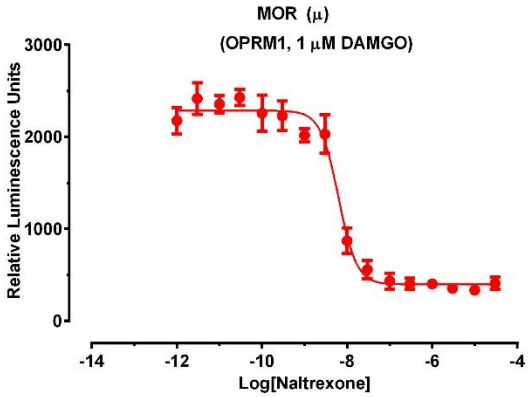

5-HT6
(HTR6, 1 μM 5-HT)
Relative Luminescence Units
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Clozapine]

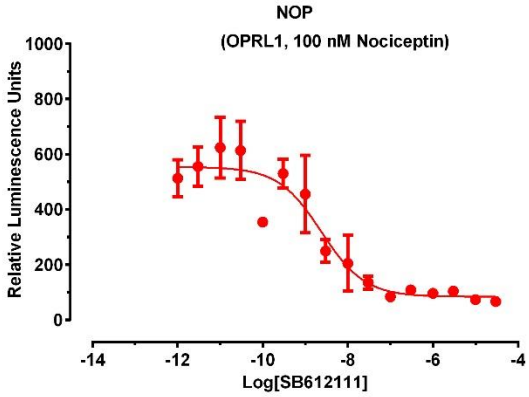

5-HT6
(HTR6, 1 μM 5-HT)
Relative Luminescence Units
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[SB399885]

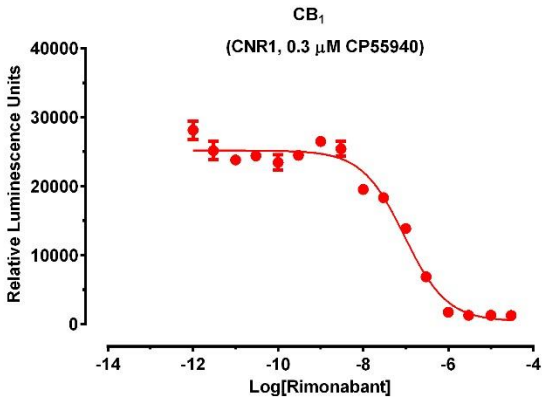

5-HT7A
(HTR7A, 3 μM 5-HT)
Relative Luminescence Units
200000
150000
100000
50000
0
-14
-12
-10
-8
-6
-4
Log[SB269970]

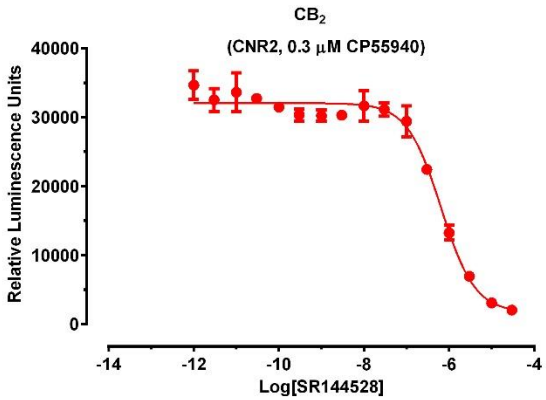

DOR (δ)
(OPRD1, 20 nM DADLE)
Relative Luminescence Units
10000
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Naltrindole]

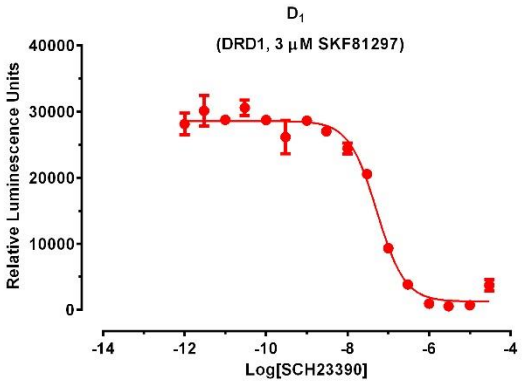

KOR (κ)
(OPRK1, 60 nM Sal A)
Relative Luminescence Units
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[GNTI]

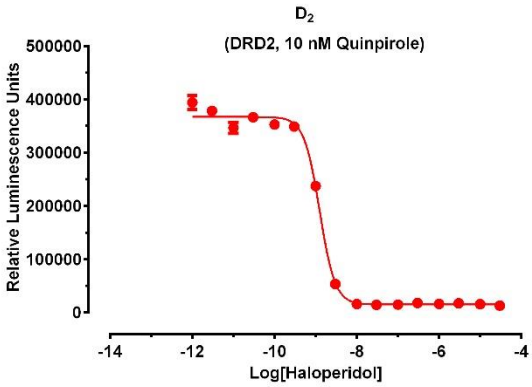

MOR (μ)
(OPRM1, 1 μM DAMGO)
Relative Luminescence Units
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Naltrexone]

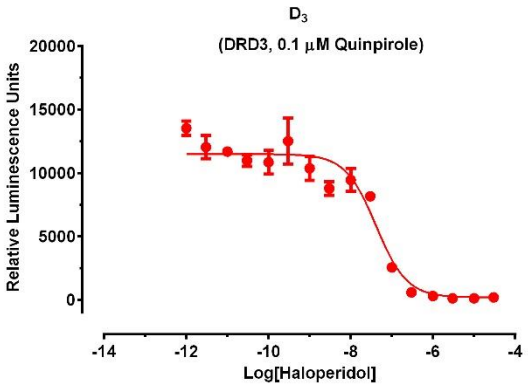

NOP
(OPRL1, 100 nM Nociceptin)
Relative Luminescence Units
1000
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[SB612111]

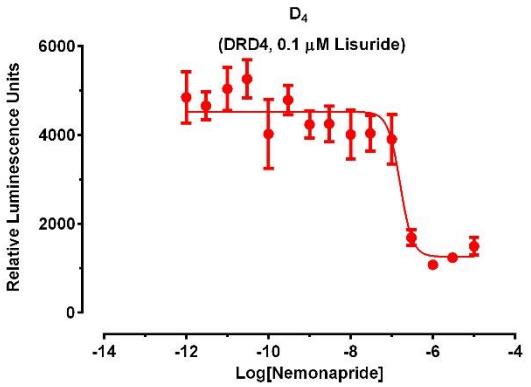

CB1
(CNR1, 0.3 μM CP55940)
Relative Luminescence Units
40000
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[Rimonabant]

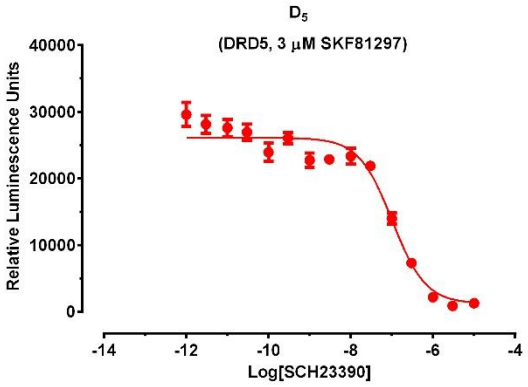

CB2
(CNR2, 0.3 μM CP55940)
Relative Luminescence Units
40000
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[SR144528]

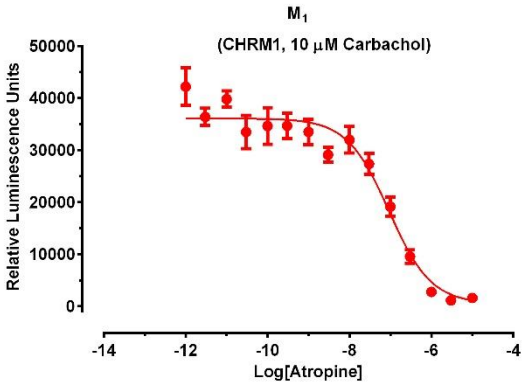

D1
(DRD1, 3 µM SKF81297)
Relative Luminescence Units
40000
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[SCH23390]

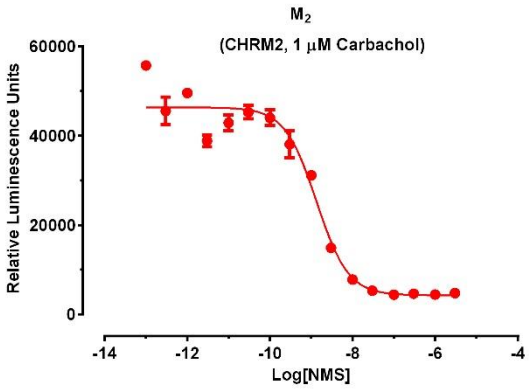

D2
(DRD2, 10 nM Quinpirole)
Relative Luminescence Units
500000
400000
300000
200000
100000
0
-14
-12
-10
-8
-6
-4
Log[Haloperidol]

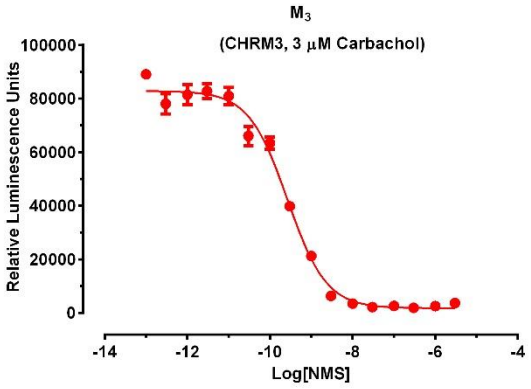

D3
(DRD3, 0.1 µM Quinpirole)
Relative Luminescence Units
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Haloperidol]

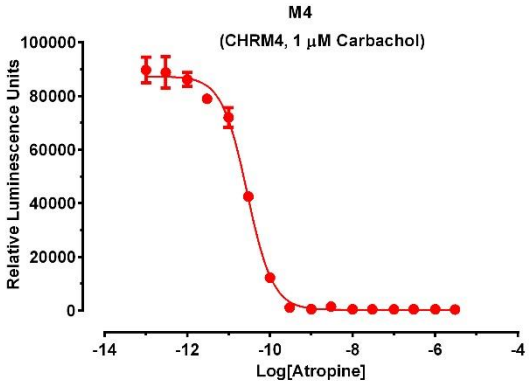

D4
(DRD4, 0.1 µM Lisuride)
Relative Luminescence Units
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Nemonapride]

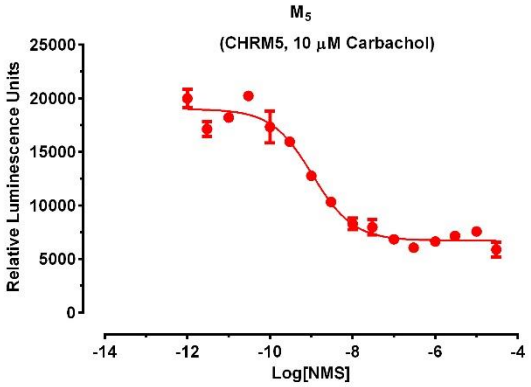

D5
(DRD5, 3 µM SKF81297)
Relative Luminescence Units
40000
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[SCH23390]

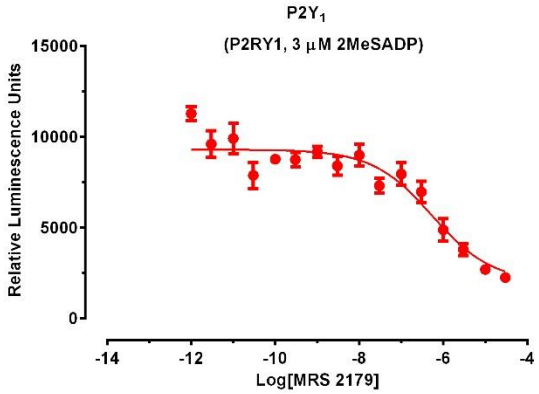

M1
(CHRM1, 10 μM Carbachol)
Relative Luminescence Units
50000
40000
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[Atropine]

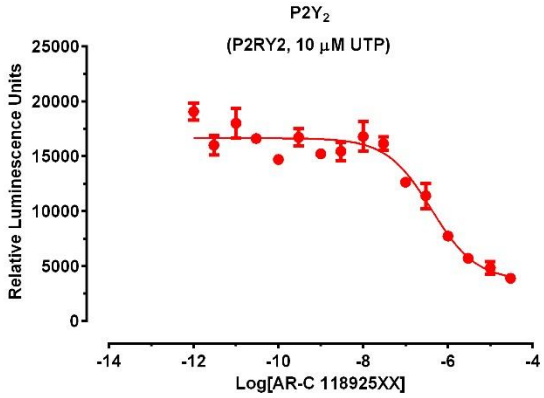

M2
(CHRM2, 1 μM Carbachol)
Relative Luminescence Units
60000
40000
20000
0
-14
-12
-10
-8
-6
-4
Log[NMS]

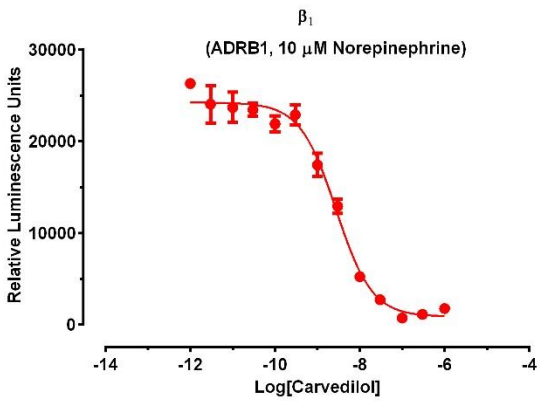

M3
(CHRM3, 3 μM Carbachol)
Relative Luminescence Units
100000
80000
60000
40000
20000
0
-14
-12
-10
-8
-6
-4
Log[NMS]

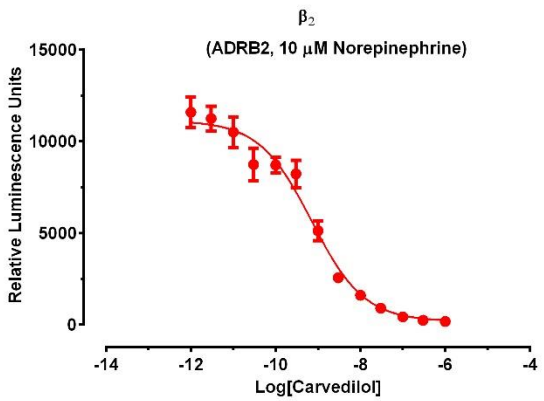

M4
(CHRM4, 1 μM Carbachol)
Relative Luminescence Units
100000
80000
60000
40000
20000
0
-14
-12
-10
-8
-6
-4
Log[Atropine]

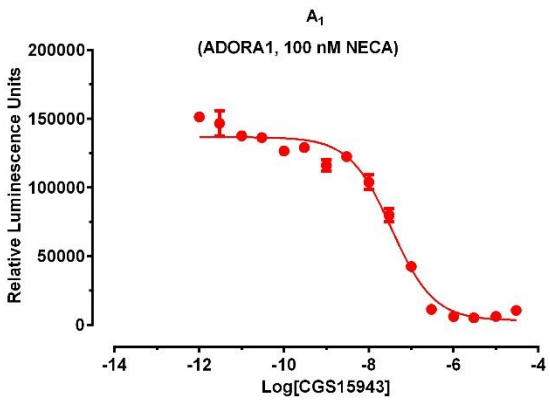

M5
(CHRM5, 10 μM Carbachol)
Relative Luminescence Units
25000
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[NMS]

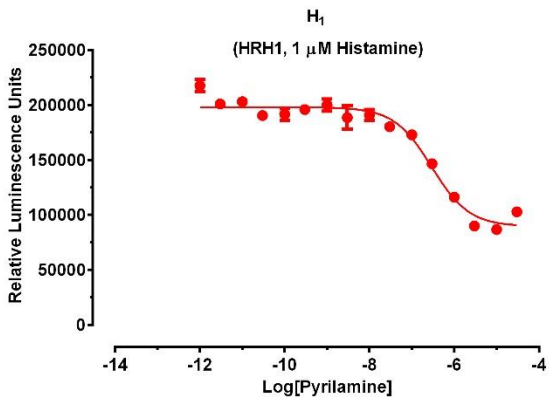

P2Y1
(P2RY1, 3 μM 2MeSADP)
Relative Luminescence Units
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[MRS 2179]

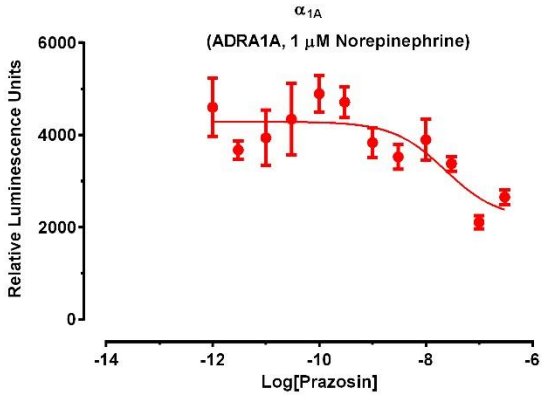

P2Y2
(P2RY2, 10 μM UTP)
Relative Luminescence Units
25000
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[AR-C 118925XX]

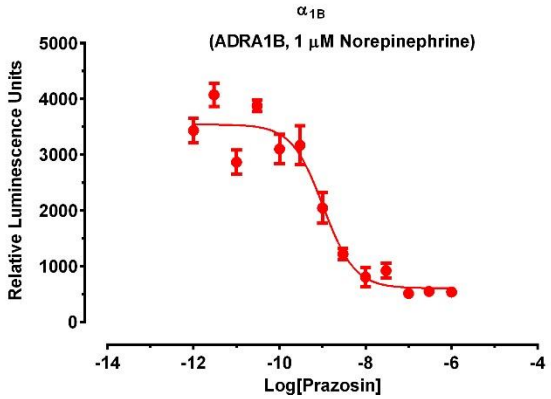

β1
(ADRB1, 10 μM Norepinephrine)
Relative Luminescence Units
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[Carvedilol]

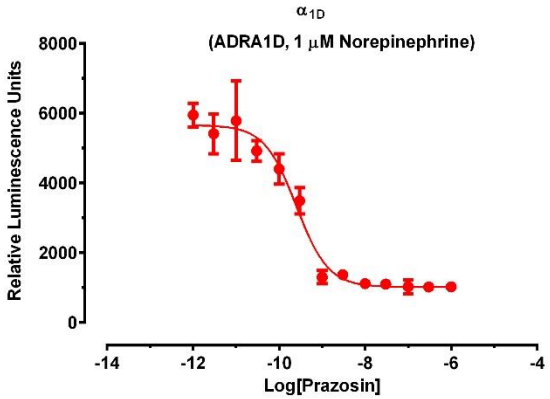

β2
(ADRB2, 10 μM Norepinephrine)
Relative Luminescence Units
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Carvedilol]

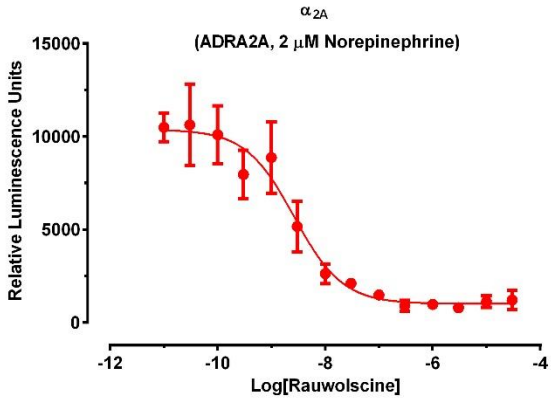

A1
(ADORA1, 100 nM NECA)
Relative Luminescence Units
200000
150000
100000
50000
0
-14
-12
-10
-8
-6
-4
Log[CGS15943]

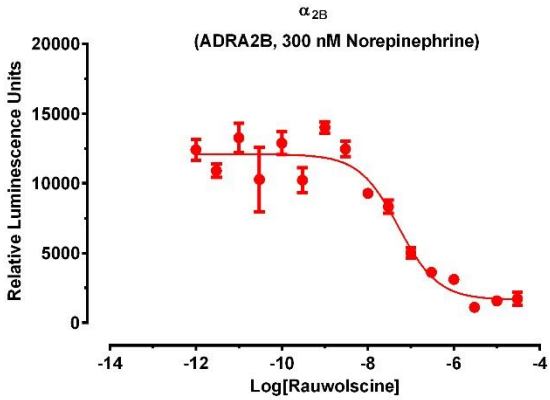

H1
(HRH1, 1 μM Histamine)
Relative Luminescence Units
250000
200000
150000
100000
50000
0
-14
-12
-10
-8
-6
-4
Log[Pyrilamine]

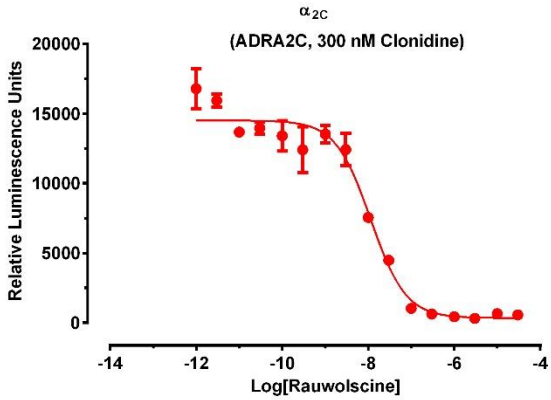

α1A
(ADRA1A, 1 μM Norepinephrine)
Relative Luminescence Units
6000
4000
2000
0
-14
-12
-10
-8
-6
Log[Prazosin]

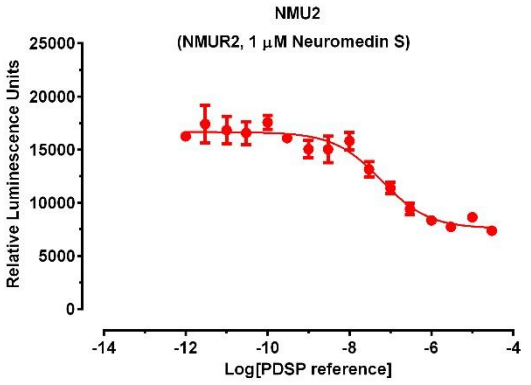

α1B
(ADRA1B, 1 μM Norepinephrine)
Relative Luminescence Units
5000
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Prazosin]

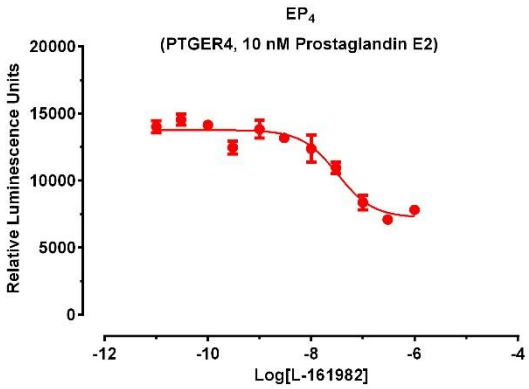

α1D
(ADRA1D, 1 μM Norepinephrine)
Relative Luminescence Units
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Prazosin]

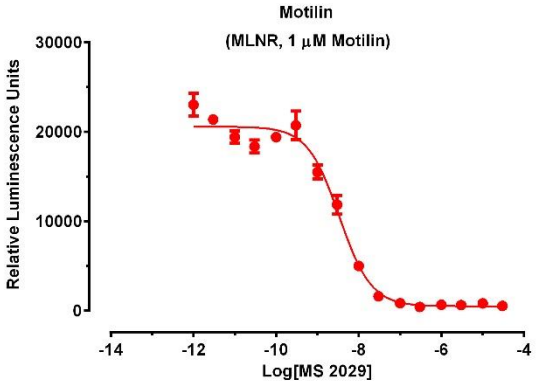

α2A
(ADRA2A, 2 μM Norepinephrine)
Relative Luminescence Units
15000
10000
5000
0
-12
-10
-8
-6
-4
Log[Rauwolscine]


α2B
(ADRA2B, 300 nM Norepinephrine)
Relative Luminescence Units
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Rauwolscine]


α2C
(ADRA2C, 300 nM Clonidine)
Relative Luminescence Units
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Rauwolscine]


NMU2
(NMUR2, 1 μM Neuromedin S)
Relative Luminescence Units
25000
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[PDSP reference]


EP4
(PTGER4, 10 nM Prostaglandin E2)
Relative Luminescence Units
20000
15000
10000
5000
0
-12
-10
-8
-6
-4
Log[L-161982]


Motilin
(MLNR, 1 μM Motilin)
Relative Luminescence Units
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[MS 2029]

2.7. PRESTO-Tango GPCRome screening

Main equipment: Liquid handling workstation for 96- and 384-well plates, luminescence counter

Main reagent: BrightGlo® from Promega

Assay buffer: 20 mM HEPES, 1x HBSS, pH 7.40

2.7.1. Cell culture. HTLA cells (a gift from Dr. Richard Axel), stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV protease fusion gene, are maintained in DMEM supplemented with 10% FBS, pen-strep, and 2 μg/ml Puromycin and 100 μg/ml Hygromycin. To set up the cells for transfection, HTLA cells are plated in DMEM supplemented with 10% dialyzed FBS in Poly-L-Lys (PLL)-coated 384-well white clear bottom cell culture plates at a density of 10,000 cells in a volume of 40 μl per well and incubated overnight. At least 1hr before transfections (below), we feed the cells with 10 μl/well DMEM supplemented with 50% FBS, using a Multidrop automated liquid dispenser.

