National Institute of Mental Health Psychoactive Drug Screening Program

(NIMH PDSP)

ASSAY PROTOCOL BOOK

Version II

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This is an updated version as of March 2013 of the previous PDSP assay protocol book, and contains detailed descriptions of experimental procedures and data analysis for radioligand binding and functional assays as 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 standard compounds; a retrieval module which selects, retrieves, 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 run 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 the ASM server computer.
4. The assay method corresponding to the assay to be run is selected; the ASM server and store, 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 selection begins in the ASM Store, which sends messages to the appropriate liquid handling station.

A. The ASM Store selects and retrieves samples using the 2-dimensional bar code on each tube.
B. Samples are collected in an empty rack, inside the ASM Store
C. 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.
D. Samples are thawed with forced hot air according to protocol with an option to enter non-default 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(s) and pipetting tips. (Steps 8-14 are performed in parallel to step 7.)
9. The STAR computer run control (this is a separate instance from the ASM run control) prompts the user for tip-counting verification; the user also affirms 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 per final plate); the plate barcode is recorded and used throughout our process to verify the integrity of sample tracking.
12. 97.5 microliters of buffer is added to each of the wells in rows A and E of each assay plate.
13. DMSO (2.5 microliters, vehicle control) is added to A1 & E1 of each assay plate.
14. The STAR platform pauses with user input, waits for drug delivery from the ASM (step 7E above).
15. Samples are mixed after step 7F is completed, and the sample rack is delivered to the STAR deck.
16. 2.5 microliters of control compound is 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 their 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 are added and the receptor to be assayed is assigned.
21. Steps 16-20 are repeated for each assay plate. (See Figure 1 for primary drug plate map).

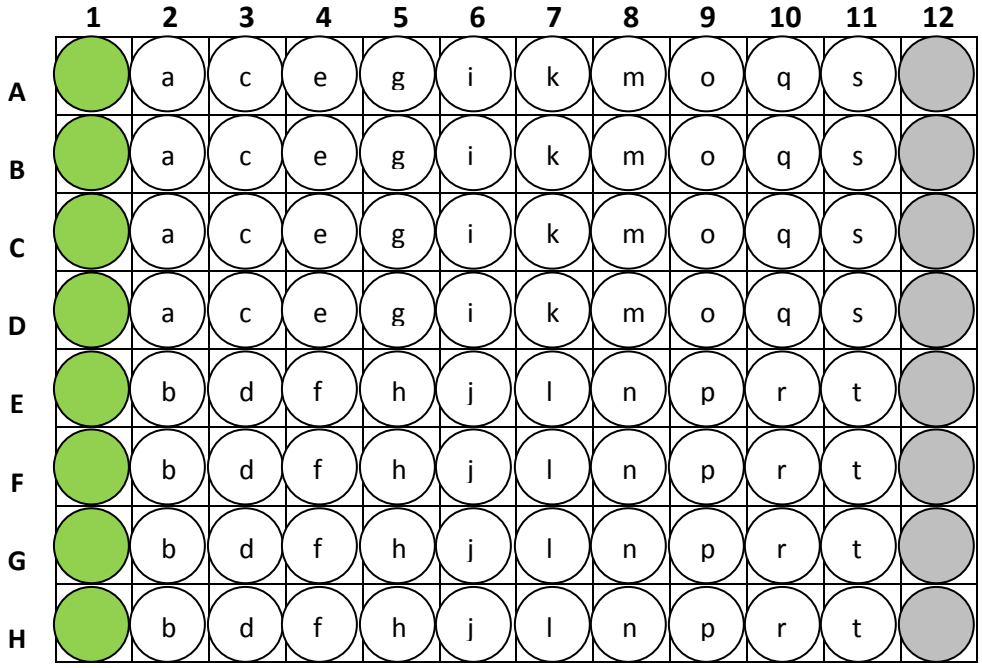
B. Secondary Plate Preparation:

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 & store, 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 the thaw time variables or defaults.
7. Sample selection and retrieval begin in the ASM Store, which sends messages to the appropriate liquid handling station.
    A. The ASM Store selects samples using the 2-dimensional bar code on each tube
    B. Samples are collected in an empty rack, inside the ASM Store
    C. 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.
    D. Samples are thawed with forced hot air according to protocol with an option to enter non-default thaw times.
    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(s) and pipetting 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; the user also affirms 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 barcode is recorded and used throughout our process to verify the integrity of sample tracking.
12. 180 microliters of buffer is added to all wells of each assay plate with the 96CORE Head.
13. 15 microliters of buffer is added to column 12, 40 microliters is aspirated from column 11.
14. The STAR platform pauses and waits for drug delivery from the ASM (step 7F above).
15. Samples are mixed.
16. 5 microliters of sample (or control) is aspirated from the appropriate sample tube and distributed into the first dilution plate in column 12 using the eight single-channel pipetters.
17. 40 microliters of buffer is removed from column 11 of the dilution plate.
18. After mixing, 60 microliters is aspirated from column 12 and dispensed into column 11 of the dilution plate.
19. A serial dilution is performed; 20 microliters is aspirated from/dispensed to column 12 to 10, column 10 to 8, column 8 to 6, column 6 to4, column 4 to 2, column 11 to 9, column 9 to 7, column 7 to 5 and, column 5 to 3, resulting in a half-log dilution series across the plate.
20. The entire plate is mixed and then three replicates containing 25 micoliters of drug solution per well are made.
21. Steps 16-20 are repeated for each additional set of plates.
22. Plate maps are created corresponding to the plate barcode, the samples added and the 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, that is, what assays to select, when to make assay plates and, assigning assays to a research technician 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 research technician. 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 and consumables ready prior to start, as well as the sequence of primary versus secondary methods to minimize transition time between runs, are all required have the system operate for >35 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 (upper panel) and an actual barcoded 96-well drug plate (lower pannel) for primary radioligand binding assays. The drug plate is designed to contain 20 compounds (compounds “a” to “t” in Columns 2 - 11, each in quadruplicate) and one reference compound (Column 12), 25 µl per well at 5x of the final concentration of 10 µM. Column 1 contains buffer only, and therefore is designated as “total binding” (0% inhibition). Column 12 contains a reference compound (Histamine in this case) and is designated as nonspecific binding (100% inhibition).

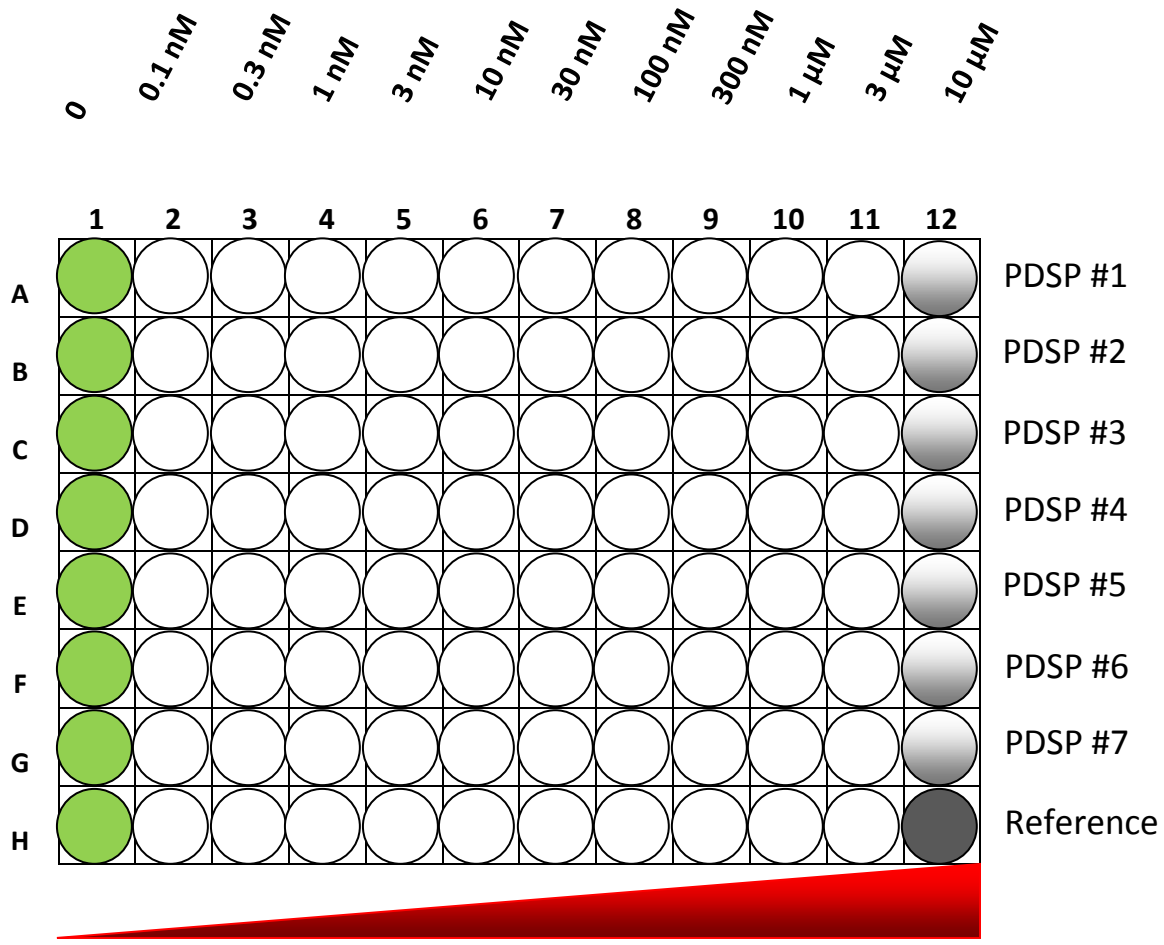


Figure 2a. 96-well drug plate map for secondary radioligand binding assays. The drug plate is designed to contain 7 PDSP compounds (Rows A to G) and 1 reference compound (Row H) as indicated at right of the plate, one compound per row and 25 µl per well at 5x of the final concentrations as indicated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

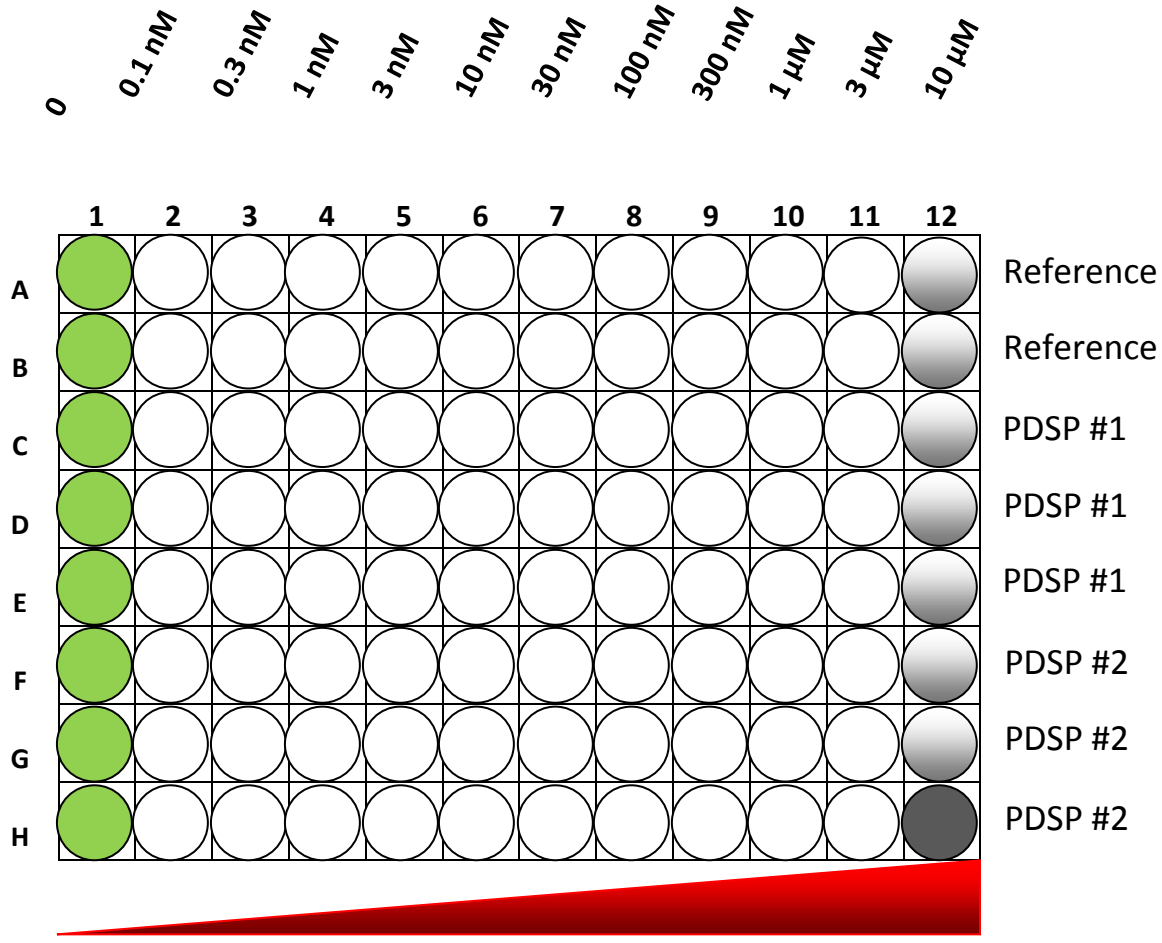


Figure 2b. 96-well drug plate map for secondary radioligand binding assays. The drug plate is designed to contain 2 PDSP compounds (Rows C to H) and 1 reference compound (Rows A and B) as indicated at right of the plate, therefore the PDSP compounds are assayed in triplicate and the reference compound 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 assays performed by the NIMH PDSP staff employ transfected (primarily stably 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.

Reference: Jordan M, Schallhorn A, Wurm FM (1996). Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res* **24**(4): 596-601.

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). In brief, HEK293 T cells are subcultured in 15-cm dishes at a density of 8 – 10 million cells per dish and incubated overnight. For each 15 cm dish, 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. 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 fetal bovine serum (FBS) overnight before harvesting. Cells are usually harvested 48 – 72 hours after transfection.

1.2.2. Membrane preparations from stably transfected cells. To make membrane fractions from stably transfected cell lines, cells are subcultured in 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 FBS before harvesting. 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 at 4°C to obtain a crude membrane fraction pellet. The fresh membrane pellet is then resuspended in 3 volumes of cold lysis buffer, and is immediately subjected to the Bradford

protein assay to determine protein concentration, followed by a saturation binding assay (see following section for detail) to determine receptor expression level and the affinity of a selected radioligand. Based on the receptor expression level and the K_d value, the fresh membrane suspension is stored at -80 °C freezer in small aliquots, such that one aliquot is sufficient for one 96-well plate to have at least 500 cpm/well when assayed at 0.5 – 1.0x K_d value of the appropriate radioligand.

1.2.3. Membrane preparationss from transiently transfected cells. To make membrane fractions from transiently transfected cells (usually HEK T cells), cells are transfected as described in **Section 1.2.1**. Transfected cells are usually harvested 48 – 72 hours after transfection, and processed as in **Section 1.2.2** to make membrane pellets.

1.2.4. Membrane preparations from tissues. To make membrane fractions from tissues, crude membrane fractions are prepared from rodent (typically rat or guniea pig) brain or kidney (purchased from PelFreeze Biologicals). The following is the general procedure used 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 at 4°C, and the resulting supernatant is decanted and replaced with the same 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 binding assays). The final pellet is resuspended in the same buffer and homogenized one last time. The fresh suspension is subjected to protein concentration measurement and saturation binding assay, and is then aliquoted for storage at -80°C for future use. Aliquots are also made such that each aliquot is sufficient 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 appropriate hot ligand.

1.2.4.1. Membrane preparations from rat brain for NMDA radioligand binding assays. The protocol is adapted from Chiu et al., 1999 (Alcohol 17:215-221). In brief, rat brains are 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-code SHE buffer per gram of wet tissue using a glass homogenizer (at least 6 strokes). The homogenate is centrifuged at 1,000 x g for 20 min at 4°C to collect supernatant. The supernatant is then centrifuged at 16,000 x g for 1h at 4°C. The pellet is suspended in the same cold SHE buffer and stored in aliquots (10 mg per aliquot after protein concentration determination) at -80°C until use. The 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 (16,000 x g) briefly in a benchtop centrifuge at 4°C to eliminate sucrose. The pellet is then resuspended in 0.5 ml HE buffer and incubated at 37°C for 30 min. The suspension is then centrifuged again 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. The final pellet is resuspended to about 2 mg/ml concentration after protein concentration determination, and is then ready for binding assays at 100 μg per well, using the HE buffer supplemented with 100 μM Glutamate Sodium and 100 μM Glycine.

Table 1. List of cell lines and targets used by the PDSP to make membrane pellets for binding assays. All clones are stable lines, whereas transiently transfected cells are marked with "*". Clones are human unless noted. Detailed information regarding culture media is listed **Section 1.2.5**.

Receptor	Note	Parental cells	Media (see detail below the table)
Serotonin (5HT)			
5-HT1A		stable CHO	500 G418
5-HT1B		stable HEK	500 G418
5-HT1D	*	HEKT	COS/HEK
5-HT1E		stable HEK	500 G418
5-HT2A (rat)		stable 3T3	500 G418
5-HT2A	*	HEKT	COS/HEK
5-HT2B		stable HEK	2 μg/ml Puromycin
5-HT2C		Flp-IN HEK	DMEM 100 μg/ml Hygromycin B
5-HT3	*	HEKT	COS/HEK
5-HT5A		Flp-In CHO	DMEM/F-12 200 μg/ml Hygromycin B
5-HT6		stable HEK	500 G418
5-HT7A		stable HEK	2 μg/ml Puromycin
Dopamine			
D1	*	HEKT	COS/HEK
D2		stable fibroblast	COS/HEK
D2L		stable CHO	F-12/10%FBS 400G418
D3 (rat)	*	HEKT	COS/HEK
D3	*	HEKT	COS/HEK
D4		stable	DMEM/F12 10% CS Fe+
D5	*	HEKT	COS/HEK
Opioid			
Mu, MOR		stable HEK	200 G418
Delta, DOR		stable HEK	200 G418
Kappa, KOR (rat)		stable HEK	500 G418
Kappa, KOR		stable HEK	500 G418
Nociceptin, NOP	*	HEKT	COS/HEK
Neurotransmitter Transporters			
SERT		stable HEK	500 G418
NET		stable HEK	hNET (250 G418)
DAT		stable HEK	hDAT (350 G418)

Receptor	Note	Parental cells	Media (see detail below the table)
Vasopressin and Oxytocin			
V1A		stable CHO	V1A & OT media
V2		stable CHO	V2 & V1B media
V1B		stable CHO	V2 & V1B media
OT		stable CHO	V1A & OT media
Prostaglandin			
EP-3	*	HEKT	COS/HEK
EP-4	*	HEKT	COS/HEK
Adrenergic			
alpha 1A		stable	500 G418
alpha 1B	*	HEKT	
alpha 1D		stable	500 G418
alpha 2A		stable MDCK	500 G418
alpha 2B	*	HEKT	COS/HEK
alpha 2C		stable MDCK	500 G418
beta 1		CHO Flp-In	DMEM/F12 200 μg/ml Hygromycin B
beta 2		HEK Flp-In	DMEM 100 μg/ml Hygromycin B
beta 3		HEK Flp-In	DMEM 100 μg/ml Hygromycin B
Muscarinic acetylcholine			
M1		stable CHO	500 G418
M2		stable CHO	500 G418
M3		stable CHO	500 G418
M3D		CHO Flp-In	DMEM/F12 100 μg/ml Hygromycin B
M4		stable CHO	10% FBS F12
M5		stable CHO	500 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			
H1		stable HEK	500 G418
H2 (in progress)		stable HEK	500 G418
H3		HEK Flp-In	DMEM 100 μg/ml Hygromycin B
H4 (in progress)			500 G418

Receptor	Note	Parental cells	Media (see detail below the table)
		Cannabinoid	
CB1 (in progress)		HEK	500 G418
CB1		HEK Flp-In	DMEM 100 µg/ml Hygromycin B
CB2		HEK Flp-In	DMEM 100 µg/ml Hygromycin B
		Adenosine	
A1	*	HEKT	COS/HEK
A2A	*	HEKT	COS/HEK
A2A		HEK	500 G418
A2B	*	HEKT	COS/HEK
A3	*	HEKT	COS/HEK
		Melanocortin	
MC-1	*	HEKT	COS/HEK
MC-2	*	HEKT	COS/HEK
MC-3	*	HEKT	COS/HEK
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
		Lysophospholide (LPA)	
LPA1	*	HEKT	COS/HEK
LPA2	*	HEKT	COS/HEK
LPA3	*	HEKT	COS/HEK
		Tachykinin (NK)	
NK1		HEK	500 G418
NK2		HEK	500 G418
NK3		HEK	500 G418
		mGluRs	
mGluR1 (in progress)	*	HEKT	500 G418
mGluR2 (in progress)	*	HEKT	500 G418
mGluR3 (in progress)	*	HEKT	500 G418
mGluR4 (in progress)	*	HEKT	500 G418

Receptor	Note	Parental cells	Media (see detail below the table)
mGluR5		CHO	2 μg/ml Puromycin
mGluR5 (in progress)	*	HEKT	500 G418
mGluR6 (in progress)	*	HEKT	500 G418
mGluR7 (in progress)	*	HEKT	500 G418
mGluR8 (in progress)	*	HEKT	500 G418
		Others	
Ghrelin		HEK Flp-In	DMEM 100 μg/ml Hygromycin B
PAR1		Lung Fibroblast, PAR1 KO	500 G418
SMO	*	HEKT	COS/HEK
SMO (in progress)		HEK	500 G418
CCK2		CHO	500 G418
Orexin-2	*	HEKT	COS/HEK
GPR58	*	HEKT	COS/HEK
GPR61	*	HEKT	COS/HEK
GPR62	*	HEKT	COS/HEK
GPR40	*	HEKT	COS/HEK
GPR41	*	HEKT	COS/HEK
GPR43	*	HEKT	COS/HEK
Il-1 imidazoline	*	HEKT	COS/HEK
HERG-K^+ Channel		HEK	500ug/ml G418
PC12			COS/HEK

1.2.5. Media Recipes

Details of media composition for cells are as follows. 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).

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

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

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

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

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

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

d. Standard Puromycin Medium +G418 Selection Medium

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

e. Dialyzed Medium (1% dialyzed serum) – 1 L

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

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

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

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

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

h. 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

i. 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

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

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

k. 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

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

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

m. D4 medium (10% serum) – 1L

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

n. FLP-In CHO medium (10% serum) – 1L

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

o. FLP-In HEK medium (10% serum)– 1L

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

p. PC-12 medium (10% serum) – 1L

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

q. HTLA medium – 1L

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

r. Inositol/Inositol-free medium

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

s. 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 amino acids, 25 mM HEPES, 1 mM Sodium Pyruvate, 10 ml Pen/Strep

1.3. Saturation binding assays

Saturation binding assays are usually performed immediately after the membrane fraction is obtained and protein concentration is determined (see above section for membrane preparations) to measure receptor expression level (B_{max}) and binding affinity (K_d) of a selected radioligand. **Tables 2-24** list 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 each well of a 96-well plate according to the setup as in **Figure 3**; followed by addition of 25 µl binding buffer (for total binding) or 25 µl reference compound at final 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 Filtermate harvester, followed by three washes with cold wash buffers (for detail, see **Tables 2-24**). Scintillation cocktail is then melted onto the microwave-dried filters on a hot plate and radioactivity is counted in a Microbeta counter.

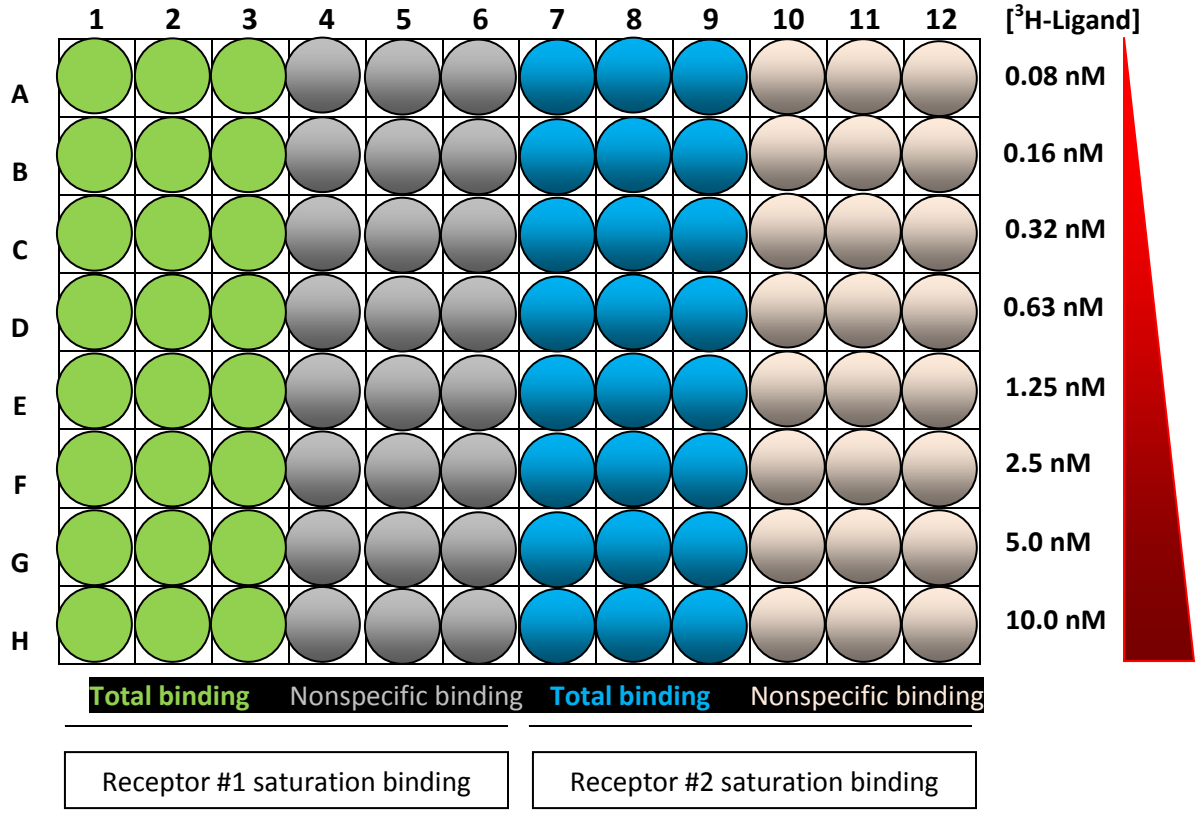


Figure 3. Radioligand saturation binding plate set-up in a 96-well plate format. Total and nonspecific binding are determined in the absence and presence of 10 µM of appropriate reference compound, respectively, in the indicated final concentrations of hot ligand (nM). Final radioligand concentrations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

1.4. Primary and secondary radioligand binding assays

Compounds are typically subjected to primary radioligand binding assays at targets selected by investigators and approved by the PDSP Director, Dr Bryan Roth. In the primary binding assays, compounds are usually tested at single concentrations (10 µM) in quadruplicate in 96-well plates (see **Figure 1** for drug plate map). Compounds that show a minimum of 50% inhibition at 10 µM

are tagged for secondary radioligand binding assays to determine equilibrium binding affinity at specific targets. In the 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 compounds and 1 reference compound. The drug map in **Figure 2a** uses resources more efficiently than the one in **Figure 2b**; drug plates such as those in **Figure 2b** are 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 buffer (for details, see **Tables 2-24**). 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 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 buffer (for detail, see T**ables 2-24**). 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 (details for radioligand binding assays for nAChRs are described in a separate section).

1. Retrieve barcoded primary or secondary drug plates from the Hamilton STAR® or cold room
2. Check out drug plates and mark them “in progress” in the PDSP database
3. Prepare appropriate binding and wash buffers
4. Check out membrane pellets
5. Check pellet box for K_d value and concentration to be used for the radioligand
6. Prepare 2.5x of final concentration of radioligand working solution
7. Count 50 µl of radioligand working solution to confirm 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 period of 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. Microwave-dry the filters
15. Melt scintillation cocktail on top of filters
16. Wrap up filters in plastic wrap
17. Count radioactivity
18. Download results, process and upload to the PDSP database
19. Preview and submit results
20. Report progress in the PDSP database (completion or redo or repeat) after receiving approving 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.

Main references for radioligand binding assays with nAChRs

- Xiao Y, Meyer EL, Thompson JM, Surin A, Wroblewski J, Kellar KJ (1998). Rat alpha3/beta4 subtype of neuronal nicotinic acetylcholine receptor stably expressed in a transfected cell line: pharmacology of ligand binding and function. Mol Pharmacol 54(2): 322-333.
- Xiao Y, Fang H, Musachio JL, Wei ZL, Chellappan SK, Kozikowski AP, Kellar KJ (2006). Sazetidine-A, a novel ligand that desensitizes alpha4beta2 nicotinic acetylcholine receptors without activating them. Mol Pharmacol 70(4): 1454-1460.

1.5.1. Cell culture. The six stable cell lines expressing human $\alpha2\beta2$, $\alpha2\beta4$, $\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$, or $\alpha4\beta4$ nAChR subtypes 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 α7 nAChRs was established by stably transfecting HEK293 cells with the rat α7 nAChR subunit. Cells are grown in minimum essential medium (MEM) supplemented with 10% FBS, 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. Nicotinic receptor radioligand binding assays. Radioligand binding assays of nAChRs to [^{3}H]-epibatidine use the above stably expressed nAChRs, or 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 x 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. Examples are listed in Table 4 and Figure 12.

1.5.3. Primary and secondary nAChR binding assays. Primary nAChR binding assays are performed with 100 pM [^{3}H]-epibatidine and a single concentration of PDSP 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 test PDSP compounds in singlets are tested to generate a competition binding curve. Results are analyzed in Prism 5.0 by nonlinear least-squares regression to obtain K_i values. Nicotine at 300 μM is included in all assays to define nonspecific binding, and a nicotine concentration-response curve is included as a positive control in all 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 **Section 1.6.2**, and the K_i values are then manually

entered into the PDSP database. Figures and raw data for these assays are available to appropriate investigators (who submitted the compounds) upon request.

1.6. General binding data entry, analysis, and quality control for targets other than nAChRs.

Except radioligand binding results with nAChRs, whose analysis is outlined in **Section 1.5**, binding assays for targets other than nAChRs are analyzed and reported as outlined in the following sections. Raw cpm data from the Microbeta counters 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 analysis and reporting of binding results, using both built-in tools in the PDSP database, or third party software (Microsoft Excel and GraphPad Prism).

1.6.1. Results analysis using built-in tools in the PDSP database. The PDSP database is coded 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 the built-in tools in the PDSP database to analyze binding assay results, technicians upload raw counting results in Excel spreadsheets from Microbeta counters directly to 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 and streamline both the data processing and quality control.

1.6.1.1. Analysis of saturation binding results. Saturation binding assays are carried out whenever a new batch of membrane preparations is made. The protein amount per well is adjusted such that less than 10% total added radiolactivity is bound. Upon completion of saturation binding assays, technicians can upload 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 concentrations. An automatic email is 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 coded to analyze saturation binding results and generate a hyperbolic curve using non-linear least squares curve-fitting algorithm to determine B_{max} and K_d values. The PDSP administrator reviews and analyzes 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 during review of a saturation binding assay. When the data set is approved, the PDSP administrator decides on (1) amount of protein per well and (2) radioligand concentration to be used for competition binding assays. Upon approval, the saturation binding curve is immediately available in the "approved membrane pellet" section with unique timestamp associated with each batch of membrane preparation. Technicians make aliquots accordingly so that each aliquot is sufficient for one plate of a binding assay at the decided concentration of radioligand. Aliquots are stored in 1.5 ml Eppendorf tubes at -80°C in assorted boxes until use at specified concentration of radioligand, with one aliquot being used per assay plate.

1.6.1.2. Analysis of primary binding assay results. Upon completion of primary binding assays, technicians upload raw results in Excel spreadsheets directly to the PDSP database via the primary binding assay reporting menu. When uploading primary binding results, technicians must identify plates by barcodes and match the barcodes generated by the Hamilton STAR liquid handling system when the drug plates were made. The designated PDSP administrator double checks the plate identity and sample order before making the results available online for reviewing. The PDSP database is coded to calculate percentage inhibition in each assay plate with total binding (with buffer) as 0% inhibition and nonspecific binding (in the presence of 10 μM of a reference compound) as 100% inhibition. It also calculates average values from 4

replicates for each sample. The PDSP database automatically marks those entries with 50% or higher inhibition from secondary binding assays and also highlights those with variances greater than 20% among the quadruplicate determinations for further inspection. In addition, those compounds with negative inhibition (more binding in the presence of test compound) by over 20% are also highlighted for inspection. For the results of any compound, there are four options available: "Redo" deletes the data from database and puts the compound back on the primary schedule list; "Repeat" accepts the results and puts the compound back on the primary schedule list for a repeat assay; "Force Secondary" accepts the primary results and adds the compound on the secondary schedule list for secondary assays, even if the primary percent inhibition is less than 50%; "Approve" accepts the results, and the database puts the compound on the 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. Analysis of secondary binding assay results. Upon completion of secondary binding assays, technicians upload raw results in Excel spreadsheets directly to the PDSP database via a secondary binding assay reporting menu. In contrast to the reporting of primary binding results, the PDSP database analysis tools can accept two types of 96-well drug plate layouts for this purpose, as indicated in **Figures 2a and 2b**, with the plates being made either manually or by the Hamilton STAR®. When uploading and submitting secondary binding results, technicians must match drug plate maps and barcodes with those generated by Hamilton STAR® liquid handling system when the drug plates were made. They also must choose buffers, radioligand identity, and reference compound from pull-down menus and enter total radioligand activity (dpm), specific activity, and the K_d value (from a saturation binding assay, see above) for the radioligand; the later values will be used by the PDSP database to calculate the radioligand concentration to be used for the assay, and convert IC_{50} to K_i values. A representative screen capture (**Figure 7**) is shown below as it appears while technicians are uploading secondary binding results for

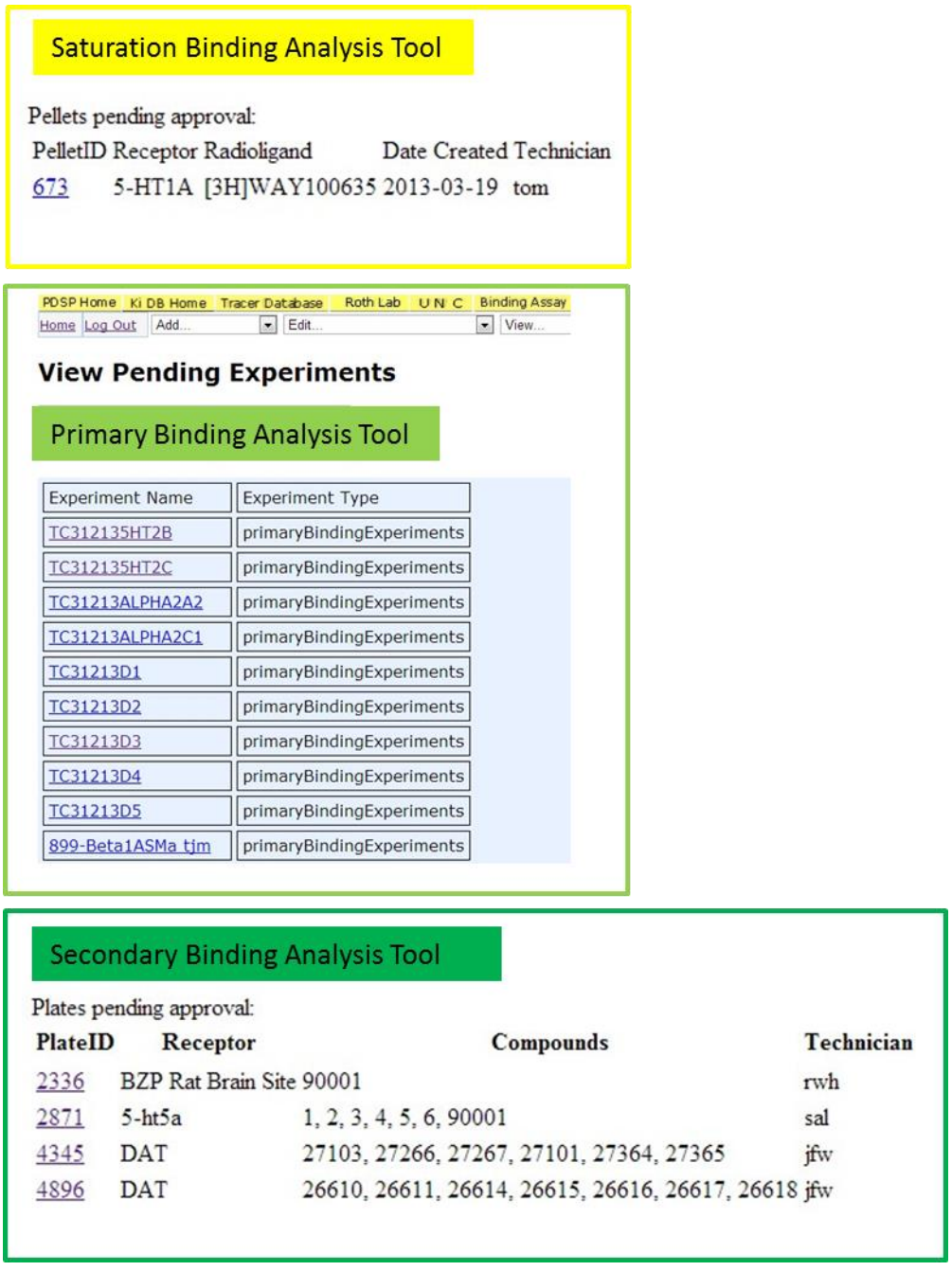
submission. Technicians can view and examine the preliminary curves on a plate or individual compound basis before submission.