2.7.2. DNA plate. Each single DNA plasmid is plated using the liquid handling workstation in one well of a 96-well plate at 0.5 μg/well (20 ng per well in 384-well plate and 8 wells per DNA, usually for 10x 384-well plates, see **Fig 50 and 51** for DNA plate designs). Each plate includes 80 receptor DNA samples, assay controls in column 1 with DRD2 and negative controls in column 2 with buffer. DNA plates, if not used immediately, can be dried in a cell culture hood for storage at (-20°C). Immediately before transfection (see below), DNA samples are resuspended in 0.25 M $CaCl_2$ and manually transferred into 384-well plates with an equal volume of 2x HBS for transfection (see **Figs 50 and 51** for DNA map in 96-and 384-well plates) followed by the next step for transfection (see below).

2.7.3. Transfection using Calcium phosphate precipitation protocol. HTLA cells are set up as indicated above overnight before transfection. The following protocol is designed for transfection on the following scale: one 384-well DNA plate (**Fig 51**) provides DNA for a total of 10 384-well cell plates. Briefly, the plated DNA in a 96-well plate (**Fig 50**) is first resuspended with 0.25 M $CaCl_2$ to a final volume of 380 μl/well and manually aliquoted into a 384-well plate (**Figure 51**), 45 μl/well for a total of 8 wells, to mix with equal volume of 2x HBS (50 mM HEPES, 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, pH 7.00). We then use Hamilton Microlab Star with a 384-well pipette head to transfer

6 μl/well of DNA/$CaCl_2$/HBS mixture into each of 10 384-well cell plates. A mixing step (10 μl x 5-time up and down pipetting) is programmed into the transfection procedure before each transfer. Each 384-well DNA plate is usually used within 20 min after being mixed with 2x HBA solutions. Transfected cells are returned to the incubator for overnight incubation. Under this setting, DNA samples from rows A-D in a 96-well DNA plate are made into a 384-well plate, therefore each 96-DNA plate is sufficient for 2 different 384-well plate layouts. One GPCRome (up to 320 Tango constructs) screening for each compound consists of a total of 8 384-well assay plates, with each receptor being screened in quadruplicate. Thus, a total of eighty 384-well assay plates are needed for a routine screening with 10 PDSP compound samples.

2.7.4. Compound addition and incubation. After overnight transfection and incubation, cells are removed from medium and receive 40 μl/well fresh DMEM supplemented with 1% dFBS about 2 hrs before compound stimulation. To make drug plates (**Fig 52**), the assay control quinpirole is added into column 1 (where the dopamine DRD2 receptor is expressed), the medium negative control is in columns 2-4 and all the odd numbered columns in a 384-well plate, while test sample is added into all the even numbered columns from 6-24 in the 384-well plate. Compounds and assay control (quinpirole) are made in DMEM with 1% dFBS at 5x working concentration, 105 μl/well. Drugs are transferred using the Hamilton Microlab Star with a 384-well pipetting head again, 10 μl/well, one drug plate for eight 384-well cell plates. Assay plates are then incubated overnight at 37°C. The following day, medium and drug solutions are removed and 20 μl per well of BrightGlo reagent (diluted 20-fold with Tango assay buffer) are added. Plates are incubated for 20 minutes at room temperature in the dark before luminescence is measured.

2.7.5. Data processing and analysis. The luminescence counter records relative luminescence units (RLU) and saves files in 384-well format in Excel sheets for easy processing. The GPCRrome screening assay is designed to have 4 replicate wells for samples and 4 replicate wells for basal levels for each construct. In each assay plate, the average background (columns 3-4, usually around 50 RLU) is shared for all constructs in the same plate. Results are expressed in the fold of corresponding average basal, calculated according to the following formula:

$$Fold\ of\ basal = \frac{(\text{Sample RLU}) - (\text{Avg background RLU})}{(\text{Avg basal RLU}) - (\text{Avg background RLU})}$$

In general, the assay control, DRD2 with 100 nM quinpirole, shows 30x to 100x of basal activity, while the activity seen with most ligand-receptor combinations generally ranges from 0.5x to 2.0x fold of basal. Usually, follow-up studies are not done when the observed activity is less than 3.0x fold of basal. An activity of <0.5x fold of basal, if corresponding basal activity is relatively high, can be a sign of inverse agonist activity, and will be recommended for followup assays. See **Figures 54-55** for representative GPCRome screening results.

	1	2	3	4	5	6	7	8	9	19	11	12
A			1	2	3	4	5	6	7	8	9	10
B			11	12	13	14	15	16	17	18	19	20
C			21	22	23	24	25	26	27	28	29	30
D			31	32	33	34	35	36	37	38	39	40
E			41	42	43	44	45	46	47	48	49	50
F			51	52	53	54	55	56	57	58	59	60
G			61	62	63	64	65	66	67	68	69	70
H			71	72	73	74	75	76	77	78	79	80
	DRD2	Buffer	Tango constructs (#1 - #80)									

Figure 50. 96-well DNA map**.** Green wells (Column 1) have DRD2 plasmid as assay control. **White** wells (Column 2) have no DNA as negative controls. Grey wells (Columns 3 – 12) have DNA plasmids (DNA sample #1 to #80). A total of 4 96-well plates would be needed for a complete GPCRome Tango experiment.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A					1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9	10	10
B					1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9	10	10
C					1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9	10	10
D					1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9	10	10
E					11	11	12	12	13	13	14	14	15	15	16	16	17	17	18	18	19	19	20	20
F					11	11	12	12	13	13	14	14	15	15	16	16	17	17	18	18	19	19	20	20
G					11	11	12	12	13	13	14	14	15	15	16	16	17	17	18	18	19	19	20	20
H					11	11	12	12	13	13	14	14	15	15	16	16	17	17	18	18	19	19	20	20
I					21	21	22	22	23	23	24	24	25	25	26	26	27	27	28	28	29	29	30	30
J					21	21	22	22	23	23	24	24	25	25	26	26	27	27	28	28	29	29	30	30
K					21	21	22	22	23	23	24	24	25	25	26	26	27	27	28	28	29	29	30	30
L					21	21	22	22	23	23	24	24	25	25	26	26	27	27	28	28	29	29	30	30
M					31	31	32	32	33	33	34	34	35	35	36	36	37	37	38	38	39	39	40	40
N					31	31	32	32	33	33	34	34	35	35	36	36	37	37	38	38	39	39	40	40
O					31	31	32	32	33	33	34	34	35	35	36	36	37	37	38	38	39	39	40	40
P					31	31	32	32	33	33	34	34	35	35	36	36	37	37	38	38	39	39	40	40
	DRD2		Negative		Tango constructs																			

Figure 51. 384-well DNA plate for calcium precipitation transfection. Tango constructs are transferred from 96-well plates into 384-well plates in the above layout. Green wells (Columns 1-2) are DRD2-transfected wells as assay control. **White wells** (Columns 3-4) have no DNA as negative controls (background). Grey wells (Columns 5-24) are for Tango constructs, each construct is transfected in 8 wells as highlighted in a block. In this setting, one 96-well DNA plate will need two 384-well plates, therefore, the entire GPCRome experiment requires a total of eight 384-well plates.

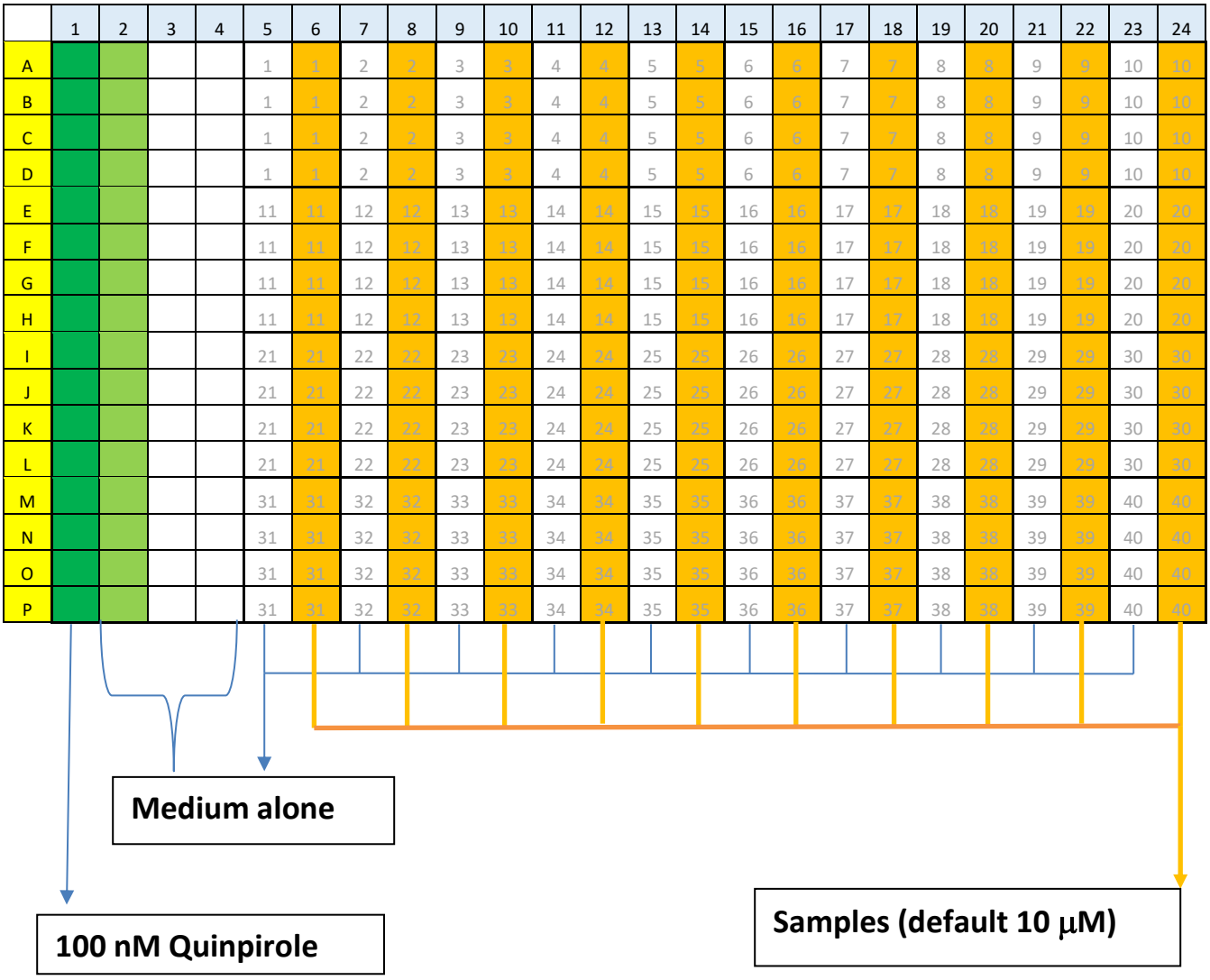


Figure 52. 384-well drug plate layout for stimulation. Column 1 receives 100 nM quinpirole, while Column 2 receives medium only (DMEM + 1% dFBS) to serve as "DRD2 basal". Columns 3 and 4 receive medium only to serve as background activity. For the rest of the plate, odd numbered columns receive medium (DMEM + 1% dFBS) and even numbered columns receive test sample (prepared in DMEM + 1% dFBS). Therefore, each Tango constructs has 4 sample wells and 4 basal wells. Results are expressed as fold of average basal for each construct.

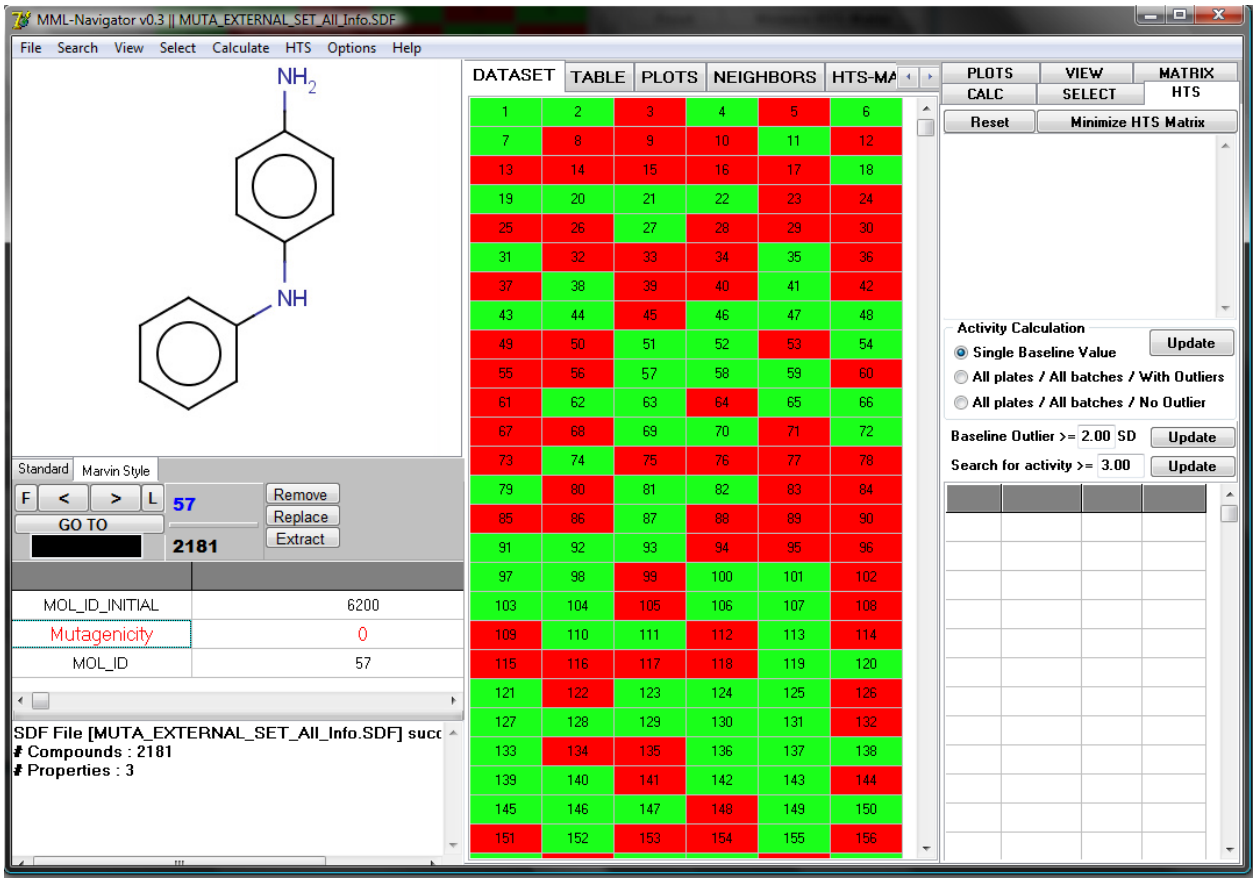


Figure 53. Navigator interface**.** This software readily calculates activation (%) relative to positive control as well as relative to baseline. Compound structures, number of plates and compounds tested per assay, and calculated values for every receptor/compound pair can be viewed on the main screen.

Alternatively, to calculate and report these values, we also use the Navigator software (custom made and developed in-house by the Molecular Modeling Laboratory, College of Pharmacy, UNC at Chapel Hill). The Navigator takes raw ouput files in Excel sheets and calculates relative activation as indicated above. A screen shot of the Navigator interface is shown in **Figure 53**.

2.7.6. Representative figures. As a proof of concept, we screened three compounds (LSD, Rimonabant, and Fluoxetine) against 143 GPCRs (non-orphan, non-olfacotry GPCRs) at final concentration of 1 μM. Results (fold of baseline) are reported below in **Figure 54**. Representative GPCRome screening with ergotamine is shown in **Figure 55**.

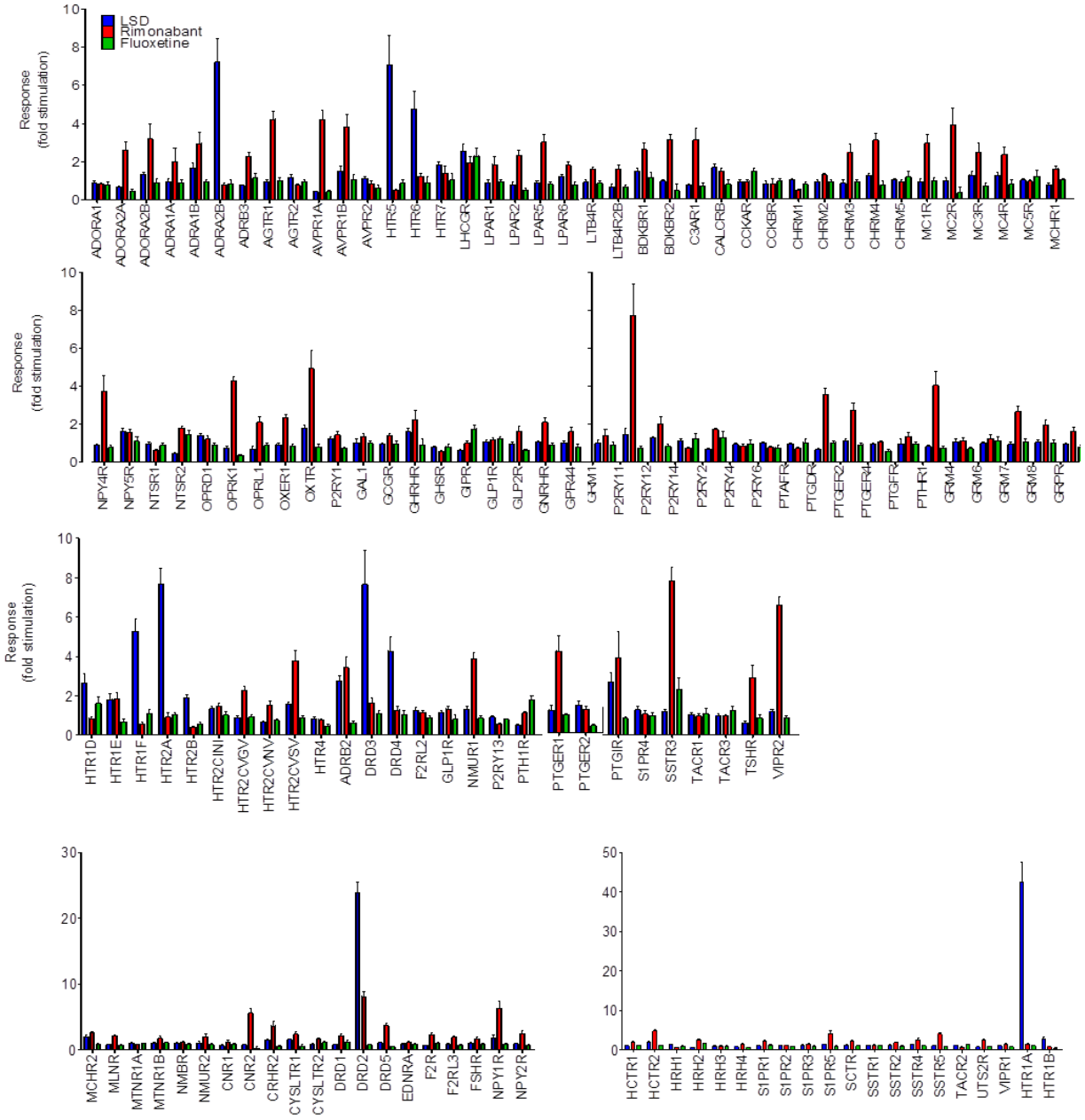


Figure 54. Sample parallel primary screening for 143 non-orphan non-olfactory GPCRs using β-arrestin recruitment assay (Tango). Activity is reported as Response (fold stimulation over baseline) for LSD (blue), rimonabant (red), and fluoxetine (green).

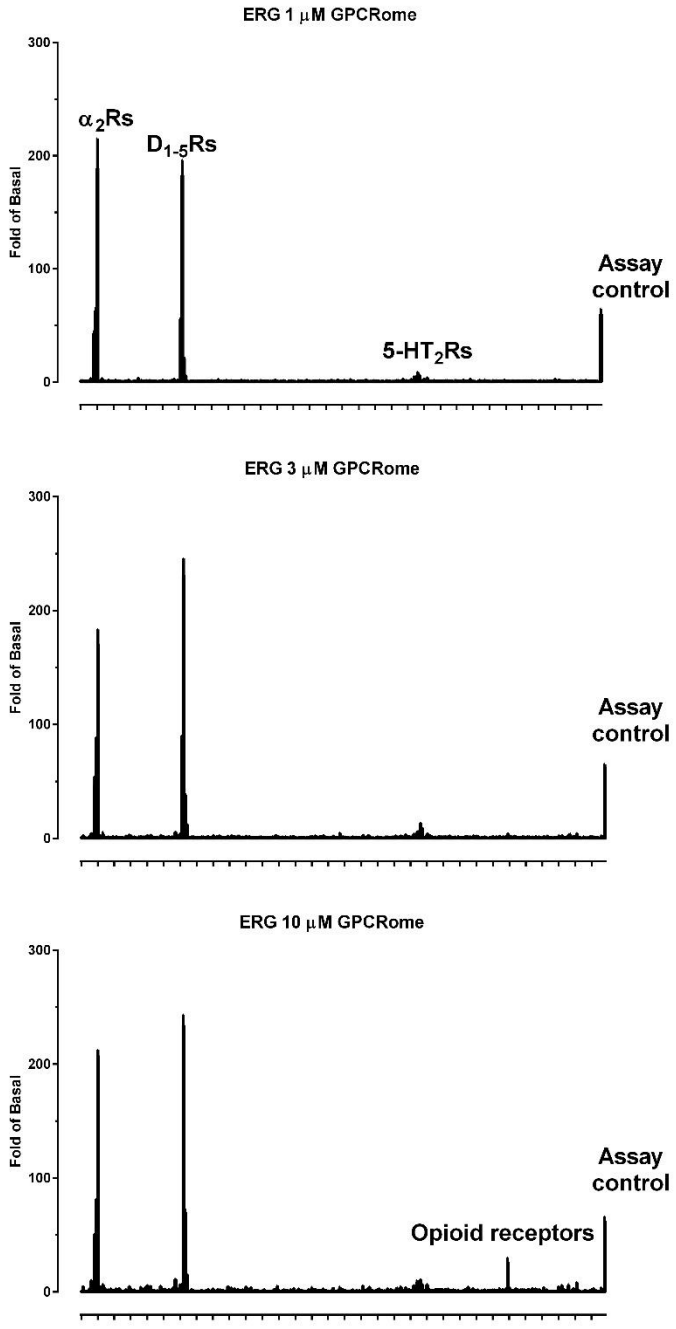


Fig 55. GPCRome screening with ergotamine (ERG) at 1, 3, and 10 μM. The assay control is 100 nM quinpirole at DRD2. Results were reformatted from a recent paper (172).

2.8. BRET-based transducerome assays

2.8.1. Introduction: G Protein Coupled Receptors (GPCRs) are membrane proteins that transduce extracellular signals to the intracellular environment through the activation of a heterotrimeric complex consisting of an α-subunit (G_{α}) and a dimerized pair of β and γ subunits ($G_{\beta\gamma}$). Upon receptor activation by an agonist, the receptor mediates an exchange of Guanosine Diphosphate (GDP) for a Guanosine Triphosphate (GTP) in the G_{α} subunit, leading to dissociation from the receptor/G_{α}/$G_{\beta\gamma}$ complex and subsequent activation of downstream effectors by these G proteins. Traditionally, GPCRs have been classified partially by the set of G_{α} proteins activated by the receptors. These are grouped into families based on the similarity of their downstream effector pairings (**Table 32**). The degree to which one receptor may activate one or multiple species of G proteins is of considerable physiological importance, as different cellular contexts, containing different G protein complements, may produce markedly different responses following the activation of the same receptor. Because of the similarity of signaling pathways within G protein families, the ability to differentiate relative specific efficacies has been fraught with complications. Moreover, the different cell types used in functional assays can produce different responses, depending on whether the full complement of the effector pathway is intact. For example, the Adenosine 2_A receptor (A_{2A}) can couple effectively to G_{olf}, but measuring its stimulation of cAMP production depends on the presence of adenylyl cyclase V (173), which is not present in all cell types (e.g. HEK293, **Fig 56**). Reconstitution of this complete effector pathway allows for a functional G_{olf} readout, but requires *a priori* knowledge of these relevant details.

Table 32. G-protein alpha subunits and their primary effectors

G-protein α subunits	Primary effector pathways
G_q, G_{11}, G_{14}, G_{15}	Activation of PLC/Trio/P63RhoGEF
G_{i1}, G_{i2}, G_{i3}, G_z	Inhibition of Adenylyl Cyclases
G_s long, G_s short, G_{olf}	Activation of Adenylyl Cyclases
G_{12}, G_{13}	Activation of Rho/Rac GTPases

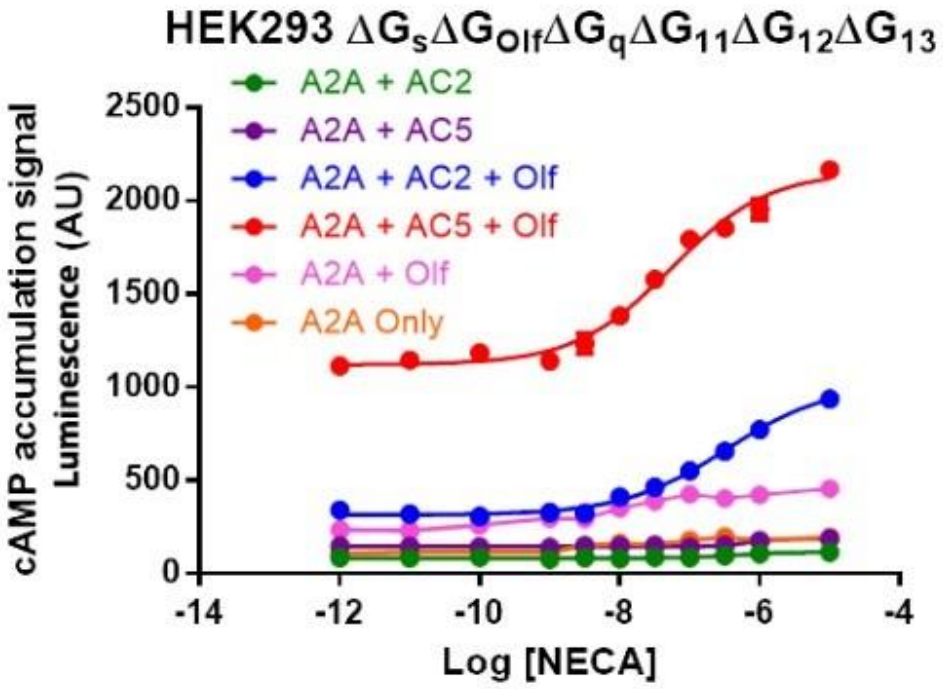


Figure 56. Adenosine 2A (A_{2A}) mediated G_s-cAMP production signaling in HEK293 cells lacking G_s, G_q, G_{12} family G-proteins (174) requires Adenylyl Cyclase 5 (AC5). G_s-cAMP production was measured using GloSensor cAMP method.

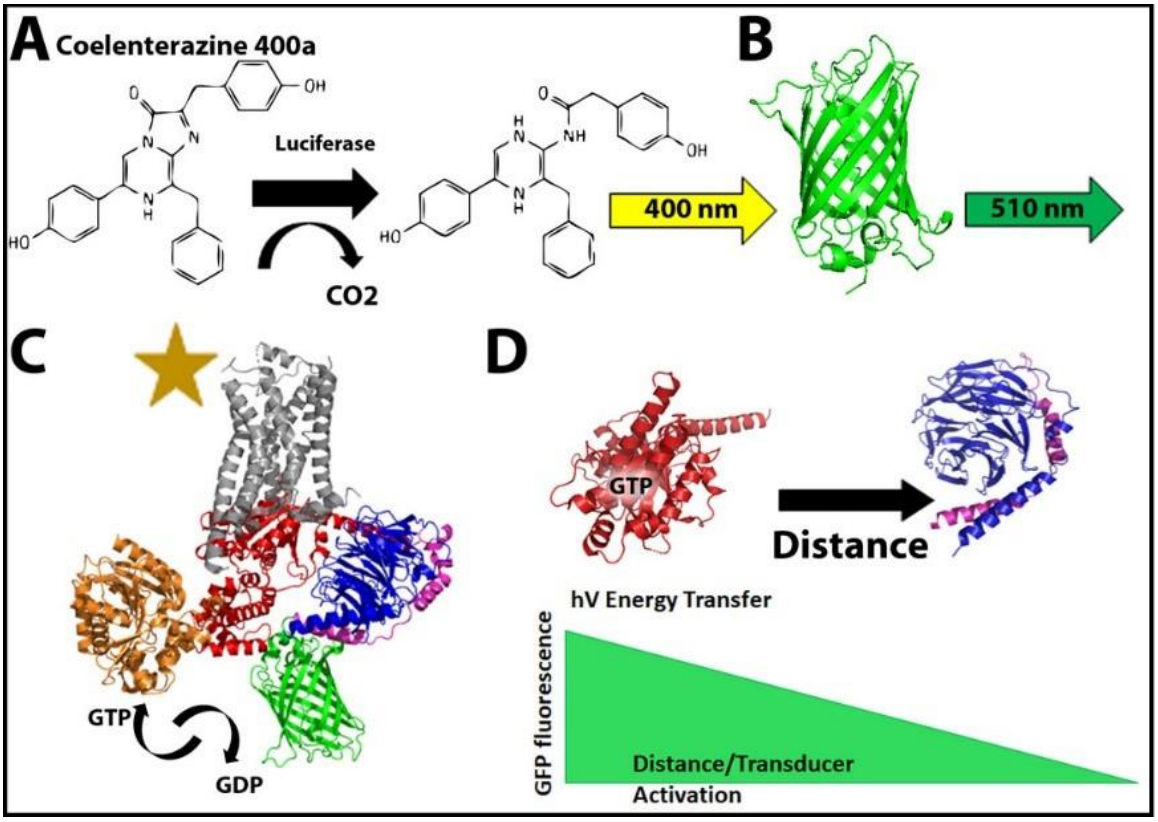


Fig 57. (**A**) Colenterazine 400a is oxidized to produce luminescence at 400 nm. (**B**) This wavelength excites the fluorophore GFP[2] which emits a fluorescent signal at 510 nm. (**C**) An active GPCR facilitates the exchange of GDP for GTP in its G_{α} subunit, leading to the dissociation of the $G_{\alpha\beta\gamma}$ and the subsequent activation of their effectors. (**D**) Labeling the heterotrimeric subunits with the luciferase and GFP[2] allows measuring the association of the inactive trimer, and the dissociation as a proxy for GPCR-mediated activation.

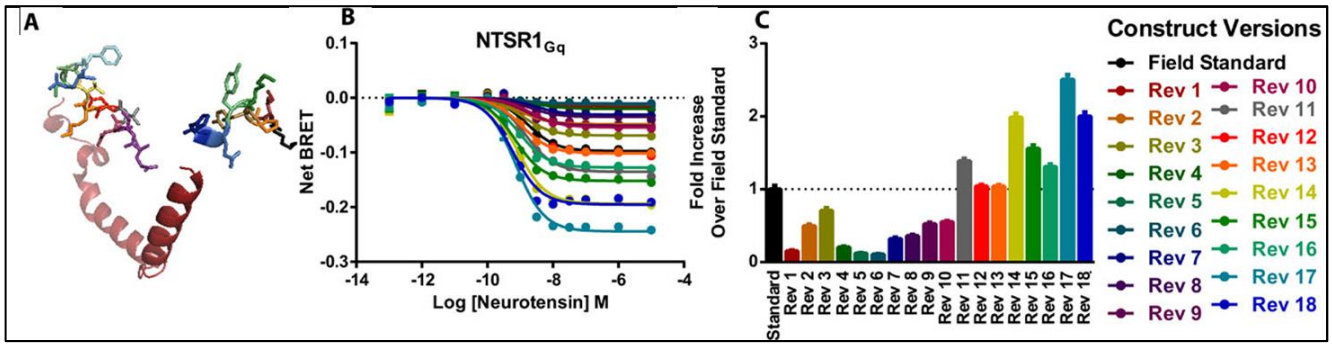


Fig 58. (**A**) rLuc was inserted at positions in the G_α subunit (green) most proximal to the c-terminus of the G_γ subunit (burgundy), which is fused to GFP[2]. (**B**) Function and dynamic range of the BRET pairs were assayed using the human Neurotensin 1 receptor (NTSR1). (**C**) Dynamic range measured as the change in BRET following receptor activation was used to assess the optimal construct (greatest improvement over the current published standard).

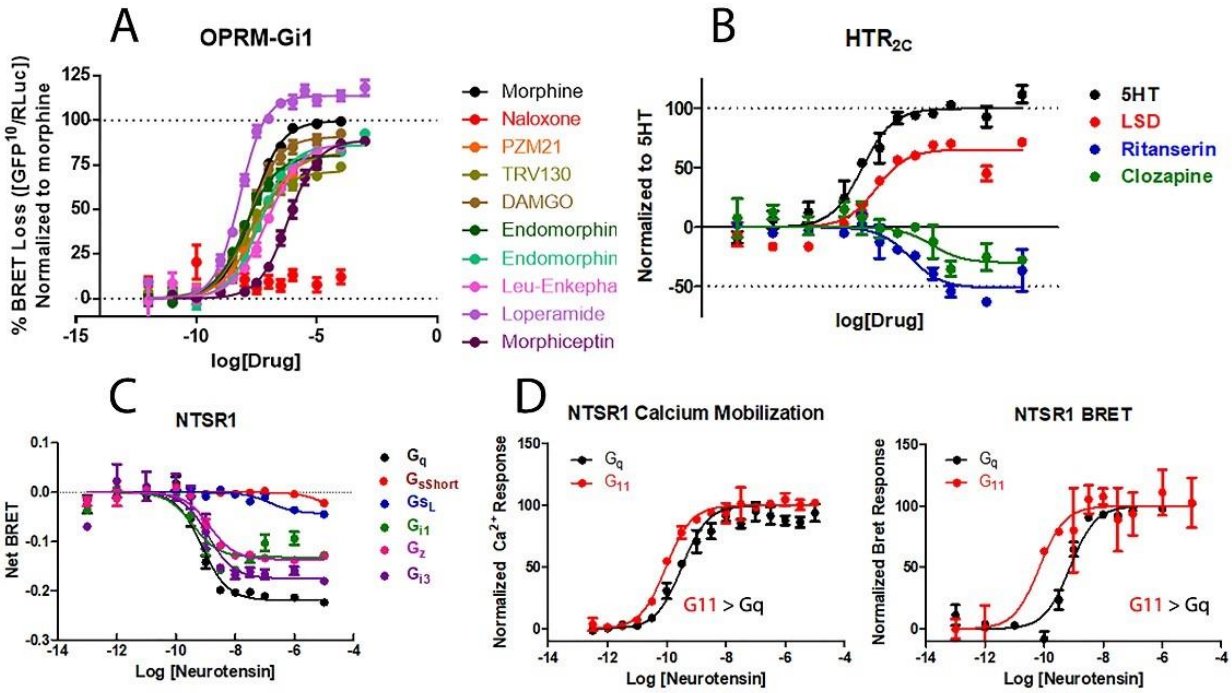


Fig 59. (**A**) Relative potencies and efficacies for μ opioid (MOR, OPRM1) receptor agonists through the G_{i1} pathway. (**B**) Full, partial, and inverse agonism at the HTR2C (5-HT_{2C}) receptor. (**C**) Relative potencies through multiple G protein families at the neurotensin 1 receptor (NTS_1). (**D**) Using G protein-deficient CRISP HEK293 cells, increased potency at the G_{11} pathway relative to its familial G_q counterpart can be confirmed.

Bioluminescence Resonance Energy Transfer (BRET) (175, 176) is an experimental tool used to study the association of two or more proteins. Briefly, an enzyme (Renilla luciferase) catalyzes a reaction with a chemical substrate (**Fig 57A**) to produce luminescence, which excites a proximal fluorophore when the emission/excitation of the substrate/ fluorophore are resonant (**Fig 57B**). Thus, the ratio of the fluorescent signal to the (constant) luminescent output can be used as a proxy for the degree of association between the two proteins. In the case of GPCR signaling, this can be used to study the association of the G_{α} and $G_{\beta\gamma}$ subunits, which make up the primary signaling complex downstream of receptor activation. An agonist-bound GPCR facilitates the exchange of Guanosine Diphosphate (GDP) for a Guanosine Triphosphate (GTP) in the G_{α} subunit (**Fig 57C**), which stabilizes its active state and subsequent dissociation from the $G_{\beta\gamma}$ subunit. The G_{α} is then free to activate its downstream effectors. By labeling the G_{α} and the $G_{\beta\gamma}$ with the luciferase and its cognate fluorophore (GFP^2), the change in their resonance can be assessed as a measure of primary signal transduction of the GPCR (**Fig 57D**). We have developed a full panel of G_{α}-luciferase fusion proteins and a cognate panel of GFP^2-labeled and unlabeled G_{β} and G_{γ} subunits. Although some versions of these constructs have been explored (177) in the literature, many of those exhibited relatively restricted dynamic ranges and thus, questionable utility. Recently, some groups have been attempting to rationally design similar biosensors, but no full panel of academic open-source freely available $G_{\alpha}/G_{\beta\gamma}$ tools exist yet.

2.8.2. Assay design and optimization. We utilized published crystal structures and modeling approaches to improve and optimize existing luciferase-tagged $G_{\alpha}/G_{\beta\gamma}$ probes, and expanded the available library to the entire $G_{\alpha}/G_{\beta\gamma}$ family. Throughout this process, we have generated and tested >400 unique constructs, selecting those showing the greatest dynamic range. These new biosensors exhibit between a 2 and 10-fold increase in dynamic range over the currently published probes, thus greatly enhancing the utility of this platform.

The optimized BRET probes can assay relative efficacies and potencies within transducer/receptor pairs (**Fig 58A**). Additionally, because the system is at equilibrium and is not subject to amplification, basal

activity and inverse agonism can also be measured (**Fig 58B**). Relative potencies among transducers can be assayed (**Fig 58C**), and can be confirmed using orthologous assays (**Fig 58D**).

2.8.3. Assay procedure

Day 1: HEK293T cells are plated at ~60% confluency in DMEM containing 10% dialyzed FBS (dFBS).

Day 2: Receptor + G_α-RLuc + G_β + G_γ-GFP^2 plasmids are co-transfected at a ratio of 1:1:1:1.

Day 3: Cells are removed from the plate using versene (0.5 mM EDTA in PBS pH 7.4) and plated at a density of 25 to 50K cells/well in poly-lysine-coated 96-well plates in DMEM containing 1% dFBS.Day 4: DMEM is aspirated or shaken out of the wells, followed by a wash in assay buffer (1x HBSS + 20 mM HEPES pH 7.4). Subsequently, 60 μl of assay buffer is added to the wells. 10 μL of 50 μM Coelenterazine 400a (Nanolight technology, Cat#340) is added to each well and incubated for 5 minutes while protected from light. 30 μL of drug dilutions (done in assay buffer + 0.3 mg/ml ascorbic acid and 0.3% BSA) are added to the wells and incubated for an additional 5 minutes. At this point, the plate is read using a LB940 Mithras multimode microplate reader (Berthold Scientific) at 1 s per read, using 400 and 510 nm emission filters. BRET is computed as the ratio of GFP^2 fluorescence (GFP) to coelenterazine signal (rLuc). Signal is calculated as the net decrease in BRET following agonist addition (net BRET, **Fig 59C**) and can be normalized to a reference ligand (**Fig 59A, B, D**).

2.8.4. Additional resources available: In addition to the G_α-rLuc probes, we have identified optimal βγ GFP-tagged acceptors. Throughout the process we have accumulated a complete set of tagged and untagged G_γ and G_β proteins that can be used to customize assays as requested (**Table 33**).

We plan to expand and validate this approach further, including GPCRs coupled to all four major G_α protein families. Once validation is complete, we will make all relevant constructs available for non-commercial use through **AddGene**.

Table 33. Complete list of tagged and untagged donor and acceptor constructs.

Donor	Optimal Acceptor
G_{i1}	γ_2-GFP
G_{i2}	γ_2-GFP
G_{i3}	γ_2-GFP
G_z	γ_2-GFP
G_{11}	γ_{12}-GFP
G_{12} (optimization in progress)	γ_2-GFP
G_{13}	γ_2-GFP
G_{14}	β_4-GFP
$G_{15/16}$ (optimization in progress)	β_4-GFP
G_q	γ_1-GFP
$G_{s\ Short}$	γ_1-GFP
$G_{s\ Long}$	γ_1-GFP
G_{olf}	γ_3-GFP, γ_8-GFP, or β_4-GFP

2.9. FluxOR assays for hERG activity

Main equipment: FLIPR^{TETRA} from Molecular Devices (Sunnyvale, CA)

Main reagents: FluxOR kit from Invitrogen (Carlsbad, CA)

FluxOR assay buffer: 20 mM HEPES, 1x HEBSS, 2.5 mM Probenecid, pH 7.40

The following protocol was adapted from a previously published peper(131).

2.9.1. Cell culture: HEK293 cells stably expressing hERG channels were purchased from ChanTest (Cleveland, OH) and maintained accordingly in DMEM supplemented with 10% FBS and 500 µg/ml G418. The hERG HEK293 cells are subcultured when they reach 80-90% confluency.

2.9.2. FluxOR assays for hERG inhibitors: Thallium (Tl^+) Flux assays are carried out using the FluxOR Potassium Ion Channel Assay kit from Invitrogen according to the manufacturer's instructions with a few modifications. In detail, HERG HEK293 cells are plated into PLL-coated 384-well black clear bottom cell culture plates in DMEM supplemented with 1% dialyzed FBS at a density of 15,000 cells in a final volume of 40 µl per well. The plated cells are incubated overnight before being used for assay. On the day of the assay, FluxOR dye reagents are reconstituted by mixing Component A (1/1000 dilution) and PowerLoad (1/100 dilution) using FluxOR assay buffer and loaded into cells with 20 µl/well of FluxOR reagent for 90 min in the dark. During incubation, drug solutions and stimulation solution are prepared. Stimulation solution is as follows: 2.5ml deionized water, 1ml FluxOR chloride-free buffer (Component E), 1 ml K_2SO_4 (125 mM, Component F), and 0.5 ml Tl_2SO_4 (50 mM, Component G). At the end of the dye loading period, dye is removed, and the FLIPR^{TETRA} is programmed to transfer drugs from drug plates into cell plates (25 µl per well). The cells are incubated with the drugs for 15 min at room temperature in the dark. Stimulation solution (6.3 µl per well) is added with the FLIPR^{TETRA}. The fluorescence intensity (excitation at 490 nm and emission at 525 nm) in each well is measured every second for 10 seconds before addition of stimulation solution (as baseline), and for 90 seconds thereafter by the FLIPR^{TETRA} using ScreenWorks 2.0 software. Alternatively, the FluxOR assays can be performed with cryopreserved hERG HEK293 cells. In brief, frozen cells are washed with growth media once to remove DMSO in the freezing media, and then

plated at 20,000 cells per well as above and assayed 5 hours later. Results using cryopreserved cells are not different from those obtained using fresh cells.

2.9.3. Assay procedure for hERG trafficking modulators: The above protocol was designed to measure the acute effect of drugs on hERG channel activity (such as hERG channel inhibitors or activators). For those drugs without acute inhibitory effect in the Tl^+ flux assays, we modified the standard protocol to conduct a longer chronic study to identify compounds that might be acting through indirect mechanisms (e.g., hERG trafficking inhibition, hERG internalization, and Na^+-K^+ ATPase inhibition). Briefly, cells are first treated with drugs for the desired time period (up to 16 hours) and are washed once with assay buffer before dye loading. The dye solution is then replaced with assay buffer and the fluorescence intensity is measured upon addition of stimulation solution per the standard protocol.

2.9.4. Data processing and analysis: The fluorescence intensity time course of each well in the 384-well assay plate is processed using ScreenWorks 2.0 software to export the slope of curve for the first 15 seconds after addition of the stimulation buffer. The initial slopes are then plotted against drug concentrations and analyzed in Prism 5.0 as outlined in **Section 2.3**.

Figure 60. Raw fluorescence signal traces recorded on FLIPR with HEK293 cells stably expressing hERG channels. Recording started 10 seconds before addition of stimulation solution and continued for another 90 seconds. Cisapride was shown to reduce signals in a dose-dependent manner (error bars not shown for clarity).

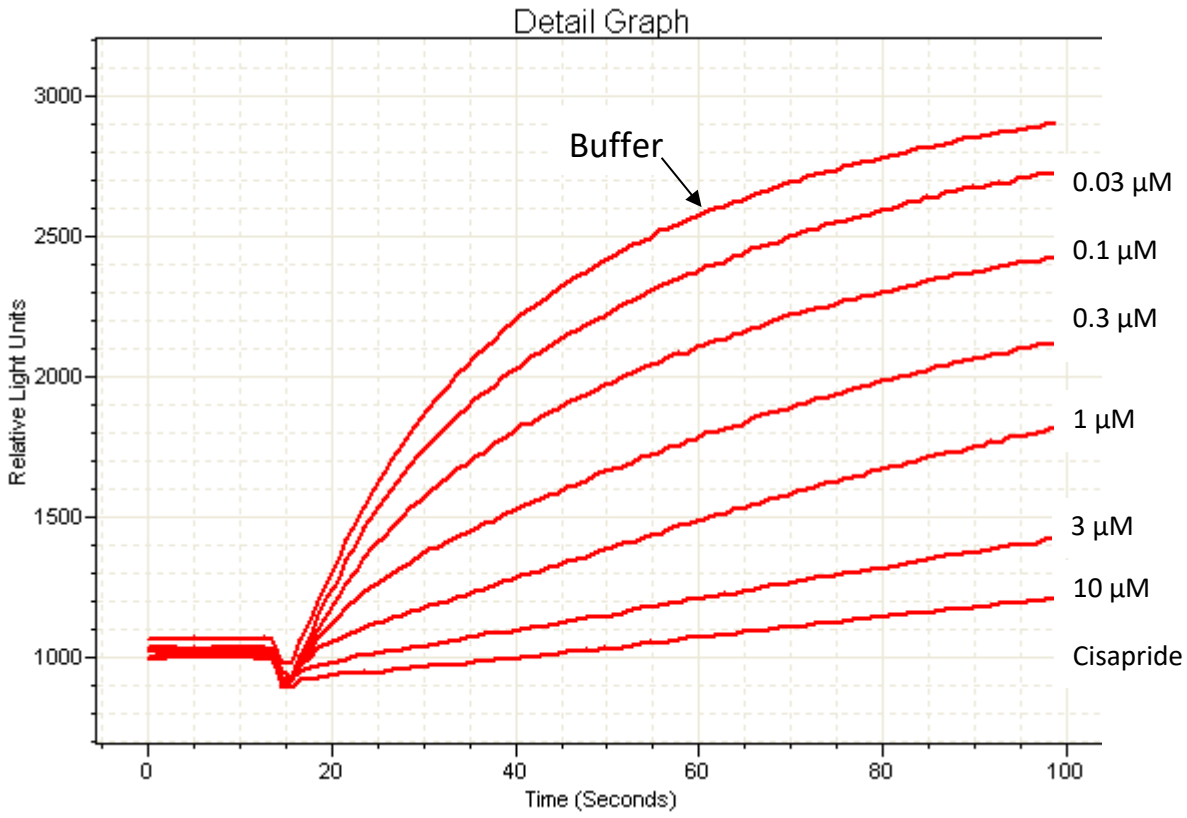


Figure 61. Initial slope of curves from above Figure 30 was exported from FLIPR ScreenWorks, plotted again corresponding concentrations of cisapride, and fitted to a four-parameter logistic function using GraphPad Prism 5.0.