After secondary binding results are submitted, the PDSP database pools 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 parses the uploaded file, and generates an editable table along with a preliminary graph for analysis purpose (**Figure 8**). Clicking on individual PDSP compound 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 reference compound are shown in tables at left of the screen. The PDSP database is also coded to run the **R**obust regression and **Out**lier removal **(ROUT**) algorithm to identify and highlight potential outliers. PDSP administrators decide whether to exclude or include the potential outliers when reviewing each data set. The raw data tables are also editable and allow for exclusion of points that are known to be errors, for example, if a mistake is noticed in pipetting radioligand or membrane by a technician.

As an internal control, each set of secondary binding assays contains a reference concentration-response curve, also done in triplicate. The reference K_i value is automatically compared with its historical average value and/or the value in our K_i database. If the reference $\mathbf{K_i}$ is more than 3 fold away from its historical average value, the entire data set is flagged for further inspection. Senior PDSP scientists work with technicians to discover the underlying causes for such discrepancies, and arrive at solutions. For example, the secondary binding assay may be repeated, or the old stock of reference compound is replaced with a fresh one, if the same reference compound has low affinity in multiple data sets.

Each curve has its K_i value listed at the right side of the screen 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 a reference curve and status of the assay is flagged as “completed”; the result is immediately available to investigators online. Otherwise, “Redo” puts the compound back on to the secondary schedule list. When an assay is approved, the PDSP database checks whether that compound has been previously tested at the same receptor. If so, the database compares the results of the assays, and generates a warning email in the case that it finds that one K_i value is at least 4x different (0.6 log unit) from another one. All of the secondary assays with the same compound are put onto the “Experiments under review” list. PDSP administrators review the results, and decide how to proceed 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 further action, since lowest K_i value could be 4x away from highest K_i value, but still be less than 3x away from mean value. If the PDSP database has multiple accepted entries for the same compound, the database calculates 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-HT1E secondary binding).



Saturation Binding Analysis Tool

Pellets pending approval:

PelletID	Receptor	Radioligand	Date Created	Technician
673	5-HT1A	[3H]WAY100635	2013-03-19	tom

PDSP Home | Ki DB Home | Tracer Database | Roth Lab | U N C | Binding Assay

Home | Log Out | Add... | Edit... | View...

View Pending Experiments

Primary Binding Analysis Tool

Experiment Name	Experiment Type
TC312135HT2B	primaryBindingExperiments
TC312135HT2C	primaryBindingExperiments
TC31213ALPHA2A2	primaryBindingExperiments
TC31213ALPHA2C1	primaryBindingExperiments
TC31213D1	primaryBindingExperiments
TC31213D2	primaryBindingExperiments
TC31213D3	primaryBindingExperiments
TC31213D4	primaryBindingExperiments
TC31213D5	primaryBindingExperiments
899-Beta1ASMa tjm	primaryBindingExperiments

Secondary Binding Analysis Tool

Plates pending approval:

PlateID	Receptor	Compounds	Technician
2336	BZP Rat Brain Site	90001	rwh
2871	5-ht5a	1, 2, 3, 4, 5, 6, 90001	sal
4345	DAT	27103, 27266, 27267, 27101, 27364, 27365	jfw
4896	DAT	26610, 26611, 26614, 26615, 26616, 26617, 26618	jfw

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.

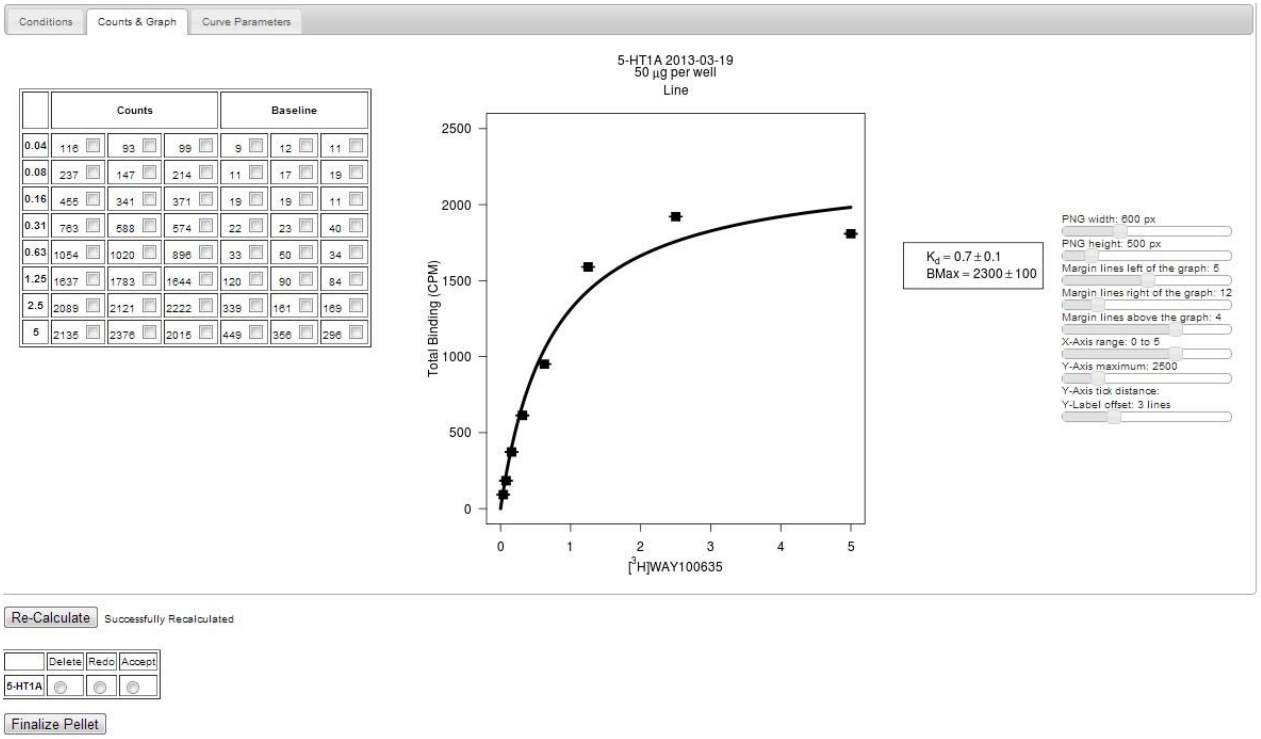


Figure 5. A representative screen capture showing a reviewing screen of saturation binding results. Both total and nonspecific binding (cpm/well) are shown in the table on the left side of the screen. Specific binding is fitted to a hyperbolic curve to determine B_{max} (cpm/well) and K_d (nM); both values are listed at the right side of the curve. The pellet information is listed above the figure, showing that this is a 5-HT1A membrane pellet, made from a stable line, assayed at 50 µg/well protein, on March 19, 2013.

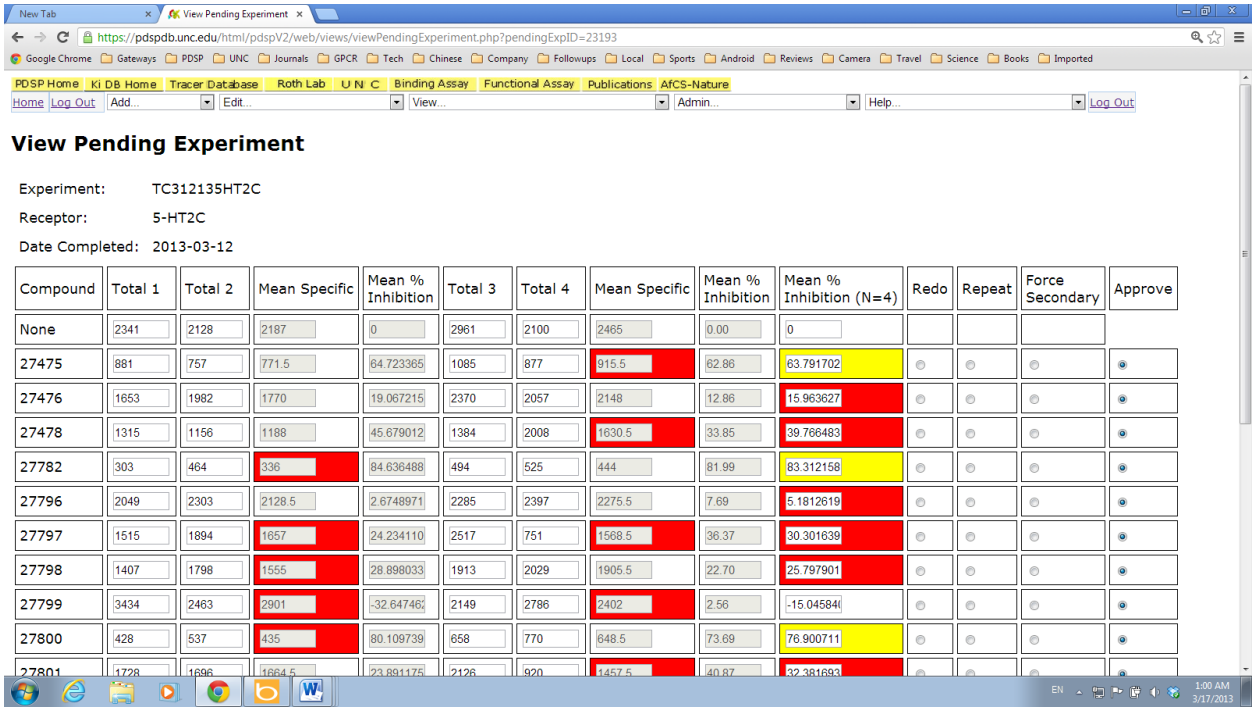
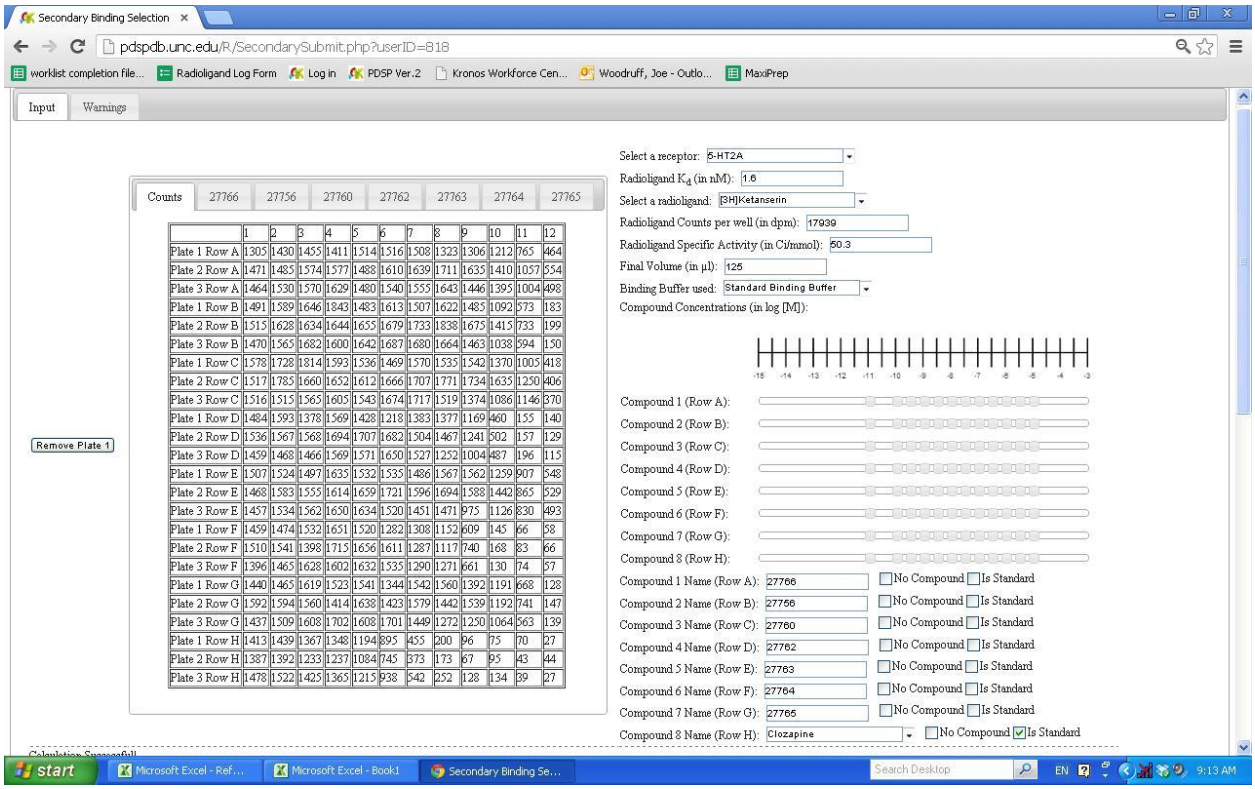


Figure 6. A representative screen capture showing a reviewing screen of primary binding assay results. The barcoded plate is for a 5-HT2C binding assay carried out on March 12, 2013. Total binding values are shown in the top row and PDSP compounds are shown in subsequent rows. The nonspecific binding values are at the bottom of the page and do not appear in the Figure. Highlighted in yellow are the samples with more than 50% inhibition and these samples are automatically tagged for secondary binding assays. Highlighted in red are the ones with more 20% variations among 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	1583	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 from three 96-well plates (Plate 7, Plate 8, and Plate 9), and organized from Rows A to G (in triplicate) from top to bottom with PDSP compound # showing above the table. On the upper right, detailed information for the binding assay is shown, including target receptor identity (5-HT2A 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 compound with corresponding concentrations) are shown. The concentrations for each compound are set at default values, but can be changed individually by sliding the corresponding bar button, if necessary.

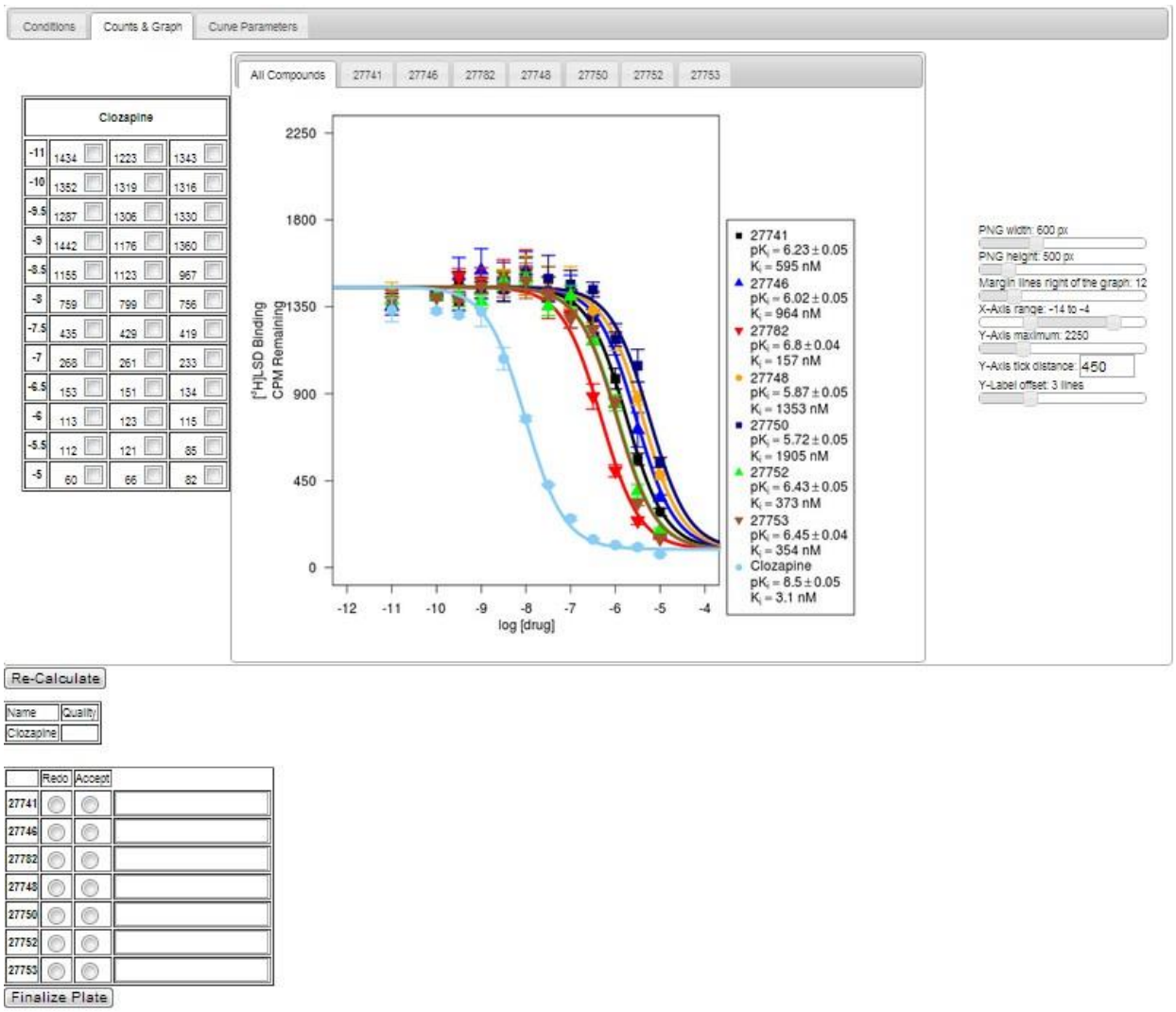


Figure 8. A screen capture showing a representative overview of a set of secondary binding results. On the left, counts (cpm/well) for the reference compounds are shown in a table. In the middle, up to 8 curves are shown on the same graph with a pre-determined color scheme with PDSP numbers above the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

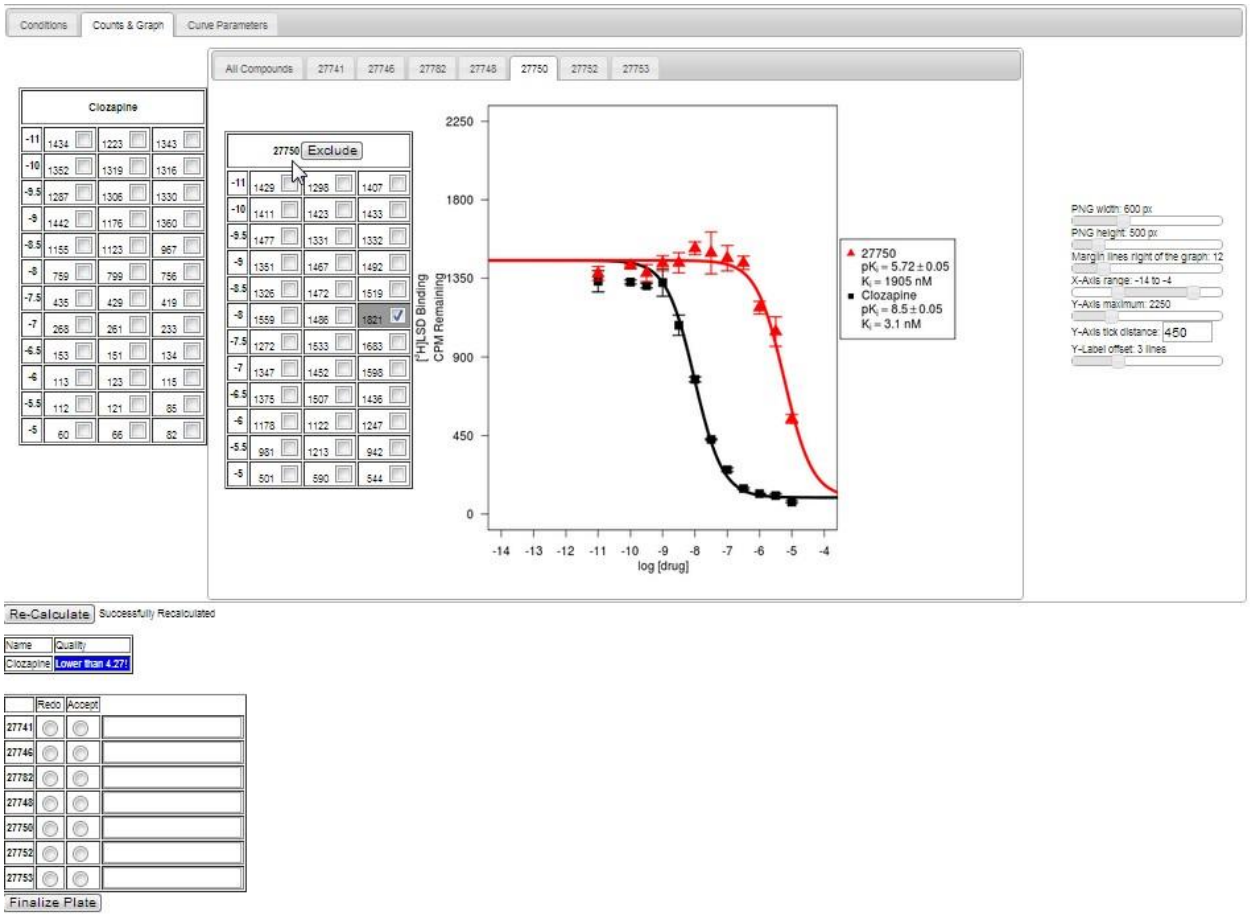


Figure 9. A screen capture showing a representative reviewing process of a secondary binding assay. On the right, counts (cpm/well) for both a reference compound and a selected PDSP compound are shown in corresponding tables. One potential outlier has been highlighted (in dark gray with a checkmark "√") 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, and corresponding pK_i and K_i values are listed on the right side. At the lower left corner, each compound can be marked either "Redo" or "Accept", and the long box can accommodate a brief text note.

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

1.6.2.1. Analysis of saturation binding assays. 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 in cpm is entered in column A and nonspecific binding in cpm is entered in column B, corresponding to concentrations of 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 of a reference compound, respectively, at the corresponding concentration [**X**] in nM. $\mathbf{B_{max}}$ is the receptor expression level in cpm/well or fmol/mg membrane protein; $\mathbf{K_d}$ is the equilibrium binding affinity, corresponding to the radioligand concentration at which point 50% of $\mathbf{B_{max}}$ is bound to the radioligand; **NS** and **Background** are two fitting values for nonspecific binding and background counts.

1.6.2.2. Analysis of primary binding assays. 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 percent inhibition. The normalization process is carried out in Prism or Excel.

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

1.6.2.3. Analysis of secondary binding assays. 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 total binding in the presence of corresponding concentration of testing drug (**X**); **Top** and **Bottom** are the total and nonspecific binding in the absence and presence of 10 μM of the reference compound; **IC_{50}** is the concentration at which 50% observed binding is inhibited and is converted to K_i according to Cheng-Prusoff equation,

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

in which **L** is the radioligand concentration used in the competition binding assay; **K_d** is the radioligand equilibrium binding affinity determined in the 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

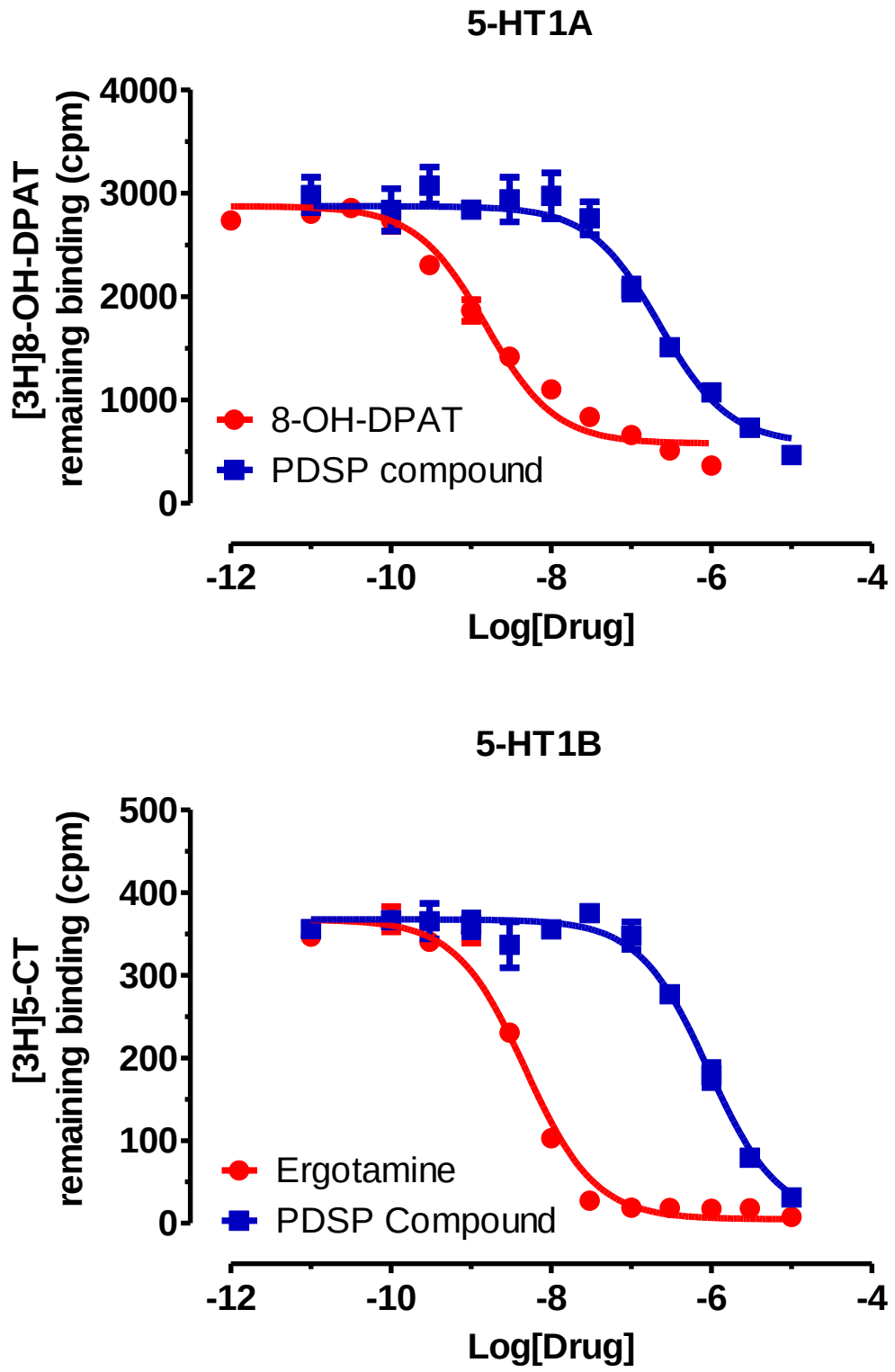
Table 2. 5-HT receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually at or near the K_d value. The K_d values listed in this table are the mean ± SEM from a minimum of 3 saturation binding assays from 2011 to 2012. BB for binding buffer; WB for wash buffer. Historical reference K_i values from the last 6 months are also listed.

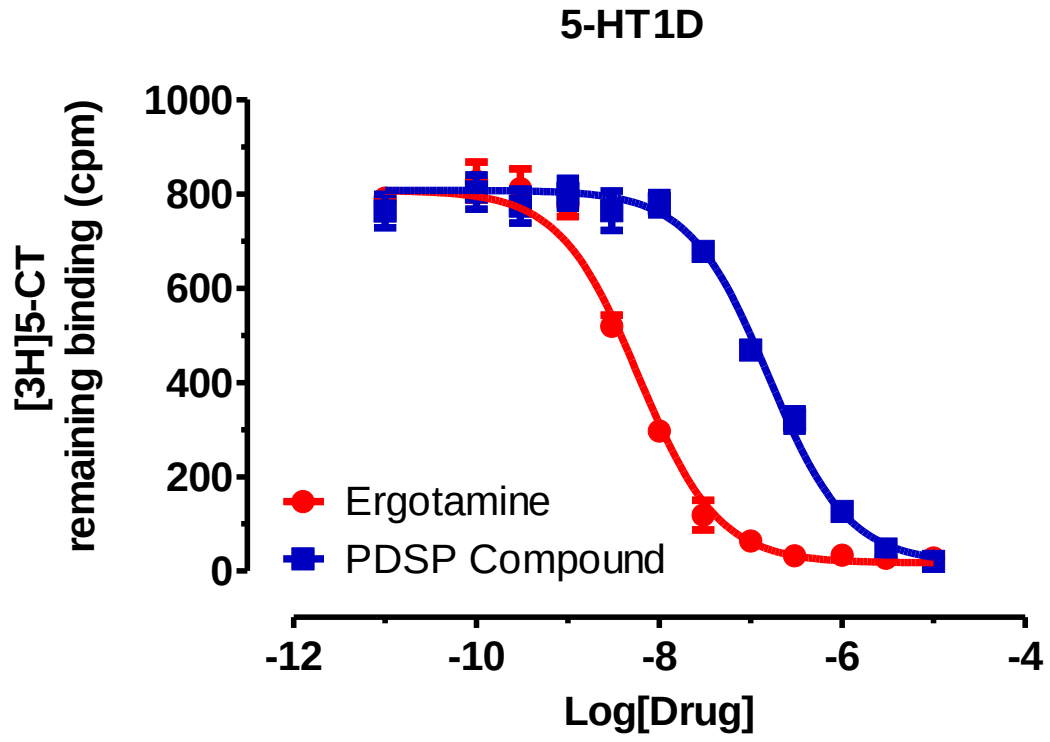
5-HT receptors

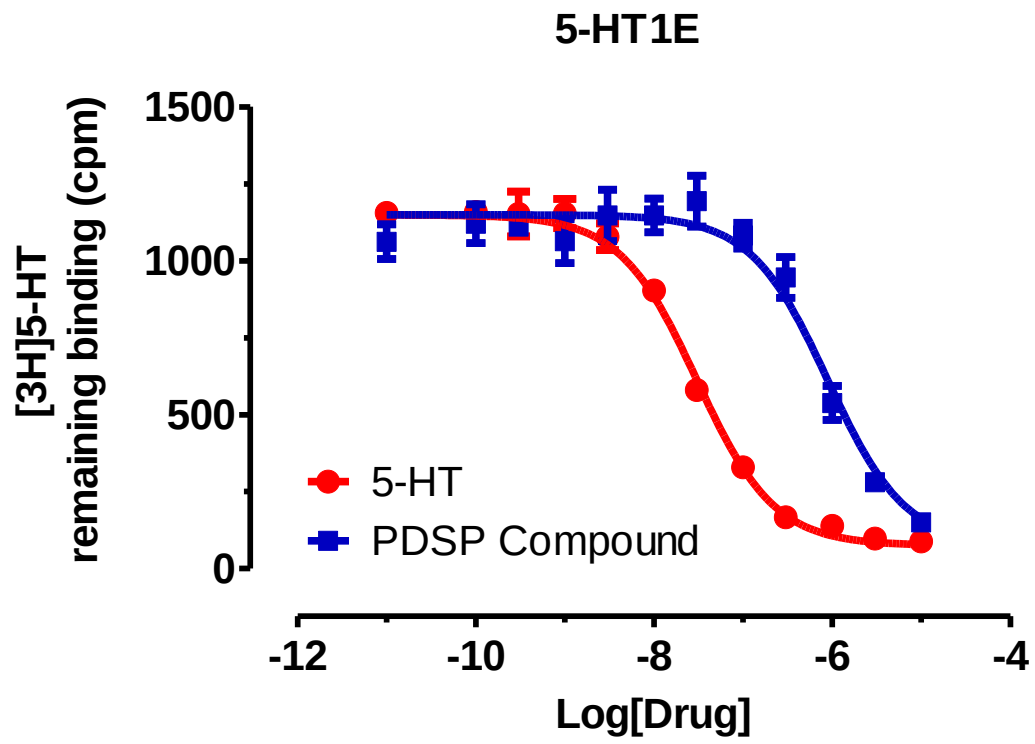
Standard BB (SBB): 50 mM Tris HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4, RT
Standard WB (SWB): 50 mM Tris HCl, pH 7.4, cold

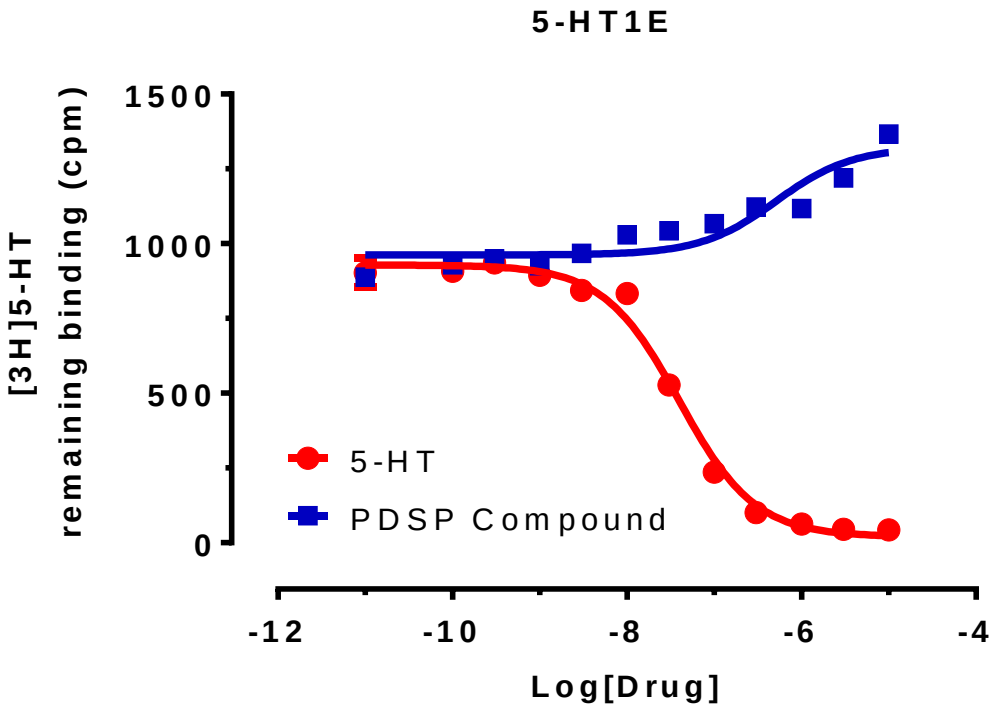
Target	Radioligand	K_d or [^{3}H] for binding in nM (N)	Reference Compound	Reference K_i (nM)
5-HT1A	[^{3}H]Way100635	0.43 ± 0.06 (21)	8-OH-DPAT	0.60 ± 0.10
5-HT1B	[^{3}H]5-CT	1.91 ± 0.29 (8)	Ergotamine	4.75 ± 0.51
5-HT1D	[^{3}H]5-CT	1.47 ± 0.24 (13)	Ergotamine	5.04 ± 0.47
5-HT1E	[^{3}H]5-HT	3.36 ± 0.46 (8)	5-HT	11.8 ± 1.2
5-HT1F				
5-HT2A	[^{3}H]Ketanserin	1.57 ± 0.26 (19)	Clozapine	9.50 ± 1.03
5-HT2B	[^{3}H]LSD	1.04 ± 0.15 (19)	SB206553	18.2 ± 2.3
5-HT2C	[^{3}H]Mesulergine	2.92 ± 0.83 (13)	Ritanserin	2.12 ± 0.22
5-HT3	[^{3}H]GR65630	1.0 for binding	Zacopride	0.65 ± 0.08
5-HT4	[3H]GR113808	0.2 – 0.5 for binding	SD205557	36.5 ± 8.5
5-HT5A	[^{3}H]LSD	2.32 ± 0.14 (36)	Ergotamine	36.5 ± 3.9
5-HT6	[^{3}H]LSD	3.97 ± 0.46 (10)	Chlorpromazine	10.5 ± 1.6
5-HT7A	[^{3}H]LSD	5.30 ± 0.70 (2)	Chlorpromazine	12.9 ± 0.9