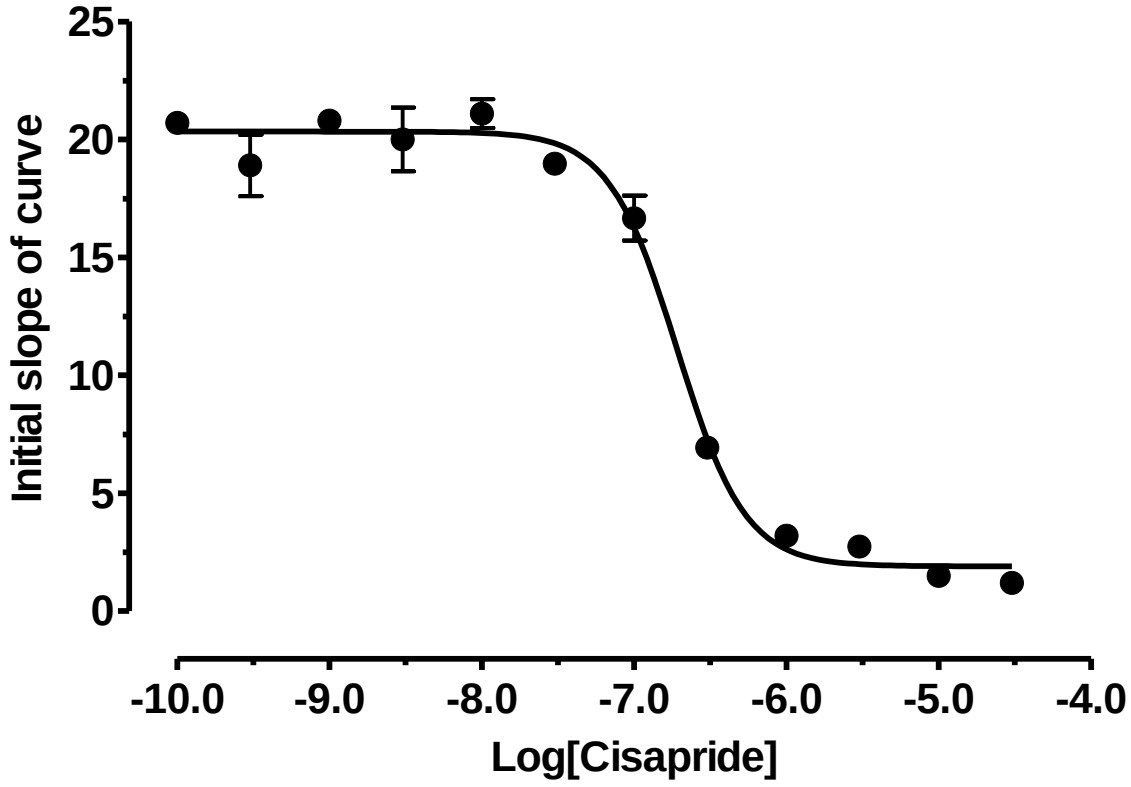


Figure 62. Acute and chronic effect of the hERG trafficking inhibitor ouabain on hERG channel activity. Activity of ouabain was determined with acute Tl^+ flux assay (upper) and chronic Tl^+ assay (lower). HEK293 cells stably expressing hERG channels were first incubated with ouabain for desired time at 37°C in the incubator; the remaining hERG activity was determined.

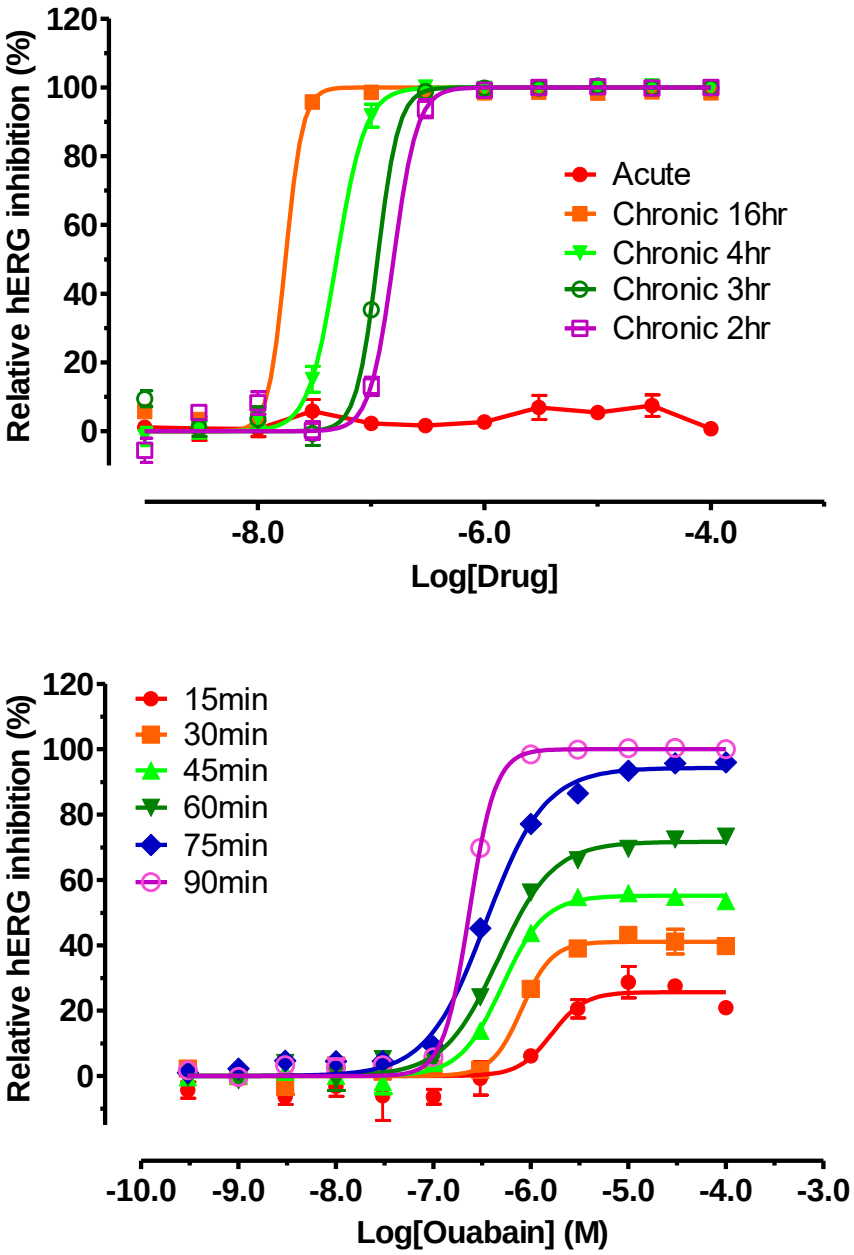
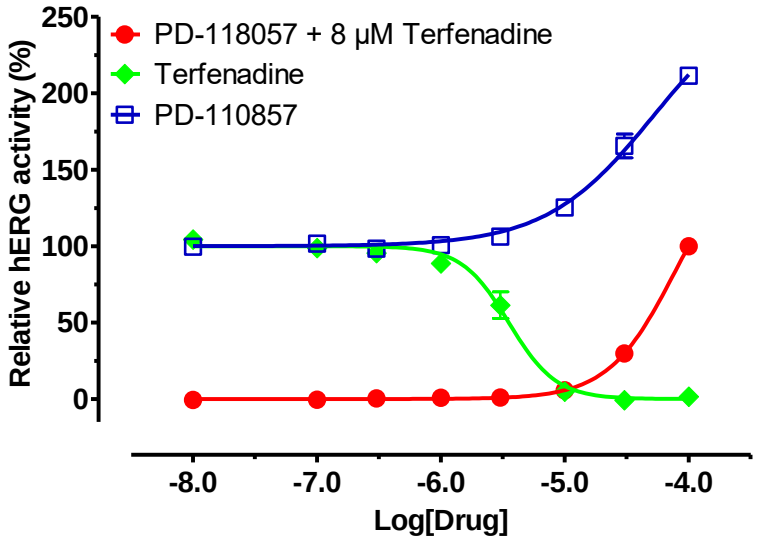
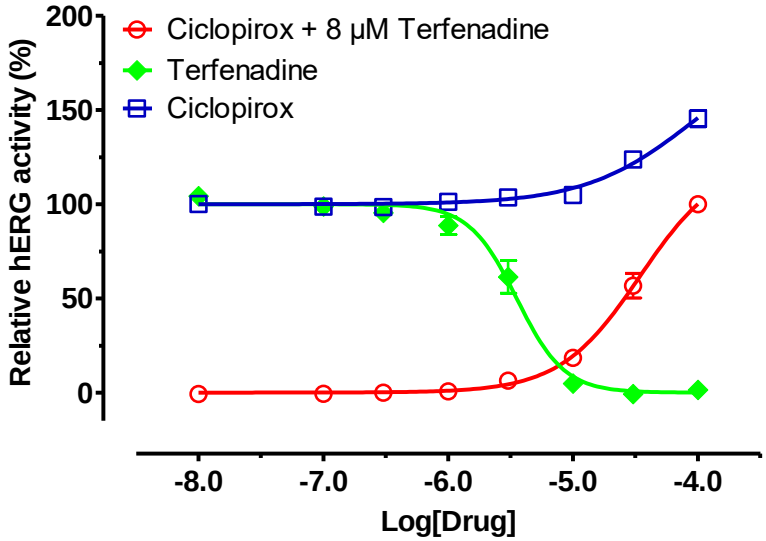


Figure 63. Activation of hERG channels in Tl^+ flux assays. Terfenadine completely inhibited hERG channel activity, whereas PD-118057 (upper panel) and ciclopirox (lower panel) were able to activate the hERG channel. For assays done in the presence of terfenadine, terfenadine was added 5 min before PD-118057 or ciclopirox. For hERG activator activity, values were normalized to their corresponding basal activities in percentage: basal activity as 100% in the absence of terfenadine and basal activity as 0% in the presence of terfenadine.





2.10. PatchXpress assays for hERG activity

Main equipment: PatchXpress 7000A (MDS Analytical Technologies, Sunnyvale, CA)

PatchXpress external solution: 137mM NaCl, 4mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 10mM Glucose, pH7.4 (adjusted with NaOH).

PatchXpress internal buffer: 15mM NaCl, 70mM KF, 60mM KCl, 1mM $MgCl_2$, 5mM HEPES, 5mM EGTA, 4mM ATP, and 0.4mM GTP, pH 7.2 (adjusted with KOH).

Automated planar patch clamp (APPC): PatchXpress electrophysiology

2.10.1. External and Internal solutions: The PatchXpress assay procedure is adopted from Huang et al., (2010) (131). Fresh external and internal solutions are prepared at room temperature on the day of the assay. The KF-based internal buffer was adopted from a report by Zeng et al., (2009) (178), in which they reported a greater success rate with a KF-based internal buffer than with a traditional KCl-internal solution. We add ATP, GTP, and Mg^{2+} to the KF-based internal solution to prevent potential current run-down. Osmolarity of the buffers is determined with a VAPRO 5520 Vapor Pressure Osmometer (Wescor, INC, Logan UT). The osmolarity is usually at 285 ± 10 mmol/Kg for the external buffer and 295 ± 10 mmol/Kg for the internal buffer. The buffers are vacuum-filtered to remove any air bubbles or small particles.

2.10.2. Cell preparation: For patch clamp assays, hERG HEK 293 cells are maintained as described above and subcultured into 10-cm dishes two days before scheduled assays at 1 – 2 million cells per dish in growth media without G418. Cells are not used if they reach more than 90% confluency. To prepare the cells for patch clamping, we followed ChanTest's recommendations with minor modifications. The goal is to prepare a clean, fresh cell suspension immediately before loading cells onto the PatchXpress. Cells are briefly washed with PBS, treated with Accutase (Sigma, 2.5ml per 10-cm dish) for 4 min at room temperature to detach them, gently transferred and suspended in 20 ml growth media without G418 in a 50-ml centrifuge tube, and allowed to recover from detachment for 30 min at 37°C in an incubator. At the end of the incubation period, 1 million cells are transferred into another 50ml centrifuge tube and pelleted by centrifugation at 250 x g for 2.5 min at room

temperature. The cell pellet is then gently re-suspended into 170 µl of the external buffer, transferred into a 1.5ml mcirocentrifuge tube, and loaded into the PatchXpress 7000A (MDS Analytical Technologies, Sunnyvale, CA) in 'waiting mode' for cells. To minimize delay and cell clumping, the APPC system is started well in advance of use and primed with fresh external and internal solutions before preparing cells. The patch clamping procedure is started with a new SealChip right at the end of the 30-min incubation and recovery period. By the time the cell suspension is ready to load into the APPC system, the system has reached the 'waiting mode' for receiving the cells.

2.10.3. Drug plate preparation: While cells are in the incubation and recovery period, drug solutions are prepared in 0.5ml polypropylene round-bottom 96-well drug plate (Fisher Scientific). Drugs in 10 mM DMSO stocks were diluted in the external buffer at a final volume of 360 µl per well, more than enough for the drug to be tested in two cells (triple addition at each of 50 µl for one cell at each concentration). The final concentration of DMSO is 0.3% (v/v) for all dilutions (except for several drugs that required 3% DMSO as indicated in Results). For initial assays, 8-point (ranging from 30 nM to 30 µM with 0.3% DMSO in external buffer as a negative control) concentration-response curves are generated. For subsequent assays, the concentration range can be adjusted as necessary to give full concentration-response curves (0 to 100% inhibition). The drug plate setup information is manually entered into the APPC system before starting the procedure. The assay is started within 15 minutes, and the highest drug concentration is tested within 90 minutes. A new drug plate is prepared for each new SealChip.

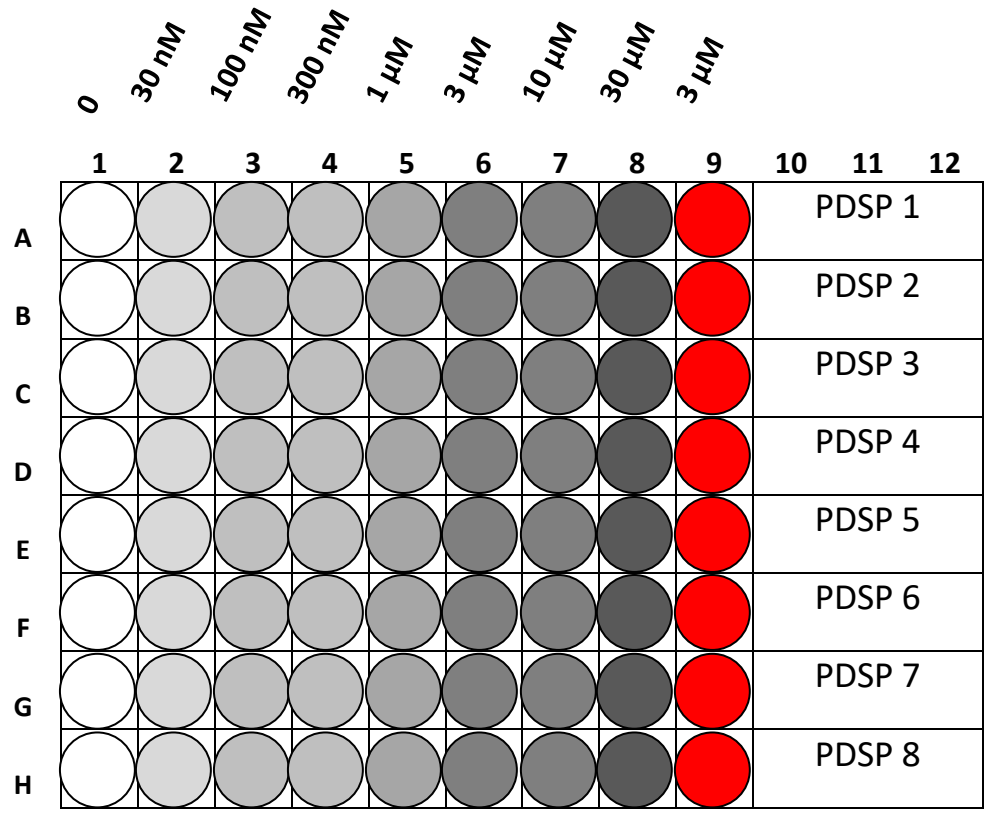


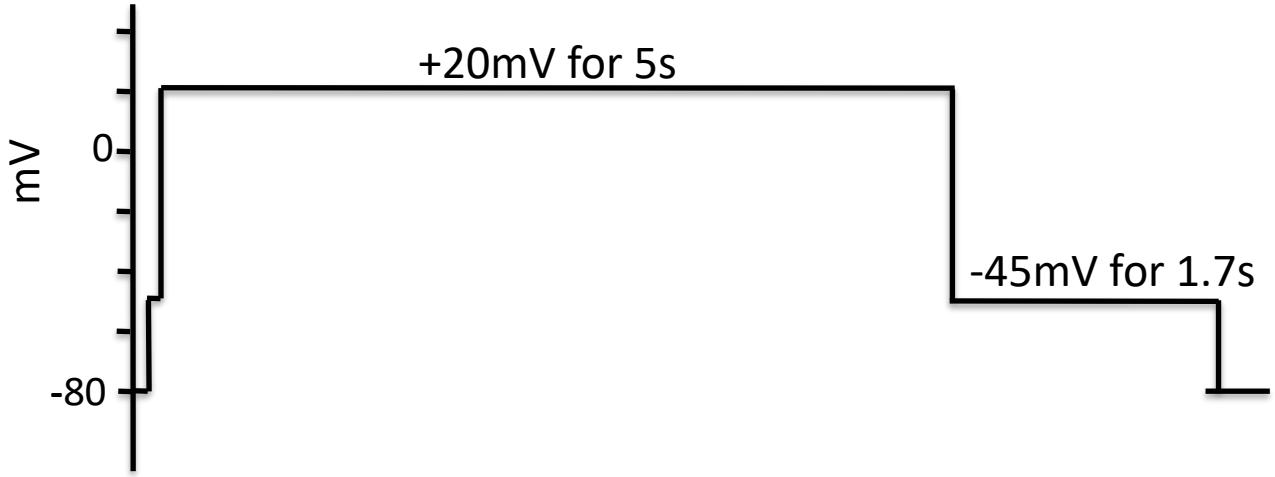
Figure 64. A typical APPC drug plate map (minimum of 360 µl per well). Each drug has eight serial dilutions 0.5 log unit apart as indicated in the plate map, starting with a buffer control on the left and ending at 30 µM, followed by a positive control (**red**) on the right, usually cisapride at 3 µM.

2.10.4. APPC procedures: The APPC procedure starts with the manual loading of a SealChip$_{16}$™ (AVIVA Biosciences, San Diego, CA) and is executed automatically at room temperature. The system requires approximately 7 min to (1) dry and load the SealChip onto the recording station; (2) external and internal solutions are then added; (3) the quality of each of the 16 chambers is confirmed; (4) the machine enters waiting mode and prompts for cell loading, at which point the cell suspension prepared above is immediately loaded. After loading, the single cell suspension is triturated briefly and aliquoted (35 µl, ~ 20,000 cells) by the on-board robotic Cavro pipette to each chamber on the SealChip. First, positive pressure (6 mmHg) then brief suction with negative pressure (-45 mmHg) are

applied to help the cells descend quickly, and a single cell is drawn onto the top of the electrode hole of each chamber. A negative pressure ramp to 75mmHg (at 5mmHg per second) is then applied repeatedly until the seal resistance reaches 1GΩ (Gigaohm) or greater for Giga-seals and over 300 MΩ (Megaohm) for 2nd seals. After obtaining a seal, another negative pressure ramp from -40mmHg to -250mmHg is applied repeatedly to rupture the patched membrane and achieve the whole cell configuration. Chambers with low seal resistances (i.e. no cell detection) are terminated within 90 seconds; chambers that cannot form seals within 5 minutes are terminated by either a built-in script or user intervention. After achieving whole cell configuration, the voltage protocol is activated, beginning with a 2-minute wash-out and a 5-minute stabilization period. After stabilization, cells with less than 0.2 nA tail current amplitude are terminated. The voltage protocol consists of the following steps: (1) depolarization from holding potential of -80mV to -50mV for 50 milliseconds to measure leak current without activation of the hERG channel; (2) further depolarization to +20mV for 5 seconds to activate the hERG channel (hERG channels are activated and quickly inactivated); (3) repolarization to -50mV for 1.7 seconds to remove inactivation and elicit outward hERG tail current; (4) repolarization to holding potential at -80mV to keep hERG channels closed. This pulse pattern is applied repeatedly every 10 seconds (0.1 Hz). The instantaneous current at -50mV before stepping to +20mV is designated as leak current and is subtracted from corresponding peak tail current for data processing. Each concentration-response study starts with a buffer control to determine the maximal hERG tail current (0% inhibition) in the absence of drugs. Each dose is applied three times (50 ul each at 25µl per second) with 11 seconds between additions, and each addition is preceded by aspiration of buffer or previous drug solution from the chamber down to a 5 µl dead volume. This triple addition and aspiration protocol fully exchanges the solution in the chamber with negligible dilution within one minute. The built-in DataStable script is activated between each dose. When either drug effect reaches steady state (less than 0.1% difference from last measurement), or after a maximum of 5 minutes, the next dose is queued for delivery. Right after each concentration-response trial, a 5-minute wash-out is applied to monitor recovery of the hERG channels from inhibition. At the end of wash-out, a positive control (usually cisapride at 3 µM) is applied to inhibit any recovered hERG channel activity or remaining hERG channel activity.

2.10.5. Data processing and analysis: APPC data are collected and automatically deposited into the database program DataXpress V2.0 (MDS Analytic Technologies, Sunnyvale, CA). If a cell has a leak current higher than 1/3 of the total tail current, the data for that cell is excluded. If a cell shows current run-down of more than 25% of the initial total tail current at the first (lowest) drug concentration, the data for that cell is excluded. The drug concentration range is usually adjusted after an initial assay so that, in subsequent assays, the lowest drug concentration inhibits hERG by less than 10%. The hERG tail currents are transformed and normalized to percent inhibition with a built-in script in the DataXpress program (total initial tail current = 0% inhibition, no current = 100% inhibition). Normalized results from multiple assays are pooled and analyzed with Prism's built-in four-parameter logistic functions as outlined in **Section 2.3**. A built-in statistical comparison function in Prism is then used to determine if a model with a variable Hill slope fit the data better than a model with a standard Hill slope (slope of 1); a p value less than 0.05 is considered significant. Finally, the potency value and corresponding Hill slope value from the best-fit model are reported.

Figure 65. Voltage protocol (upper) and corresponding hERG tail current recording (lower) for the automated planar patch clamp assays.



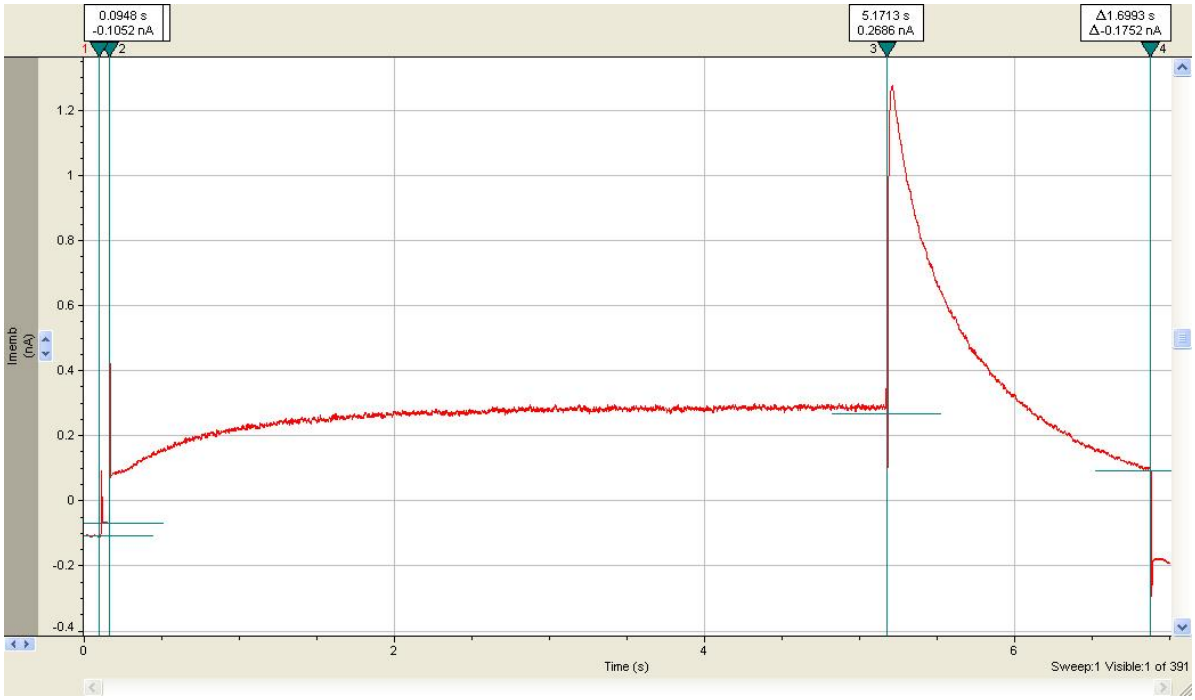


Figure 66. A representative whole-cell patch clamp recording showing inhibition of hERG channel current (nA) by a PDSP compound in a dose-dependent manner, which was partially recovered during a wash-out cycle, and then completely inhibited again by a positive control (3 µM cisapride). The figure is a captured screen in the DataXpress program showing hERG tail currents in the absence and presence of a PDSP compound. The red vertical line (left) indicates addition of buffer control, green vertical lines indicate triple additions of drugs with concentrations (µM) above the green lines, the dark blue vertical lines indicated wash-out with buffer, and the pink vertical lines indicate the positive control cisapride. Red crosses indicate the times when the hERG tail current was measured by the system.

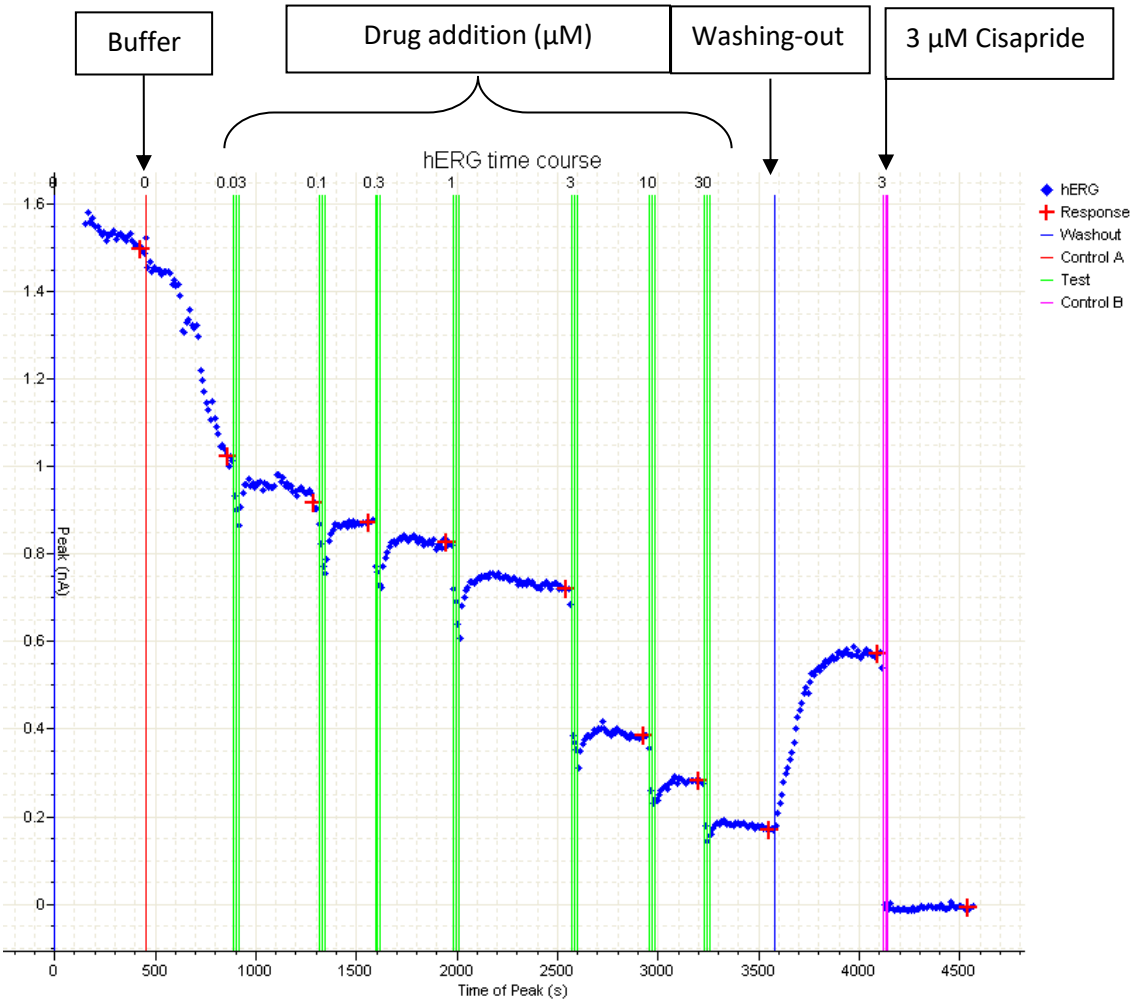


Figure 67. The captured screen in the DataXpress program shows a representative whole-cell patch clamp recording of hERG tail currents in the absence and presence of cisapride. The red vertical line indicates addition of buffer control and the green vertical triple lines indicate triple additions of cisapride with concentrations (µM) listed above the green lines. Red crosses indicate the times when hERG tail current was measured by the system.

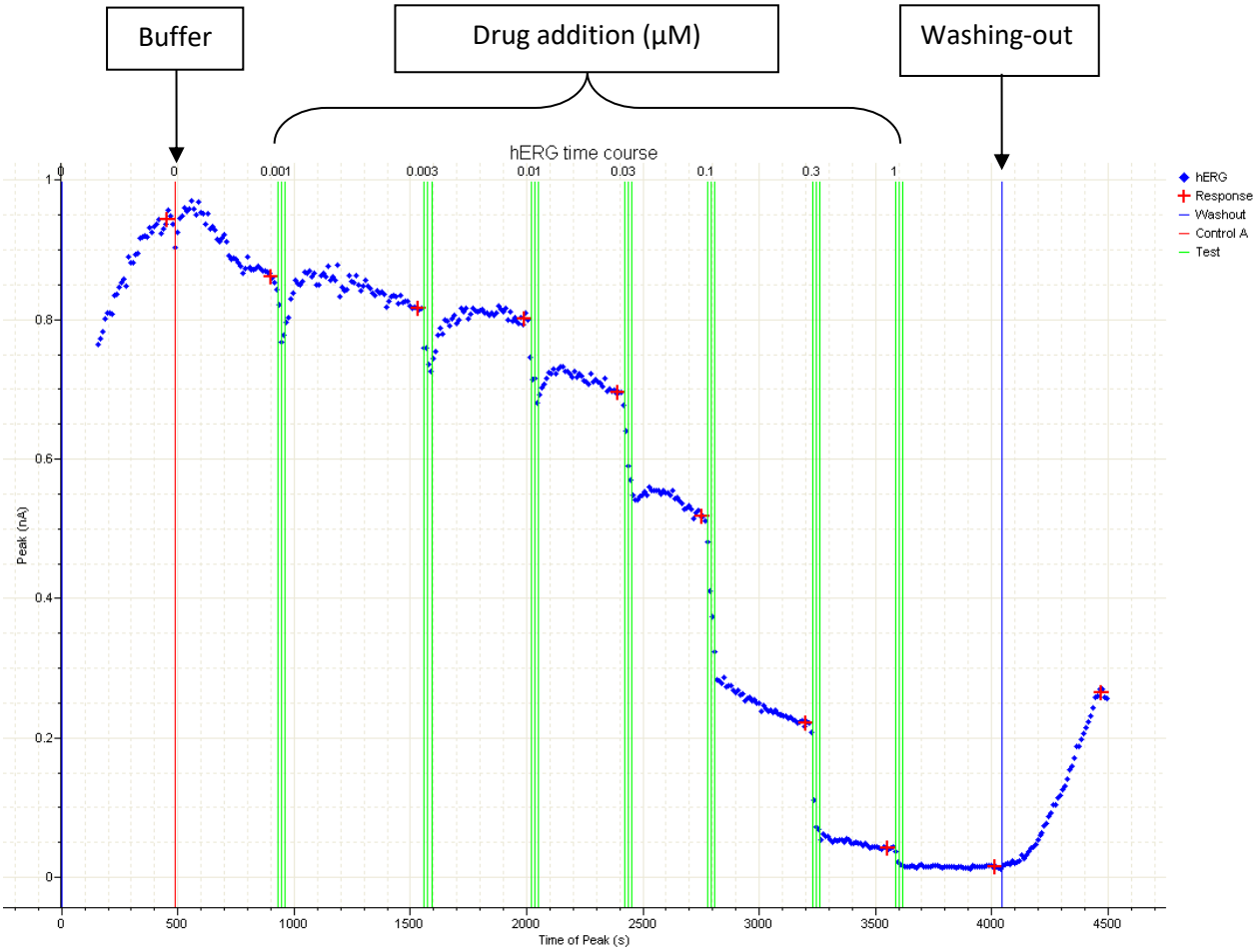


Figure 68. The hERG tail currents in Figure 67 were extracted from the program DataXpress, plotted against cisapride concentrations, and fitted to a four-parameter logistic function using GraphPad Prism 5.0 (131).

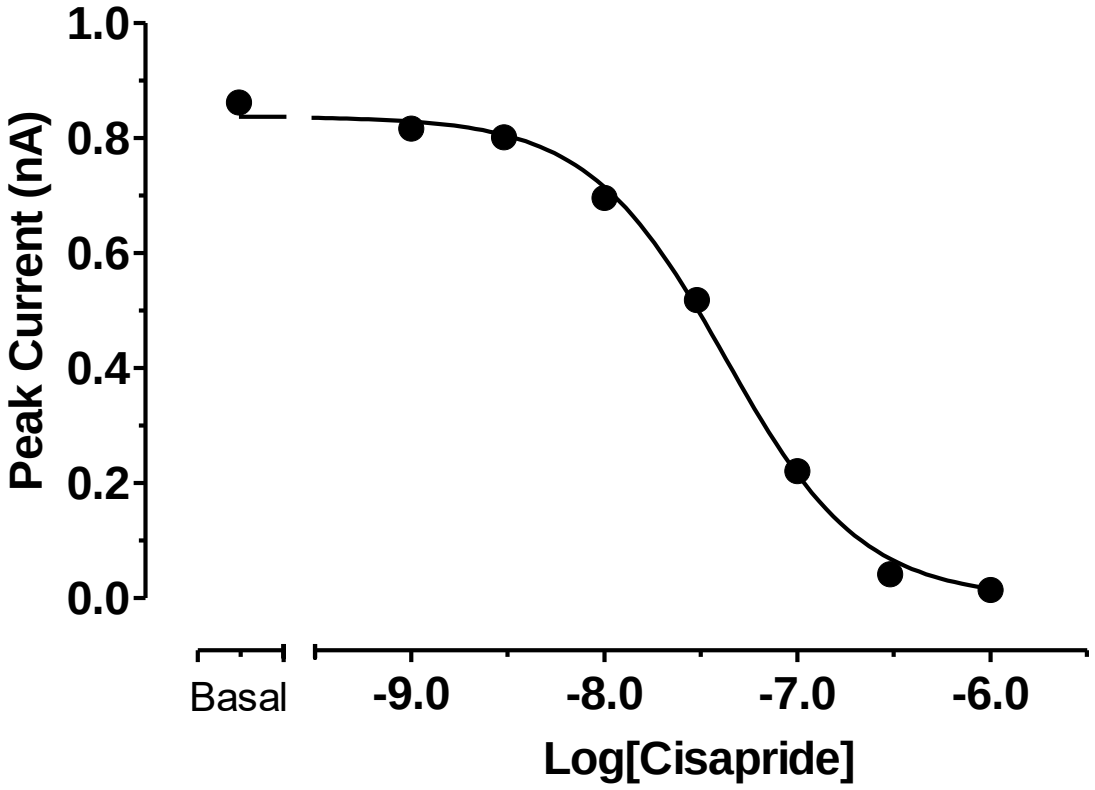
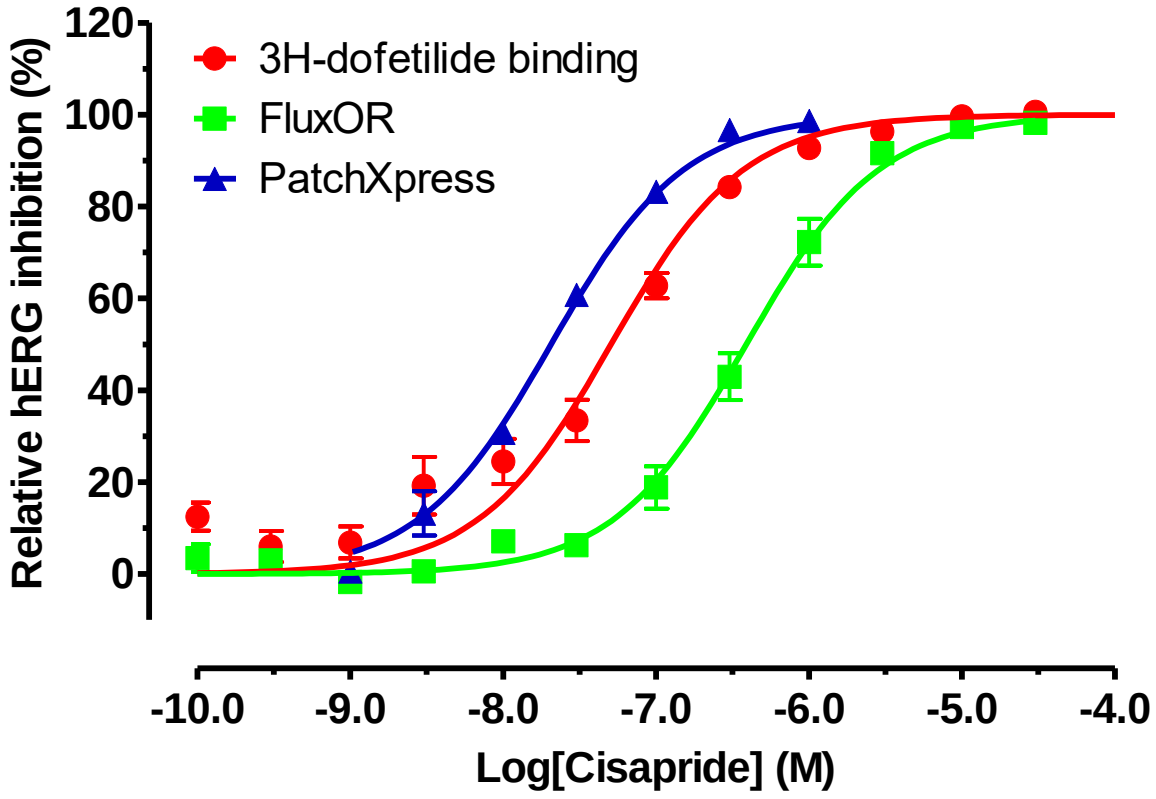


Figure 69. Side-by-side comparison of dose-dependent relations of cisapride in 3H-dofetilide competition binding assay; Tl^+ flux assay, and APPC assay. Results from multiple assays ($n \geq 2$) were normalized to percentage inhibition and pooled for curve-fitting in GraphPad Prism 5.0. Results indicated that the PatchXpress assay was the most sensitive, and the Tl^+ flux assay was the least sensitive (131).



2.11. Neurotransmitter transporter assays for DAT (Dopamine transporter), NET (norepinephrine transporter), and SERT (Serotonin transporter).

Main equipment: FlexStation II (Molecular Devices, Sunnyvale, CA)

Main reagent: Neurotransmitter transporter uptake assay kit (R8174) from Molecular Devices

Assay buffer: 20 mM HEPES, 1x HBSS, pH 7.40, RT

2.11.1. Background and principle: The assay system is designed to use the same fluorophore to measure norepinephrine (NET), dopamine (DAT), and serotonin (SERT) transporter activity (179–184). The proprietary fluorophore mimics biogenic neurotransmitters and is actively transported into the cell through the NET, DAT, or SERT. After incubation with test compounds, the dye solution is added to cells and the fluorescent dye is transported into the cell. External fluorescence is quenched with a masking dye, which cannot enter cells. Therefore, fluorophore fluoresces when it enters the cell and the fluorescence intensity is proportional to the transporter activity. The assay can be performed without a wash step and the fluorescence intensity can be monitored in kinetic mode or end-point modes.

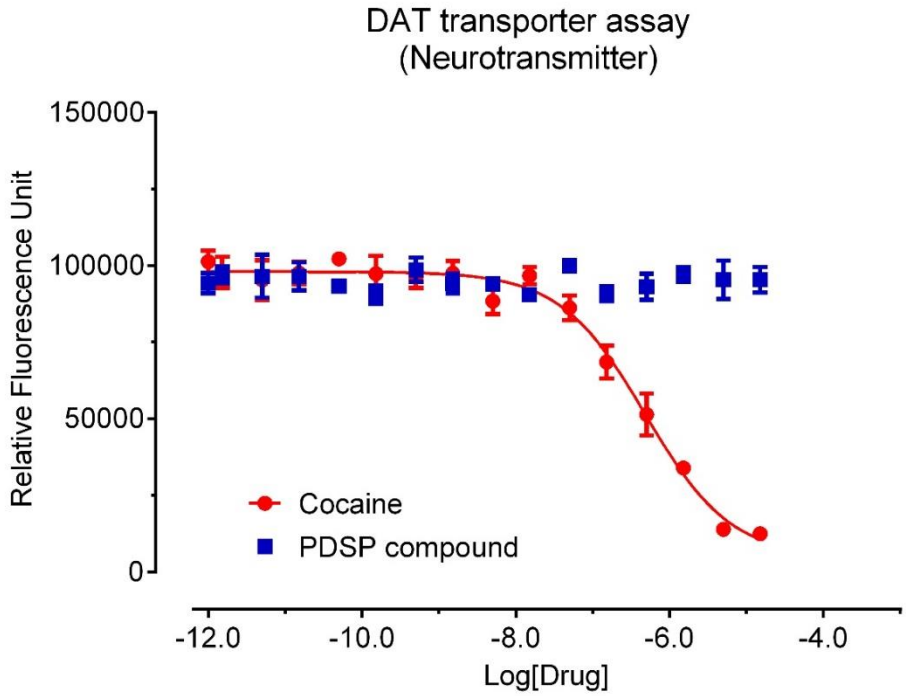
2.11.2. Assay procedure: The neurotransmitter transporter assays are conducted using Molecular Devices' Neurotransmitter Transporter Uptake Assay Kit (R8174) with HEK293 cells stably expressing human DAT, NET, or SERT. In brief, cells are plated in Poly-L-Lys (PLL)-coated 384-well black clear bottom cell culture plates in DMEM + 1% dialyzed FBS, at a density of 15,000 cells per well in a total volume of 40 ul. The cells are incubated for a minimum of 6 hours before use in assays. First, medium is removed, 20 μl of assay buffer (20 mM HEPES, 1x HBSS, pH 7.40) is added, followed by 5 μl of 5x drug solutions (384-well drug plate map #1 or #2, Figure 22, for primary assays and see 384-well drug map #4, Figure 25, in Section 2.1). Plates are incubated at 37°C for 30 min. After incubation, 25 μl of dye solution is added and fluorescence intensity is measured after 30 min at 37°C, using the FlexStation II (bottom read mode, Excitation at 440 nm, Emission at 520 nm with 510 nm cut-off). Results (Relative Fluorescence Units, RLU) are exported and plotted against drug concentrations in

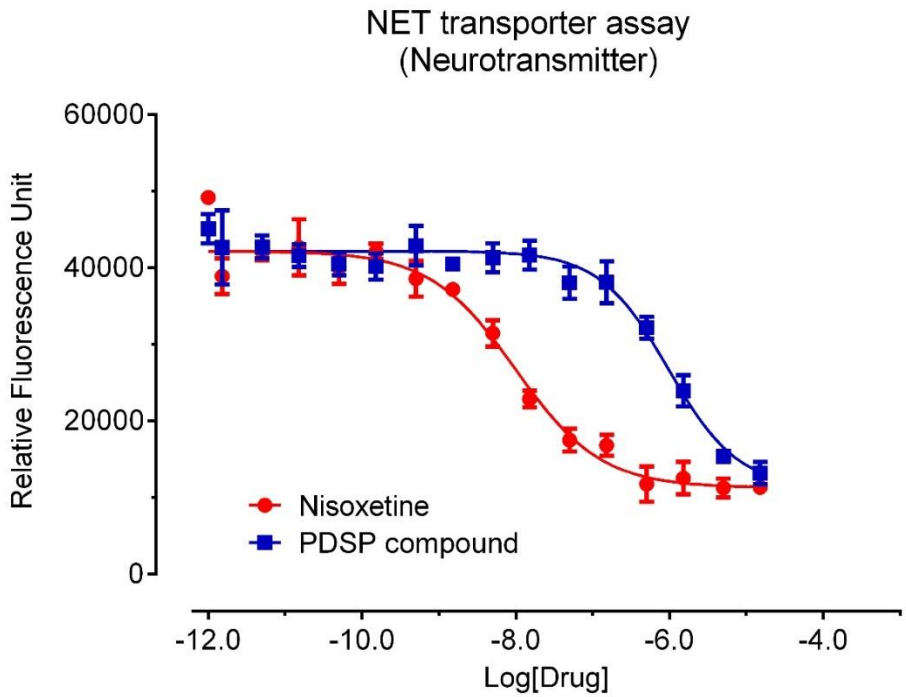
Prism 5.0 for nonlinear regression to obtain inhibitory potency. Cocaine, nisoxetine, and fluoxetine serve as positive controls for DAT, NET, and SERT, respectively.

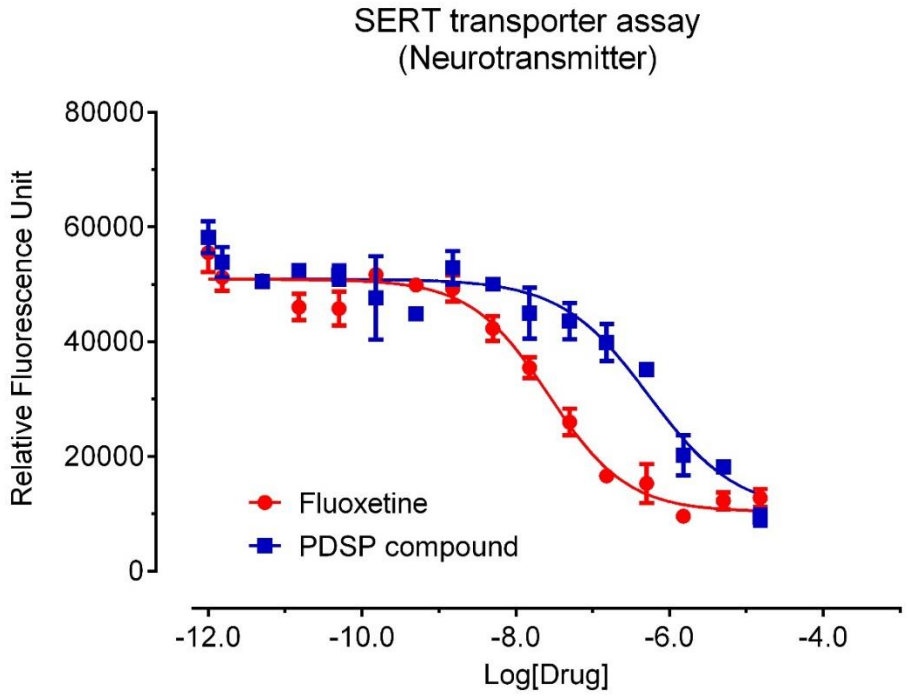
2.11.2. Data processing and analysis. Fluorescence intensity is exported and analyzed in Prism 5.0 to obtain IC_{50} values using non-linear least-squares curve fitting.

2.11.3. Representative figures

Figure 70. Representative curves of neurotransmitter reuptake inhibition at DAT, NET, and SERT.






SERT transporter assay
(Neurotransmitter)
Relative Fluorescence Unit
80000
60000
40000
20000
0
Fluoxetine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

2.12. Multidrug Resistance Transporter (MDR-1) assay.

Main equipment: FlexStation II (Molecular Devices, Sunnyvale, CA)

Assay buffer: Dulbecco's PBS, 10 mM Glucose

Protocol: The MDR assay protocol is adapted from PubChem BioAssay ID 377 (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=377)

2.12.1. MDR assay Background: Assays for modulation of MDR activity are performed using Caco-2 cells, a cultured line derived from human colonic epithelium. The assay relies on the fluorescent dye calcein an an indicator. Lipophilic pro-dye calcein acetoxymethyl ester (Calcein-AM) is not fluorescent, and easily gets across the plasma membrane, entering cells by passive diffusion. Calcein-AM is then converted by cellular esterases into highly fluorescent calcein, which is negatively charged and stays inside the cell. MDR can actively transport calcein-AM, but not fluorescent calcein, out of the cells; therefore intracellular fluorescent calcein would be low. Hihger MDR activity leads to lower fluorescence. Compounds that compete with calcein-AM for MDR will prevent the removal of calcein-AM from cells, leading to increased fluorescence. Therefore, fluorescence intensity can be used to estimate interaction between compounds and MDR (185–189).

2.12.2. MDR assay procedure: The assay monitors the time-dependent increase in calcein fluorescence in live cells in 96 well plates. This is carried out using a FlexStation II fluorimeter (Molecular Devices). Cells are seeded into glass-bottom 96-well plates one day before assay (80,000 cells per well). On the day of the assay, the medium is removed and replaced with 50 µl of D-PBS, 10 mM glucose containing no additional compound (negative control), test compound (25 µM), or reference compound (cyclosporin A) (25 µM). The cells are incubated for 30 min at 37°C, and then the instrument adds calcein-AM to the cells (500 nM final concentration). The instrument monitors fluorescence over a 4-min period and calculates the slope of the fluorescence increase. All compounds are assayed in quadruplicate and each assay contains wells with no test compound (negative control) and wells with 25 µM cyclosporin A, an efficient MDR inhibitor as a positive control. Results for test compounds are calculated from the slope of the fluorescence increase and are normalized so the value from untreated cells is 0% and the value for cyclosporin A is 100%.