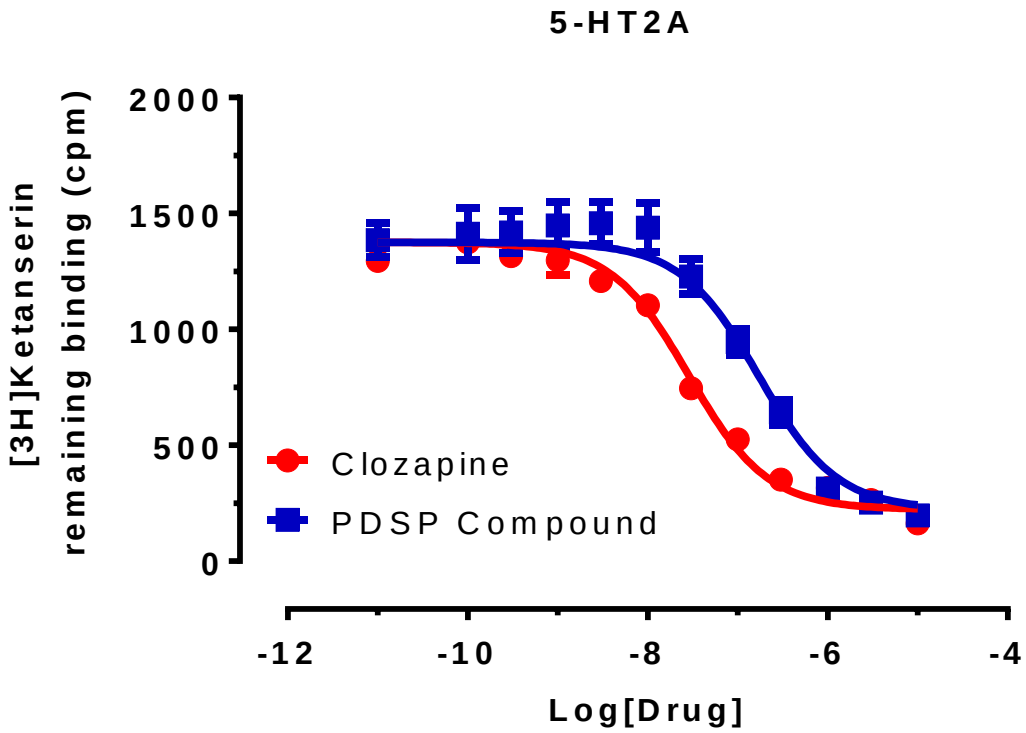
Figure 10. Representative competition binding curves with 5-HT receptors

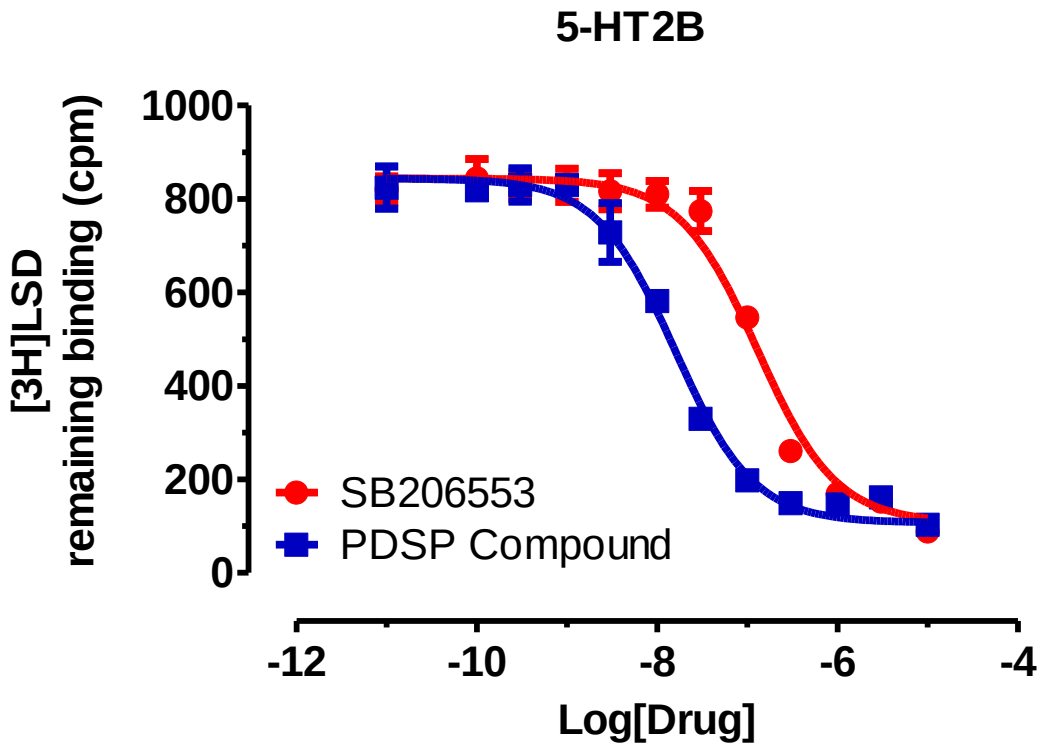


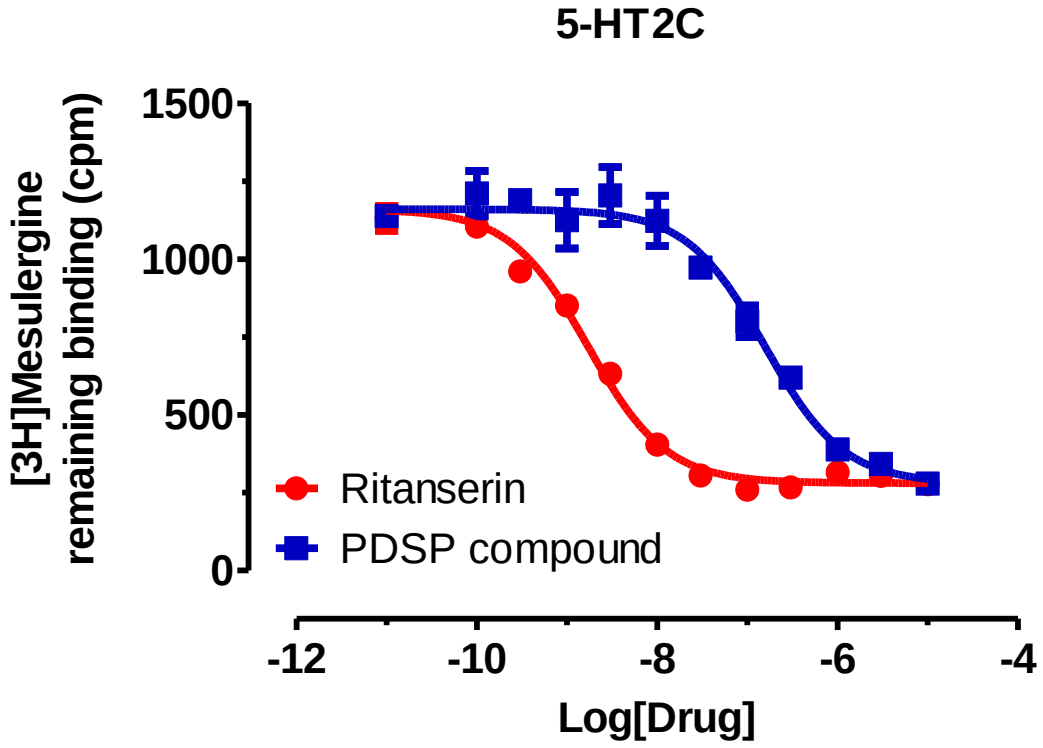

5-HT1D
[3H]5-CT
remaining binding (cpm)
1000
800
600
400
200
0
Ergotamine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


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


5-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
[3H]Mesulergine
remaining binding (cpm)
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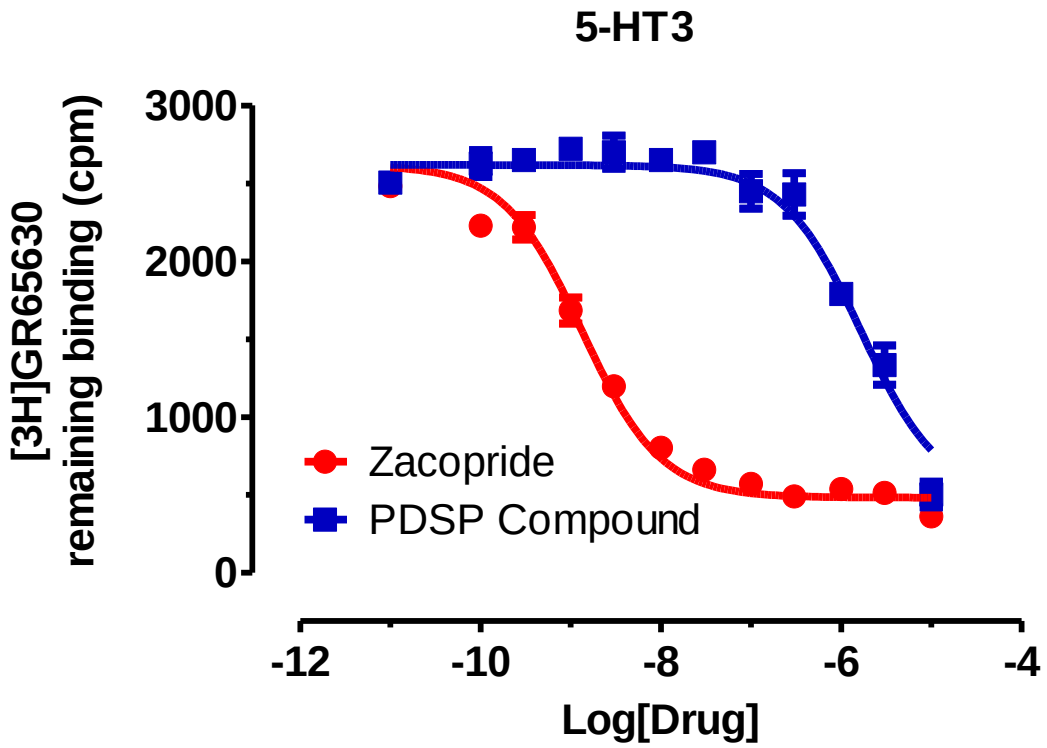
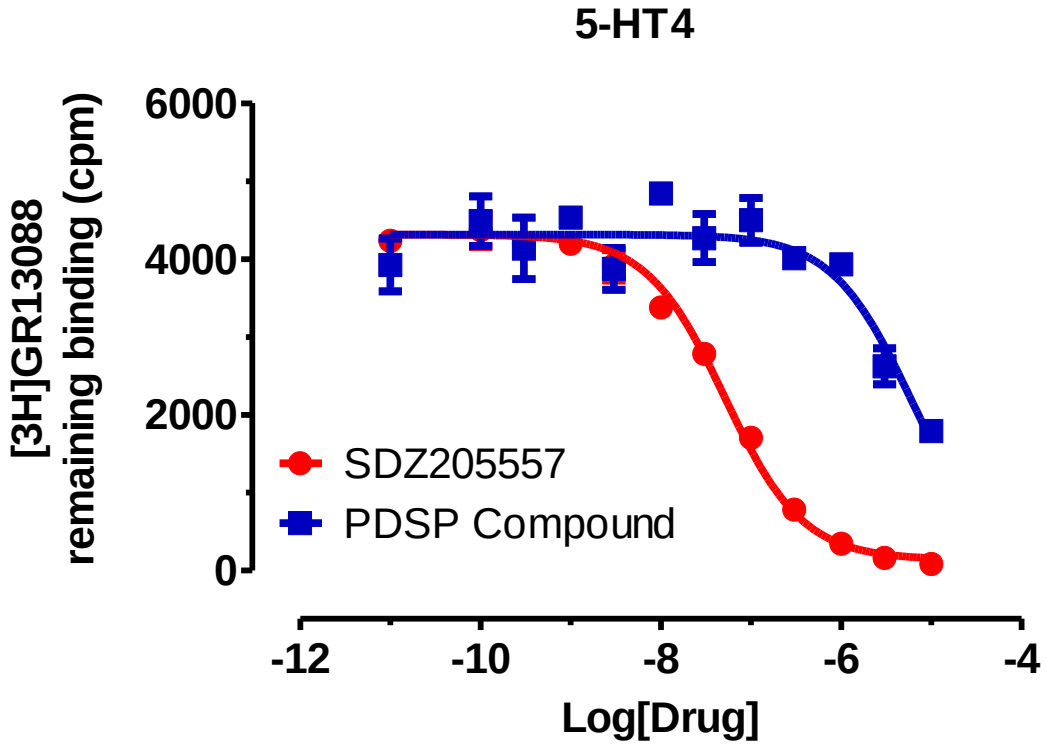
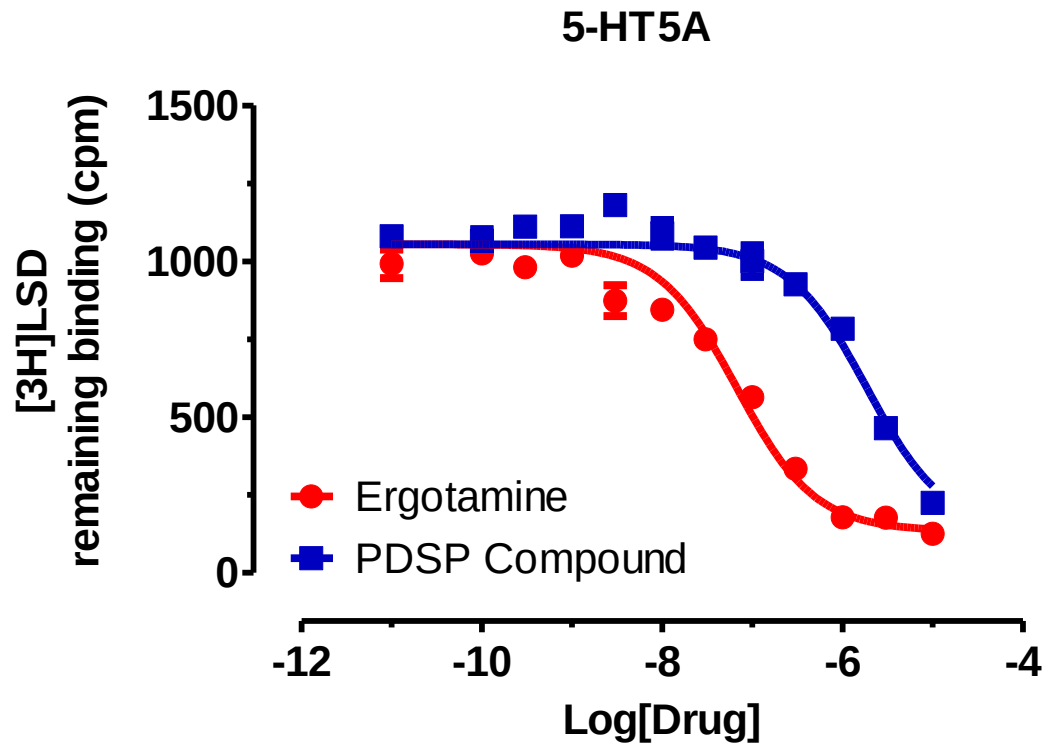
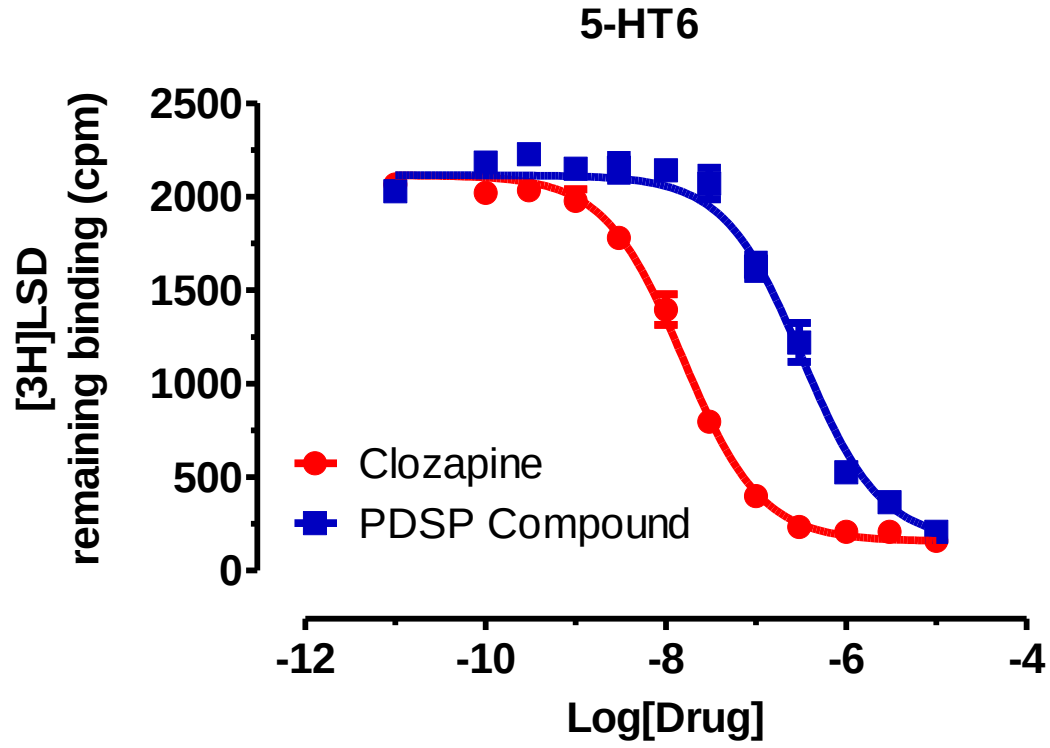
1000
500
0
Ritanserin
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]


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


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


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


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

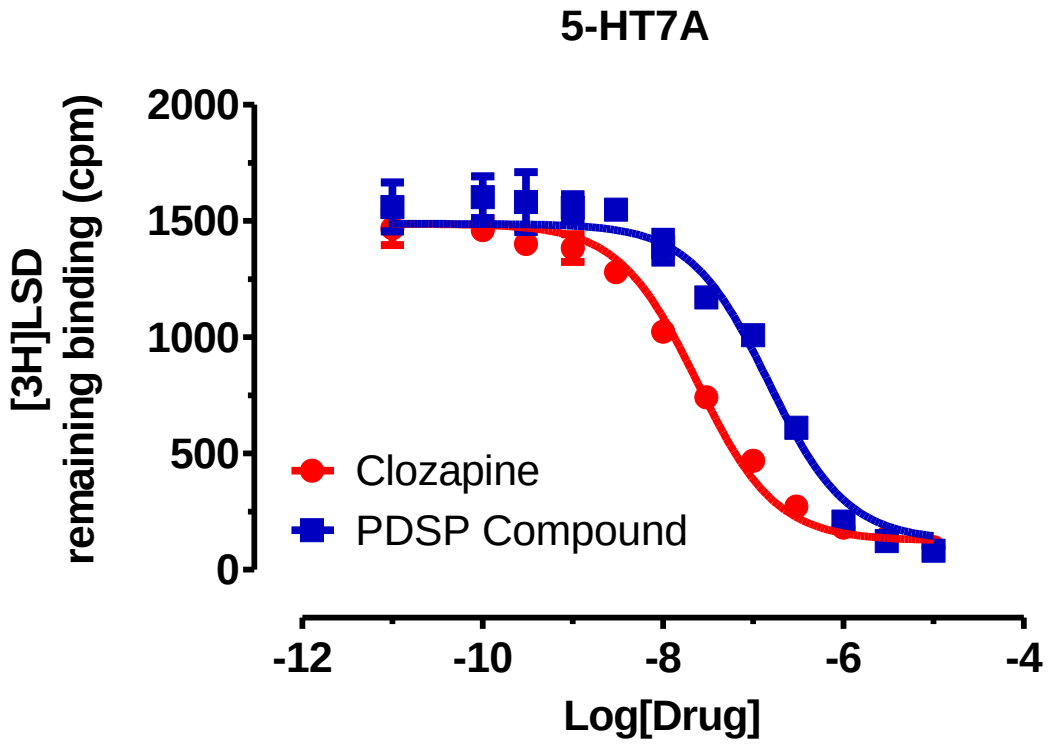

5-HT7A
[3H]LSD
remaining binding (cpm)
2000
1500
1000
500
0
Clozapine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

Table 3. Muscarinic acetylcholine receptors, radioligands and corresponding K_d values, reference compounds, and buffers for primary and secondary radioligand binding assays. For muscarinic receptors, we usually use 0.5 – 1.0 nM ^{3}H-QNB or ^{3}H-NMS for inhibition binding assays. The K_d values listed in this table are the average ± SEM from a minimum of 3 saturation binding assays from 2011 to 2012. BB for binding buffer; WB for wash buffer. Historical reference K_i values from last the 6 months are also included for quality control.

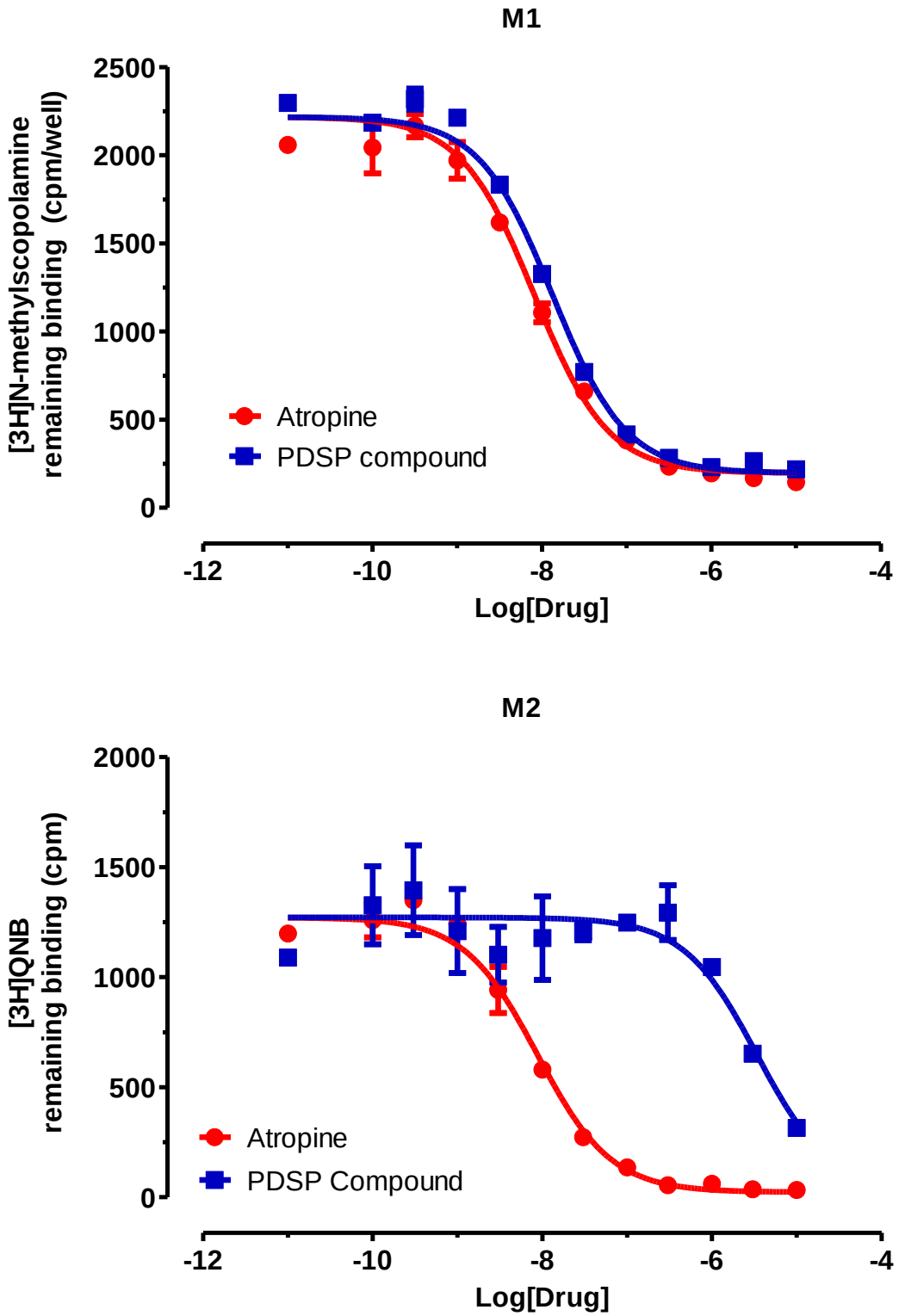
Muscarinic acetylcholine receptors (mAChRs)

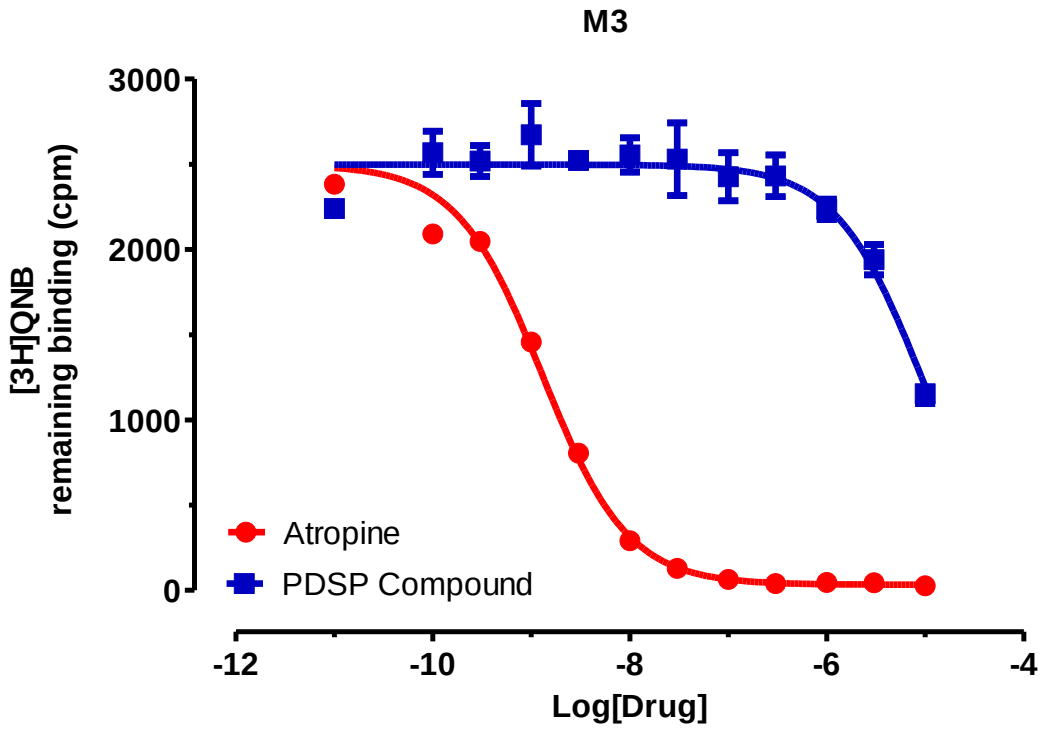
Muscarinic BB (MBB) #1: 50 mM Tris HCl, pH 7.7, RT + SWB
Muscarinic BB (MBB) #2: 25 mM Sodium Phosphate, 5 mM $MgCl_2$, pH 7.4, RT (cold for washing)
Muscarinic wash buffer #2: Same as muscarinic binding buffer #2, cold

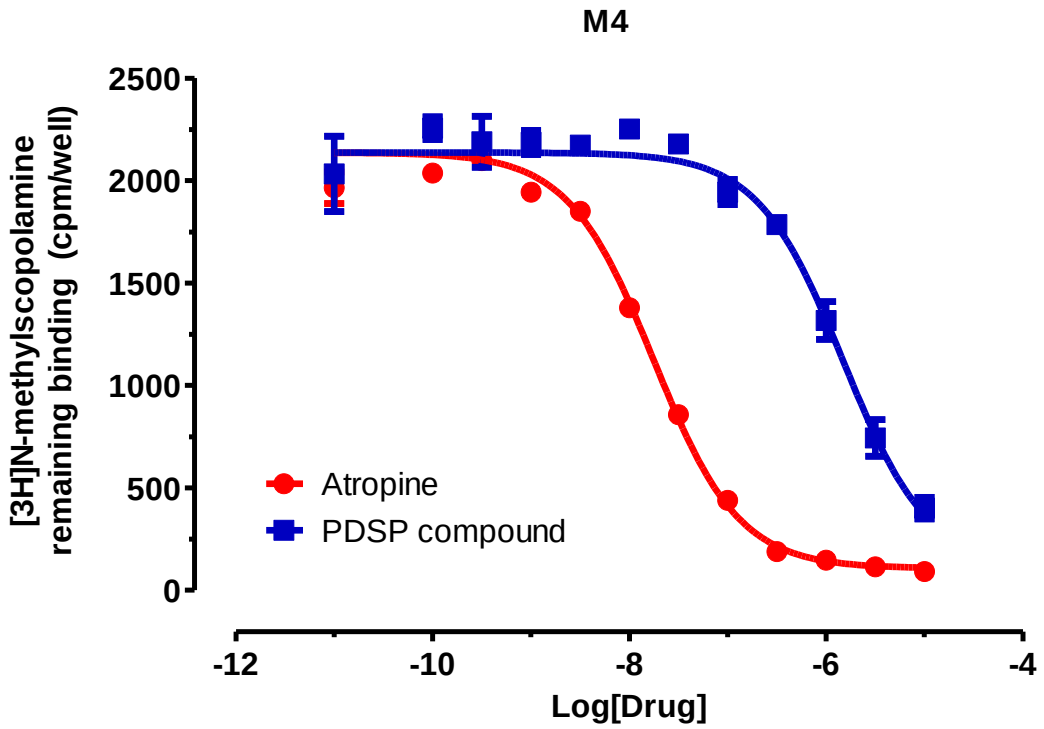
Use 0.5 – 1.0 nM hot ligand for binding assays

Target	Radioligand	K_d in nM (N)	References	Reference K_i (nM)
M1	[^{3}H]QNB	1.02 ± 0.17 (9)	Atropine	
M1	[^{3}H]NMS	0.90 ± 0.11 (5)	Atropine	1.80 ± 0.12
M2	[^{3}H]QNB	0.34 ± 0.06 (28)	Atropine	
M2	[^{3}H]NMS	0.34 ± 0.03 (5)	Atropine	1.52 ± 0.42
M3	[^{3}H]QNB	0.57 ± 0.05 (17)	Atropine	
M3	[^{3}H]NMS	0.36 ± 0.10 (7)	Atropine	0.86 ± 0.12
M4	[^{3}H]QNB	0.27 ± 0.05 (28)	Atropine	
M4	[^{3}H]NMS	0.19 ± 0.07 (7)	Atropine	0.86 ± 0.13
M5	[^{3}H]QNB	0.67 ± 0.13 (21)	Atropine	
M5	[^{3}H]NMS	0.45 ± 0.08 (12)	Atropine	0.88 ± 0.08

Figure 11. Representative competition binding curves with muscarinic acetylcholine receptors




M3
[3H]QNB
remaining binding (cpm)
3000
2000
1000
0
Atropine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


M4
[3H]N-methylscopolamine
remaining binding (cpm/well)
2500
2000
1500
1000
500
0
Atropine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

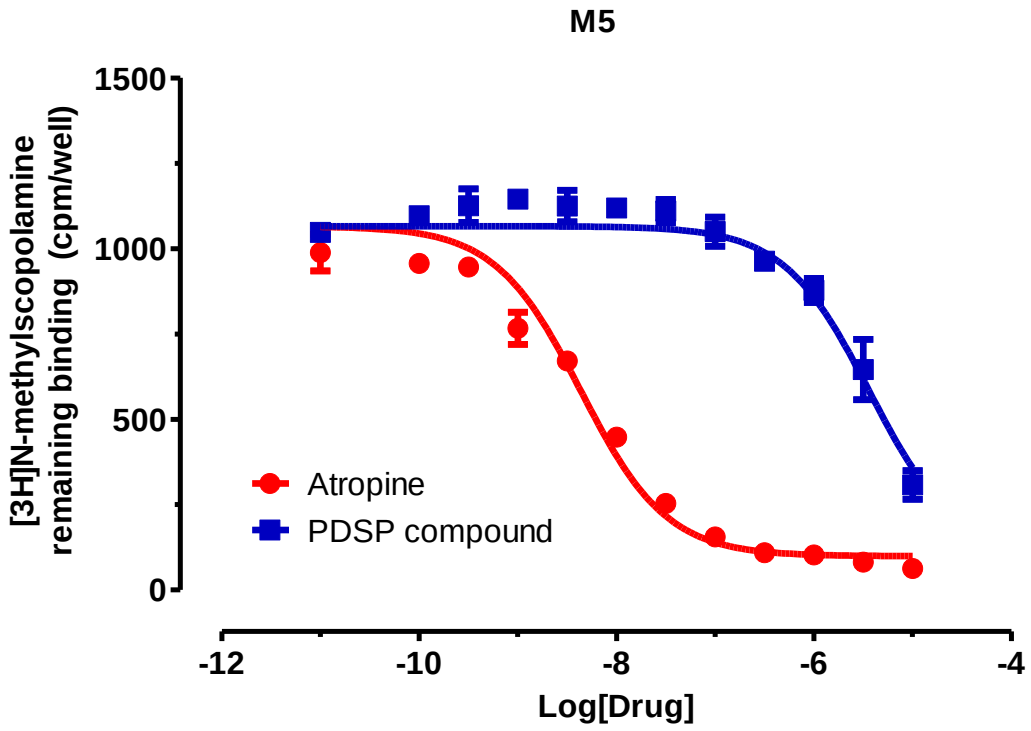

M5
[3H]N-methylscopolamine remaining binding (cpm/well)
1500
1000
500
0
Atropine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

Table 4. Radioligand binding assays for nicotinic acetylcholine receptors (nAChRs)**.** Primary binding assays use 100 pM of [^{3}H]-epibatidine and secondary binding assays use 0.5 nM [^{3}H]-epibatidine. Historical reference K_i values from the last 6 months are also included for quality control.