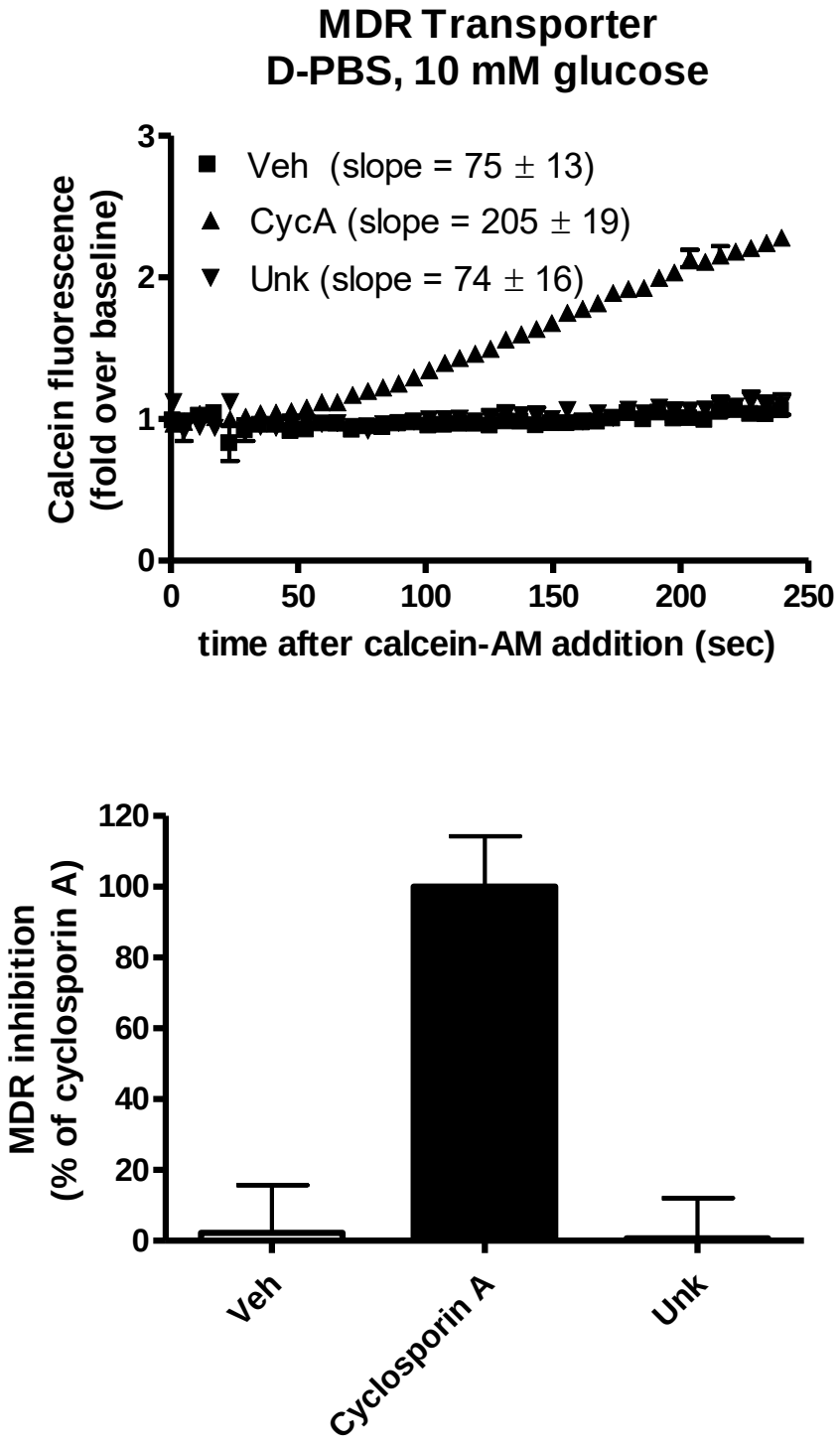
This assay has several features that make it ideal for initial screening of compounds for interaction with MDR. Because the assay is carried out in live cells, compounds must diffuse across lipid bilayers to interact with MDR sites on the cytoplasmic face of the protein. This is similar to the situation in vivo, where compounds must diffuse into the cytoplasm where they interact with MDR. Similarly, the assay provides a means for assessing not only interactions with MDR but also partitioning across cell membranes and thus hydrophobicity.

Although this assay is excellent for initial screening, users should be aware that the assay has several drawbacks. i) The assay does not distinguish between MDR substrates and inhibitors. Both will give similar signals in the assay because they prevent the transport of calcein-AM. ii) Some compounds may give spurious results by inhibiting the esterases that convert calcein-AM to calcein. Finally, (iii) activity depends on the cytoplasmic concentration of the compounds. For a compound that is an MDR substrate, this concentration depends on the rate of diffusion across the plasma membrane and the rate at which MDR pumps the compound out of the cells. At steady state, the cytoplasmic concentration will be lower than the extracellular concentration, but it cannot be measured easily. Consequently, this assay is not the best choice for determining half-maximal concentrations for interacting compounds.

2.12.3. Data process. Fluorescence instensity is exported and analyzed in Prism 5.0 uising non-linear least-squares curve fitting.

2.12.4. Representative figures

Figure 71. Representative data from an MDR inhibition assay.



2.13. Histone Deacetylase (HDAC) inhibition assay.

Main equipment: FlexStation II (Fluorescence plate reader) (Molecular Devices, CA)

Assay buffer: 50 mM Tris HCl, 137 mm NaCl; 2.7 mM KCl, 1 mM $MgCl_2$, pH 8.0

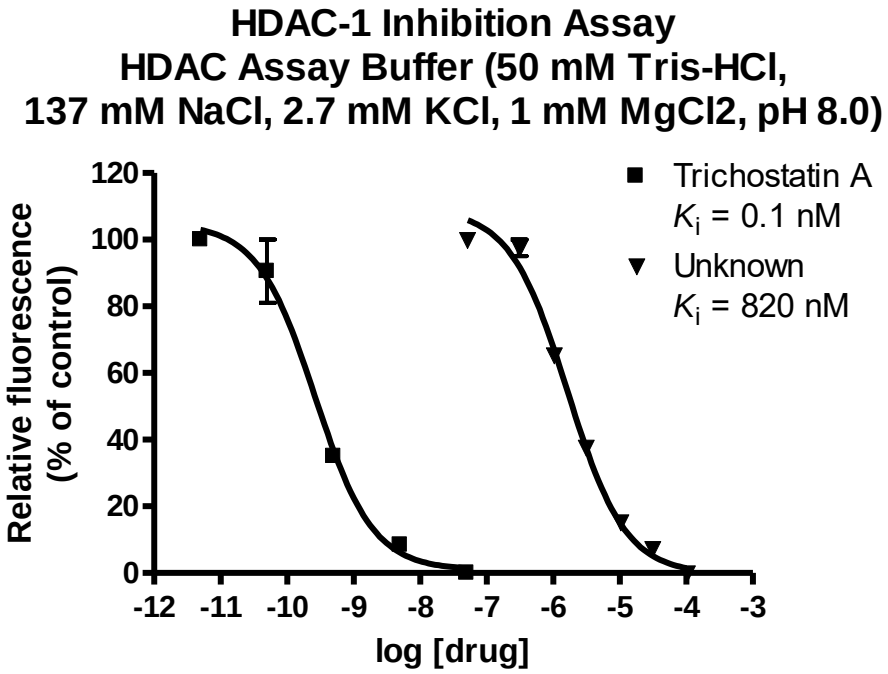
Main reagent: The HDAC assay protocol was adapted from BioMol Fluor de Lys assay system (Plymouth Meeting, PA)

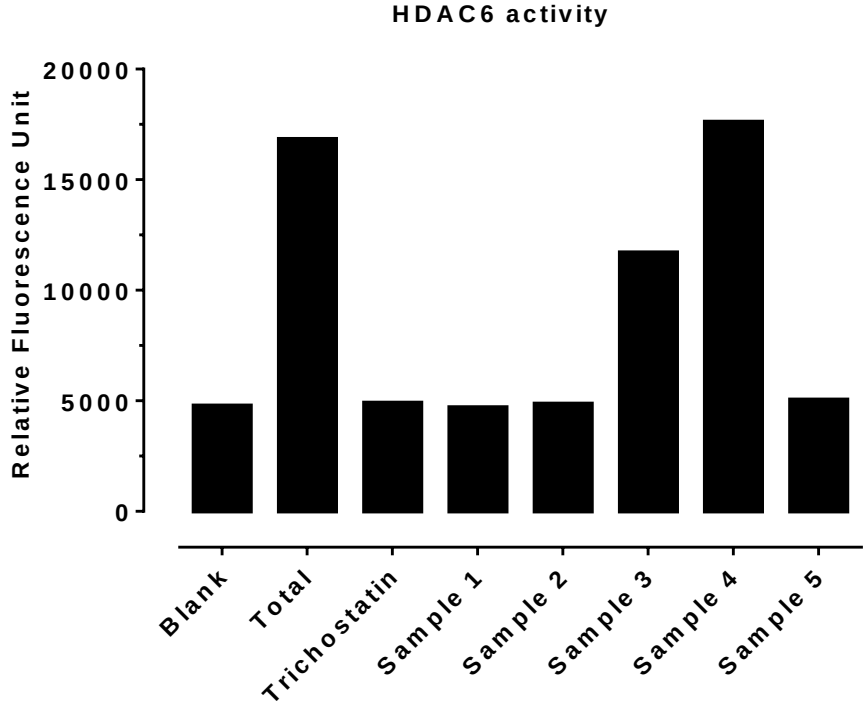
2.13.1. General Assay Procedure. To identify potential inhibitors of HDAC, we utilize the fluorimetric Fluor de Lys HDAC Assay Kit from Biomol as instructed by the manufacturer. Similar procedures have been modified for high throughput screening assays (190–193). Briefly, 4X dilutions of test compound or reference compound (trichostatin A) are prepared (final assay concentrations span from 0.1 nM to 10 µM) in Assay Buffer, and 12.5 µl are added to the wells of a 96-well plate (particular to this assay; Biomol). Nuclear extracts containing HDAC activity (procured from Biomol) are diluted to 4X and 12.5 µl are added to the wells containing test or reference compound (each concentration assayed in triplicate). The samples are incubated at room temperature for 10 min to equilibrate the temperature. Then, 25 µl of 2X Fluor de Lys HDAC substrate (final HDAC substrate concentration is typically a value between one half its apparent K_M and the apparent K_M; for HDAC1 a concentration of 50 µM is used, for HDAC6 a concentration between 10 and 30 µM is used) are added to each well. Deacetylation of the substrate, which generates a product that can be made fluorescent, is allowed to proceed for 30 min. Next, the reactions are stopped and the fluorescence of the deacetylated product is developed by adding 50 µl of 2X Assay Developer and incubating at room temperature for 15 min. Finally, fluorescence is read on a FLEXStation II plate reader (Molecular Devices) (excitation 350-380 nm, emission 440-460 nm).

2.13.2. Data processing. Raw data (RFUs) representing deacetylated substrate fluorescence is plotted as a function of the logarithm of the molar concentration of the test or reference compound. Non-linear regression of the normalized (to the fluorescence measured in the absence of HDAC inhibitor and test compound) raw data is performed in Prism 5.0 using the built-in three parameter logistic model describing competitive inhibition (one-site):

2.13.3. Representative figures.

Figure 72. Representative HDAC 1 inhibition curves (upper) and HDAC 6 inhibition in the absence and presence of 10 μM compounds (bar graph, lower)





2.14. Monoamine Oxidase (MAO) A and B assays.

Main equipment: FlexStation II (Fluorescence plate reader) (Molecular Devices, CA)

Main reagent: Monoamine A and B Detection Kit from Cell Technology (Mountain View, CA)

2.14.1. Background. Monoamine oxidase (MAO) is a flavin-containg enzyme that catalyses the oxidation of amine-containing neurotransmitters such as serotonin, norepinephrine, epinephrine, and dopamine to yield the corresponding aldehydes. MAO has two isoforms, MAO A and MAO B. They exhibit different specificities to substrates and inhibitor selectivities. MAO A acts preferentially on serotonin and norepinephrine and is inhibited by clorgyline. MAO B acts preferentially on 2-phenylethylamine and benzylamine and is inhibited by deprenyl and pargyline. Several fluorescence-based screening assays have been developed over the years (194–198) to screen for inhibitors or to measure MAO activities.

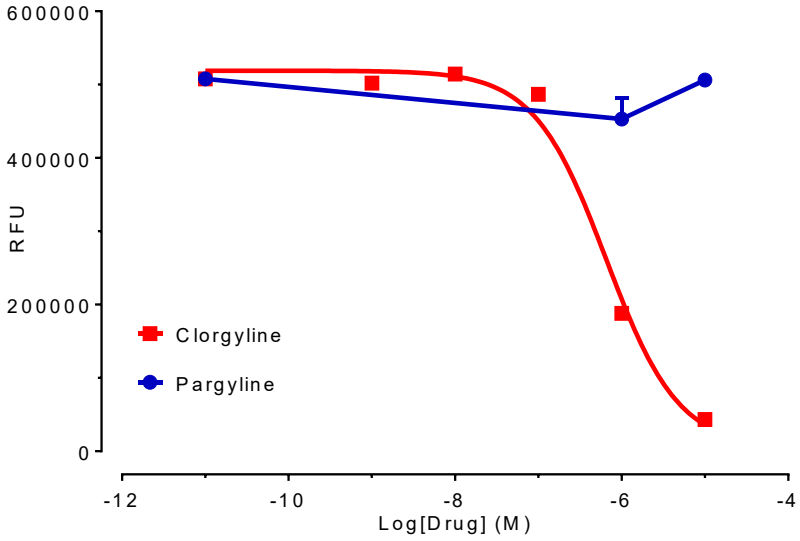
We use the MAO A and B Detection Kit from Cell Technology for Monoamine A and B enzyme assays. The assay system utilizes a non-fluorescent proprietary substrate to detect H_2O_2 released from the conversion of the MAO substrate (Benzylamine for MAO A and Tyramine for MAO B) to its aldehyde. The reaction of H_2O_2 with the non-fluorescent substrate is catalyzed by peroxidase in 1:1 stoichiometry to produce a fluorescent product with emission at 590 – 600 nm and excitation at 530 – 571 nm.

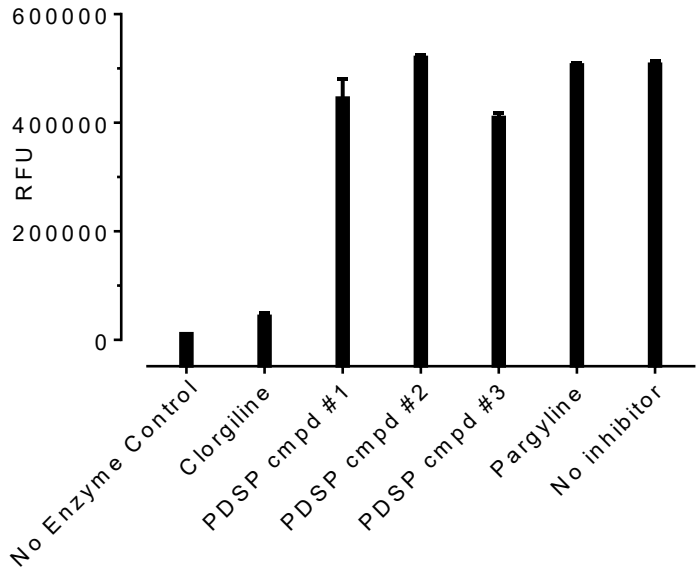
2.14.2. Assay procedure. The MAO A and B assays were performed in 96-well plates according to manufacturer's instructions. In brief, samples (test drugs and controls) are added to designated wells, followed by reaction cocktail containing reaction buffer, non-fluorescent substrate for H_2O_2, HRP, and substrate for MAO A or B. The reactions are allowed to proceed for 30 minutes at room temperature in the dark. At the end of the 30-minute incubation period, plates are read in the FlexStation II (Molecular Devices, CA) using excitation 570 nm and emission at 590 nm.

2.14.3. Data processing. Fluorescence intensity is exported and analyzed in Prism v 5.0 uising non-linear least-squares curve fitting.

2.14.4. Representative figures

Figure 73. Representative curves for MAO A activity in the presence of clorgyline and pargyline (upper) and bar graph showing MAO A activity in the presence of 10 μM different test compounds (lower).





2.15. PKC activity assay

Main equipment: FlexStation II (Fluroescence plate reader) (Molecular Devices, CA)

Main reagent: Omnia Ser/Thr Recombinant Kit 8 (#KNZ2081) from Invitrogen (Carlsbad, CA)

PKC isoforms: purchased from Invitrogen or Sigma (St. Louis, MO)

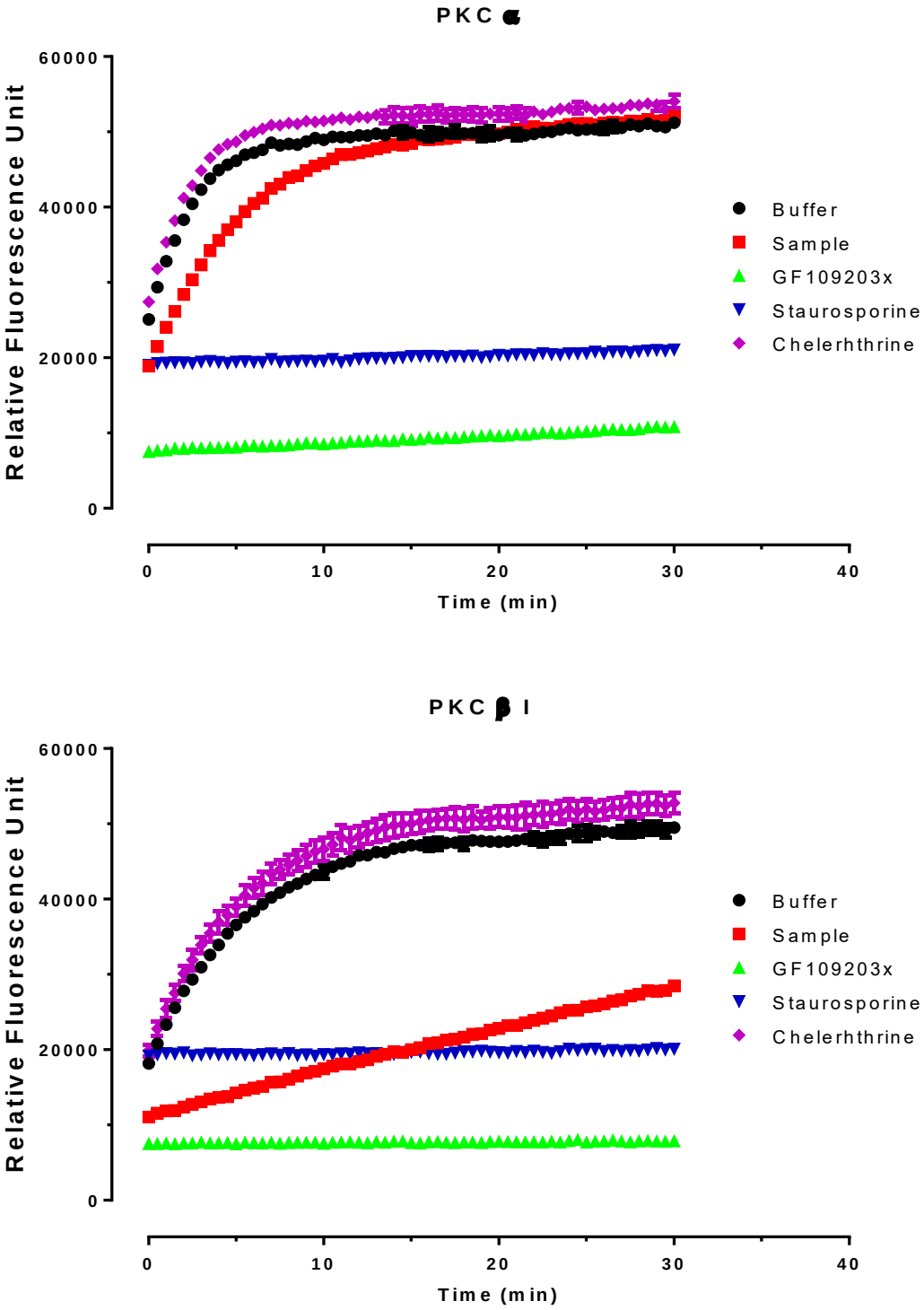
2.15.1. Background. Protein Kinase C (PKC) has 12 isoforms and is classed into three groups (conventional PKCs, Novel PKCs, and Atypical PKCs) based on their requirement of activators (Calcium ion lipids). Conventional PKC isoforms (α, βI, βII, and γ) require calcium, DAG, and phospholipid as activators; novel PKC isoforms (δ, ε, η,θ, and μ) require DAG but not calcium; atypical PKC isoforms (ζ and ι) require neither DAG nor calcium. We use Invitrogen's PKC assay kit (Kinase Activity Assay Kit, KNZ2081, aka The Omnia(R) Ser/Thr Recombinant Kit 8) for PKC inhibitor screening assays. The assay uses a Ser/Thr containing peptide substrate conjugated with the chelation-enhanced fluorophore (CHEF) 8-hydro-5-(N,N-dimethylsulfonamido)-2-methylquinoline (Sox). Phosphorylation of the peptide substrate results in Mg^{2+} chelation and formation of an ion-ion interaction bridge between the Sox moiety and phosphate group, leading to an increase in fluorescence at 485 nm when being excited at 360 nm.

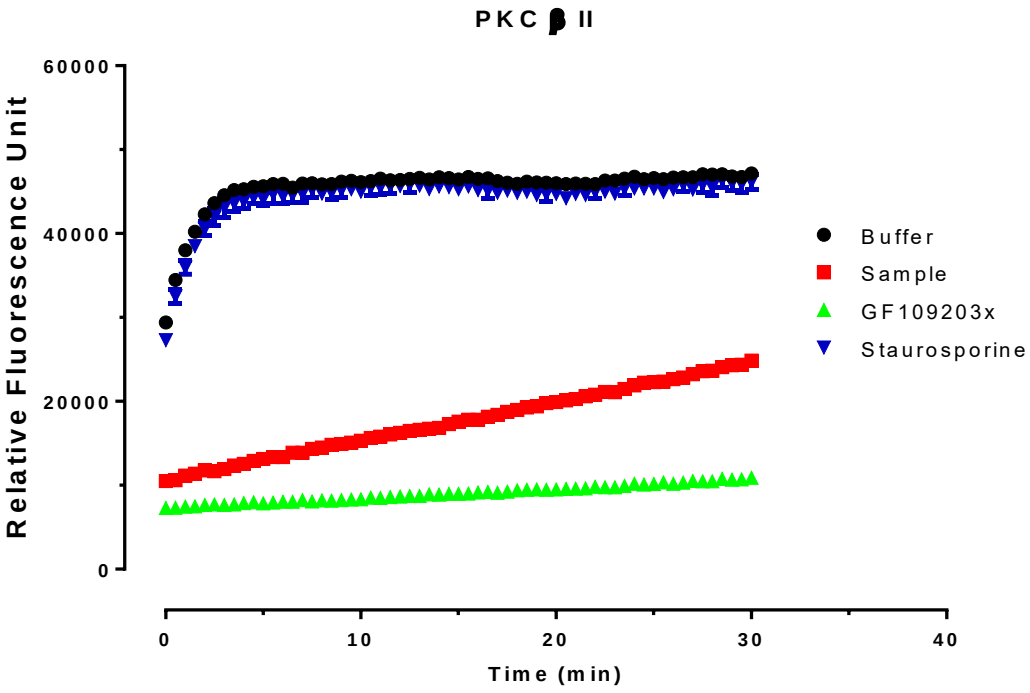
2.15.2. Assay procedure. PKC kinase activity assays are performed in 96-well plates (1/2 area and low protein binding surface) according to the manufacturer's suggested procedure. In brief, a master reaction mix is made with the following components: assay buffer, peptide substrate, ATP, DTT, calcium and/or lipid activator (use buffer for atypical PKC isoforms), and aliquotted to corresponding wells; this is followed by addition of drug working solutions (samples) or buffer (as negative control) or known inhibitors (as positive control). The mixture is warmed up in the FlexStation to 30°C for about 10 min. The reaction starts when PKC isoform is added, and plates are read every minute for 60 min, with excitation at 360 nm and emission at 485 nM.