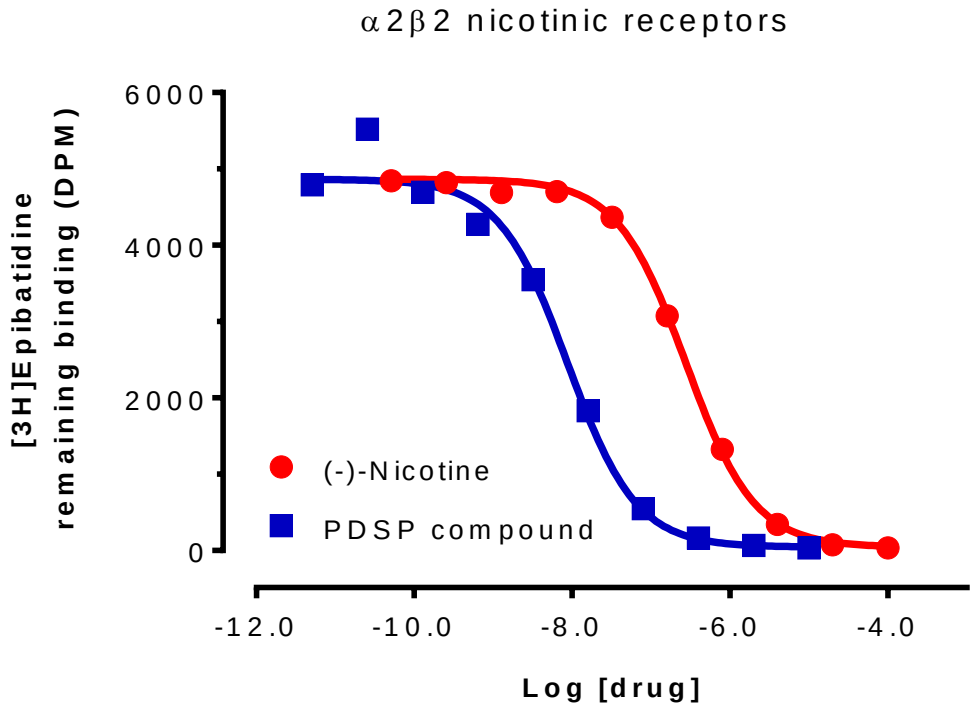
Nicotinic acetylcholine receptors (nAChRs)

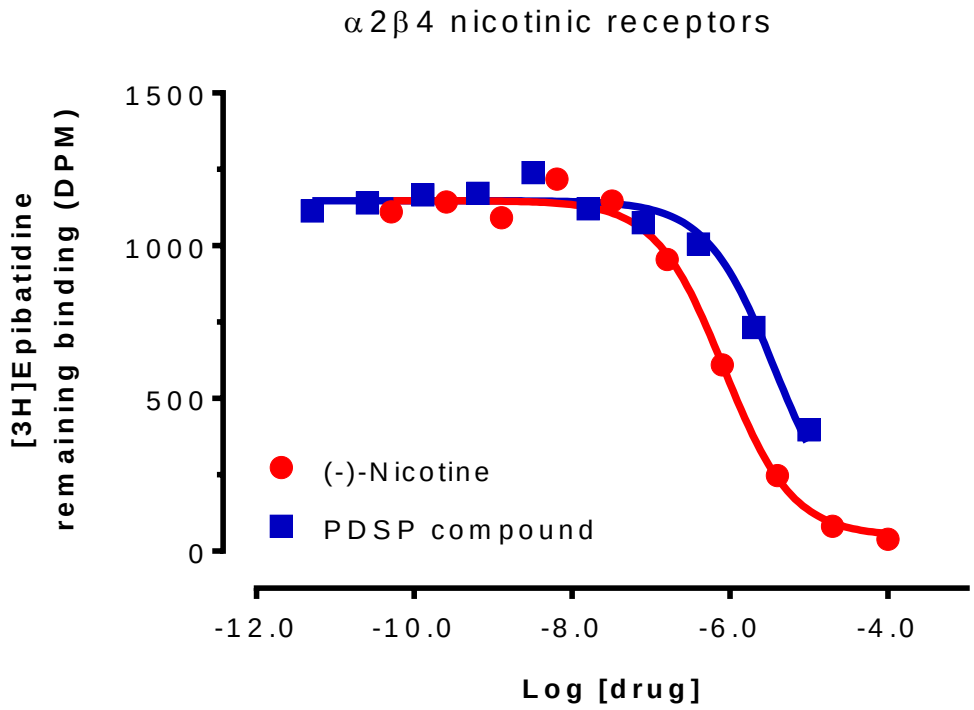
Nicotinic acetylcholine receptor binding buffer: 50 mM Tris HCl, pH 7.4, RT
Standard wash buffer: 50 mM Tris HCl, pH 7.4, cold

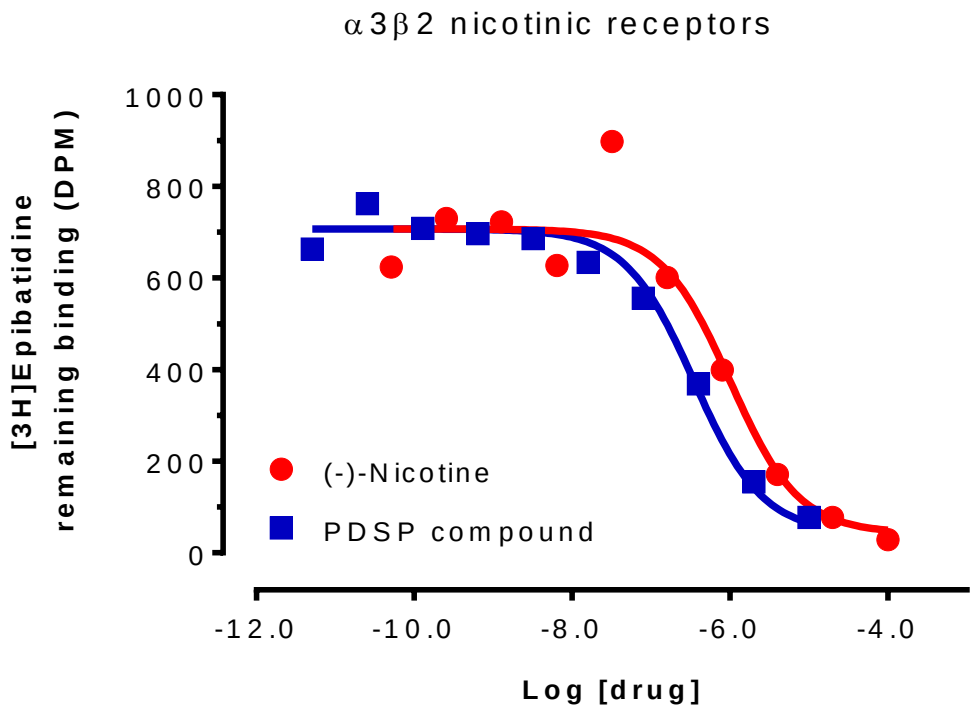
Target	Radioligand	[^{3}H] for binding	Reference	Reference K_i (nM)
α2ß2	[^{3}H]Epibatidine	0.5 nM	Nicotine	10.4 ± 2.2
α2ß4	[^{3}H]Epibatidine	0.5 nM	Nicotine	113.0 ± 7.4
α3ß2	[^{3}H]Epibatidine	0.5 nM	Nicotine	62.8 ± 5.4
α3ß4	[^{3}H]Epibatidine	0.5 nM	Nicotine	466.2 ± 6.4
α4ß2	[^{3}H]Epibatidine	0.5 nM	Nicotine	10.5 ± 1.3
α4ß2*	[^{3}H]Epibatidine	0.5 nM	Nicotine	10.9 ± 1.0
α4ß4	[^{3}H]Epibatidine	0.5 nM	Nicotine	35.8 ± 2.0
α7, rat	[^{3}H]Epibatidine	0.5 nM	Nicotine	690

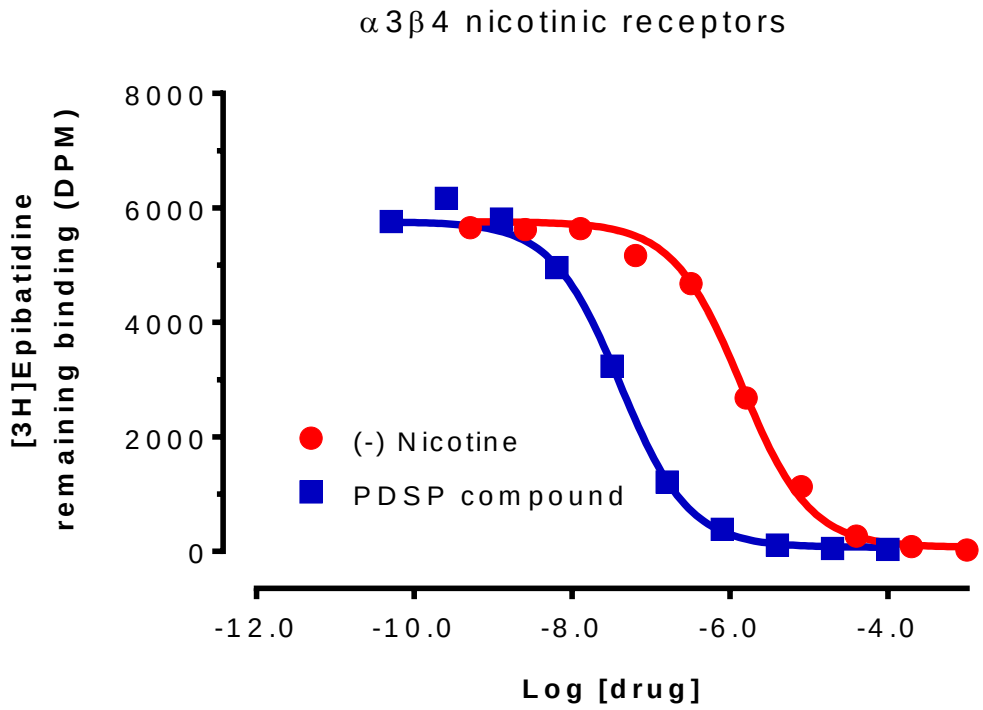
* Rat forebrain

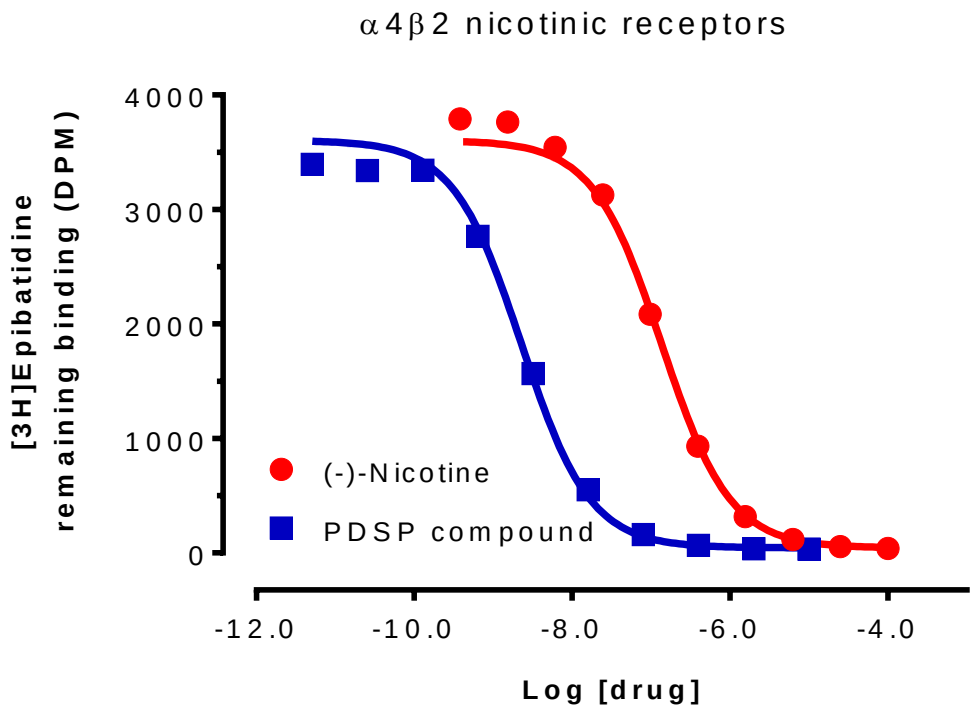
Figure 12. Representative competition binding curves with nicotinic acetylcholine receptors

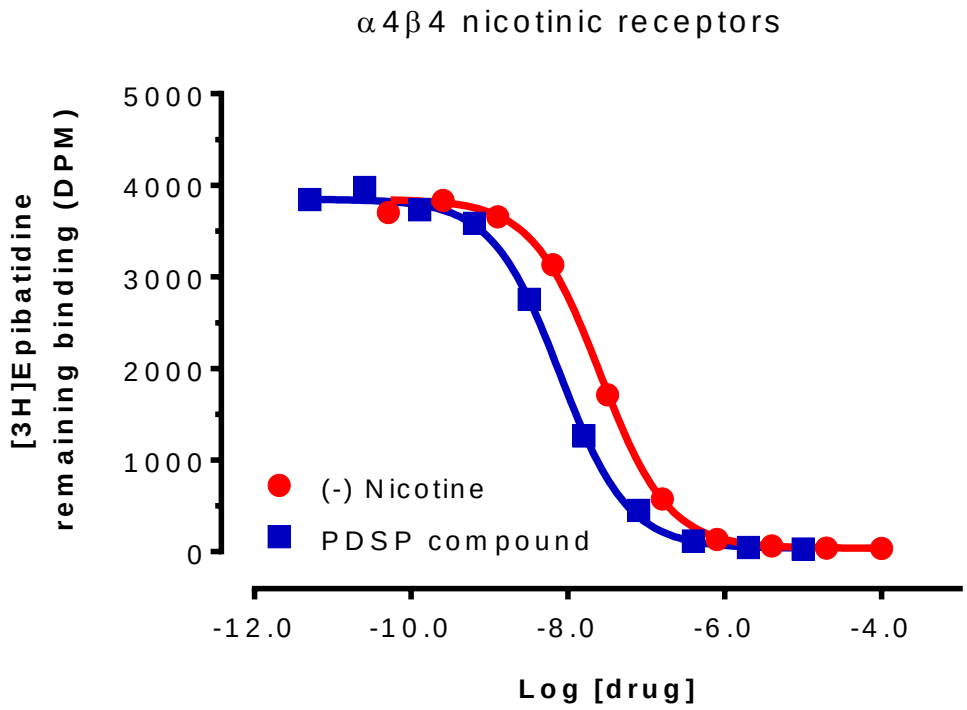


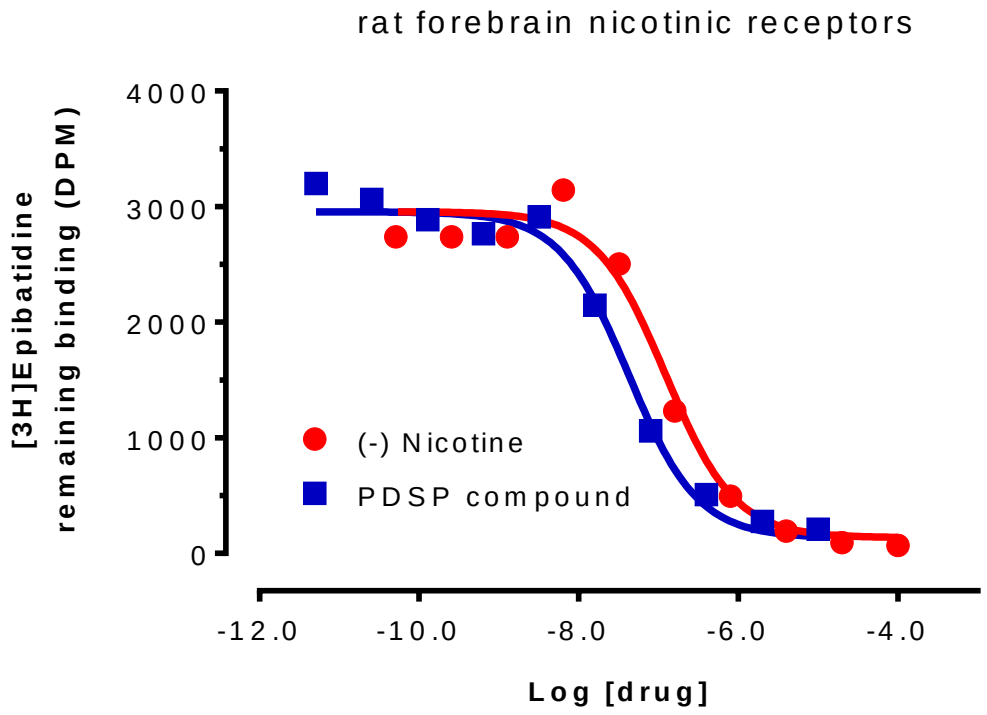



α3β2 nicotinic receptors
[3H]Epibatidine remaining binding (DPM)
1000
800
600
400
200
0
(-)-Nicotine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log [drug]


α3β4 nicotinic receptors
[3H]Epibatidine remaining binding (DPM)
8000
6000
4000
2000
0
(-) Nicotine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log [drug]


α4β2 nicotinic receptors
[3H]Epibatidine remaining binding (DPM)
4000
3000
2000
1000
0
(-)-Nicotine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log [drug]


α4β4 nicotinic receptors
[3H]Epibatidine remaining binding (DPM)
5000
4000
3000
2000
1000
0
(-) Nicotine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log [drug]


rat forebrain nicotinic receptors
[3H]Epibatidine
remaining binding (DPM)
4000
3000
2000
1000
0
(-) Nicotine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log [drug]

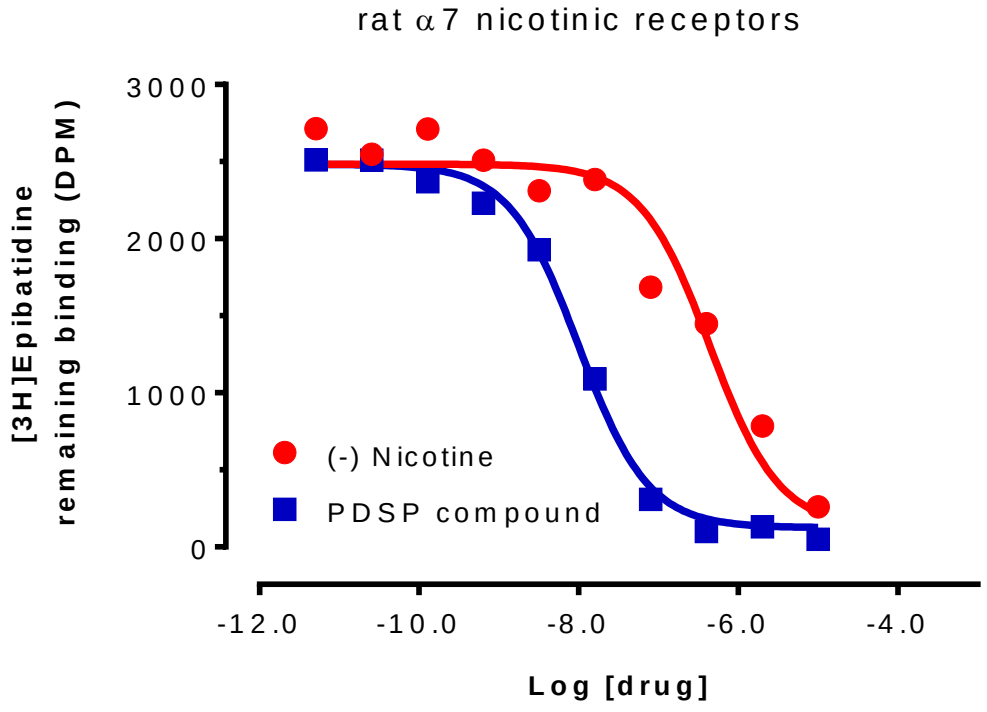

rat α7 nicotinic receptors
[3H]Epibatidine
remaining binding (DPM)
3000
2000
1000
0
(-) Nicotine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log [drug]

Table 5. Adenosine receptors, radioligand and corresponding concentration, reference compound, and buffer for primary and secondary radioligand binding assays.

Adenosine receptors Adenosine Binding Buffer: 50 mM Tris HCl, 1U/ml adenosine deaminase, pH 7.4, RT Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold				
Target	**Radioligand**	**[^{3}H] for binding in nM**	**References**	**Reference K_i (nM)**
A1	[^{3}H] NECA	3 nM	NECA	
A2A	[^{3}H] NECA	3 nM	NECA	
A2B	[^{3}H] NECA	3 nM	NECA	
A3	[^{3}H] NECA	3 nM	NECA	

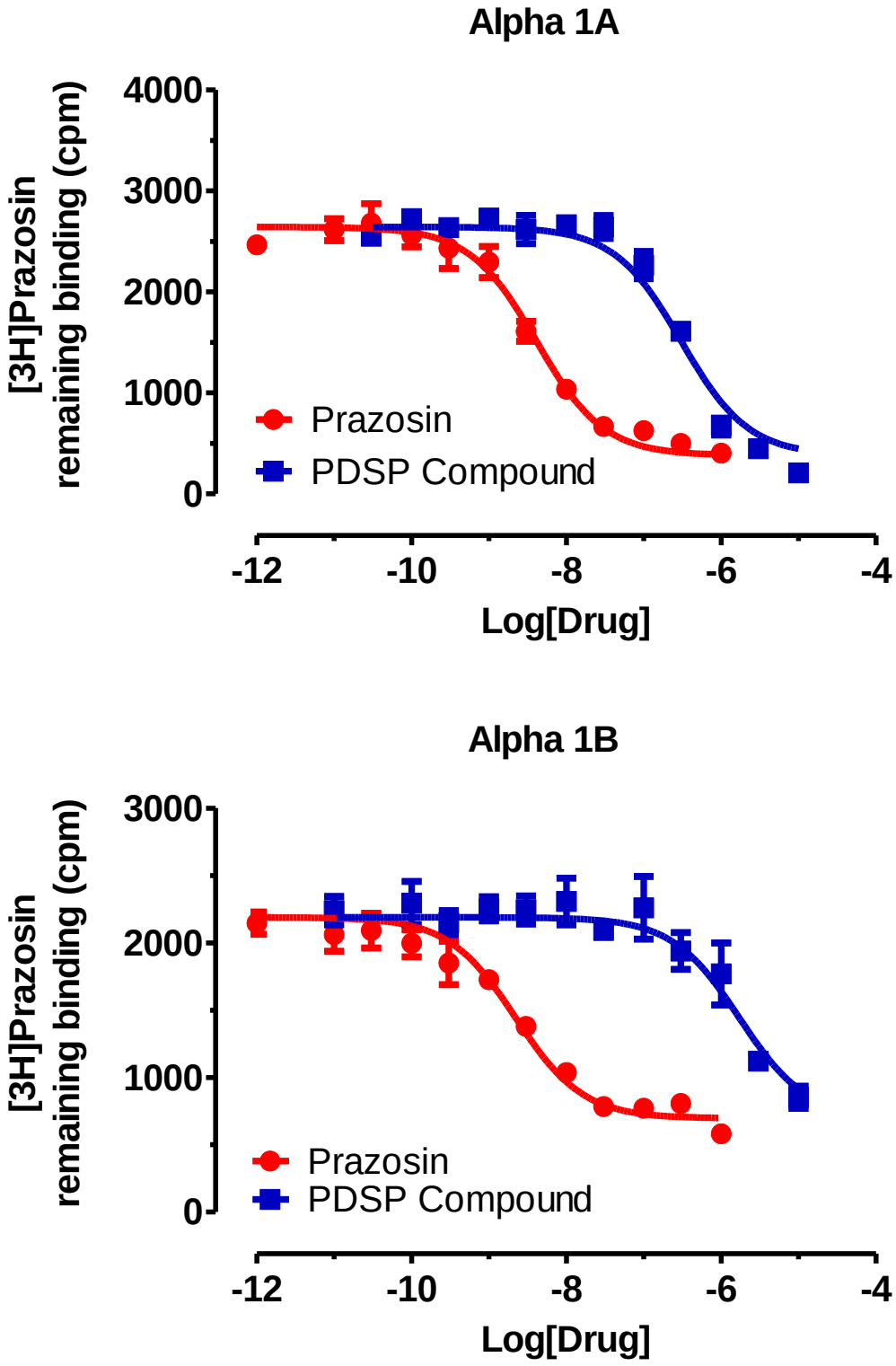
Table 6. Adrenergic receptors, radioligands and corresponding K_d values, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentrations of radioligand used for competition binding assays are usually at or near the K_d value or as listed. The K_d values listed in this table are the average ± SEM from a minimum of 3 saturation binding assays from 2011 to 2012. Historical reference K_i values from the last 6 months are also listed.

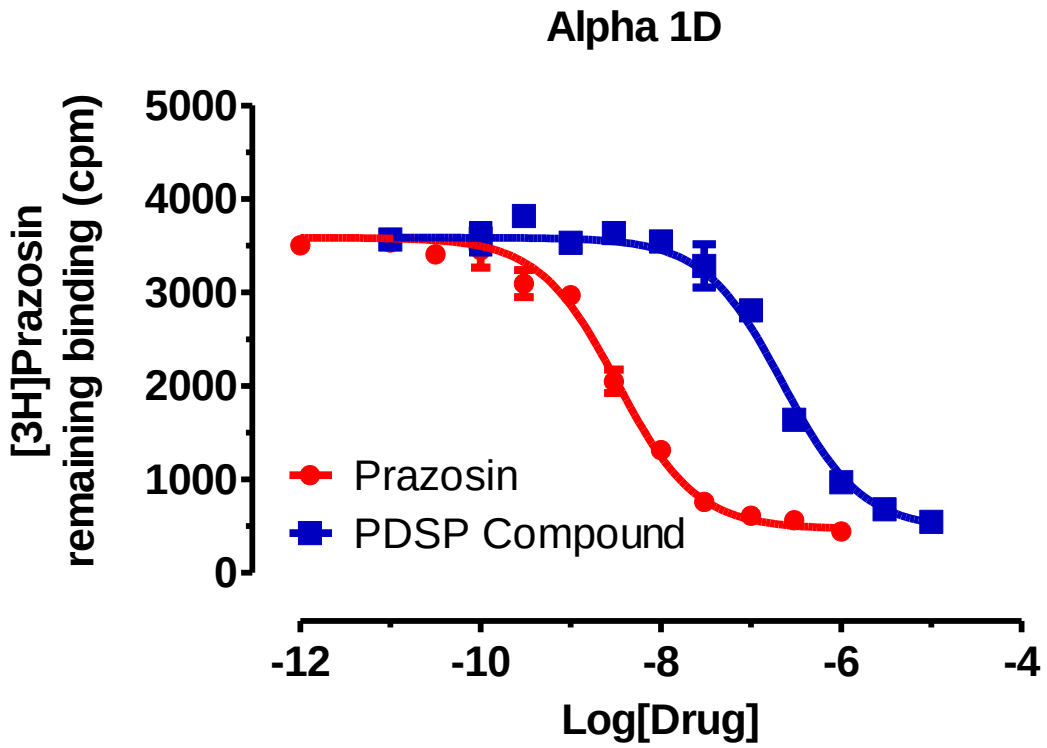
Adrenergic receptors

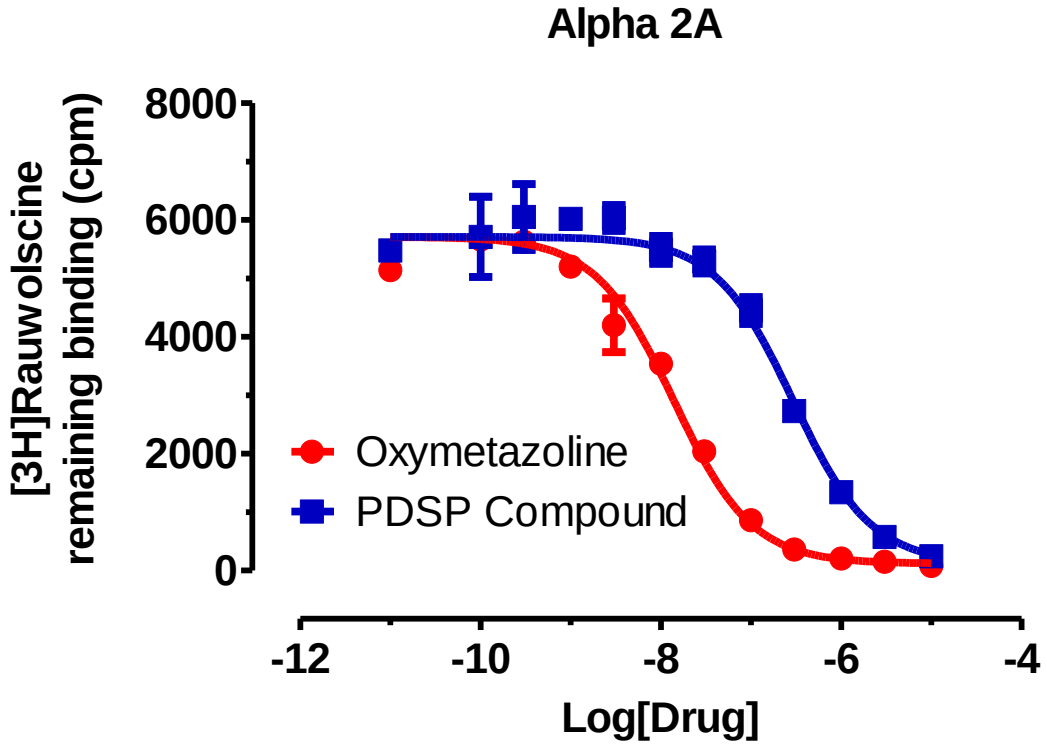
α1 Binding Buffer: 20 mM Tris HCl, 145 mM NaCl, pH 7.4, RT
α2 Binding Buffer: 50 mM Tris HCl, 5 mM $MgCl_2$, pH 7.7, RT
ß Binding Buffer: 50 mM Tris HCl, 3 mM $MgCl_2$, pH 7.7, RT
Standard Wash Buffer: 50 mM Tris HCl, pH 7.4, cold

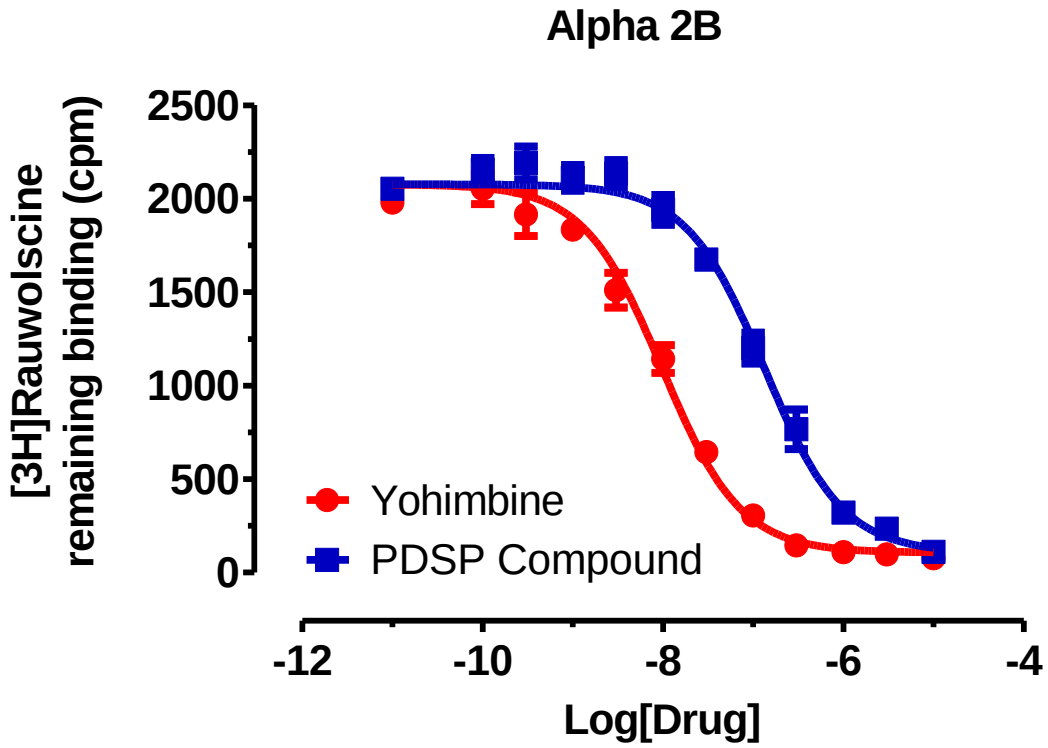
Target	Radioligand	K_d or [^{3}H] for binding in nM (N)	References	Reference K_i (nM)
α1A	[^{3}H]Prazosin	0.64 ± 0.14 (8)	Prazosin	0.95 ± 0.08
α1B	[^{3}H]Prazosin	0.5 – 1.0 nM for binding	Prazosin	1.28 ± 0.13
α1D	[^{3}H]Prazosin	0.89 ± 0.14 (7)	Prazosin	1.04 ± 0.10
α2A	[^{3}H]Rauwolscine	0.5 - 1.0 nM for binding	Oxymetazoline	5.15 ± 0.21
α2B	[^{3}H]Rauwolscine	1.36 ± 0.50 (7)	Prazosin	5.86 ± 0.52
α2C	[^{3}H]Rauwolscine	0.73 ± 0.24 (6)	Oxymetazoline	36.9 ± 3.3
ß1	[^{3}H]CGP12177	1.0 nM for binding	Alprenolol	
ß1	[^{125}I]Pindolol	0.05 nM for binding	Alprenolol	1.86 ± 0.21
ß2	[^{3}H]CGP12177	1.03 ± 0.47 (6)	Alprenolol	2.03 ± 0.28
ß3	[^{3}H]CGP12177	13.8 ± 9.1 (5) 10 nM for binding	Propanolol	49.7 ± 14.1
ß3	[^{125}I]Pindolol	0.5 nM for binding	Propanolol	

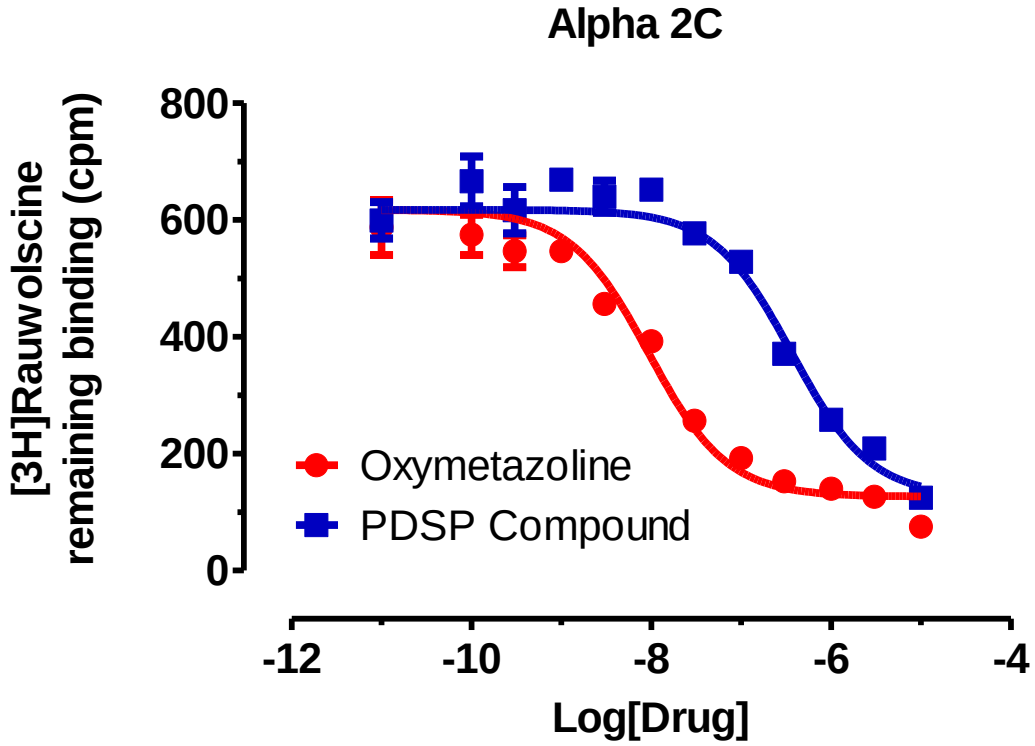
Figure 13. Representative competition binding curves with adrenergic receptors