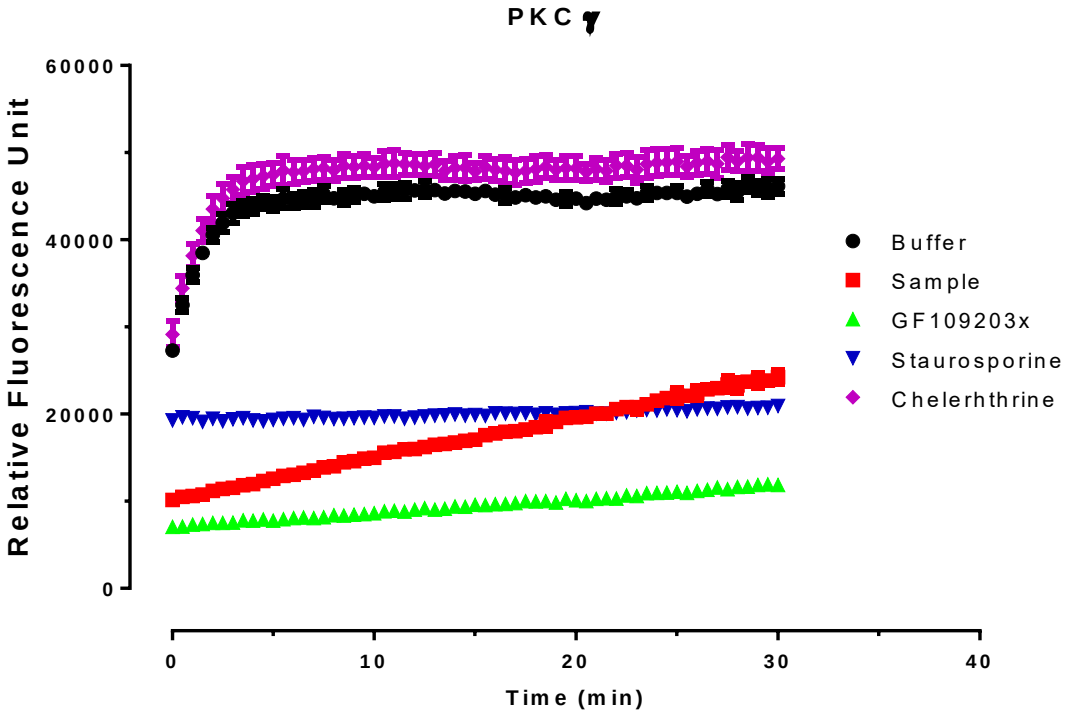
2.15.3. Data analysis. Fluorescence intensity increases over time. Intensity values are exported when the total activity reaches a plateau (or at the 30 min point) and analyzed in Prism v 5.0 using non-linear least-squares curve fitting.

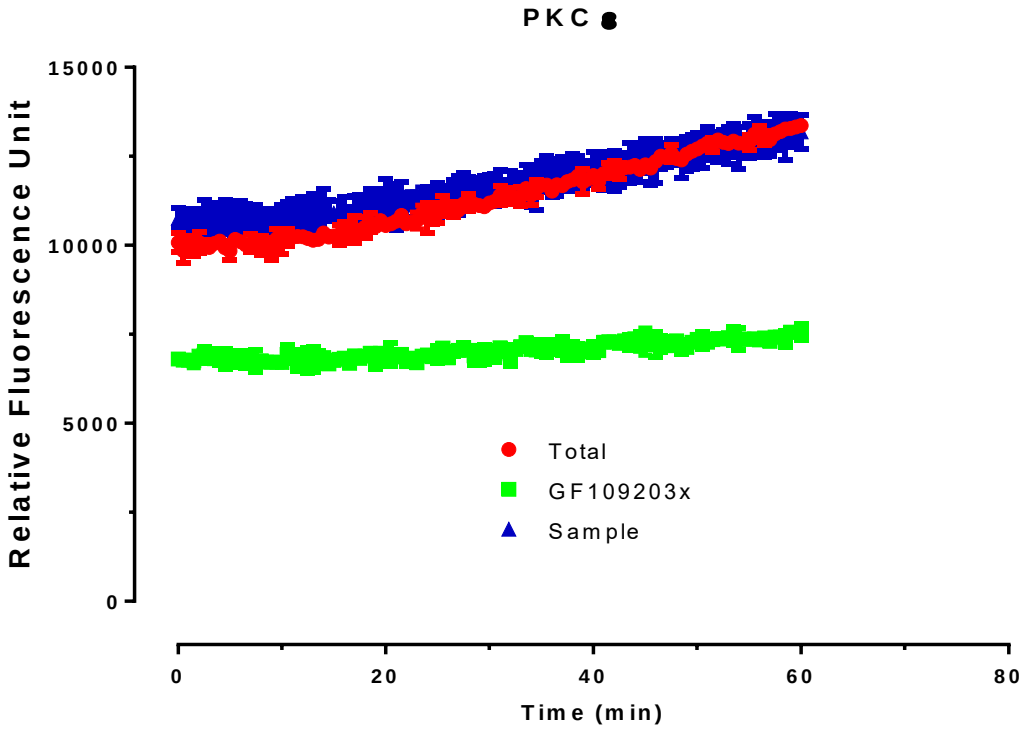
2.15.4. Representative figures.

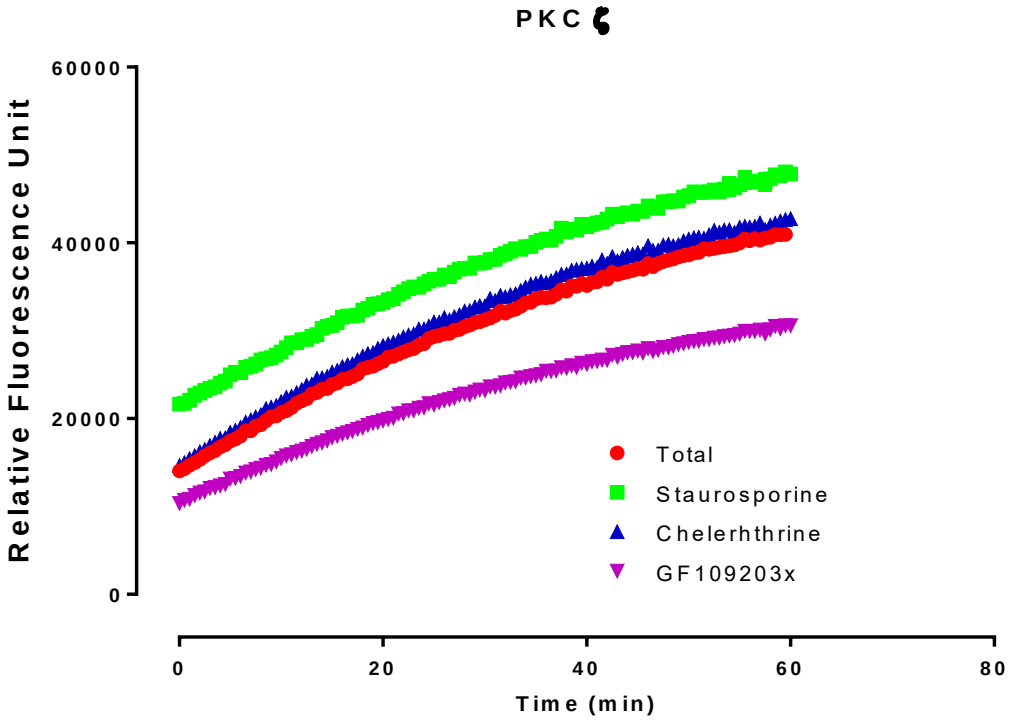
Figure 74. Time course of PKC activity in the absence and presence compounds (10 μM).

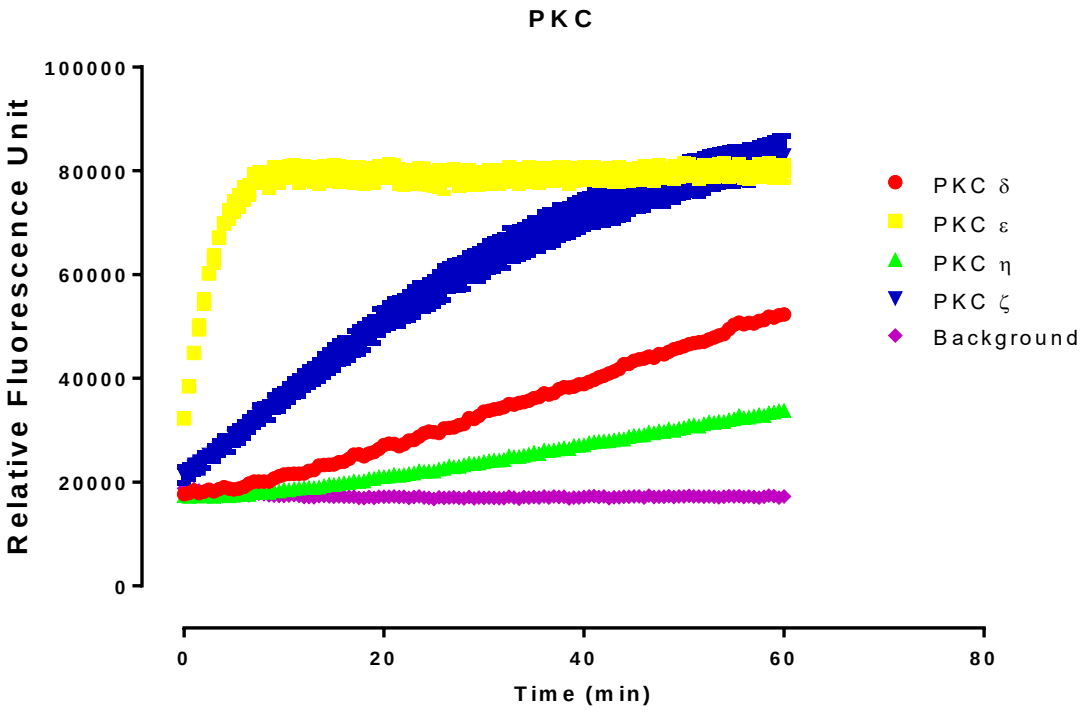


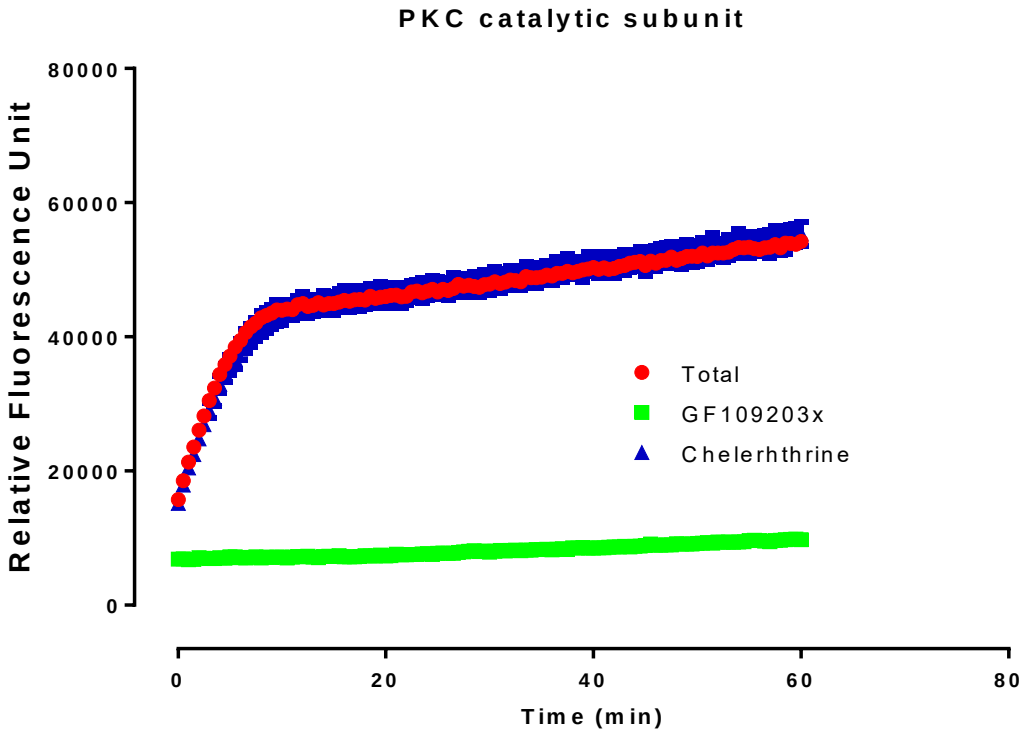

PKC β II
Relative Fluorescence Unit
60000
40000
20000
0
0
10
20
30
40
Time (min)
Buffer
Sample
GF109203x
Staurosporine

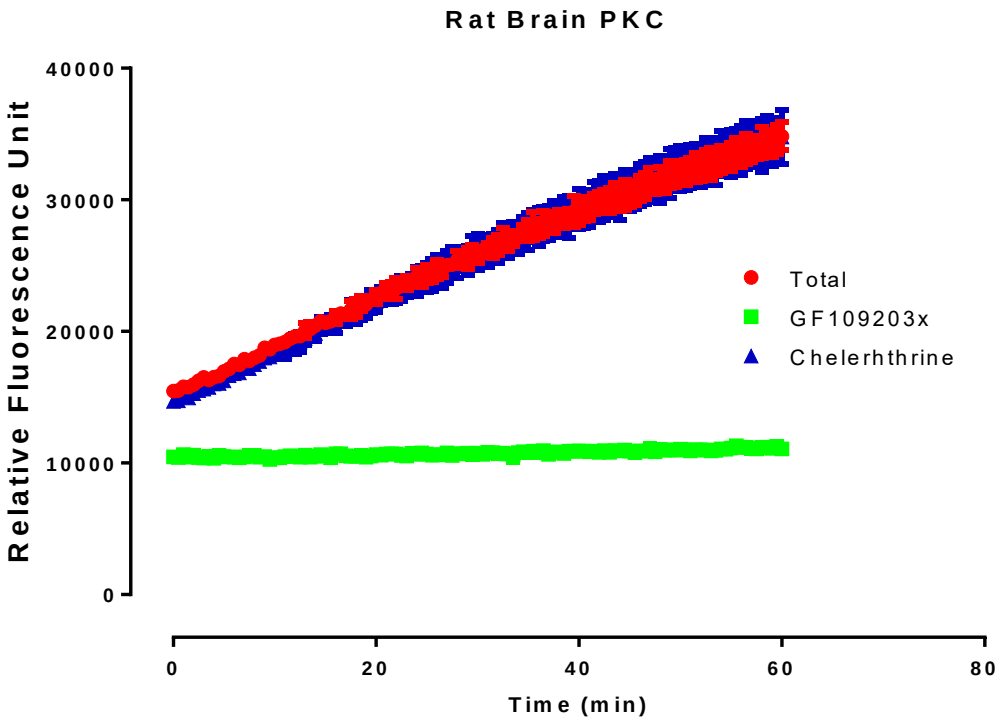

PKC γ
Relative Fluorescence Unit
60000
40000
20000
0
0
10
20
30
40
Time (min)
Buffer
Sample
GF109203x
Staurosporine
Chelerhthrine


PKC
Relative Fluorescence Unit
15000
10000
5000
0
Total
GF109203x
Sample
0
20
40
60
80
Time (min)


PKC
Relative Fluorescence Unit
60000
40000
20000
0
Total
Staurosporine
Chelerhthrine
GF109203x
0
20
40
60
80
Time (min)


PKC
Relative Fluorescence Unit
100000
80000
60000
40000
20000
0
0
20
40
60
80
Time (min)
PKC δ
PKC ε
PKC η
PKC ζ
Background


PKC catalytic subunit
Relative Fluorescence Unit
80000
60000
40000
20000
0
0
20
40
60
80
Time (min)
Total
GF109203x
Chelerhthrine


Rat Brain PKC
Relative Fluorescence Unit
40000
30000
20000
10000
0
0
20
40
60
80
Time (min)
Total
GF109203x
Chelerhthrine

2.16. Checkpoint Kinase 2 (CHK2) assay

Main equipment: FlexStation II (Fluorescence plate reader) (Molecular Devices, Sunnyvale, CA)

Main reagent: Omnia Ser/Thr Recombinant Kit 3 (#KNZ1031) from Invitrogen (Carlsbad, CA)

CHK2 enzyme (#PV3367) was purchased from Invitrogen.

2.16.1. Background. CHK2 is one of the Ser/Thr kinases phosphorylated and activated by upstream signaling apparatus (ATM and ATR) in response to DNA damage. Along with CHK1, CHK2 plays a critical role in determining cellular responses to DNA damage. Inhibitors for kinases like CHK2 could represent novel anticancer therapies.

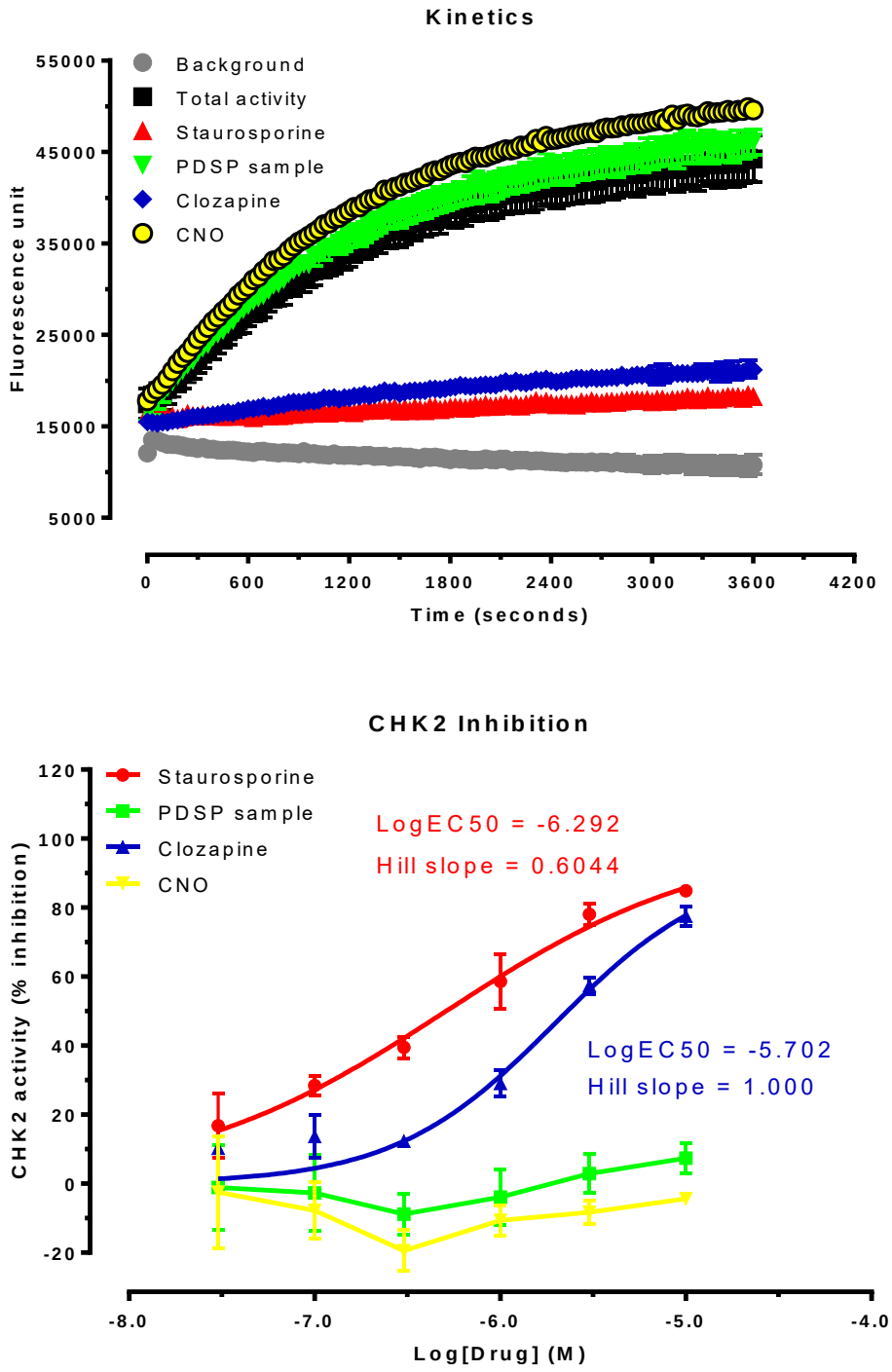
We use Invitrogen's Kinase Activity Assay Kit (#KNZ1031, Omnia Ser/Thr Recombinant Kit 3) to measure CHK2 activity (199–201). The Omnia® Kinase assay uses a Ser/Thr-containing peptide substrate conjugated with the chelation-enhanced fluorophore (CHEF) 8-hydro-5-(N,N-dimethylsulfonamido)-2-methylquinoline (Sox). Phosphorylation of the peptide sbustrate results in Mg^{2+} chelation and formation of an ion-ion interaction between the Sox moiety and phosphate group, leading to an increase in fluorescence at 485 nm when being excited at 360 nm.

2.16.2. Assay procedure. The PKC kinas activity assays are performed in 96-well plates (1/2 area and low protein binding surface) according to the manufacturer's suggested procedure. In brief, a master reaction mix is made with the following components: assay buffer, peptide substrate, ATP, DTT, calcium and/or lipid activator (use buffer for atypical PKC isoforms), and aliquotted to corresponding wells; drug working solutions (test samples) or buffer (as negative control) or known inhibitors (as positive control) are added, and the mixture is warmed up in the FlexStation to 30°C for about 10 min. The reaction starts when the PKC isoform is added, and plates are read every minute up to 60 min, with excitation of 360 nm and emission of 485 nM.

2.16.3. Data analysis. Fluorescence intensity increases over time. Intensity values are exported when the total activity reaches a plateau (or at the 30 min point) and analyzed in Prism v 5.0 uising non-linear least-squares curve fitting.

2.16.4. Representative figures

Figure 75. Representative figures for CHK2 kinase activity kinetics (upper) and percentage inhibition (lower).



2.17. Functional assays with human α3β4 and α4β2 nAChRs – $^{86}Rb^+$ efflux assay

2.17.1. Background. Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels, different from G protein-coupled muscarinic acetylcholine receptors (mAChRs). Each nAChR contains five subunits symmetrically forming a pore; each subunit has four transmembrane domains with both intracellular N- and C-terminus.

2.17.2. Assay procedure. Agonist and antagonist activities of PDSP compounds on nAChRs are assessed by measuring $^{86}RB^+$ efflux in HEK293 cells stably expressing nAChRs as described previously (5, 6). In brief, aliquots of cells in the selection growth medium are plated into Poly-D-Lysine coated 24-well plates. The plated cells are grown at 37°C for 18 to 24 h to reach 70-95% confluence. The cells are then incubated in growth medium (0.5 ml/well) containing $^{86}RBCl$ (2 μCi/ml) for 4 h at 37°C. This loading mixture is then aspirated, and the cells are washed four times with HEPES buffer (15 mM HEPES, 140 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM Glucose, pH 7.4; 1 ml/well). One ml of buffer, with or without agonists, is then added to each well. After incubation for 2 min, the assay buffer is collected and the amount of $^{86}Rb^+$ in the buffer is determined. Cells are lysed by adding 1 ml of 100 mM NaOH to each well, and the lysate is then collected for determination of the amount of $^{86}Rb^+$ in the cells at the end of the efflux assay. Radioactivity of assay samples and lysates is measured by liquid scintillation counting. The total amount of $^{86}Rb^+$ loaded (cpm) is calculated as the sum of the assay sample and the lysate of each well. The amount of $^{86}Rb^+$ efflux is expressed as a percentage of $^{86}Rb^+$ loaded. Stimulated $^{86}Rb^+$ efflux is defined as the difference between efflux in presence of nicotinic agonists and basal efflux measured in the absence of agonists.

2.17.2.1. Primary functional assay. The primary functional assay is carried out with $^{86}Rb^+$ efflux experiments. For assessing agonist activity, 4 concentrations of a test PDSP compound, 0.1, 1, 10 and 100 μM, are applied. Agonist activity is scaled as % of the stimulation by 100 μM nicotine. If a PDSP test compound shows a concentration-dependent activation, or shows 25% stimulation at any concentration, it is progressed to the secondary functional assay for agonist activity. For assessing antagonist activity, 4 concentrations of a test PDSP compound, 0.1 1, 10 and 100 μM, are applied in the presence of 100 μM nicotine. Antagonist activity is scaled as % inhibition of the $^{86}Rb^+$ efflux

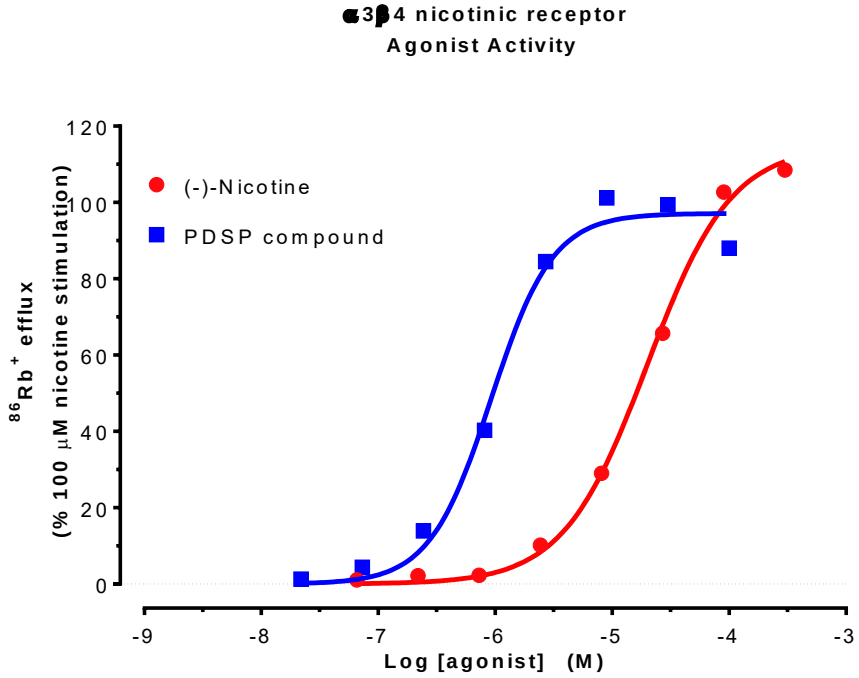
stimulated by 100 μM nicotine. If a PDSP test compound shows a concentration-dependent inhibition, and shows more than 50% of inhibition at 100 μM, it is progressed to the secondary functional assay for antagonist activity. All efflux assays are performed in quadruplicate. Nicotine is included in assays to define 100% agonist activity as well as to serve as a control.