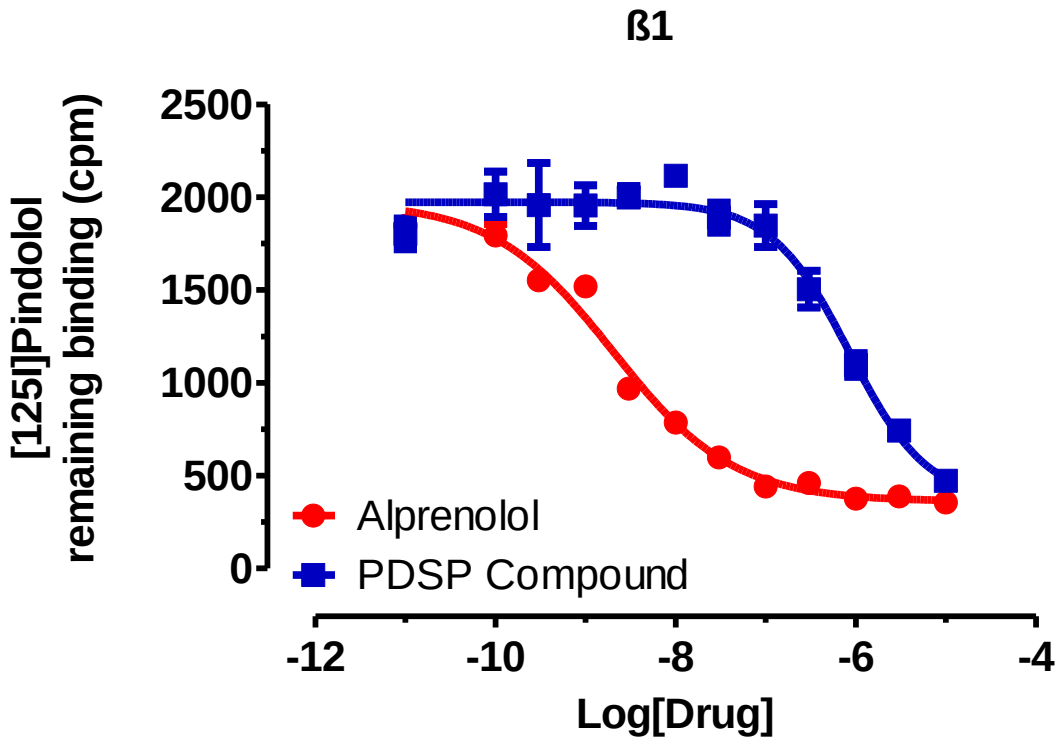


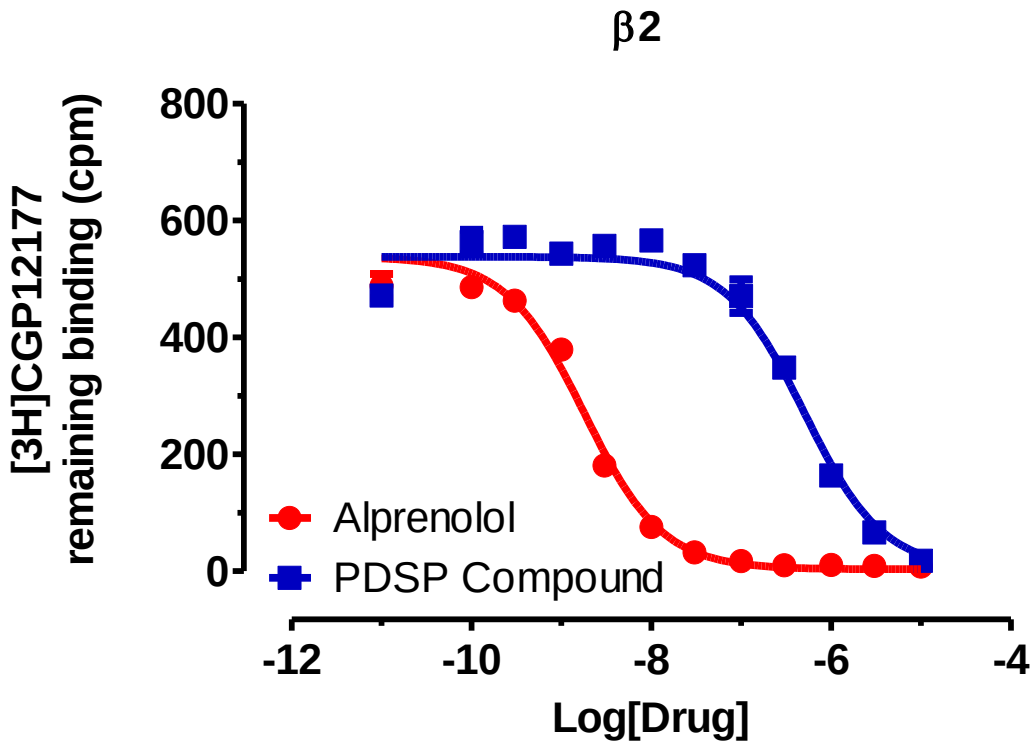

Alpha 1D
[3H]Prazosin
remaining binding (cpm)
5000
4000
3000
2000
1000
0
Prazosin
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


Alpha 2A
[3H]Rauwolscine
remaining binding (cpm)
8000
6000
4000
2000
0
Oxymetazoline
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


Alpha 2B
[3H]Rauwolscine remaining binding (cpm)
2500
2000
1500
1000
500
0
Yohimbine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


Alpha 2C
[3H]Rauwolscine remaining binding (cpm)
800
600
400
200
0
Oxymetazoline
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


ß1
[125I]Pindolol
remaining binding (cpm)
2500
2000
1500
1000
500
0
Alprenolol
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]


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

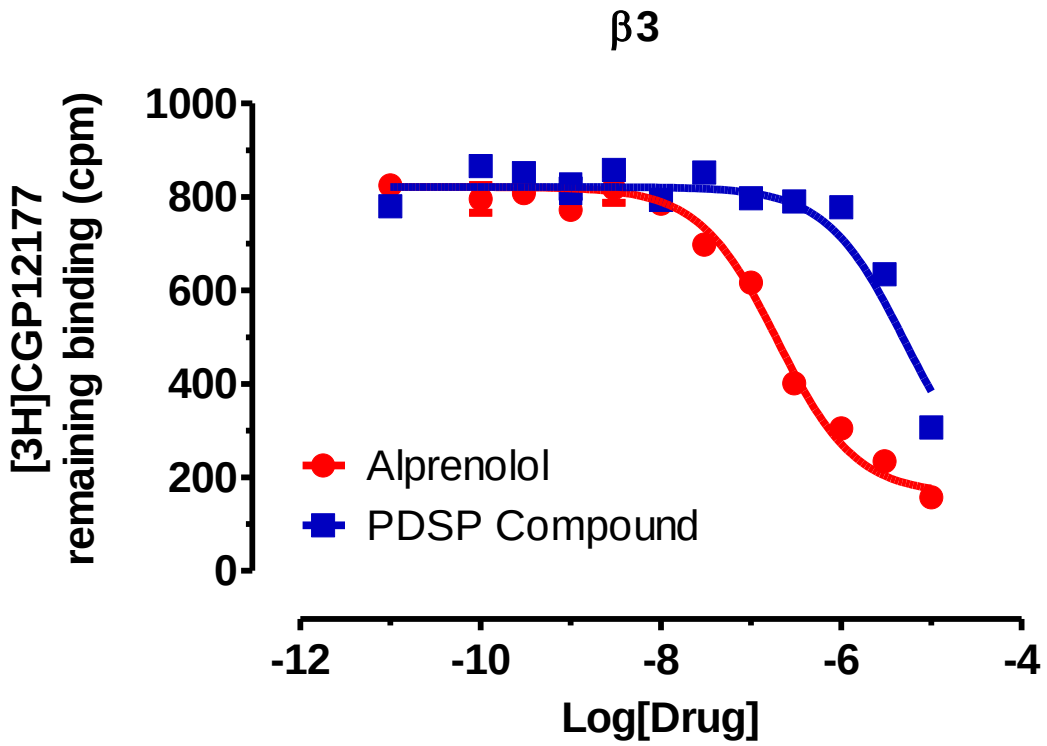

β3
[3H]CGP12177
remaining binding (cpm)
1000
800
600
400
200
0
Alprenolol
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

Table 7. Cannabinoid receptors, radioligand and corresponding concentration, reference compound, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from the last 6 months 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	[^{3}H] for binding in nM	References	Reference Ki (nM)
CB1 (rat brain)	[^{3}H]CP55940	1 nM	WIN55212-3	18.5 ± 3.0
CB2	[^{3}H]CP55940	2 nM	WIN55212-3 CP55940	9.4 ± 2.6 4.95 ± 0.55

Figure 14. Representative competitive binding curves for cannabinoid receptors.

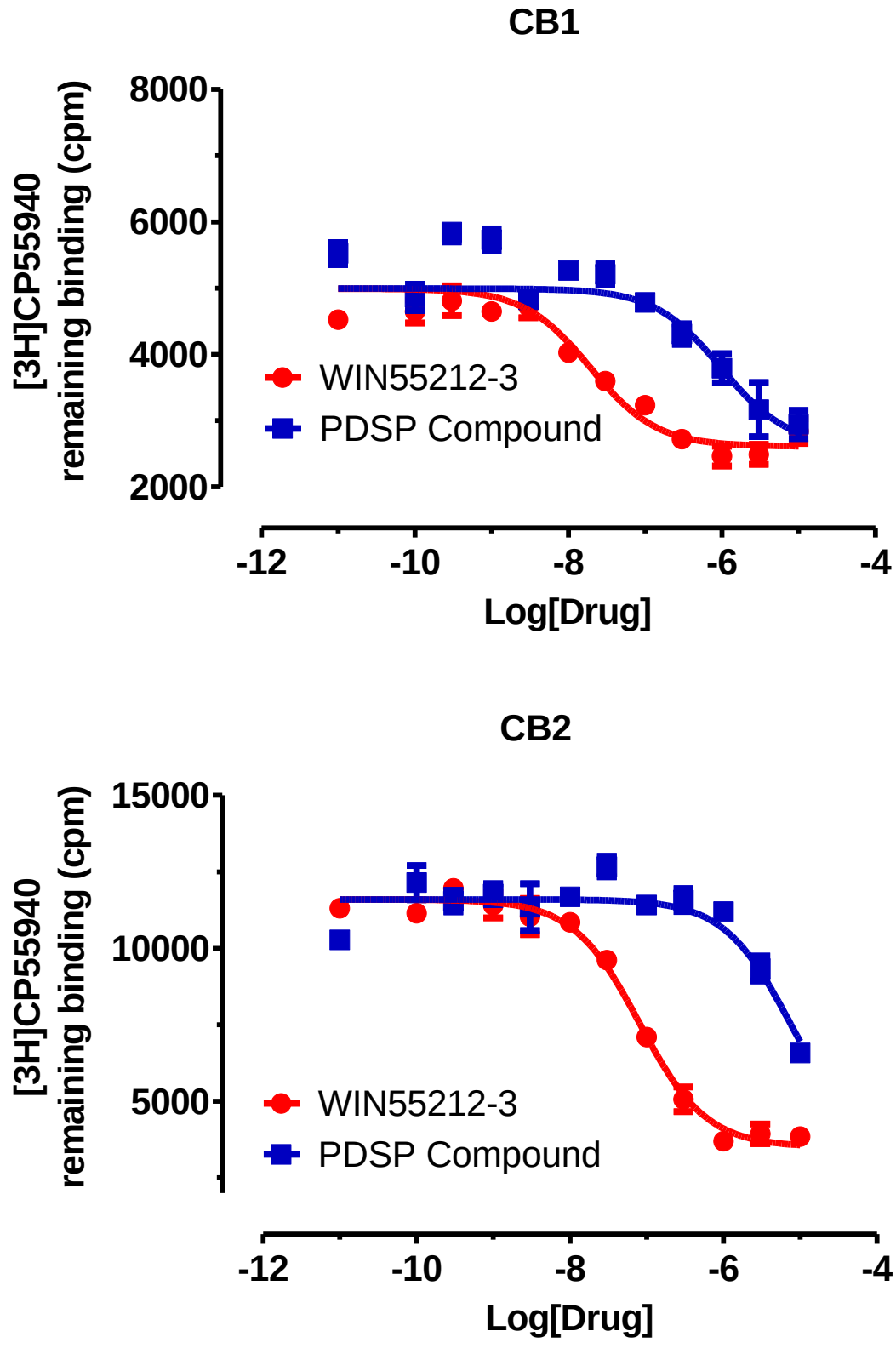


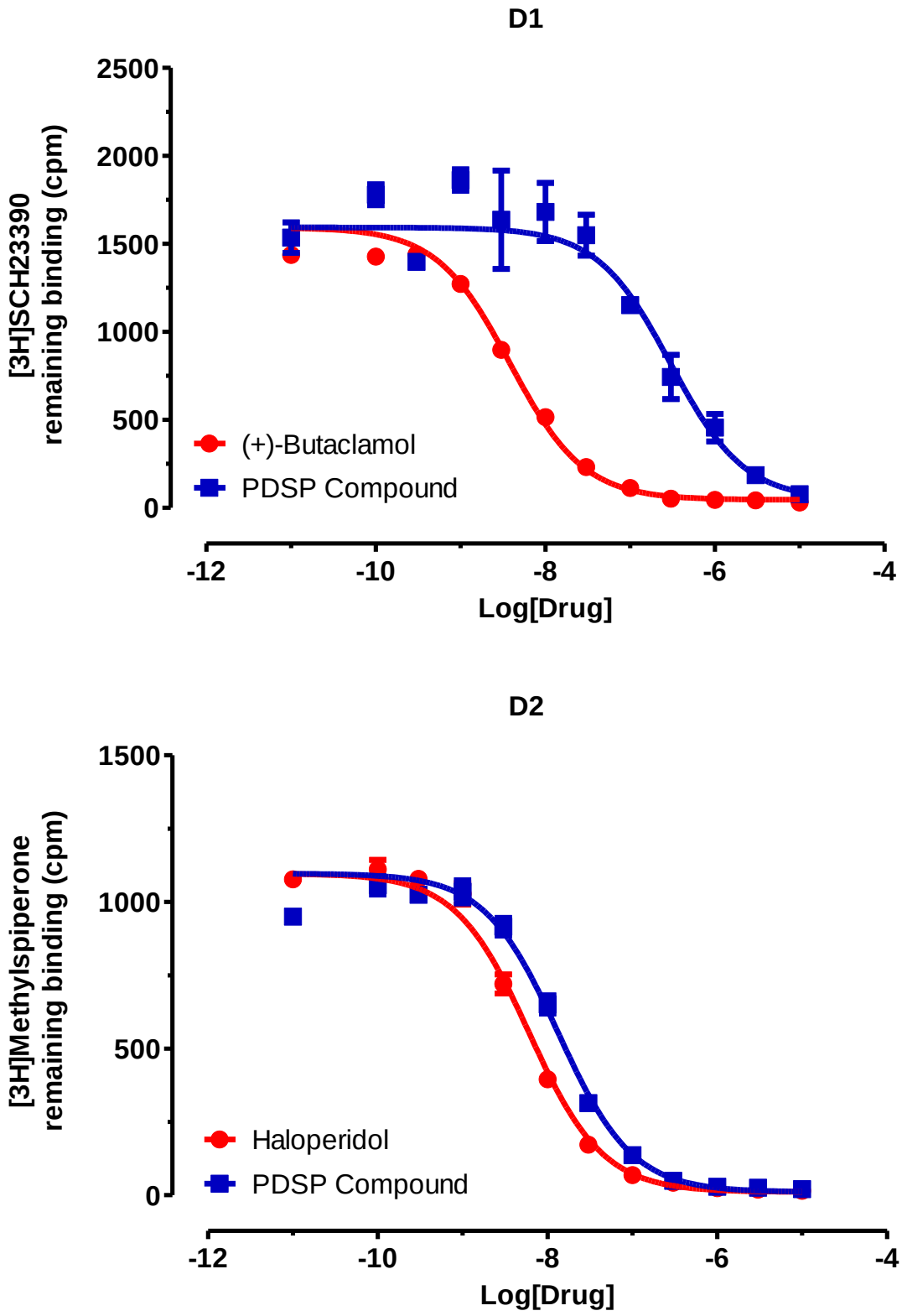
Table 8. Dopamine receptors, radioligands and corresponding K_d values, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually at or near the K_d value or as listed. The K_d values listed in this table are the average ± SEM from a minimum of 3 saturation binding assays from 2011 to 2012. Historical reference K_i values from the last 6 months are also included.

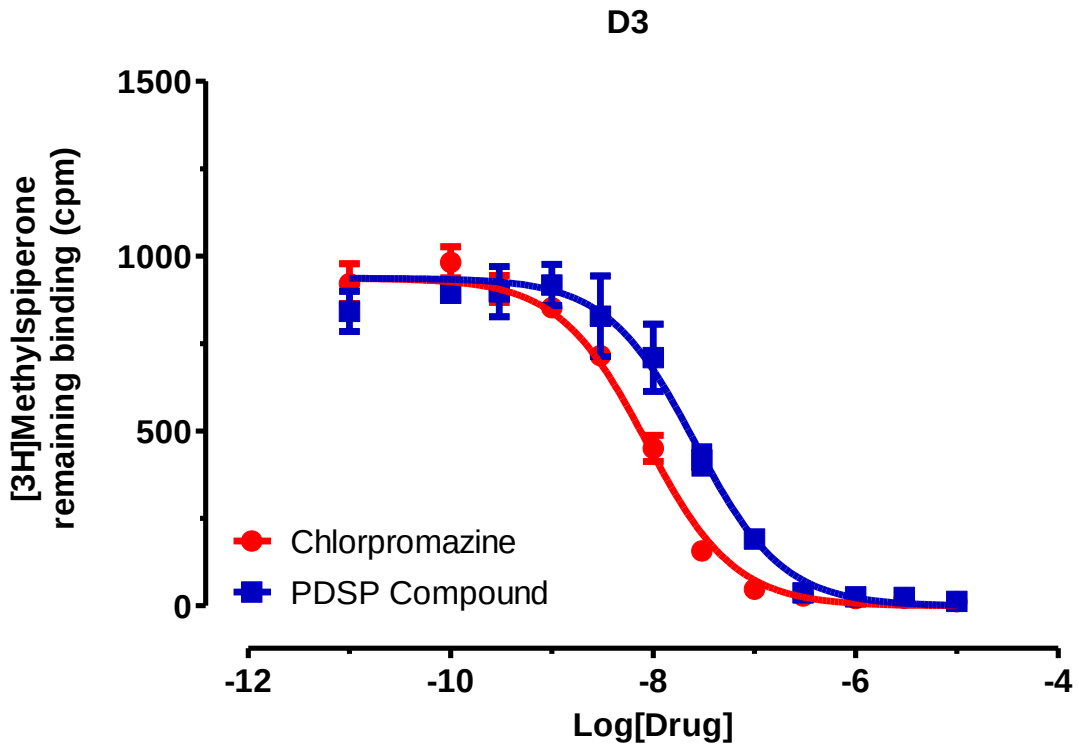
Dopamine receptors

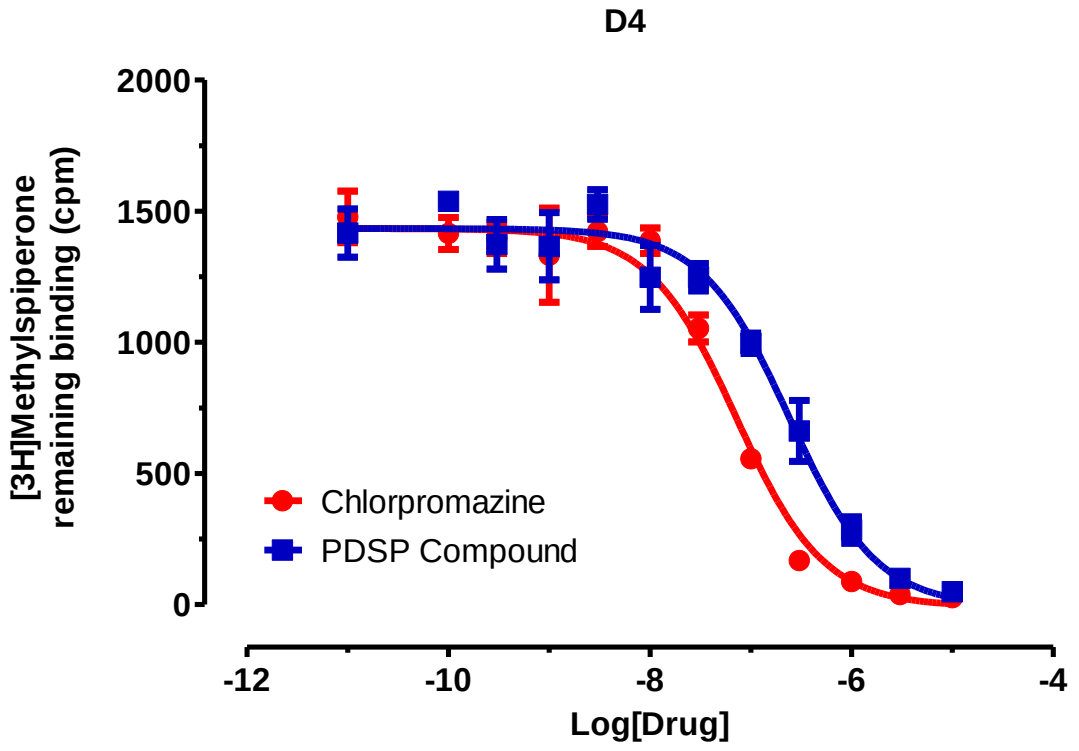
Dopamine Binding Buffer: 50 mM HEPES, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4, RT
Standard Wash buffer: 50 mM Tris HCl, pH 7.4, cold

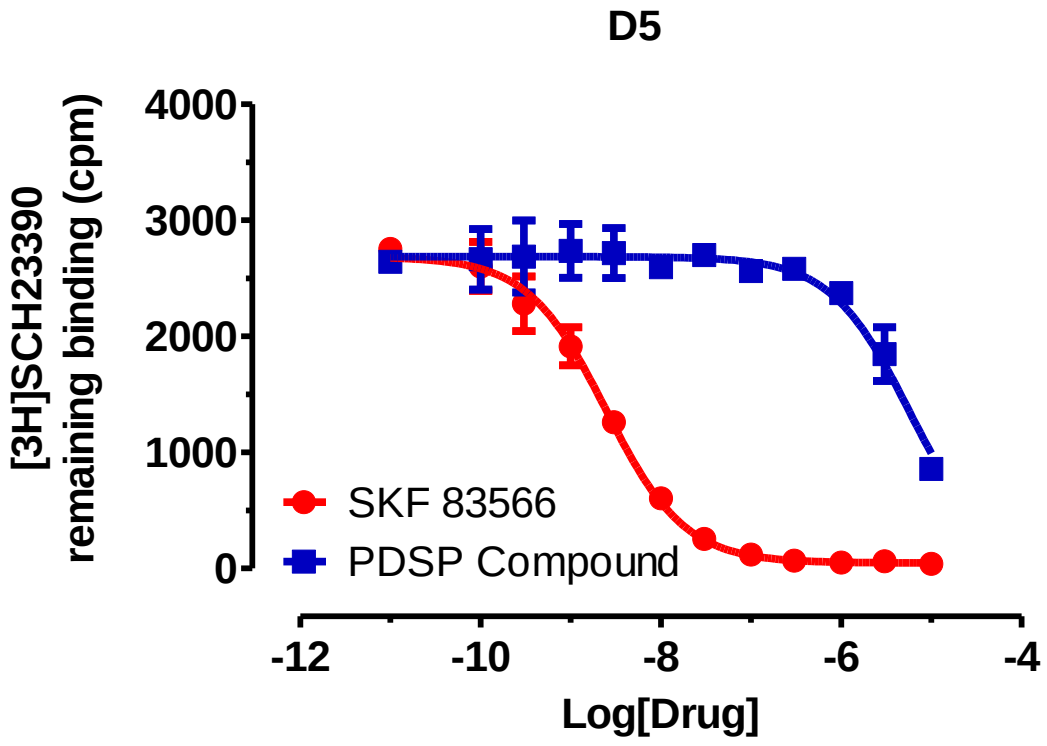
Target	Radioligand	[^{3}H] K_d in nM (N)	References	Reference K_i (nM)
D1	[^{3}H]SCH23390	0.89 ± 0.12 (16)	SKF38393	3.97 ± 0.37
D2	[^{3}H]N-methylspiperone	0.67 ± 0.11 (21)	Haloperidol	7.20 ± 0.75
D3	[^{3}H]N-methylspiperone	0.66 ± 0.24 (7)	Chlorpromazine	10.30 ± 1.08
D4	[^{3}H]N-methylspiperone	0.84 ± 0.09 (9)	Chlorpromazine	37.37 ± 4.21
D5	[^{3}H]SCH23390	2.08 ± 0.27 (18)	SKF38393	3.08 ± 0.18

Figure 15. Representative competitive binding curves with Dopamine receptors.




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


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



Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

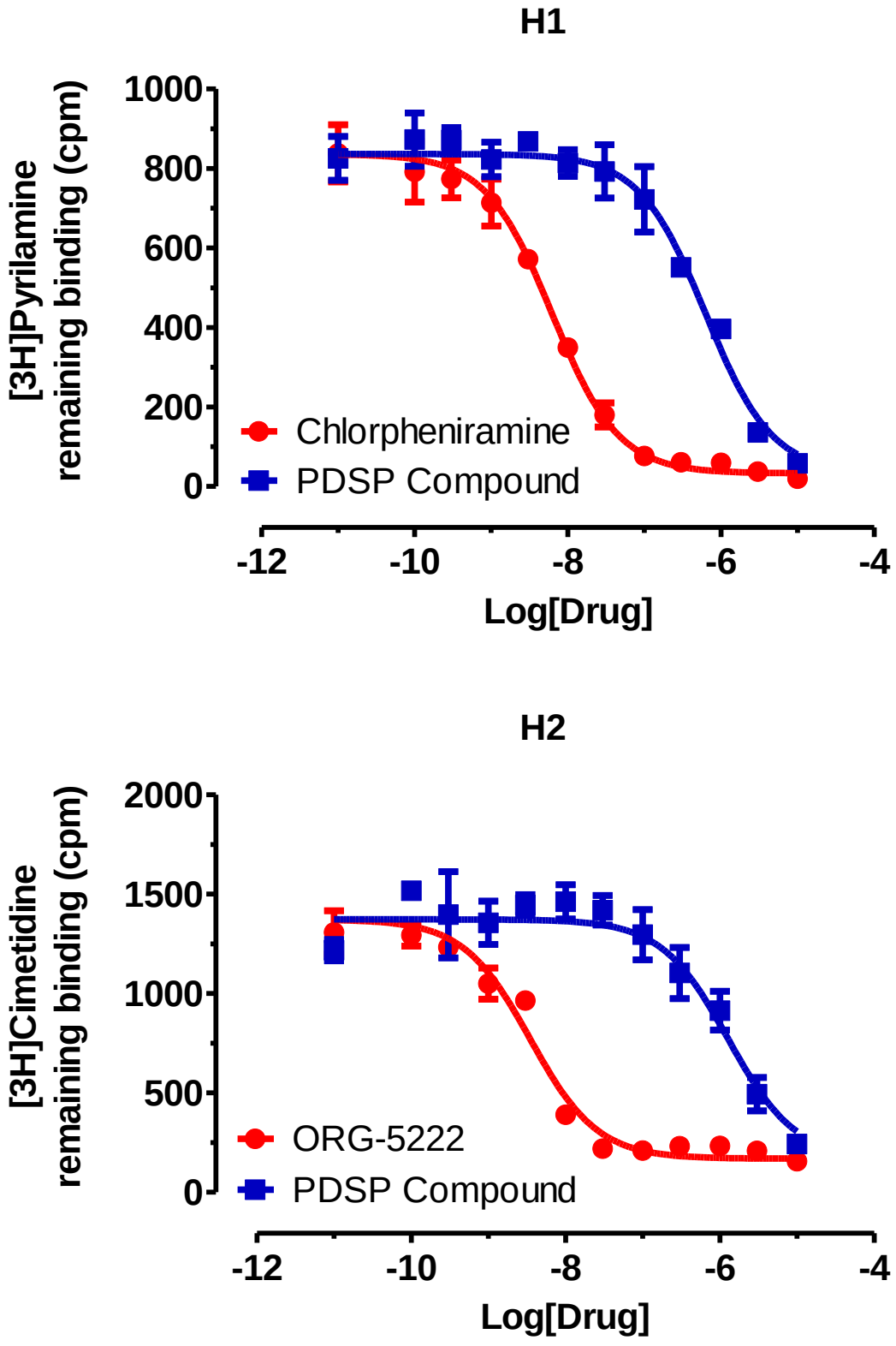
Table 9. Histamine receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually at or near the K_d value or as listed. The K_d values listed in this table are the average ± SEM from a minimum of 3 saturation binding assays from 2011 to 2012. Historical reference K_i values from the last 6 months are also included.

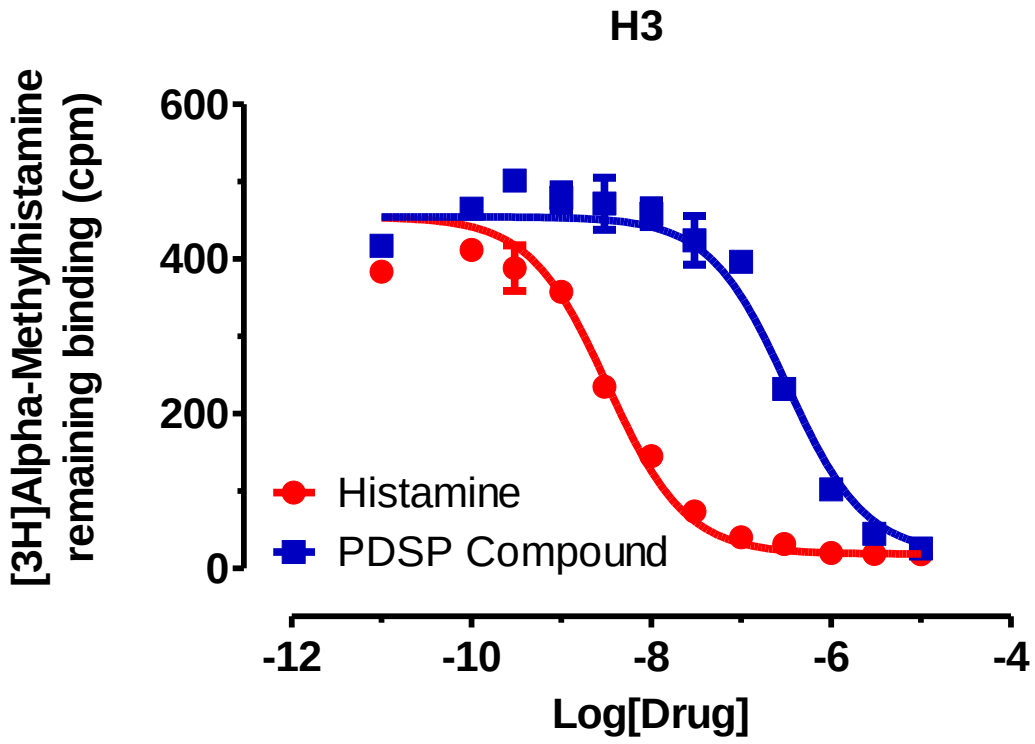
Histamine receptors

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	K_d or [^{3}H] for binding in nM (N)	References	Reference K_i (nM)
H1	[^{3}H]Pyrilamine	1.01 ± 0.13 (21)	Chlorphreniramine	3.22 ± 0.33
H2	[^{3}H]Cimetidine	3 nM for binding	ORG-5222	3.05 ± 0.47
H2	[3H]Tiotidine	2 nM for binding	Cimetidine	
H3	[^{3}H]α-methylhistamine	0.74 ± 0.11 (19)	Histamine	6.43 ± 1.60
H4	[^{3}H]Histamine	1.20 ± 0.10 (2)	Clozapine	

Figure 16. Representative competitive binding curves with Histamine receptors.




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

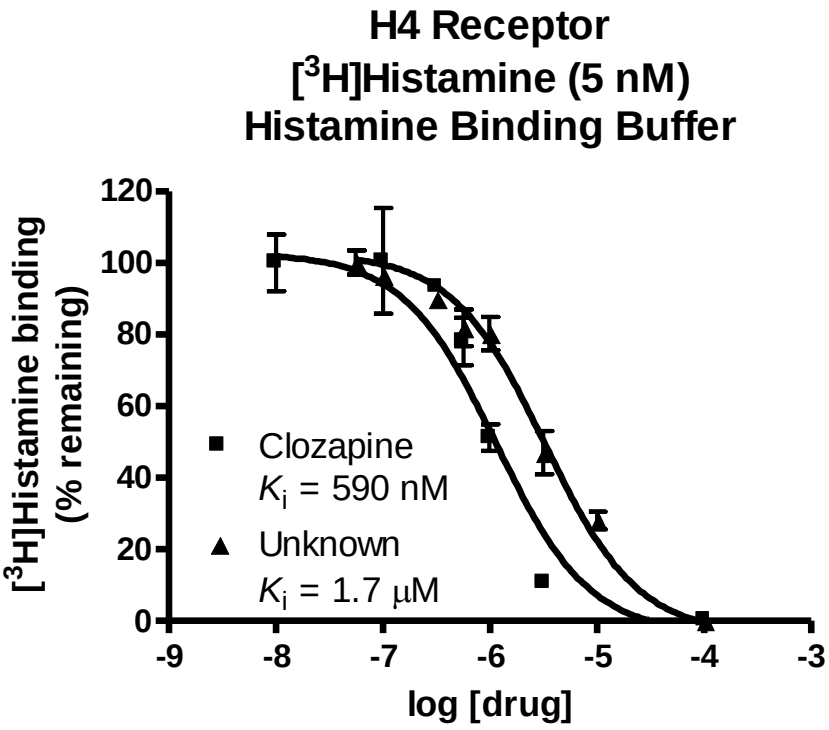

H4 Receptor
[3H]Histamine (5 nM)
Histamine Binding Buffer
[3H]Histamine binding (% remaining)
120
100
80
60
40
20
0
Clozapine
Ki = 590 nM
Unknown
Ki = 1.7 µM
-9
-8
-7
-6
-5
-4
-3
log [drug]

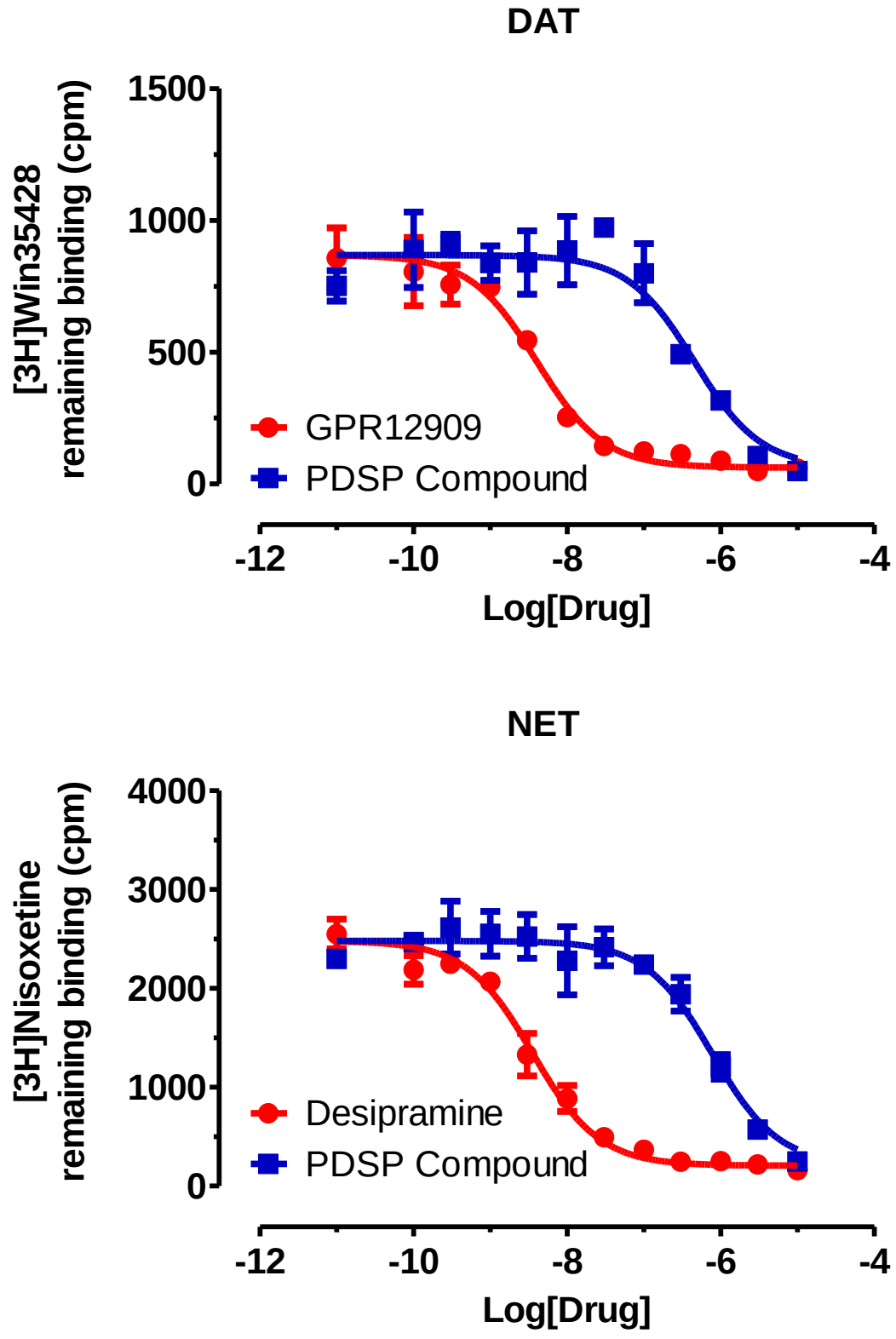
Table 10. Neurotransmitter transporters, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually at or near the K_d value or as listed. The K_d values listed in this table are the average ± SEM from a minimum of 3 saturation binding assays from 2011 to 2012. Historical reference K_i values from the last 6 months are also included. BB for binding buffer; WB for wash buffer.