2.17.2.2. Secondary functional assay. The secondary functional assay is carried out with $^{86}Rb^{+}$ efflux experiments. For assessing agonist activity, 8 concentrations of a test PDSP compound are applied. Agonist activity is scaled as % of stimulation by 100 μM nicotine. For assessing antagonist activity, 8 concentrations of a test PDSP compound are applied in the presence of 100 μM nicotine. Antagonist activity is scaled as % inhibition of the $^{86}Rb^{+}$ efflux stimulated by 100 μM nicotine. All efflux assays are performed in quadruplicate. Nicotine is included in assays to define 100% agonist activity as well as to serve as a control.

2.17.3. Data analysis. Radioactivity (cpm/well) is exported and analyzed in Prism 5.0 by nonlinear least-squares regression to estimate EC_{50} or IC_{50} values.

2.17.4. Representative figures

Figure 76. Representative figures of agonist activity at nAChRs



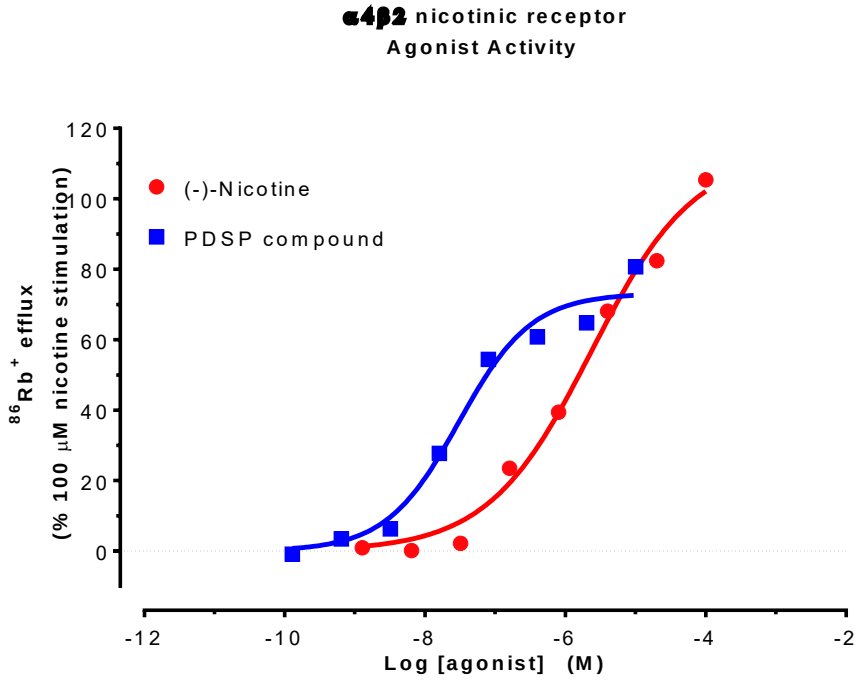
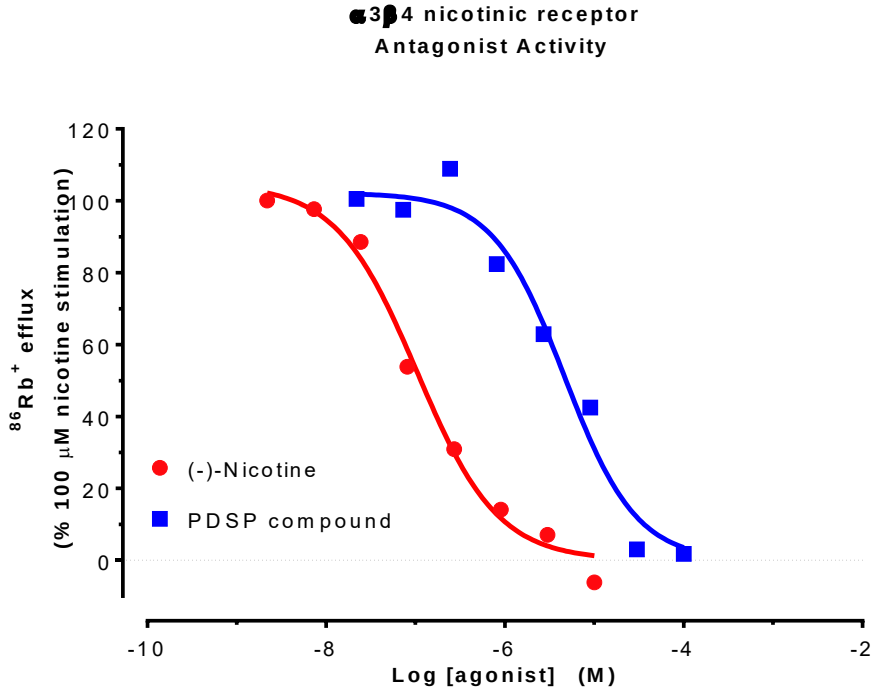


Figure 67. Representative figures of antagonist activity at nAChRs.



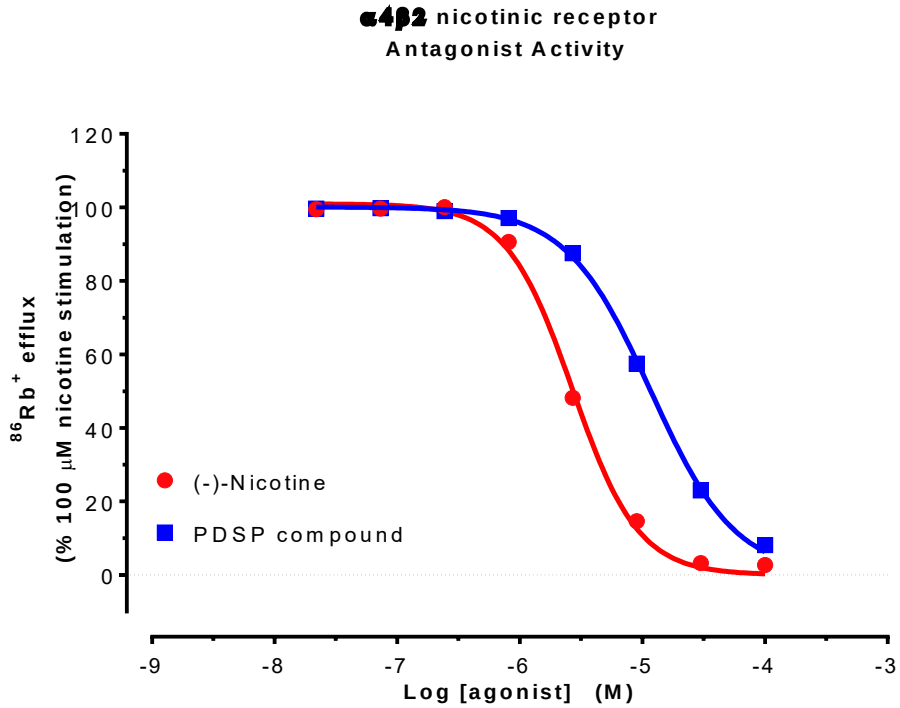


Table 34. Complete list of targets and corresponding available functional and radioligand binding assays at PDSP. ^{3}H or ^{125}I is for available radioligand binding assays. A checkmark (√) indicates that the functional assay is available: G_q = Calcium mobilization or Inositol phosphate accumulation assay; G_i or G_s = split luciferase cAMP biosensor assay; β-arrestin = GPCR mediated arrestin translocation assay; "Other Assay" indicates target-specific assays as detailed in the main text as indicated in the specific section; "TBO" indicates assays under development or verification or optimization. Many assays can be developed upon request. BRET assays are being developed and will be made available for the PDSP when they are fully optimized.

*DREADD = Designer Receptors Exclusively Activated by Designer Drugs.

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
5-HT receptors						
5-HT_{1A}	3H		√	√		
5-HT_{1B}	3H		√	√		
5-HT_{1D}	3H		√	√		
5-HT_{1E}	3H		√	√		
5-HT_{1F}			√	√		
5-HT_{2A}	3H	√		√		
5-HT_{2B}	3H	√		√		
5-HT_{2C} (INI)	3H	√		√		
5-HT_{2C} (VGI)	3H	√		√		
5-HT_{2C} (VGV)	3H	√		√		
5-HT_{2C} (VNV)	3H	√		√		
5-HT_{2C} (VSV)	3H	√		√		
5-HT_{3}	3H					
5-HT_{4}	3H		√	√		
5-HT_{5A}	3H		√	√		
5-HT_{6}	3H		√	√		
5-HT_{7A}	3H		√	√		
5-HT_{7B}						
5-HT_{7D}						
Acetylcholine (Muscarinic) receptors						
M_1	3H	√		√		
M_2	3H		√	√		
M_3	3H	√		√		
M_4	3H		√	√		

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
M_5	3H	√		√		
Acetylcholine (Muscarinic) DREADDs						
M_1 DREADD*	3H	√				DREADD
M_2 DREADD	3H		√			DREADD
M_3 DREADD	3H	√		√		DREADD
M_4 DREADD	3H		√	√		DREADD
M_5 DREADD	3H	√				DREADD
GsDREADD	3H	√	√			DREADD
Ahesion class GPCRs						
ADGRA1						GPR123
ADGRA2						GPR124
ADGRA3						GRP125
ADGRB1						BAI1
ADGRB2						BAI2
ADGRB3						BAI3
CELSR1						ADGRC1
CELSR2						ADGRC2
CELSR3						ADGRC3
ADGRD1						GPR133
ADGRD2						GPR144
ADGRE1						EMR1
ADGRE2						EMR2
ADGRE3						EMR3
ADGRE4P						EMR4, GPR127
ADGRE5						CD97
ADGRF1						GPR110
ADGRF2						GPR111
ADGRF3						GPR113
ADGRF4						GPR115
ADGRF5						GPR116
ADGRG1						GPR56
ADGRG2						GPR64
ADGRG3						GPR97
ADGRG4						GPR112
ADGRG5						GPR114
ADGRG6						GPR126
ADGRG7						GPR128
ADGRL1						LPHN1
ADGRL2						LPHN2
ADGRL3						LPHN3
ADGRL4						ELTD1
ADGRV1						GPR98
Adrenergic receptors						

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
α_{1A}	3H	√		√		
α_{1B}	3H	√		√		
α_{1D}	3H	√		√		
α_{2A}	3H		√	√		
α_{2B}	3H		√	√		
α_{2C}	3H		√	√		
$ß_1$	125I		√	√		
$ß_2$	3H		√	√		
$ß_3$	125I		√	√		
Adenosine receptors						
A_1	3H		√	√		
A_{2A}	3H		√	TBO		
Mouse A_{2A}	3H		√			
A_{2B}	3H		√	TBO		
A_3	3H			TBO		
Angiotensin II receptors						
AT_1	3H	√		√		
AT_2	3H			TBO		
Apelin receptor						
Apelin				√		
Bile Acid receptors						
GPBA				√		
Bombesin receptors						
BB_1		√		√		
BB_2		√		√		
BB_2, mouse		√				
BB_3		√		√		
Bradykinin receptors						
B_1				√		
B_2		√		√		
Calcitonin receptors						
CT				TBO		Calcitonin receptor
CT-like				TBO		CT-like receptor
Calcium sensing receptors						
CaS				√		
GPRC6				TBO		
Cannabinoid receptors						
CB_1 (rat brain)	3H					
CB_1	3H		√	√		
CB_2	3H		√	√		
GPR18				TBO		Orphan
GPR55				√		Orphan

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
GPR119				TBO		Orphan
Chemokine receptors						
CCR1				TBO		
CCR2				TBO		
CCR3				TBO		
CCR4				√		
CCR5				TBO		
CCR6				√		
CCR7				TBO		
CCR8				TBO		
CCR9				TBO		
CCR10				TBO		
CXCR1				√		
CXCR2				√		
CXCR3				TBO		
CXCR4				√		
CXCR5				TBO		
CXCR6				√		
ACKR3				√		CXCR7, CMKOR1, GPR159
CX_3CR1				√		
XCR1						
CCRL1						
CCRL2						
Cholecystokinin receptors						
CCK1		√		√		
CCK2		√		TBO		
Complement peptide receptors						
C3a				√		
C5a1				TBO		
C5a2				TBO		
Corticotropin-releasing factor receptors						
CRF_1	3H		√	TBO		CRHR1
CRF_2	3H		√	TBO		CRHR2
Dopamine receptors						
D_1	3H		√	√		
D_2	3H		√	√		
D_3	3H		TBO	√		
D_4	3H		√	√		
D_5	3H		√	√		
Endothelin receptors						
ET_A				√		

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
ET_B				TBO		
Estrogen receptor						
GPER				TBO		
Formylpeptide receptors						
FPR1				√		
FPR2/ALX				√		
FPR3				√		
Free fatty acid receptors						
FFA1		√		√		
FFA2				TBO		
FFA3				TBO		
FFA4				√		GPR120
GPR42				TBO		Orphan
Frizzled receptors						
FZD1						
FZD2						
FZD3						
FZD4						
FZD5						
FZD6						
FZD7						
FZD8						
FZD9						
FZD10						
SMO	3H					
GABAB receptors						
GABAB1						
GABAB2						
Galanin receptors						
GAL_1				√		
GAL_2				√		
GAL_3				√		
Ghrelin receptor						
Ghrelin	3H	√		√		
Glucagon receptors						
GHRH				√		
GIP						
GLP-1			√	√		
GLP-2				TBO		
Glucagon				TBO		
Secretin				√		
Glycoprotein receptors						

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
FSH				TBO		
LH				TBO		
TSH				TBO		
Gonadotrophin-releasing hormone receptors						
$GnRH_1$				√		
$GnRH_2$				TBO		
Histamine receptors						
H_1	3H	√		√		
H_2	3H, 125I	√	√	√		
H_3	3H		√	√		
H_4	3H			√		
Hydroxycarboxylic acid receptors						
HCA_1				TBO		GPR81
HCA_2	3H		√	√		GPR109A
HCA_3				TBO		GPR109B
Kisspeptin receptor						
Kisspeptin				TBO		
Leukotriene receptors						
BLT_1				√		
BLT_2				TBO		
$CysLT_1$				√		
$CysLT_2$				TBO		
OXE				TBO		
FRP2/ALX				√		
Lysophospholide (LAP) receptors						
LPA_1				√		
LPA_2				√		
LPA_3				TBO		
LPA_4				TBO		
LPA_5				√		
LPA_6				TBO		
Lysophospholipid (S1P) receptors						
$S1P_1$				√		
$S1P_2$				√		
$S1P_3$				√		
$S1P_4$				TBO		
$S1P_5$				TBO		
Melanin-concentrating hormone receptors						
MCH_1				√		
MCH_2				√		
Melanocortin receptors						
MC_1		√	√	√		
MC_2				TBO		

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
MC_3		√	√	TBO		
MC_4			√	√		
MC_5			√	√		
Melatonin receptors						
MT_1			√	√		
MT_2			√	√		
Metabotropic Glutamate receptors						
$mGlu_1$	3H TBO	√		TBO		
$mGlu_2$	3H		√	TBO		
$mGlu_3$	3H		√	TBO		
$mGlu_4$	3H TBO		√	TBO		
$mGlu_5$	3H	√		TBO		
$mGlu_5$ (rat brain)	3H		√			
$mGlu_6$	3H		√	TBO		
$mGlu_7$	3H TBO			TBO		
$mGlu_8$	3H TBO		√	TBO		
Motilin receptor						
Motilin				√		
Neuromedin U receptors						
NMU1				√		
NMU2				√		
Neuropeptide FF receptors						
NPFF1				TBO		
NPFF2				TBO		
Neuropeptide S receptor						
NPS				√		
Neuropeptide W/Neuropeptide B receptor						
$NPBW_1$			√	√		
$NPBW_2$			√	√		
Neuropeptide Y receptors						
Y_1				√		
Y_2				√		
Y_4				√		
Y_5				TBO		
Y_6				TBO		
Neurotensin receptors						
NTS1	3H	√		√		
NTS2	3H			√		
Opioid receptors						
δ (DOR)	3H		√	√		

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
κ (KOR)	3H		√	√		
μ (MOR)	3H		√	√		
NOP	3H		√	√		
MRGPRX1				√		Orphan
MRGPRX2		√		√		Orphan
MRGPRX3				TBO		Orphan
MRGPRX4		√		√		Orphan
Orexin receptors						
OX_1				√		
OX_2				√		
Oxoglutarate						
Oxoglutarate			√	TBO		
P2Y receptors						
$P2Y_1$		√		√		
$P2Y_2$		√		√		
$P2Y_4$		√		√		
$P2Y_6$		√		√		
$P2Y_8$				TBO		Orphan
$P2Y_{10}$				TBO		Orphan
$P2Y_{11}$		√		√		
$P2Y_{12}$				√		
$P2Y_{13}$				√		
$P2Y_{14}$				√		
Parathyroid hormone receptors						
PTH1				√		
PTH2				TBO		
QRFP receptor						
QRFP				√		GPR103
Platelet-activating factor receptor						
PAF	3H	√		√		
Prokineticin receptors						
PKR_1				TBO		PROKR1 (GPR73a)
PKR_2				TBO		PROKR2 (GPR73b)
Prolactin-releasing peptide receptor						
PrRP				TBO		GPR10
Prostanoid receptors						
DP_1				TBO		PTGDR
DP_2				√		PTGDR2
EP_1	3H			√		PTGER1
EP_2	3H			√		PTGER2
EP_3	3H			√		PTGER3
EP_4	3H			√		PTGER4

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
FP				√		PTGFR
IP1				√		PTGIR
TP			√	TBO		TBXA2R
Protease-activated receptors						
PAR_1		√		TBO		
PAR_2				TBO		
PAR_3				TBO		
PAR_4				TBO		
Relaxin family peptide receptors						
$RXFP_1$			√	TBO		
$RXFP_2$			√	TBO		
$RXFP_3$			√	TBO		
$RXFP_4$			√	TBO		
Somatostatin receptors						
SST_1				√		
SST_2				√		
SST_3				√		
SST_4				√		
SST_5			√	√		
Succinate receptor						
Succinate				TBO		GPR91
Tachykinin receptors (Neurokinin receptors)						
NK_1		√		√		
NK_2		√		√		
NK_3		√		√		
Thyrotropin-releasing hormone receptors						
TRH_1				TBO		
TRH_2				TBO		
Trace amine receptor						
TA_1				√		
Urotensin receptor						
UT				√		
Vasopressin and Oxytocin receptors						
V_{1A}	3H	√		√		
V_{1B}	3H	√		√		
V_2	3H	√		√		
OT	3H	√		√		
VIP and PACAP receptors						
PAC_1				TBO		ADCYAP1R1
$VPAC_1$				√		PVR2
VPAC2				√		VIPR1
	Class A orphan GPCRs					
CMKLR1				√		

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
GPR1				TBO		
GPR3				TBO		
GPR4			√	TBO		
Mouse GPR4			TBO			
GPR6				TBO		
GPR12				TBO		
GPR15				TBO		
GPR17				TBO		
GPR18				TBO		
GPR19				TBO		
GPR20				TBO		
GPR21				TBO		
GPR22				TBO		
GPR25				TBO		
GPR26				TBO		
GPR27				TBO		
GPR31				TBO		
GPR32				TBO		
GPR33				TBO		
GPR34				TBO		
GPR35				√		
GPR37				TBO		
GPR37L1				TBO		
GPR39				√		
GPR42						FFAR1L
GPR45				TBO		
GPR50				TBO		
GPR52				TBO		
GPR55				√		
GPR61				TBO		
GPR62				TBO		
GPR63				TBO		
GPR65			√	TBO		
Mouse GPR65			TBO			
GPR68		√	√	TBO		
Mouse GPR68			TBO			
GPR75				TBO		
GPR78				TBO		
GPR79						
GPR82				TBO		
GPR83				TBO		
GPR84				TBO		
GPR85				TBO		

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
GPR87				TBO		
GPR88			√	TBO		
GPR101				TBO		
GPR119				TBO		
GPR132				TBO		
GPR135				TBO		
GPR139				TBO		
GPR141				TBO		
GPR142				TBO		
GPR146				TBO		
GPR148				TBO		
GPR149				TBO		
GPR150				TBO		
GPR151				TBO		
GPR152				TBO		
GPR153				TBO		
GPR156				TBO		
GPR157				TBO		
GPR158				TBO		
GPR160				TBO		
GPR161				TBO		
GPR162				TBO		
GPR171				TBO		
GPR173				TBO		
GPR174				TBO		
GPR176				TBO		
GPR182				TBO		
GPR183				TBO		EBI2
LGR4						GPR48
LGR5						GPR49
LGR6						
MAS1				√		
MAS1L				TBO		
MRGPRD				TBO		
MRGPRE				TBO		
MRGPRF				TBO		
MRGPRG				TBO		
MRGPRX1				√		
MRGPRX2		√		√		
MRGPRX3				TBO		
MRGPRX4		√		√		
P2RY8				TBO		
P2RY10				TBO		

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
TAAR2				TBO		GPR58
TAAR3						GPR57
TAAR4P						
TAAR5				TBO		
TAAR6				TBO		
TAAR8				TBO		GPR102
TAAR9				TBO		
	Class B orphan GPCRs					
GPR56				TBO		
GPR64				TBO		
GPR97				TBO		
GPR110				TBO		
GPR113				TBO		
GPR114				TBO		
GPR115				TBO		
GPR116				TBO		
GPR123				TBO		
GPR124				TBO		
GPR125				TBO		
GRP126				TBO		
GPR128				TBO		
GPR133				TBO		
GPR144				TBO		
GPR157				TBO		
	Class C orphan GPCRs					
GPR156				TBO		
GPR158				TBO		
GPRC5A				TBO		
GPRC5B				TBO		
GPRC5C				TBO		
GPRC5D				TBO		
GPRC6A				TBO		
Opsin receptors						
OPN1LW						
OPN1MW						
OPN1SW						
Rhodopsin						
OPN3				TBO		
OPN4						
OPN5				TBO		GPR136
	Other 7TM receptors					

Target (Receptor name)	Available assays at PDSP (new system)					Note
	Binding	G_q	G_i or G_s	ß-arrestin	BRET	
GPR107						
GPR137						
OR51E1						GPR136/GPR164
TPRA1						GPR175
GPR143				TBO		
GPR157						

Table 34. PDSP targets other than 7-TMs.

Other targets	Binding	Functional assays	Note
nAChRs			
α2ß2	3H	($^{86}Rb^{+}$ efflux assay)	Section 2.17 Assays conducted at PDSP subcontractor Georgetown Univ.
α2ß4	3H		
α3ß2	3H		
α3ß4	3H	($^{86}Rb^{+}$ efflux assay)	
α4ß2	3H	($^{86}Rb^{+}$ efflux assay)	
α4ß4	3H		
α4ß2 (Rat Brain)	3H		
α2ß2	3H		
α7	3H		
Imidazoline receptors			
I1 (rat brain)	3H		
I2 (rat brain)	3H		
GABA channels			
GABAA a1	3H, BZP		
GABAA a2	3H, BZP		
GABAA a3	3H, BZP		
GABAA a5	3H, BZP		
GABAA a6	3H, BZP		
GABAA (rat brain)	3H, BZP		
GABAB (rat brain)	3H		
Ion channels			
hERG K+	3H	Tl^{+} Flux and PatchXpress	Section 2.9 Section 2.10
Ca^{2+} (rat brain)	3H		
Cav1.2, human	3H		
Na+ site II	3H		
Transporters			
DAT	3H	Neurotransmitter Transporter assay	Section 2.11
NET	3H		
SAT	3H		
VMAT1			
VMAT2	3H		
Sigma receptors			
Sigma 1 (Guinea Pig)	3H		
Sigma 1, human	3H		
Sigma 2 (PC12)	3H		
Sigma 2, human	3H		
Glutamate channels			

Other targets	Binding	Functional assays	Note
NMDA (rat brain)	3H		
AMPA (rat brain)	3H		
PCP (rat brain)	3H		
Kainate (rat brain)	3H		
NR1 (rat brain)	3H		
NR2B (rat brain)	3H		
Other targets			
MDR1		(Caco-2)	
PKCα	3H	Fluorimetric, Omnia Ser/Thr Recombinant assay kits from Invitrogen	Secton 2.15
PKCβ I	3H		
PKCβ II	3H		
PKCγ	3H		
PKCδ	3H		
PKCε	3H		
PKCη			
PKCζ			
PKCτ			
CHK2		Fluorimetric, Omnia Ser/Thr Recombinant assay kits from Invitrogen	
PBR (rat brain)	3H		
HDAC		Fluorimetric, BioMol Fluor de Lys assay	Section 2.13
MAO A		Fluorimetric, MAO detection kit	Section 2.14
MAO B		Fluorimetric, MAO detection kit	

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