Neurotransmitter transporters

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

Target	Radioligand	K_d Or [^{3}H] for binding in nM (N)	References	Reference K_i (nM)
DAT	[^{3}H]WIN35428	11.1 ± 1.4 (10)	GBR12909	3.04 ± 0.21
NET	[^{3}H]Nisoxetine	4.9 ± 0.7 (4)	Desipramine	3.16 ± 0.28
SERT	[^{3}H]Citalopram	3.8 ± 2.1 (4)	Amitriptyline	4.46 ± 0.55

Figure 17. Representative competitive binding curves with neurotransmitter transporters.



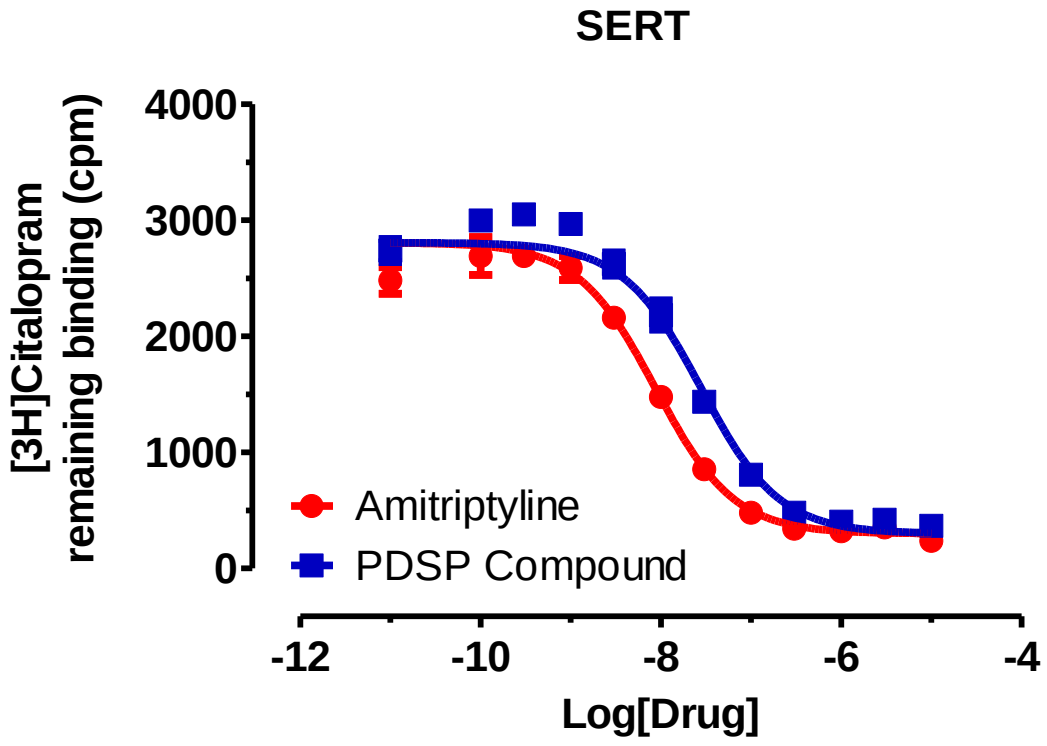

SERT
[3H]Citalopram remaining binding (cpm)
4000
3000
2000
1000
0
Amitriptyline
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

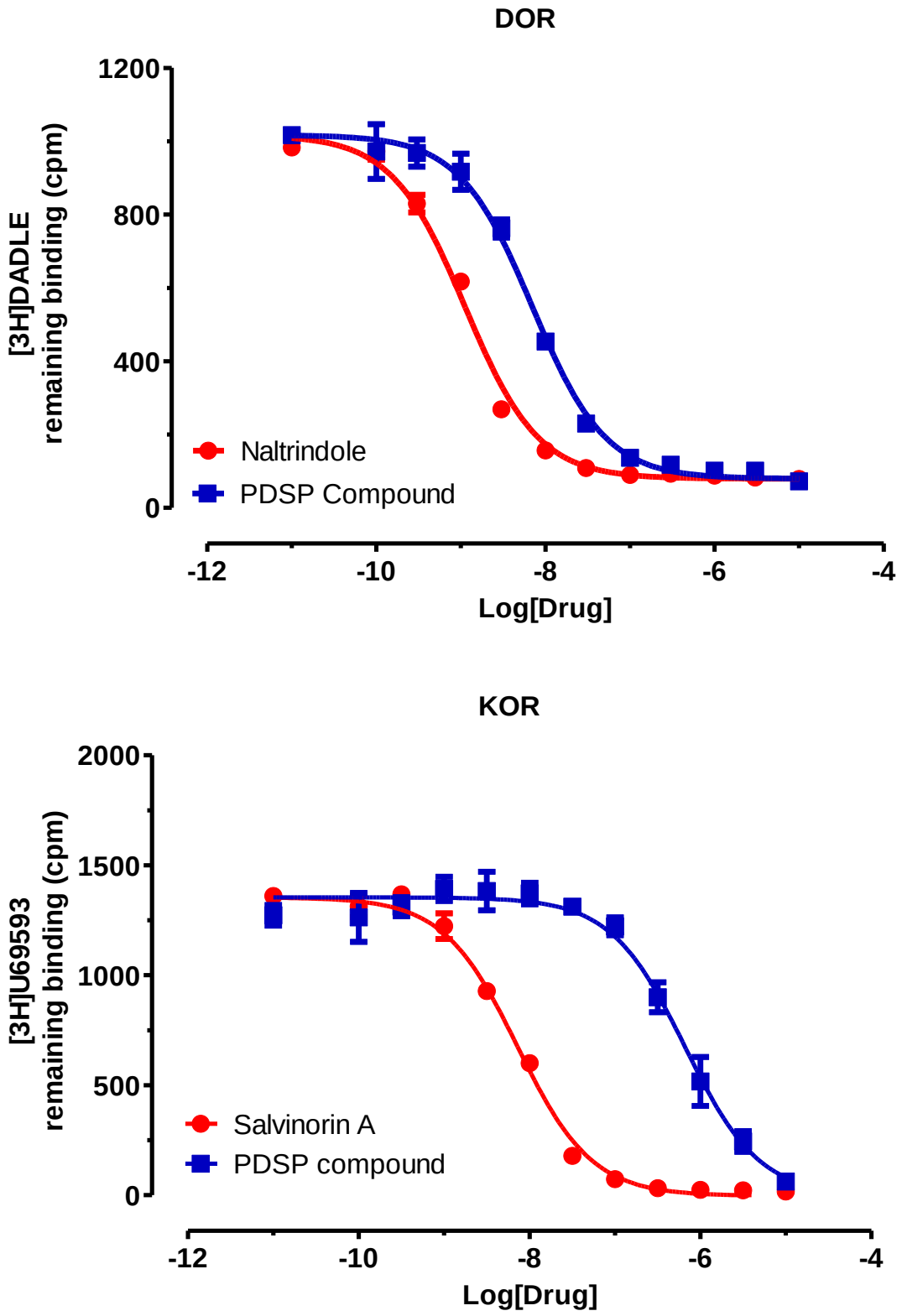
Table 11. Opioid receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentrations of radioligand used for competition binding assay are usually at or near the K_d value or as listed. The K_d values listed in this table are the average ± SEM from a minimum of 3 saturation binding assays from 2011 to 2012. Historical reference K_i values from the last 6 months are also included.

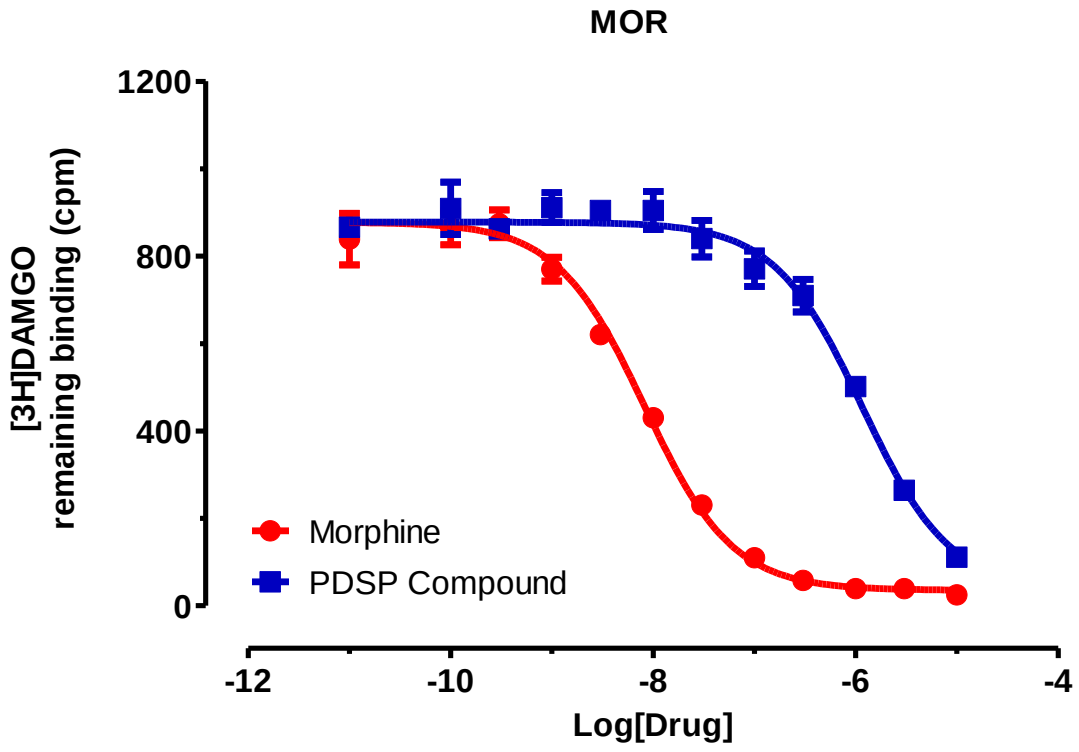
Opioid receptors

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	K_d or [^{3}H] for binding in nM (N)	References	Reference K_i (nM)
DOR	[^{3}H]DADLE	1.85 ± 0.15 (2)	Naltrindole	0.81 ± 0.08
KOR	[^{3}H]U69593	1.07 ± 0.10 (21)	Salvinorin A	1.93 ± 0.45
MOR	[^{3}H]DAMGO	1.73 ± 0.14 (6)	DAMGO, Morphine	2.62 ± 0.22
NOP	[^{3}H]N/OFQ	0.74 ± 0.22 (4)	JDTiC SB612111	12.05 ± 1.47 6.58 ± 1.42

Figure 18. Representative competitive binding curves with opioid receptors.




MOR
[3H]DAMGO
remaining binding (cpm)
1200
800
400
0
Morphine
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

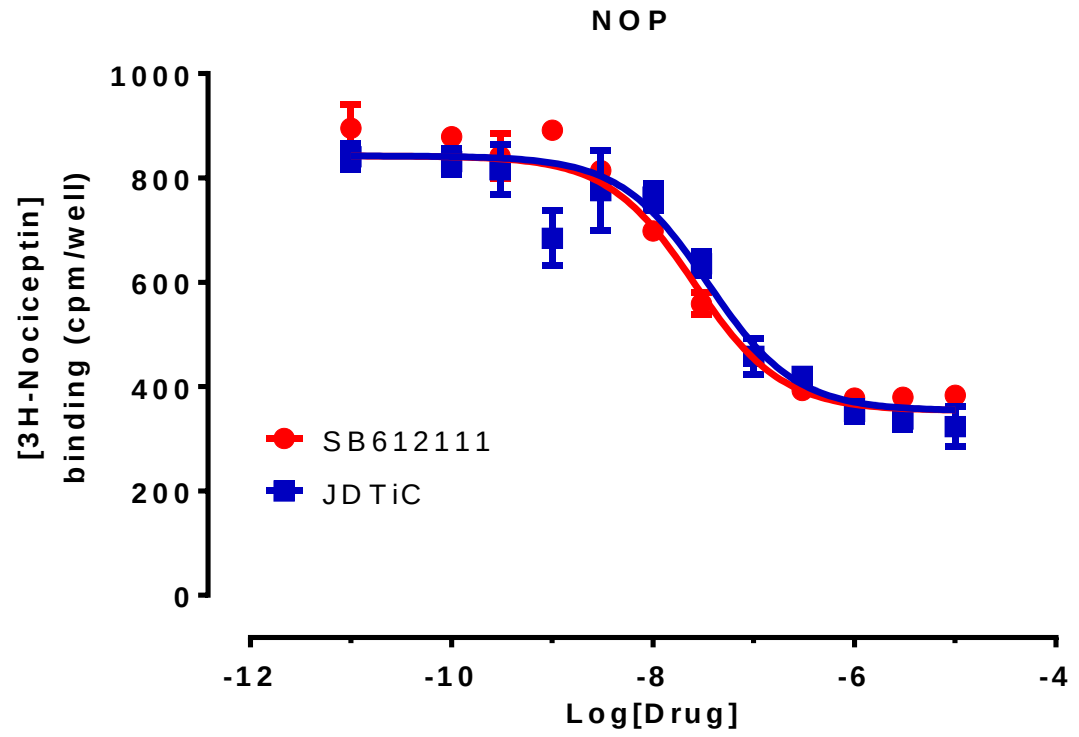

NOP
[3H-Nociceptin]
binding (cpm/well)
1000
800
600
400
200
0
SB612111
JDTiC
-12
-10
-8
-6
-4
Log[Drug]

Table 12. Oxytocin and Vasopressin receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentrations of radioligand used for competition binding assays is usually at or near the K_d value or as listed. The K_d values listed in this table are the average ± SEM from a minimum of 3 saturation binding assays from 2011 to 2012. Historical reference K_i values from the last 6 months are also included.

Oxytocin and Vasopressin receptors

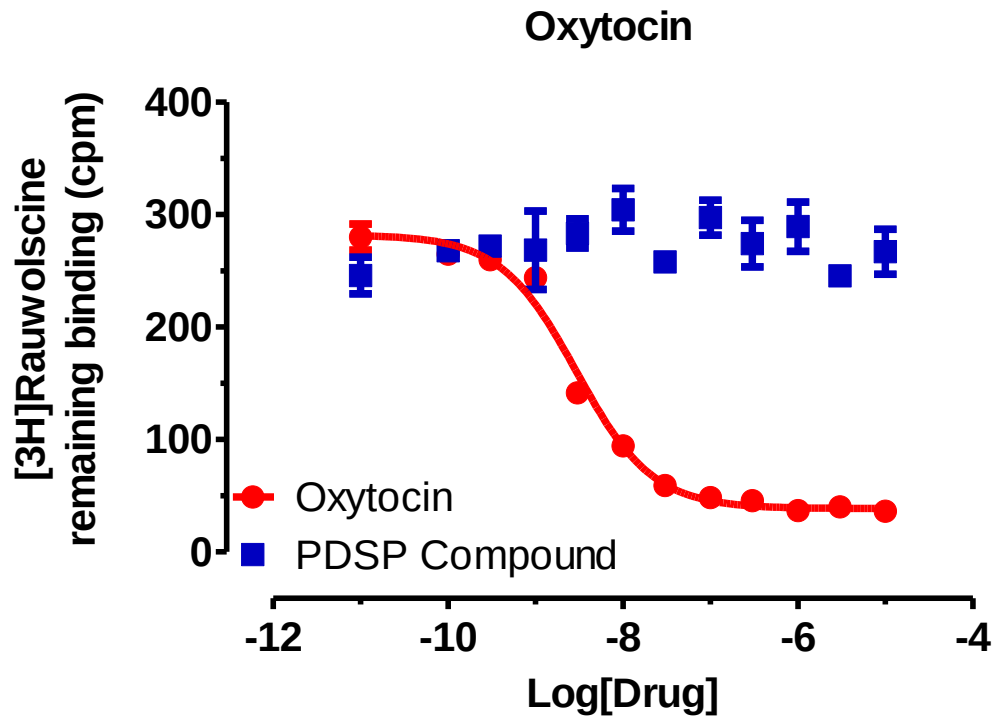
Oxytocin binding buffer: 50 mM HEPES, 10 mM $MgCl_2$, pH 7.4

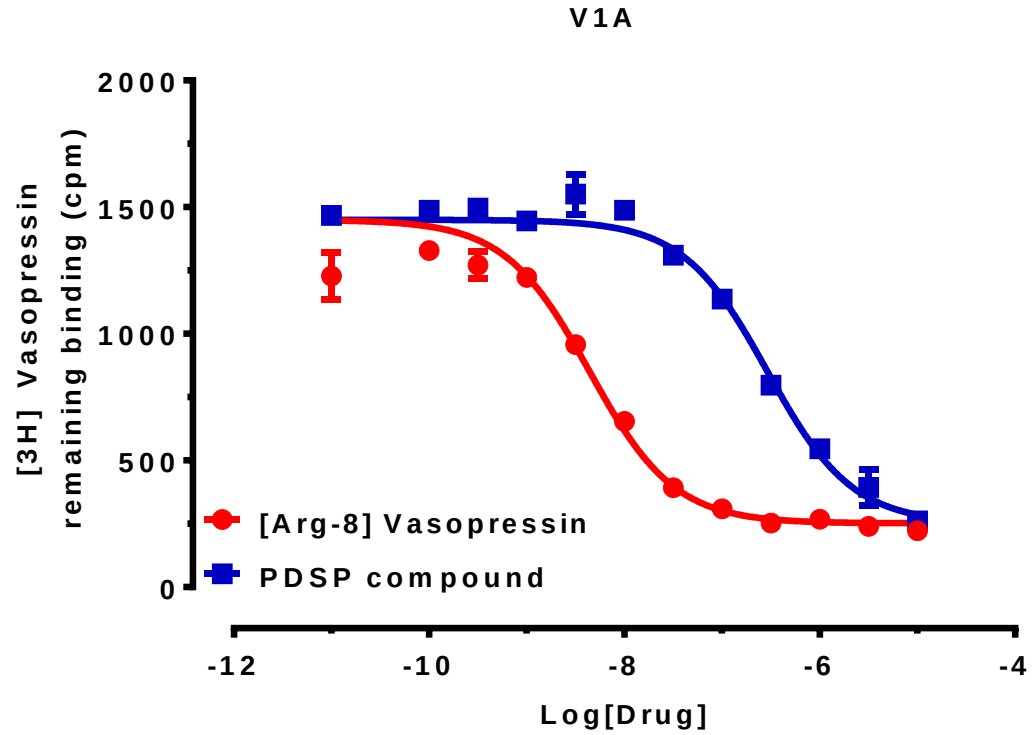
Vasopressin Binding Buffer: 20 mM Tris HCl, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/ml bacitracin, 1 mg/ml BSA, pH 7.4, RT

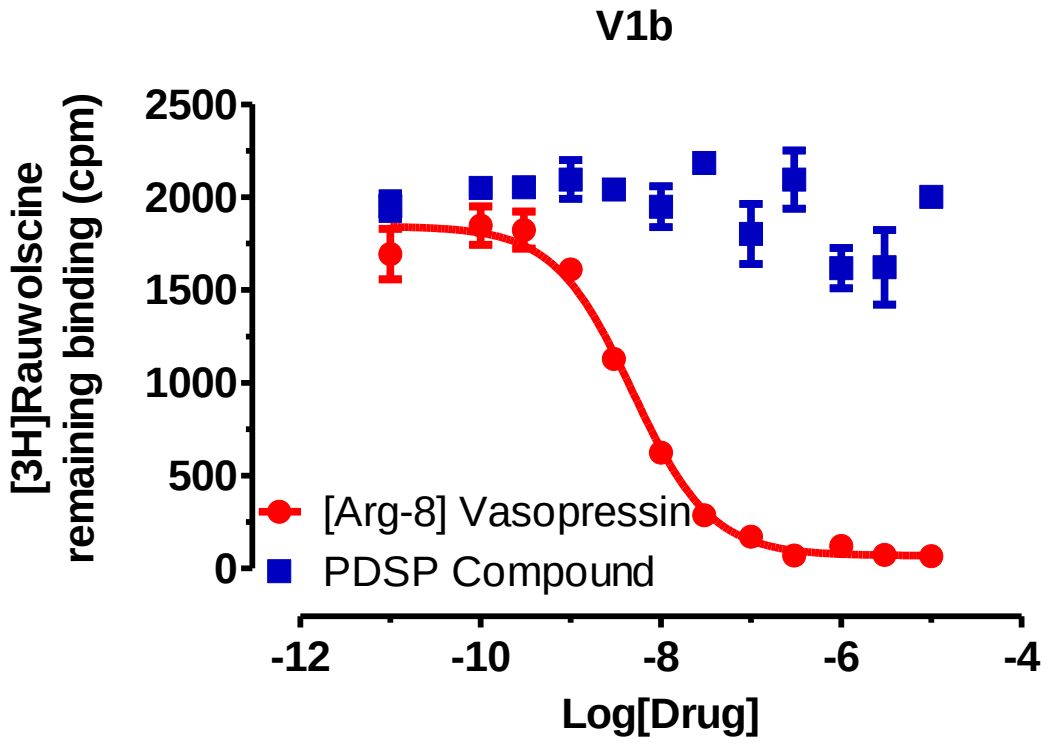
Standard wash buffer: 50 mM Tris HCl, pH 7.4, cold

Target	Radioligand	Kd in nM (N)	References	Reference K_i (nM)
Oxytocin	[^{3}H]Oxytocin	3.10 ± 0.50 (30)	Oxytocin	6.07 ± 0.78
V1a	[^{3}H]Vasopressin	2.12 ± 0.98 (32)	Vasopressin	1.40 ± 0.17
V1b	[^{3}H]Vasopressin	1.33 ± 0.15 (9)	Vasopressin	1.53 ± 0.33
V2	[^{3}H]Vasopressin	3.03 ± 0.48 (10)	Vasopressin	4.60 ± 0.57

Figure 19. Representative competitive binding curves with Oxytocin and Vasopressin receptors.






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

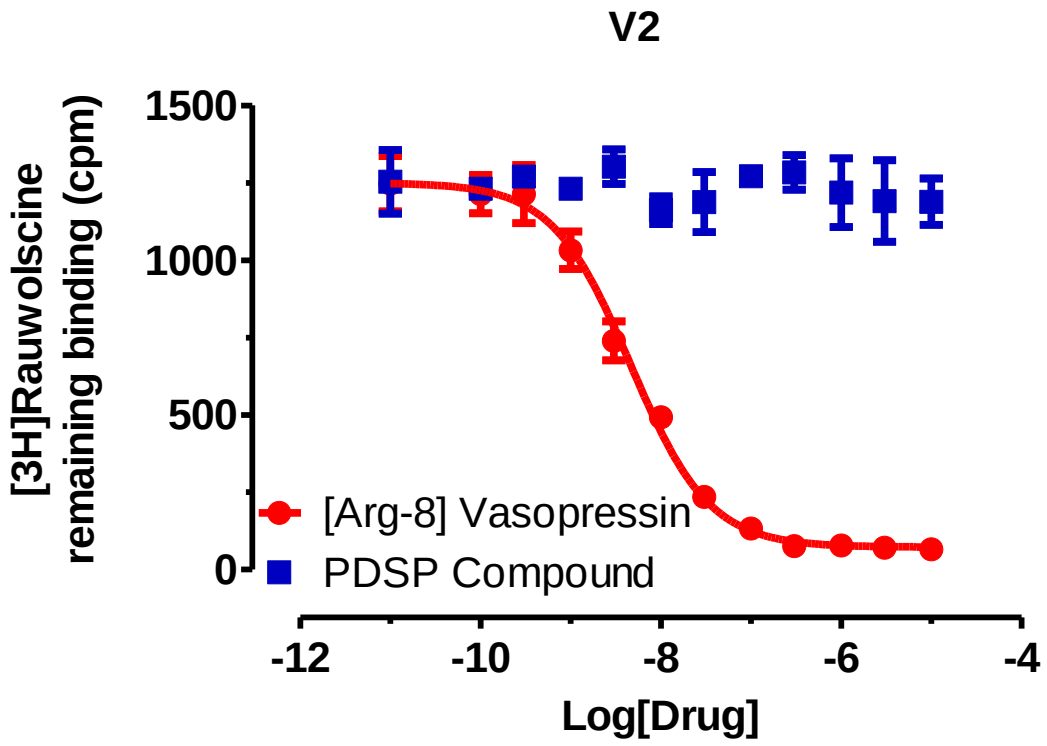

V2
[3H]Rauwolscine remaining binding (cpm)
1500
1000
500
0
[Arg-8] Vasopressin
PDSP Compound
-12
-10
-8
-6
-4
Log[Drug]

Table 13. Smoothened receptor, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from last the 6 months 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**	**[^{3}H] in nM**	**References**	**Reference K_i (nM)**
SMO	[^{3}H]Cyclopamine	3.94 ± 0.67 (3)	SAG LY2490680	12.19 ± 5.01 18.06 ± 2.86

Figure 20. Representative competitive binding curves with SMO receptors.

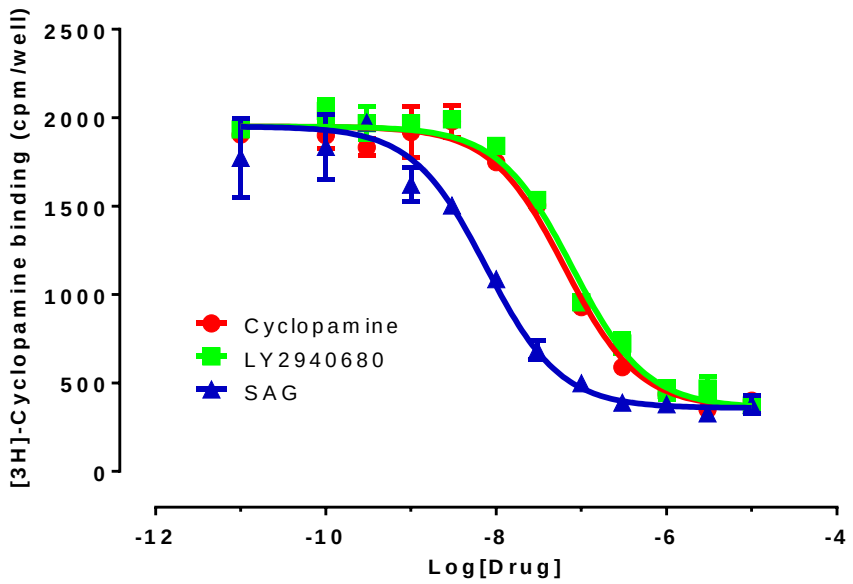


Table 14. Prostanoid receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays.

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**	**[^{3}H] in nM**	**References**
EP3	[^{3}H]PGE2	10	EP2
EP4	[^{3}H]PGE2	10	EP2

Table 15. PKC subunits, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays.

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	References
PKCα	[^{3}H]PDBU	3	PDBU
PKCβ	[^{3}H]PDBU	3	PDBU
PKCγ	[^{3}H]PDBU	3	PDBU
PKCδ	[^{3}H]PDBU	3	PDBU
PKCε	[^{3}H]PDBU	3	PDBU

Table 16. GABAA receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Historical reference K_i values from the last 6 months are also included.

GABA receptors

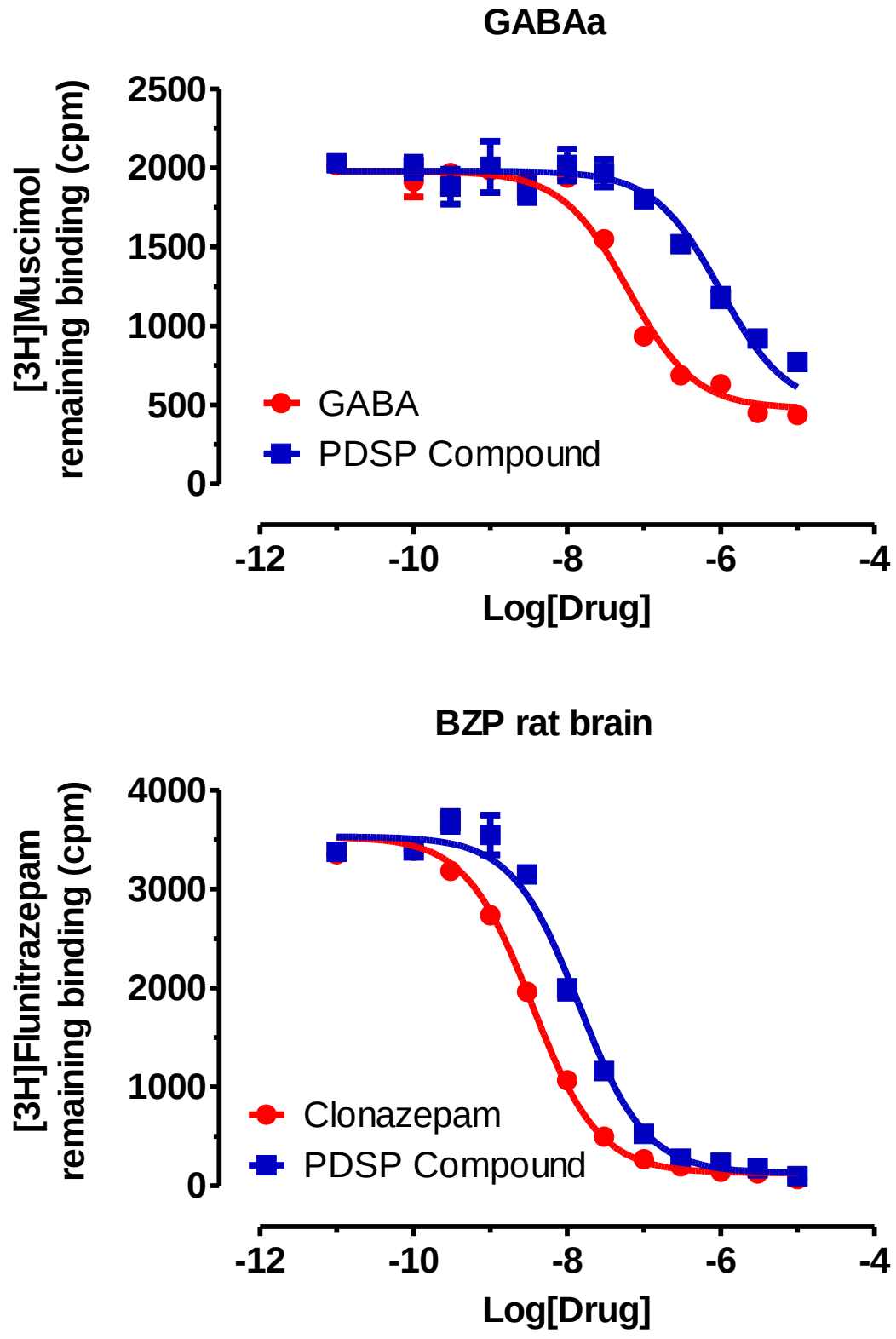
GABA/PBR binding buffer: 50 mM Tris Acetate, pH 7.4, RT
Benzodiazepine (BZP) binding buffer: 50 mM Tris HCl, 2.5 mM $CaCl_2$, pH 7.4, RT
Standard wash buffer: 50 mM Tris HCl, pH 7.4, cold

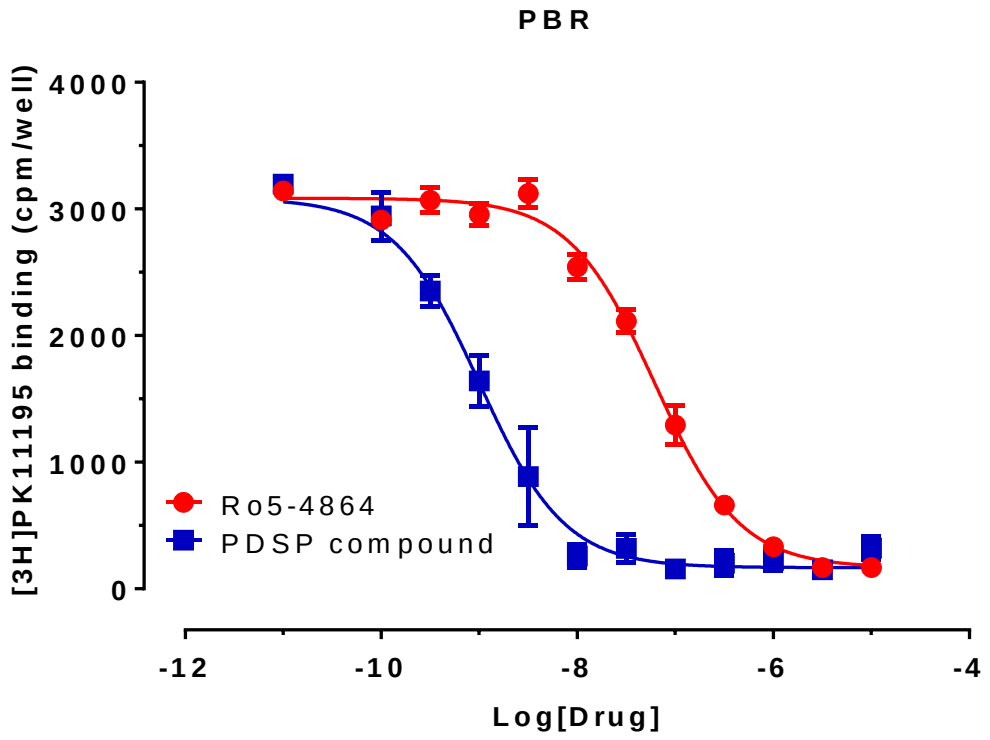
Target	Radioligand	[^{3}H] in nM	References	Reference K_i (nM)
GABA/PBR (rat brain)	[^{3}H]PK11195	1	PK11195 Ro5-4864	 27.6 ± 2.3
GABAA (rat brain)	[^{3}H]Muscimol	5.0	GABA	241 ± 26
GABAA/BZP (rat brain)	[^{3}H]Flunitrazepam	0.5	Diazepam Clonazepam	 1.50 ± 0.08
$\alpha1\beta2\gamma2$	[3H]Flunitrazepam	0.5	Diazepam	1.2
$\alpha2\beta2\gamma2$	[3H]Flunitrazepam	0.5	Diazepam	0.8
$\alpha3\beta2\gamma2$	[3H]Flunitrazepam	0.5	Diazepam	1.4
$\alpha5\beta2\gamma2$	[3H]Flunitrazepam	0.5	Diazepam	0.6

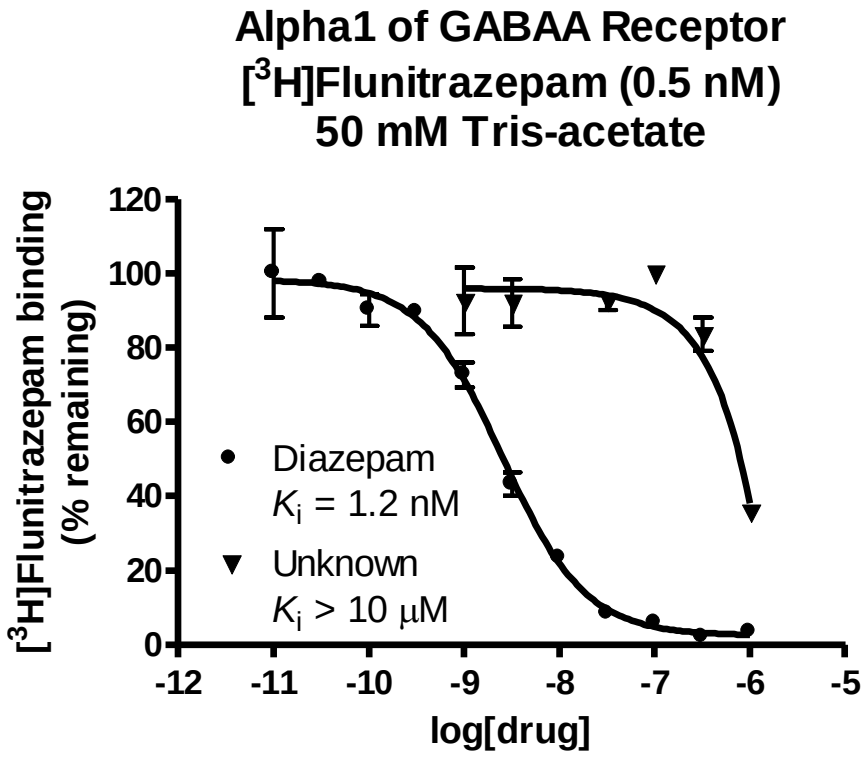
Notes:

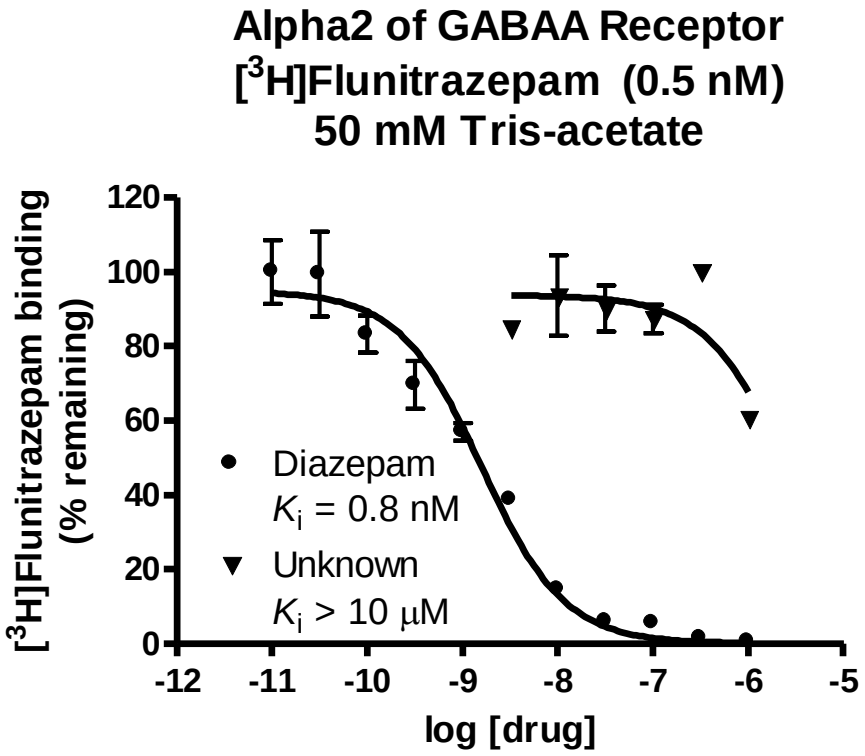
Protocol was adapted from Nadler et al., Brian Res Dev Brain Res 97(2): 216-225 (1996)

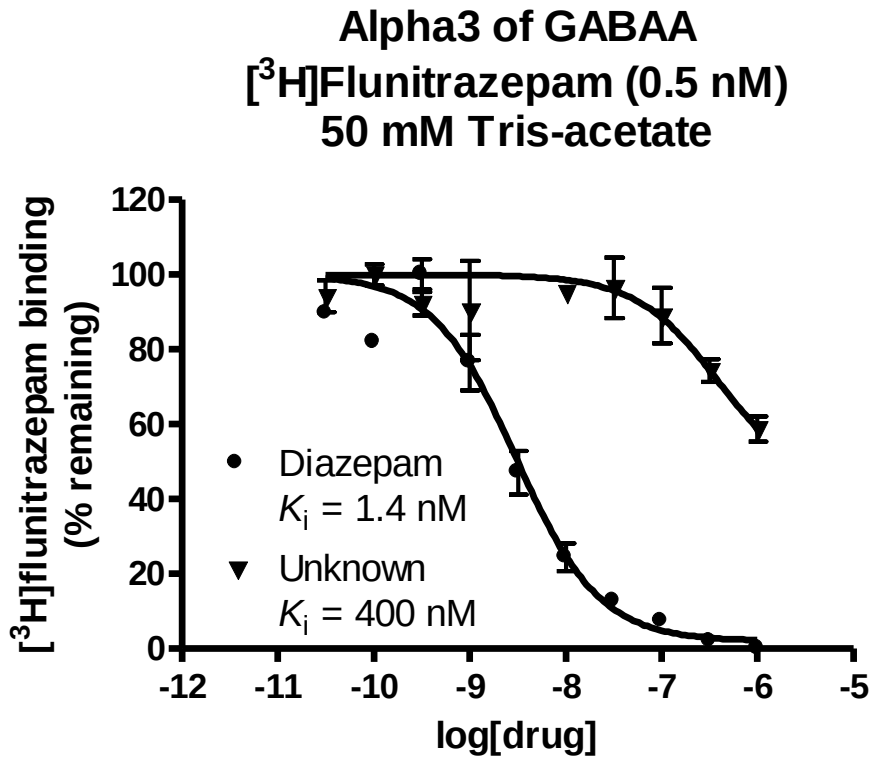
Figure 21. Representative competitive binding curves with GABA receptors.




PBR
[3H]PK11195 binding (cpm/well)
4000
3000
2000
1000
0
Ro5-4864
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]


Alpha1 of GABAA Receptor
[3H]Flunitrazepam (0.5 nM)
50 mM Tris-acetate
[3H]Flunitrazepam binding
(% remaining)
120
100
80
60
40
20
0
Diazepam
Ki = 1.2 nM
Unknown
Ki > 10 μM
-12
-11
-10
-9
-8
-7
-6
-5
log[drug]


Alpha2 of GABAA Receptor
[3H]Flunitrazepam (0.5 nM)
50 mM Tris-acetate
[3H]Flunitrazepam binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Diazepam
Ki = 0.8 nM
Unknown
Ki > 10 μM


Alpha3 of GABAA
[3H]Flunitrazepam (0.5 nM)
50 mM Tris-acetate
[3H]flunitrazepam binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log[drug]
Diazepam
Ki = 1.4 nM
Unknown
Ki = 400 nM

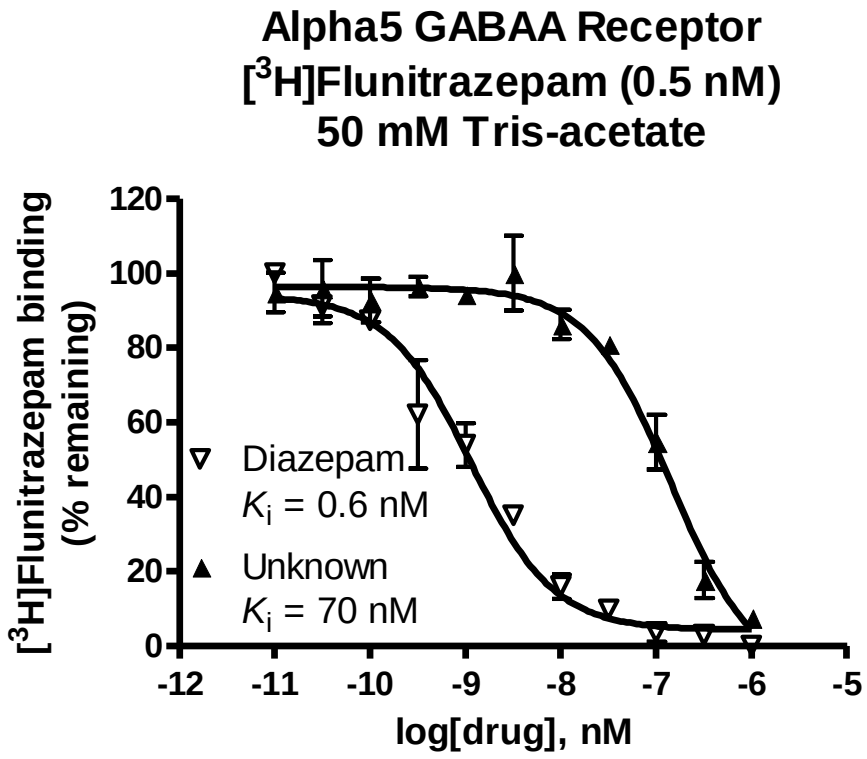

Alpha5 GABAA Receptor
[3H]Flunitrazepam (0.5 nM)
50 mM Tris-acetate
[3H]Flunitrazepam binding
(% remaining)
120
100
80
60
40
20
0
Diazepam
Ki = 0.6 nM
Unknown
Ki = 70 nM
-12
-11
-10
-9
-8
-7
-6
-5
log[drug], nM

Table 17. Glutamate receptors radioligands, corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. Membrane pellets for NMDA binding are made with rat brain cortex with a protocol modified from Chiu et al., 1999.

Glutamate receptors

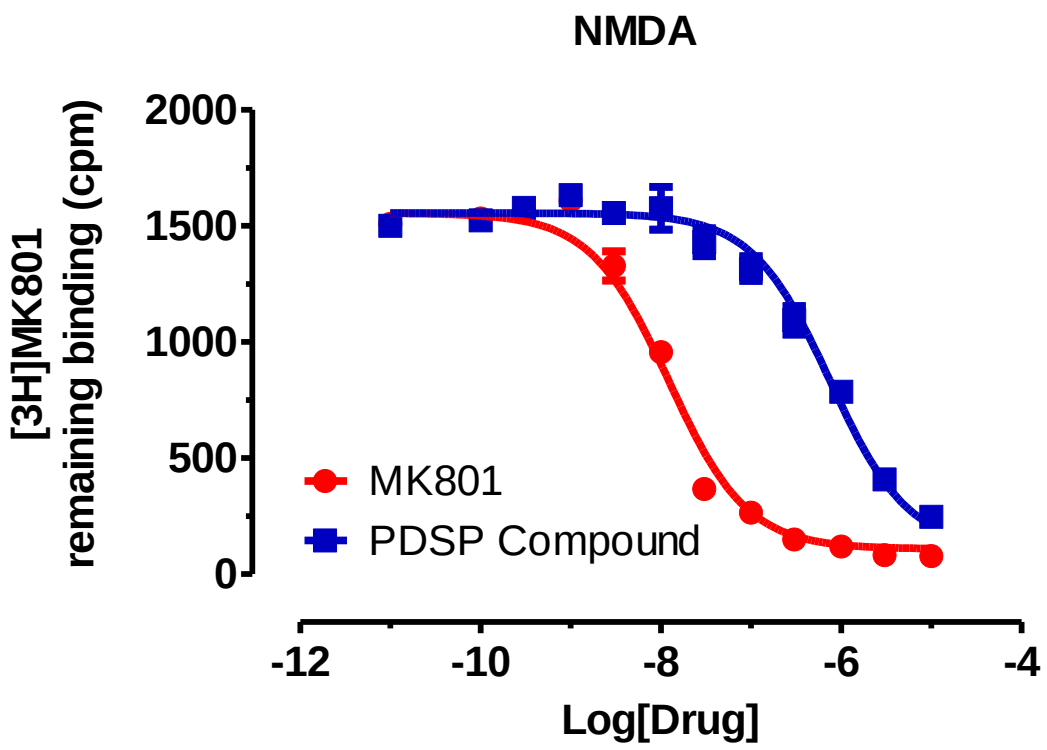
NMDA binding buffer: 20 mM HEPES, 1 mM EDTA, 100 μM L-Glu(Na), 100 μM Glycine, pH 7.0, RT
mGluR binding buffer: 50 mM Tris HCl, 10 mM MgCl2, 0.1 mM EDTA, pH 7.4, RT
Standard wash buffer: 50 mM Tris HCl, pH 7.4, cold

Target	Radioligand	[^{3}H] in nM	References	Reference K_i (nM)
NMDA (rat brain)	[^{3}H]MK801	1	MK801	4.17 ± 0.74
mGluR1				
mGluR2				
mGluR3				
mGluR4				
mGluR5	[^{3}H]MPEP	0.9	MTEP	27
mGluR6				
mGluR7				
mGluR8				

Notes:

1) The mGluR binding protocol is adapted from Kozikowski et al., J Med Chem 33(6):1561-1571 (1990)
2) The NMDA binding protocol is modified from Chiu et al., Alcohol 17:215-221 (1999).
3) The mGluR1, 2, 3, 4, 6, 7, 8 binding assays are under development

Figure 22. Representative competitive binding curves for NMDA and mGluR5 receptors.



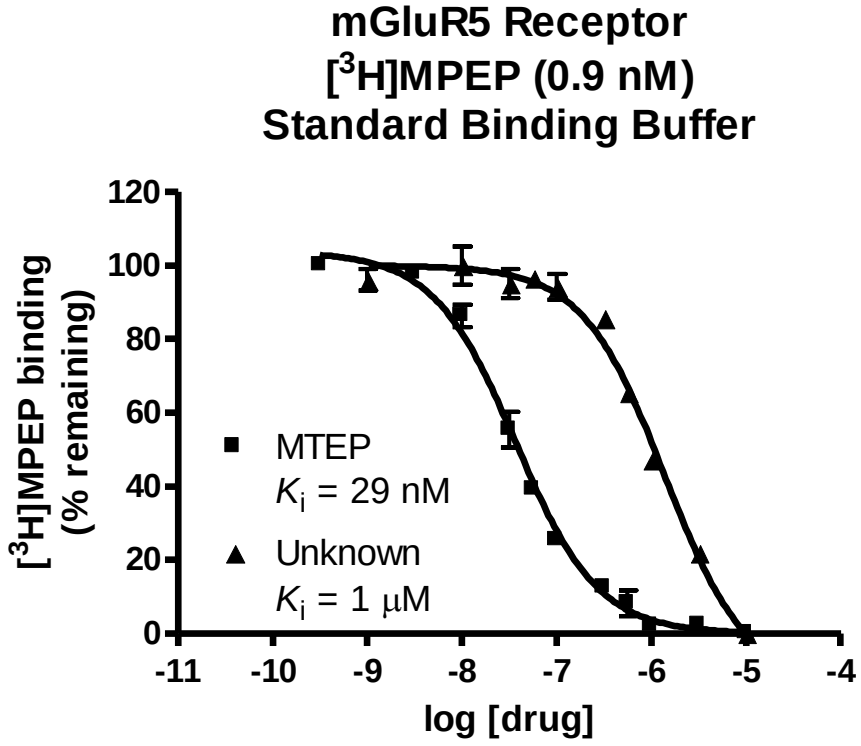


Table 18. Sigma receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually at or near the K_d value or as listed. The K_d values listed in this table are the average ± SEM from a minimum of 3 saturation binding assays from 2011 to 2012**.** Historical reference K_i values from the last 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	K_d in nM (N)	References	Reference K_i (nM)
Sigma1 (Guinea pig)	[^{3}H]Pentazocine	4.45 ± 0.55 (2)	Haloperidol	3.29 ± 0.50
Sigma2 (PC12)	[^{3}H]DTG	10.08 ± 1.38 (10)	Haloperidol	24.7 ± 1.8

Figure 23. Representative competitive binding curves with Sigma receptors.

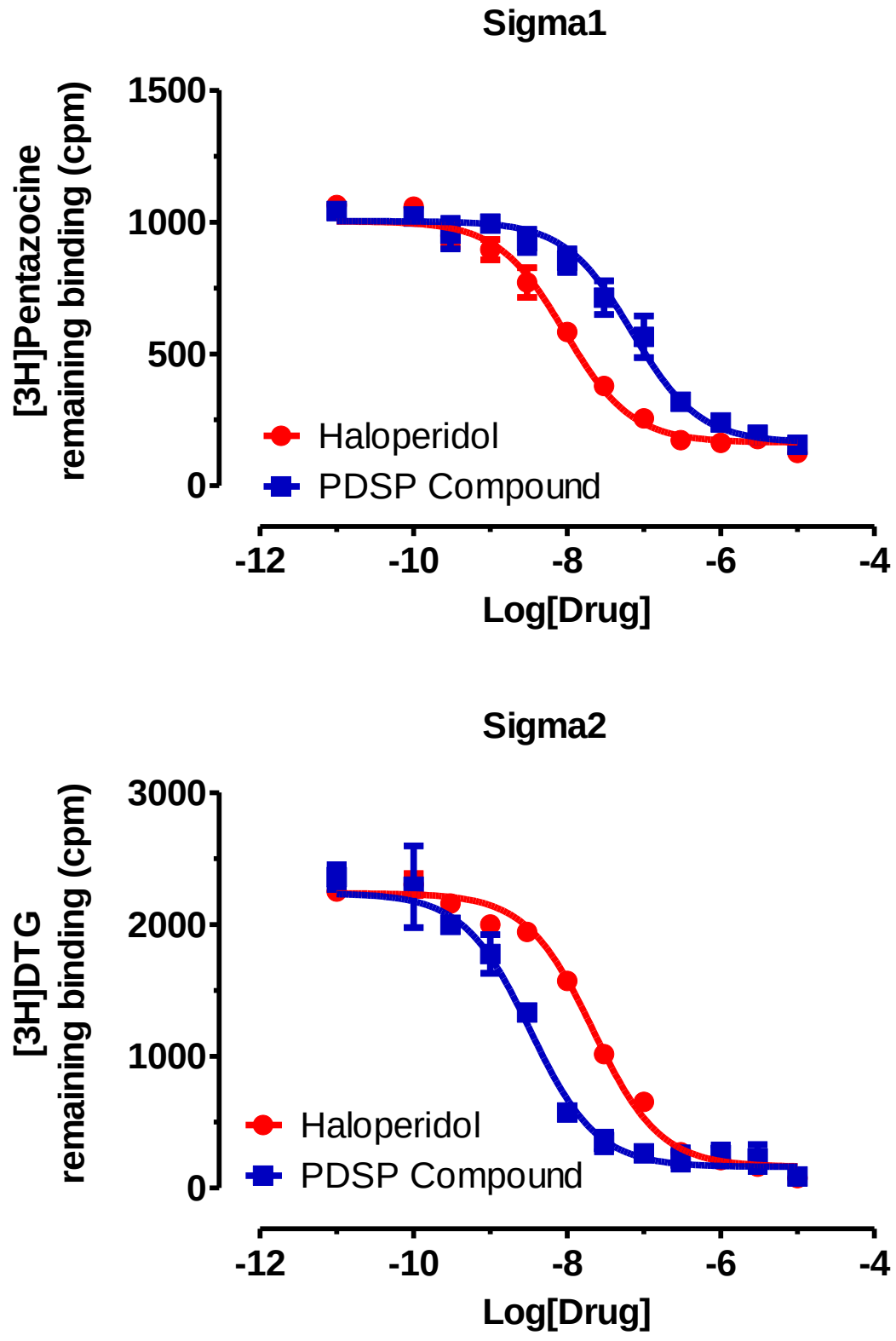


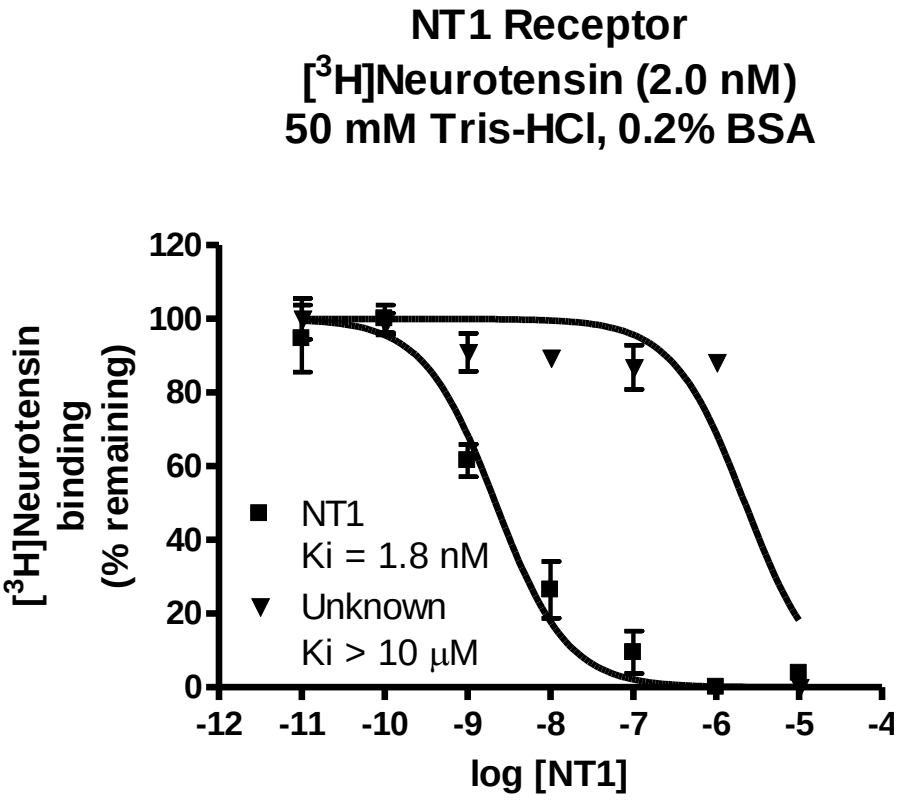
Table 19. Angiotensin II receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually ator near the K_d value or as listed.

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**	**[^{3}H] in nM**	**References**	**Reference K_i (nM)**
AT1	[^{3}H]Angiotensin II	0.1	Candesartan	
AT2	[^{3}H]Angiotensin II	0.1	PD123319	

Table 20. Neurotensin receptors, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually at or near the K_d value or as listed. The K_d values listed in this table are an average ± SEM from a minimum of 3 saturation binding assays from 2011 to 2012.

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**	**K_d or [^{3}H] in nM for binding (N)**	**References**	
NTS1	[^{3}H]Neurotensin	6.33 ± 2.03 (3)	Neurotensin	1.8
NTS2	[^{3}H]Neurotensin	2 nM	Neurotensin	

Figure 24. Representative binding curve for NTS1 receptor.



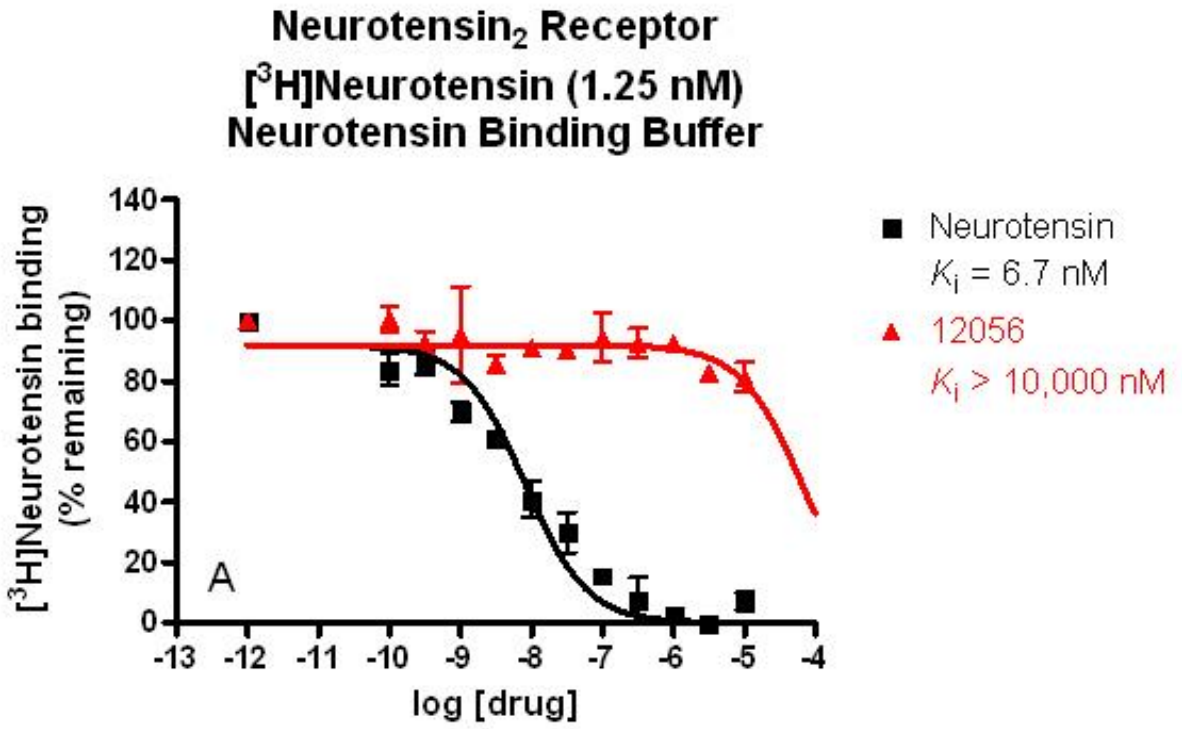


Table 21. VMAT2 transporter, radioligand and corresponding concentration, reference compound, and buffers for primary and secondary radioligand binding assays.

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**	**K_d or [^{3}H] in nM for binding (N)**	**References**	**Reference K_i (nM)**
VMAT2	[^{3}H]Tetrabenezine	1.5	Reserpine	280

Figure 25. Representative binding curve for VMAT2 transporters.

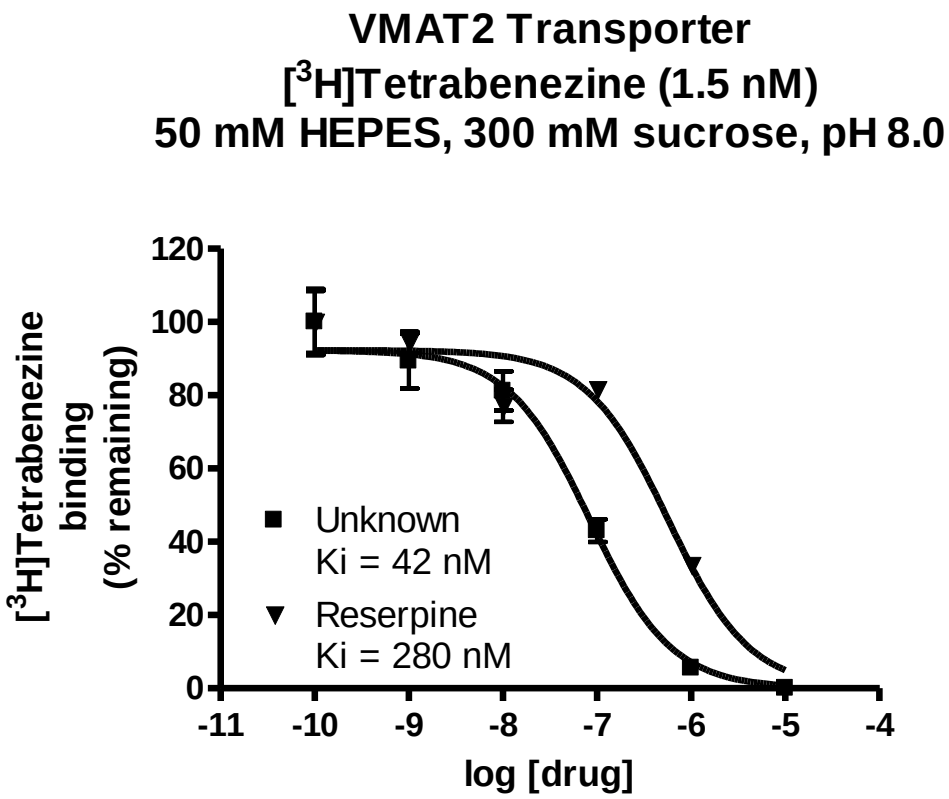


Table 22. Calcium and sodium channels, radioligands and corresponding concentrations, reference compounds, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually at or near the K_d value, or as listed. The K_d values listed in this table are an average ± SEM from a minimum of 3 saturation binding assays before 2012.

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	K_d or [^{3}H] in nM for binding (N)	References	Reference K_i (nM)
Ca^{2+} channel	[^{3}H]Nitrendipine	4.77 ± 1.75 (3)	Nifendipine	
Na^+ channel	[^{3}H]Batrachotoxin		Veratridine	

Figure 26. Representative figure for Ca^{2+} binding assay.

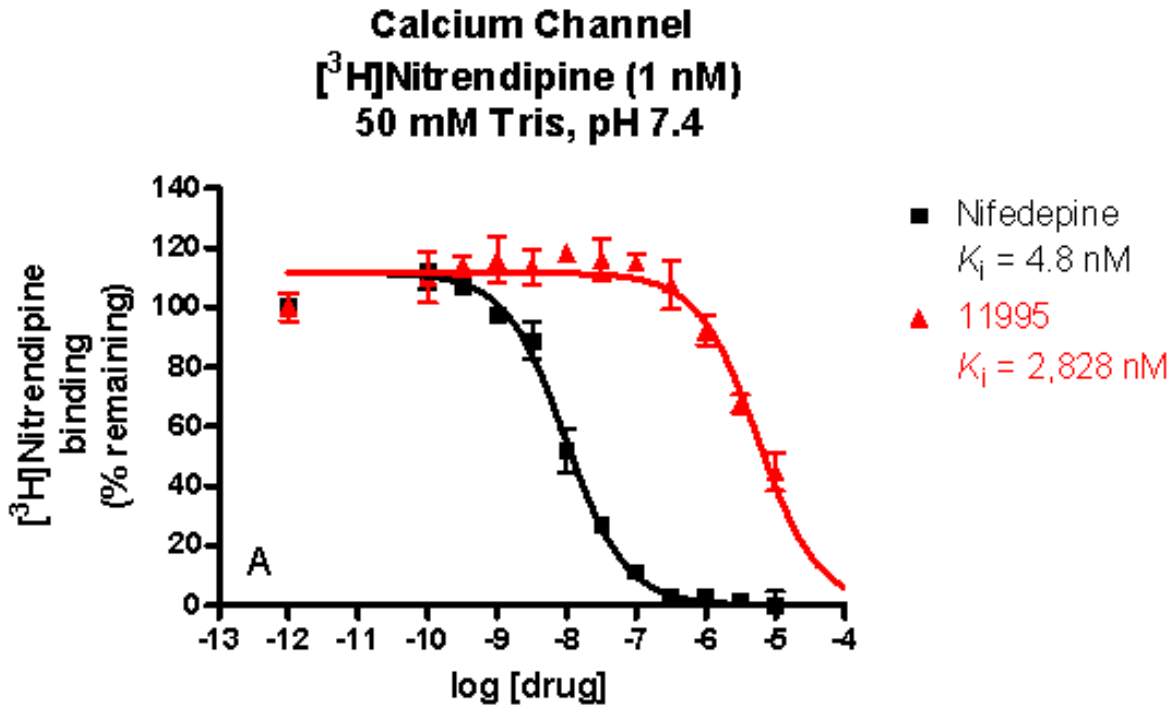


Table 23. HERG potassium channel, radioligand and corresponding concentration, reference compound, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually at or near the K_d value, or as listed. The K_d values listed in this table are an average ± SEM from a minimum of 3 saturation binding assays from 2009 to 2012. Historical reference K_i values from the last 6 months 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	K_d in nM (N)	References	Refere nce K_i (nM)
hERG	[^{3}H]Dofetilide	2.93 ± 0.55 (11)	Dofetilide Cisapride	4.1 ± 0.4 38.9 ± 3.9

Figure 27. Representative binding curve with hERG potassium channel.

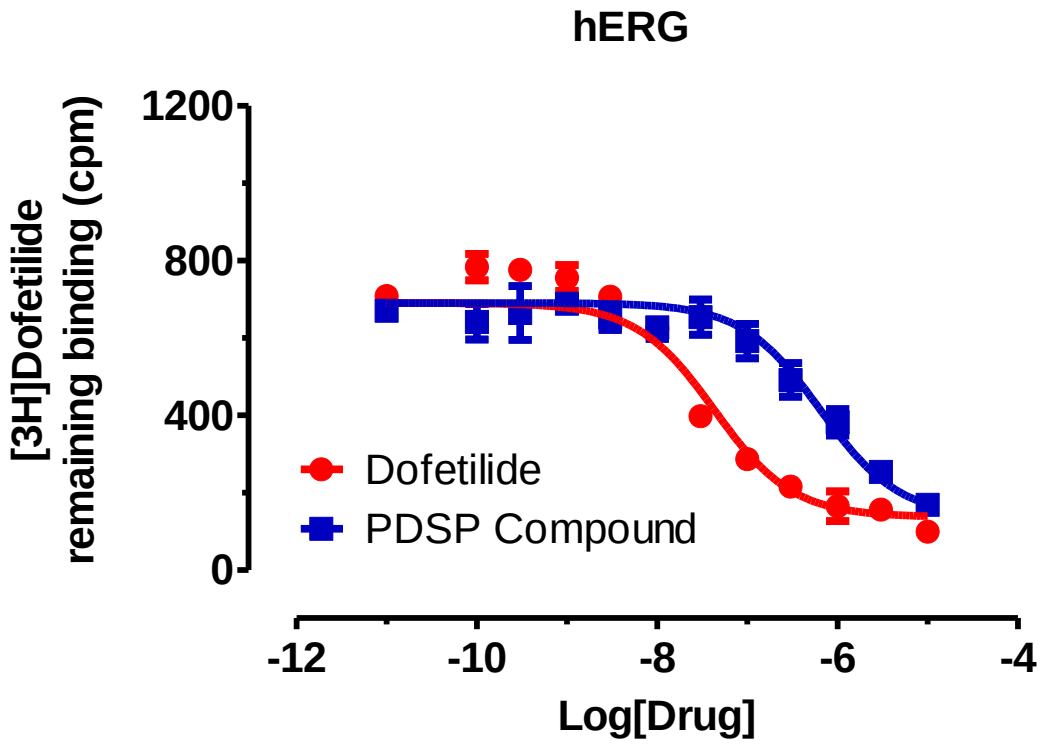


Table 24. Imidazoline receptor, radioligand and corresponding concentration, reference compound, and buffers for primary and secondary radioligand binding assays. The concentration of radioligand used for competition binding assay is usually at or near the K_d value or as listed.

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	[^{3}H] in nM for binding	References
Imidazoline 1	[^{3}H]Clonidine	0.1 nM	Naphazoline

Section 2: Functional assays

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

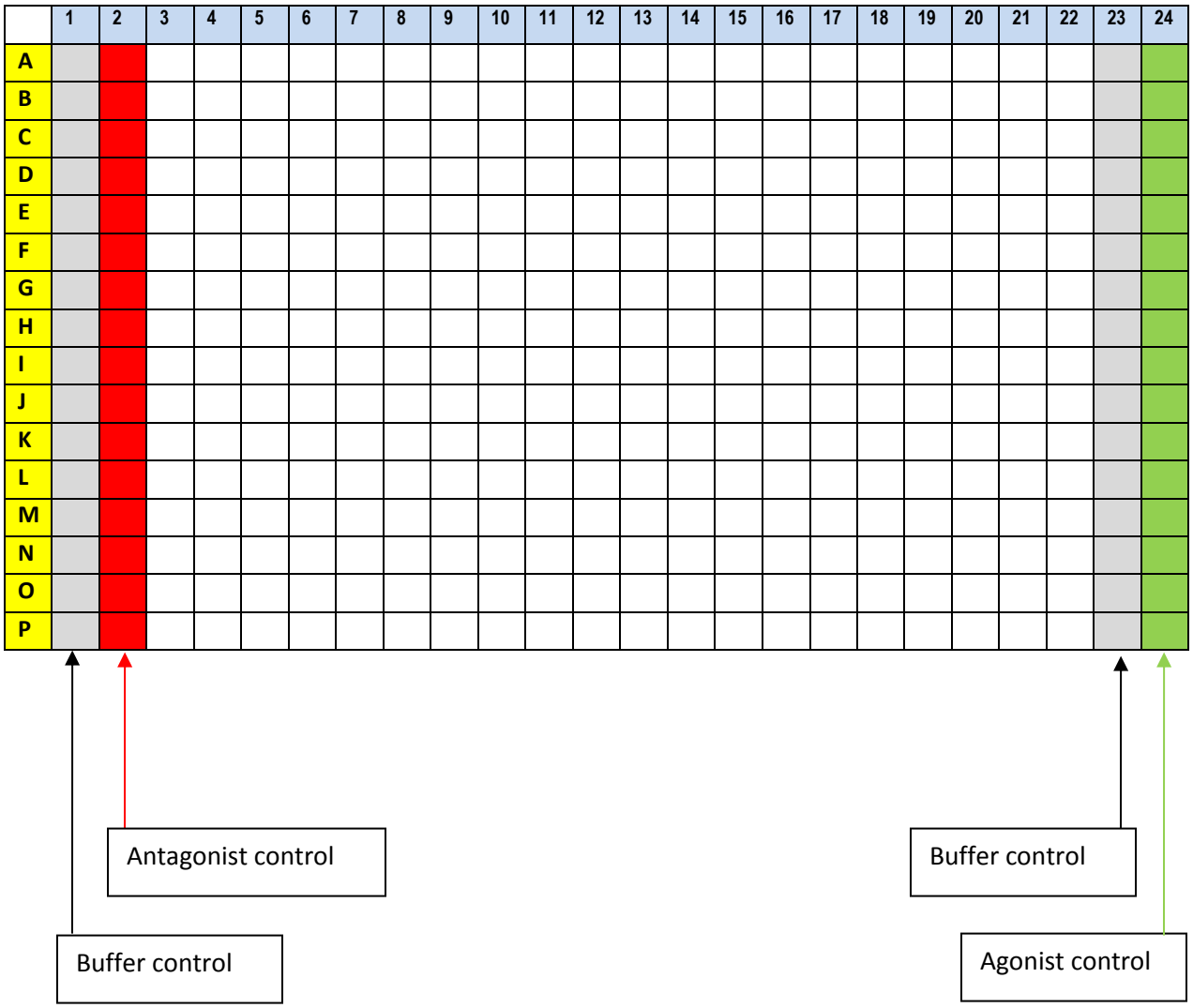


Figure 28. 384-well drug plate map #1 for primary screening: Singlet format (first addition). A total of 320 different drugs (test compounds) can be plated in singlet format in a 384-well drug plate. Columns 1, 2, 23, and 24 are usually used for positive and negative controls

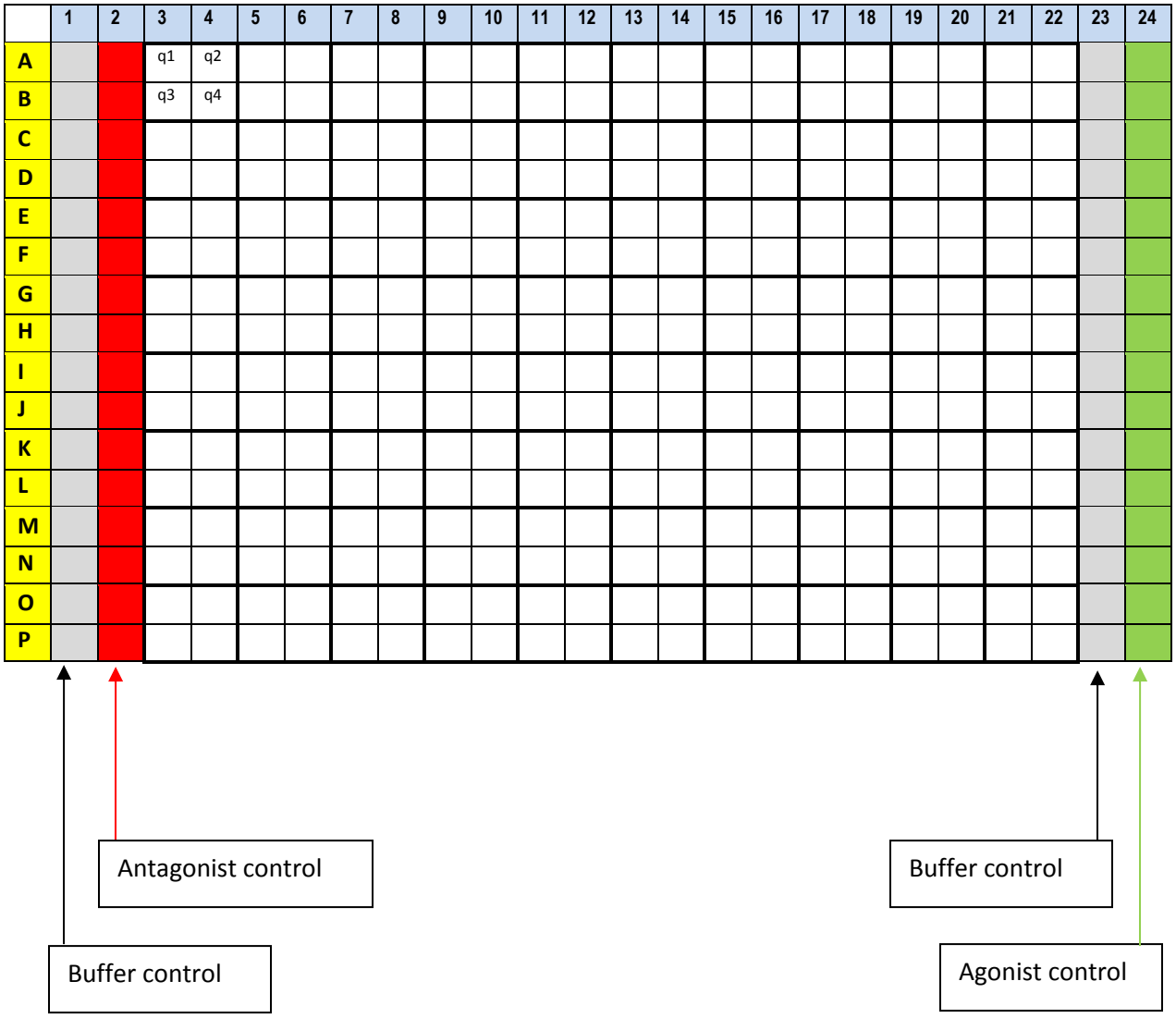


Figure 29. 384-well drug plate map #2 for primary screening: quadrant format (first addition). A total of 80 different drugs can be plated in quadrant format (q1, q2, q3, and q4) in a 384-well drug plate. Columns 1, 2, 23, and 24 are usually used for positive and negative controls.

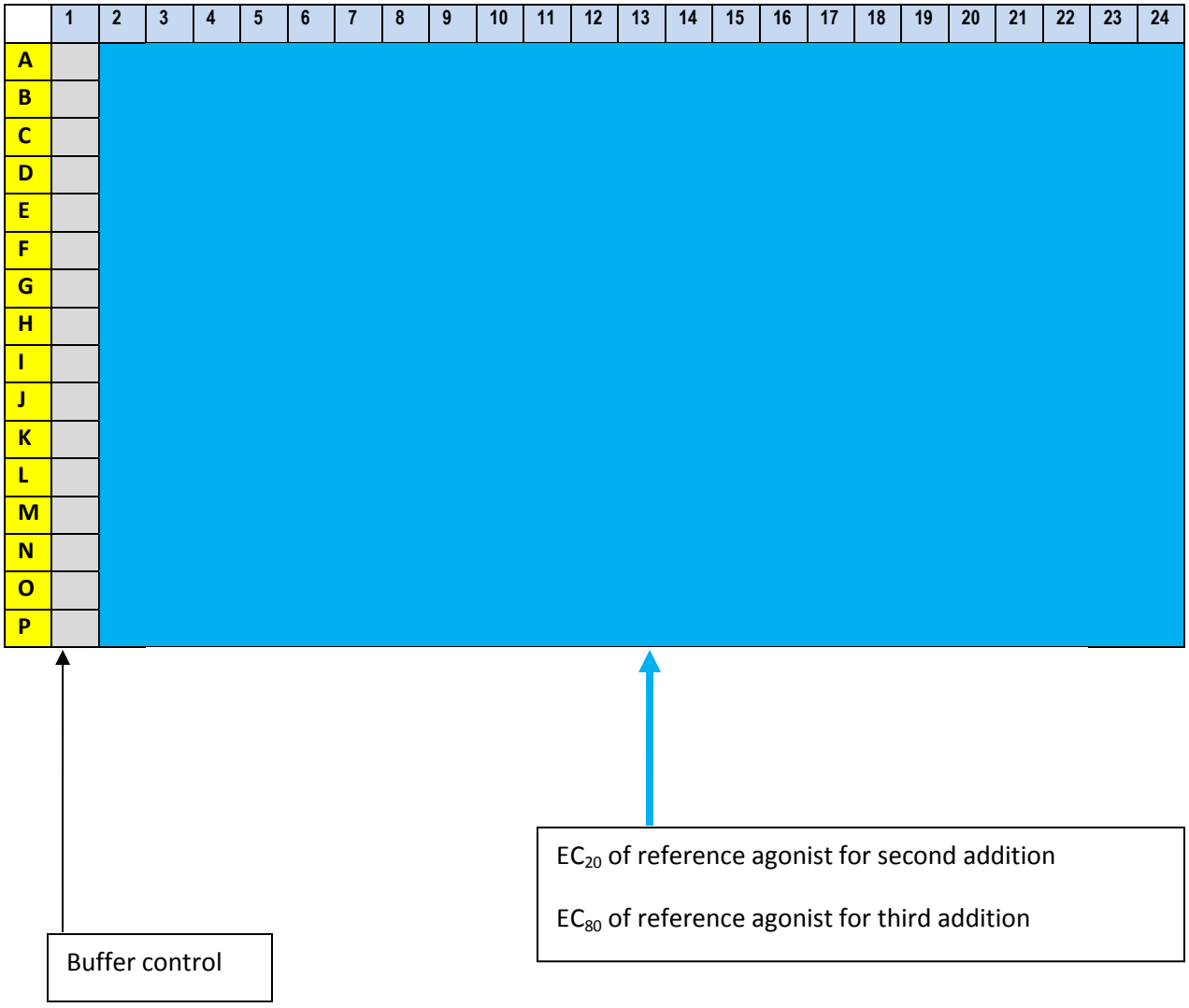


Figure 30. 384-well drug plate map #3: second or third addition in primary screening assays (second or third addition). Except for Column 1 (which serves as a negative control with assay buffer for all additions), all the other wells receive EC_{20} or EC_{80} of a reference agonist to determine allosteric moduator 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 31. 384-well drug plate map #4: agonist or antagonist concentration-responses for secondary screening (first addition): Eight serial drug dilutions are made in triplicate (Drug A to H) or six drug serial dilutions in quadruplicate (Drug A’ to F’) (indicated below the plate template) from high to low concentrations (final concentrations are indicated to the right of the plate template). Either format can be 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.

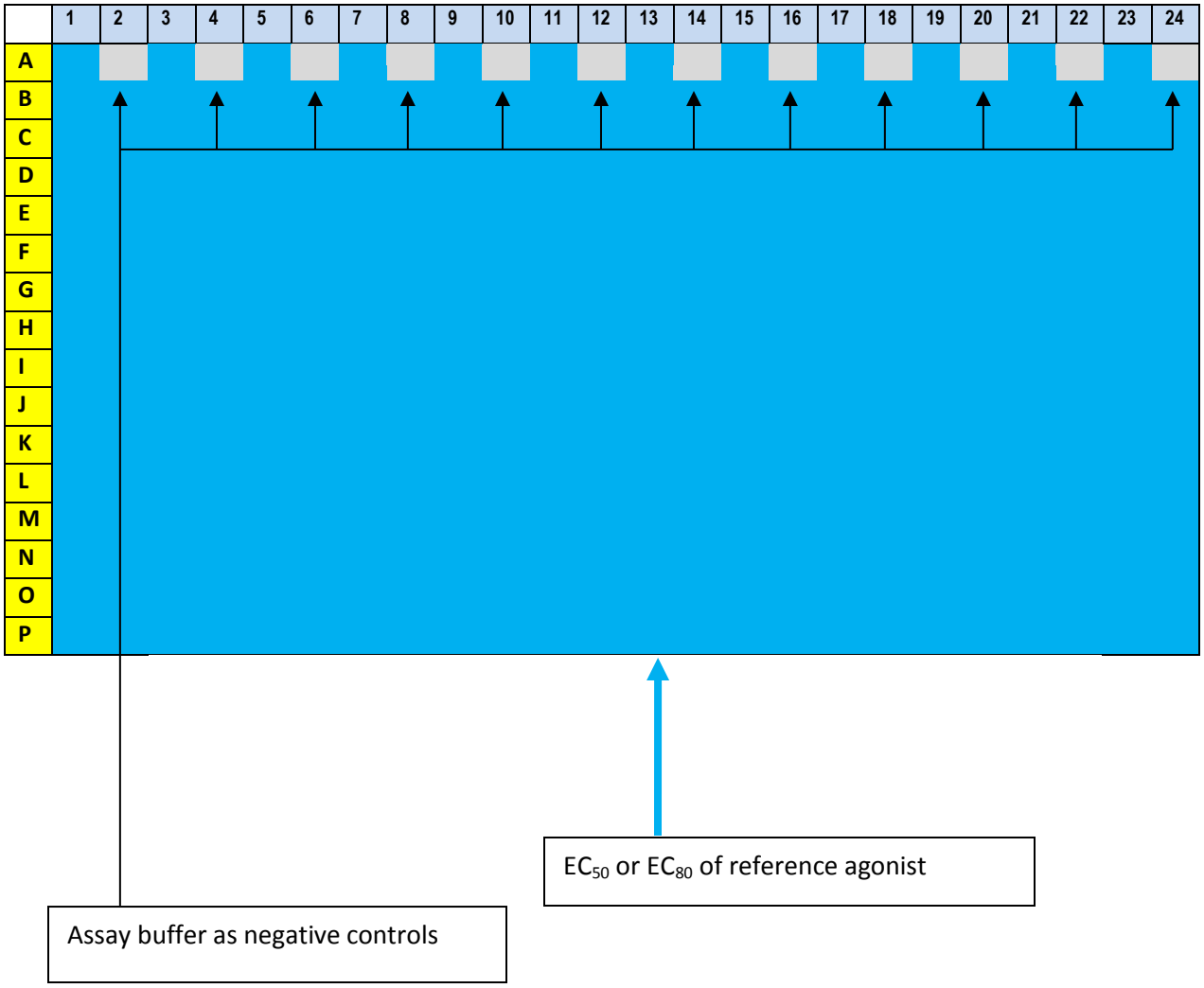


Figure 32. 384-well drug plate map #5: EC_{50} or EC_{80} of a reference agonist as second addition for antagonist activity (second addition). The even numbered wells in row A serve as negative controls with assay buffer only, while the other wells on the plate contain EC_{50} – EC_{80} concentrations of a 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 33. 384-well drug plate map #6 for Schild plot analysis (first addition): Antagonist or allosteric modulator is made in 7 concentrations as indicated at the bottom of the drug plate template. Each concentration (including 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 34. 384-well drug plate map #7 for Schild plot analysis (second addition). Reference agonist is made in the following serial dilutions (as indicated at the right of the drug plate template). Each concentration (including 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 a final concentration of 10 µM or 10 µg/ml (for crude extracts), or at specific concentrations upon request for agonist and antagonist activities (see 384-well drug plate maps #1, #2, and #3 for detailed setup). Results are normalized and transformed to percentage values. For agonists, the reference agonist activity at 10 µM is set as 100% and 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 only is set as 100% inhibition and the activity of the EC_{80} of the reference agonist as 0% inhibition. For allosteric potentiators, the activity of the EC_{20} of a 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 selected for secondary screening using concetnration-response assays.

In secondary functional assays, potential agonist hits are subjected to full concentration-response studies to determine their efficacy and potency (see 384-well drug plate map #4). Potential antagonist hits are subjected to full concentration-response studies to determine IC_{50} values against EC_{50} to EC_{80} concentrations of a reference agonist (see 384-well drug plate map #5 for detailed setup). Potential antagonist hits and positive allosteric modulators are further characterized, if necessary, by Schild plot analysis to obtain pK_B and pA_2 values (see 384-well drug plate maps #6 and #7 for detailed setup). Schild plot analysis is designed to obtain full agonist concentration-response curves in the absence and presence of increasing concentrations of potential antagonists or allosteric modulators. For those antagonists that shift agonist concentration-response curves to the right and also reduce Emax, a modified Lew and Angus analysis is applied to obtain pK_B and pA_2 values. For allosteric modulators, secondary concentration-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, concentration-response results are analyzed using the Black

and Leff operational model to quantify bias. Detailed procedures for these secondary assays are given in subsequent data analysis sections.

Secondary functional assays provide the following parameters for the PDSP database: maximum activation or inhibition after normalization (in percentage values), concentration range used in the assay (lowest and highest concentrations), Hill slope of the concentration-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 the 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 maximum response ($\mathbf{E_{max}}$) and basal activity, respectively; **X** is the agonist concentration and **n** is the Hill slope; $\mathbf{EC_{50}}$ is the concentration that generates a 50% level ofactivity.

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

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

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

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

in which **K_i** is the ligand binding affinity determined from an antagonist concentration-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 the 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 (see Figure 36 for examples): The PDSP has adopted a nonlinear regression analysis with modified Lew and Angus method to estimate antagonist potency pA_2 values. For Schild plot analysis, agonist concentration-response studies are designed and performed in the absence and presence of 7 concentrations of antagonist in a 384-well plate (see **Figures 33 and 34**). Each agonist concentration-response curve is based on triplicate values. Eight agonist concentration-response curves share the same X-axis and each set of measured responses is arranged in a Y column under antagonist concentrations (M) as a title. Results are analyzed using the agonist concentration-response function as above to determine Bottom and Top values for each concentration-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% activity) is selected for all or most curves if possible, and corresponding agonist concentration-response curves in the absence and presence of increasing concentrations of antagonist are obtained. Prism 5 has a built-in feature to provide equiactive agonist concentrations if the radio button for "Interpolate unknowns from standard curve" is selected. The 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. The results are fitted usingfollowing 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 equal-active agonist concentration values) at corresponding antagonist concentration (X); n is the Schild slope; K_B is the apparent binding affinity of testing antagonist. The pA_2 value could be calculated from pK_B/n. If n =1 or not significantly different from 1, $pA_2 = pK_B$, the testing antagonist is competitive against agonist; otherwise, i.e., if $n \neq 1$, the antagonist may not be competitive against agonist.

2.3.4. Quantifying bias and functional selectivity analysis (see Figures 39 and 40, Tables 26 and 27 for examples). To quantify functional selectivity and to calculate a bias factor, we analyze functional concentration-response results using the Black and Leff operation model as outlined by Dr. Terry Kenakin et al., 2012 (ACS Chemical Neuroscience, 3:193-203, 2012).

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

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 of the system and **basal** is the response in the absence of test drugs (i.e. buffer only). The $\mathbf{E_m}$ and **Basal** are usually 100 and 0 if responses are normalized to a percentage of the activity of a reference compound (100%). $\mathbf{K_A}$ is the equilibrium dissociation constant of the agonist (**A**), τ 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 are shared by all agonists that are being tested for the same pathway. Log(τ/K_A) is defined as "transduction coefficient" for a particular agonist at a measured signaling pathway.

To quantify ligand bias (see the following equations and steps), drug activity is measured in two or more pathways for a group of ligands (e.g., agonists) to determine their corresponding log(τ/K_A) values. For each pathway, Δlog(τ/K_A) values are calculated by subtracting the log(τ/K_A) value of the reference agonist (usually endogenous agonist). The same reference agonist should

be used for the different pathways. For each agonist, $\Delta\Delta\log(\tau/K_A)$ values are calculated by subtracting $\Delta\log(\tau/K_A)$ for pathway I from the corresponding $\Delta\log(\tau/K_A)$ for pathway II. Bias is quantified as $10^{\Delta\Delta\log(\tau/KA)}$. If the value of $10^{\Delta\Delta\log(\tau/KA)}$ is 1, the agonist is unbiased; if the value is larger than 1, it is biased towards pathway I and it is is less than 1, it is biased towards pathway II.

Step 1: Concentration-response curves are generated for 2 or more pathways to determine the 'transduction coefficient' $\text{Log}(\tau/K_A)$ value for each ligand at each pathway;

Step 2: The transduction coefficient difference $\Delta\text{Log}(\tau/K_A)$ between different ligands in the same pathway is calculated by subtracting transduction coefficient of the test sample from that of the reference compound, usually the 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: The difference between transduction coefficients $\Delta\Delta\text{Log}(\tau/K_A)$ is calculated between different pathways for the same test ligand by subtracting the $\Delta\text{Log}(\tau/K_A)$ 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: The bias factor is calculated as below

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

Step 5: to determine if this bias value is statistically significant, a statistical analysis is done with $\Delta\Delta\text{Log}(\tau/K_A)$ values. In addition to the comprehensive methods to estimate SEM values outlined by Dr. Terry Kenakin (2012), an alternative (and simpler) method to estimate SEM values is given below, as suggested by Dr. Arthur Christopoulos and his colleagues (Gregory et al., 2012, JBC 287:37066-37077).

To calculate SEM for $\Delta\log(\tau/K_A)$:

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

To calculate SEM for $\Delta\Delta\log(\tau/K_A)$:

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

2.3.5. Allosteric operational analysis (see Figure 41 for an example). For ligands with allosteric modulator activity, we apply the allosteric operational model to analyze functional results. Functional assays are carried out in the same way as for Schild plot analysis (**Section 2.3.3**), in which orthosteric agonist concentration-response curves are 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 (Trends in Pharmacological Sciences 28: 382-389).

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

In which ***Effect*** is the measured functional readout in the presence of an orthosteric agonist [**A]** and an allosteric modulator [**B]**; $\mathbf{E_{max}}$ and **Basal** are the maximal possible system activity and basal activity under control conditions; $\mathbf{K_A}$ and $\mathbf{K_B}$ are the equilibrium binding affinities 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); $\boldsymbol{\tau_A}$ and $\boldsymbol{\tau_B}$ are the capacity of the orthosteric agonist (**A**) and the allosteric ligand (**B**) to exhibit agonism, respectively; **n** is a fitting slope factor.

While doing non-linear least-squares regression curve-fitting in Prism v5.0, the $\mathbf{E_{max}}$ should be a shared value for all assays carried out under the same conditions, and should be set to a fixed value which is the maximal system efficacy. The $\mathbf{K_A}$ should be the equilibrium binding affinity and can be estimated by radioligand binding assay. If the test allosteric modulator itself has no agonist activity, then $\tau_B = 0$.

2.3.6. List of functional assays carried out routinely by the PDSP:

- 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.
- Thallium flux (FluxOR) assay for hERG potassium channel function.
- PatchXpress automated patch clamp assay for hERG potassium channels.
- Neurotransmitter transporter assays for DAT, NET, and SERT transporters.
- Multidrug resistance transporter-1 (MDR-1) assay
- Enzyme activity assays
    - HDAC assay
    - MAO-A and -B assays
    - 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) overnight, are 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 – 20,000 cells in 40 µl per well. The plates are cultured for minimum of 6 hour or overnight before assays.

2.4.1.2. Calcium Precipitation transfection: The PDSP usually uses HEK293 T cells for transient transfections. HEK293 T cells are subcultured into either 10-cm dishes (3 million cells per dish) or 15-cm dishes (8 million cells per dish) and are incubated overnight. For each 10-cm dish of HEK293 T cells, 10 µg receptor of DNA construct are mixed in 440 µl distilled water and with 60 µl of 2.5 M $CaCl_2$, then add 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, and 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}. On the day of the 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 a 10 min incubation at room temperature in the dark, and then loaded in the FLIPR. The FLIPR is programmed to take 10 readings (1 read per second) first 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 of the final concentration, and added as above for

potential effects on basal levels for 2 minutes first, followed by a 5 to 10 min incubation before addition of 10 µl of 4x of a reference agonist at a final concentration equivalent 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 at 4x of reference agonist to give a final concentration equivalent to the EC_{20} (usually the 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 addition, the FLIPR is programmed to wash tips first with 10% EtOH, and then with distilled water while recording the fluorescence intensity. Incubation times between each addition can be adjusted accordingly to accommodate any special preincubation requirements.

2.4.1.4. Schild plot analysis: To further examine antagonist activity at a particular receptor, we also carry out agonist concentration-response curves 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, more usually, overnight. Selected antagonist drug plates are prepared as shown above (384-well drug map #6) and are used for the first addition to determine if the test drug has any effect on basal levels. A reference agonist drug plate is prepared as shown above (384-well drug map #7) and is used for the second addition to measure effects of antagonists on the remaining agonist activity.

2.4.1.5. FLIPR data processing: In Calcium mobilization assays done with the FLIPR^{TETRA}, every well of a 384-well plate has its own basal level, which is defined as the average value of the 10 readings before corresponding drug addition. We measure maximal fluorescence intensity readings (RFU, Relative Fluorescence Unit) within a minute after drug addition as agonist activity, and values are exported as "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 levels are transformed to 0% and the E_{max} of the reference agonist in the same assay plate is transformed to 100%.

2.4.2. **Intracellular inositol phosphate accumulation for Gq coupled GPCRs**

Receptors: $mGluR_1$ and $mGluR_5$

Assay buffer: LiCl-containing Locke's buffer (156 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM glucose, and 20 mM HEPES, 20 mM LiCl, pH 7.4.

The protocol is adapted from: Emery AC, DiRaddo JO, Miller E, Hathaway HA, Pshenichin S, Takoudjou GR, Grajkowska E, Yasuda RP, Wolfe BB, Wroblewski JT. Ligand bias at metabotropic glutamate 1a receptors: molecular determinants that distinguish b-arrestin-mediated from G protein-mediated signaling. Mol Pharmacol 82:291-301 (2012).

2.4.2.1. General experimental procedure: Compounds are tested using cell lines stably expressing G_q protein-coupled $mGluR_1$ and $mGluR_5$ receptors in functional assays. Cells are seeded and cultured in 96-well plates to confluency (3 to 4 days), and are incubated overnight in glutamine-free culture medium supplemented with 0.625 Ci/well of *myo*-[^{3}H]-inositol (NEN) to label cell membrane phosphoinositides. After two washes with 0.1 ml of Locke's buffer, incubations with receptor ligands are carried out for 45 min at 37°C in Locke's buffer containing 20 mM LiCl to block inositol phosphate degradation. The reaction is terminated by aspiration of media and [^{3}H]inositol phosphates are extracted in 60 µl of 10 mM formic acid for 30 min. Samples (40 µl) are transferred to opaque-welled plates and incubated with 60 µl of poly lysine-coated yttrium scintillation proximity assay (SPA) beads (GE Healthcare) at room temperature for 1 hour with vigorous shaking. After an additional 8 hours of incubation with SPA beads, inositol phosphates are detected by scintillation counting. All of these studies are performed in the absence and in the presence of agonists appropriate for the different mGluRs, and used at concentrations equivalent to their EC_{50} values: 15 µM and 5 µM glutamate is used for mGluR1 and mGluR5, respectively.

2.4.2.2. Primary screening assays – Single concentration and data analysis: Each new compound is tested on selected 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 cell passages. In addition to the tested compounds, each 96-well plate contains points 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 percent 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.

2.4.2.3. Secondary screening assays: Concentration-response curves 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-responses curves are performed in duplicate twice on two separate passages 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 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.

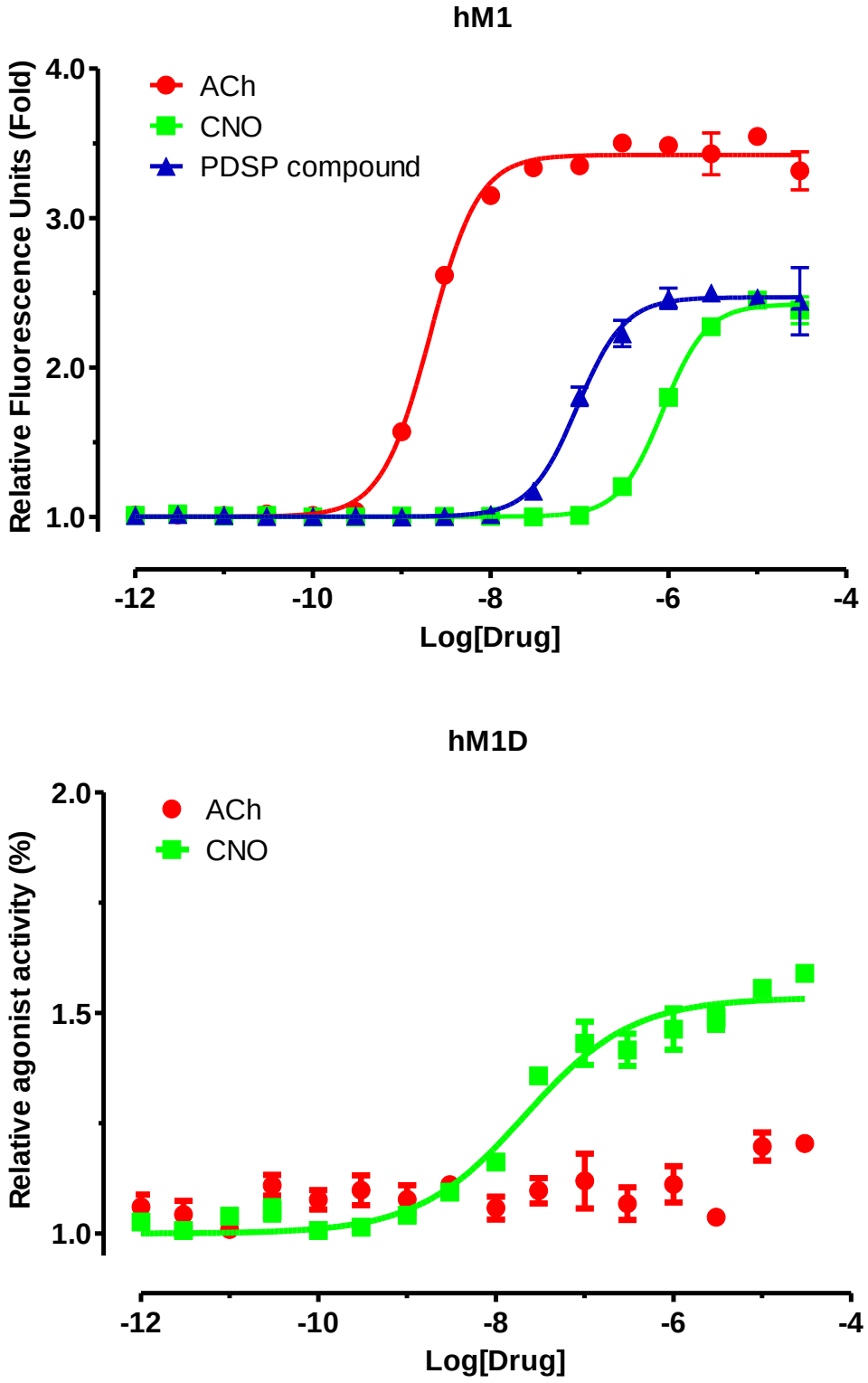
2.4.2.4. The following table lists GPCRs for which the PDSP has functional assays to measure G_q protein activation via Calcium mobilization using the FLIPR^{TETRA} or intracellular inositol phosphate accumulation. Representative results were analyzed using Prism v5.0.

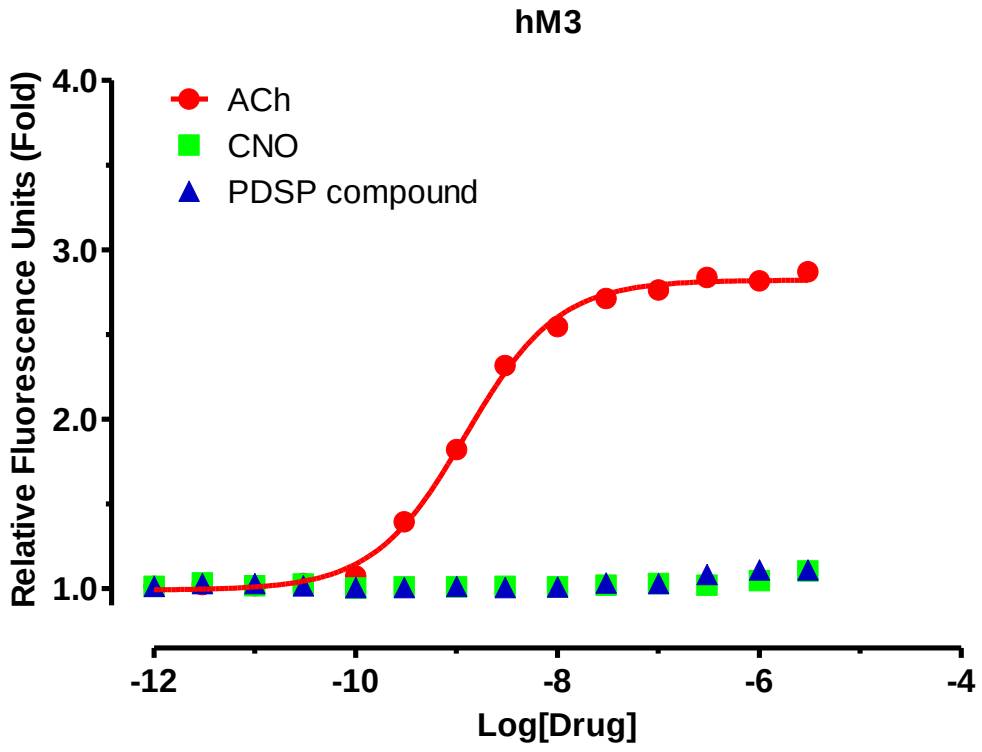
Table 25. List of GPCRs for which the PDSP has assays for G_q protein mediated Calcium mobilization or inositol phosphate (IP) accumulation and their pharmacological parameters. The PDSP will also develop Calcium mobilization assays for other GPCRs upon request and approval. Assays were carried out according to above procedures and results were analyzed in Prism. Representative results are from single assays done in either triplicate or quadruplicate.

Receptor	Cell line	Reference	Emax (fold)	pEC50	Hill slope
M1	CHO	Acetylcholine	3.4	8.68	1.59
M3	CHO	Acetylcholine	2.8	8.91	0.95
M5	CHO	Acetylcholine	3.4	8.25	1.03
M1D	CHO	CNO	1.5	7.68	0.72
M3D	Flp-In CHO	CNO	2.7	7.45	1.01
M5D	CHO	CNO	2.1	7.44	0.81
5-HT2A	Flp-In HEK	5-HT	2.4	8.82	1.19
5-HT2B	Flp-In HEK	5-HT	4.8	8.85	1.25
5-HT2C INI	Flp-In HEK	5-HT	3.3	10.11	1.92
5-HT2C VNV	Flp-In HEK	5-HT	4.6	9.17	2.32
5-HT2C VSV	Flp-In HEK	5-HT	6.0	9.06	2.12
α1A	Fibroblast, rat	Norepinephrine	3.0	8.60	1.34
α1B	Fibroblast, rat	Norepinephrine	1.6	8.72	0.92
α1D	Fibroblast, rat	Norepinephrine	4.1	8.61	1.14
AT1A	HEK	Angiotensin II	1.8	9.41	0.69
BB1	3T3 Balb-C	Bombesin	5.6	7.82	1.36
BB1	3T3 Balb-C	BIM 187	5.8	7.21	1.73
BB1	3T3 Balb-C	Neuromedin B	5.5	8.40	1.40
BB2	3T3 Balb-C	Bombesin	4.4	8.76	0.67
BB2	3T3 Balb-C	BIM 187	4.7	8.49	0.95
BB2	3T3 Balb-C	Neuromedin B	4.6	6.58	1.29
BB2	3T3 Balb-C	Gastin-releasing peptide (GRP)	4.9	8.49	0.70
BB3	3T3 Balb-C	PDSP compound	4.2	5.87	4.03
BB2 (mouse)	3T3 Balb-C	Bombesin	5.3	9.06	0.82
BB2 (mouse)	3T3 Balb-C	BIM 187	5.6	8.74	1.23
BB2 (mouse)	3T3 Balb-C	Neuromedin B	5.4	6.74	2.61
BB2 (mouse)	3T3 Balb-C	Gastrin-releasing peptide (GRP)	5.5	8.80	0.91
B2	HEK T	Bradykinin	1.7	9.57	0.88
CCK2	HEK T	Gastrin	3.1	9.83	1.29
Ghrelin	HEK	L-692,585	1.7	9.05	0.80
H1	HEK	Histamine	2.4	7.77	1.22

Receptor	Cell line	Reference	Emax (fold)	pEC50	Hill slope
H2	HEK T	Histamine	1.5	6.24	1.42
mGluR1 (IP)	CHO	Glutamate	6.4	4.66	1.20
mGluR5 (IP)	CHO	Glutamate	7.1	5.15	0.94
MC3		α-MSH	3.2	8.37	1.00
MC4		α-MSH	6.2	6.92	1.00
NK1	HEK	Substance P	2.7	7.65	0.77
NK2	HEK	Neurokinin A	4.6	7.53	0.72
NK2	HEK	Neurokinin B	4.4	6.90	0.82
NK3	HEK	Neurokinin B	4.0	8.41	0.83
NTS1	HEK	Neurotensin	3.5	8.00	0.77
P2Y1	1321N1	ADP	3.1	6.64	1.33
P2Y2	1321N1	ATP	2.1	6.87	1.32
P2Y2	1321N1	UTP	1.9	8.03	1.48
P2Y4	1321N1	UTP	2.5	7.65	1.59
P2Y6	1321N1	UDP	3.6	7.78	1.17
P2Y11	1321N1	ATP	3.6	5.70	1.78
PAR1	KOLF, mouse	Thrombin	3.9	6.86	1.17
PTAFR	HEK	PTAF	1.9	8.06	0.65
V1a	CHO	Vasopressin	3.7	8.07	0.81
V1b	CHO	Vasopressin	3.3	8.20	0.99
V2	CHO	Vasopressin	2.7	7.42	1.57
Oxytocin	CHO	Oxytocin	3.1	7.29	1.21
GPR40	HEK T	Linolenic acid	12	5.05	
GPR40	HEK T	Lauric acid	10	5.15	
GPR41	HEK T	Propionate	4.2	7.18	
GPR43	HEK T	Propionate		6.43	

Figure 35. Representative dose-response curves of Calcium mobilization assays. Some curves are presented after normalization with reference agonist activity as 100% and basal as 0%.

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ACh
CNO
PDSP compound
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1.0
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-10
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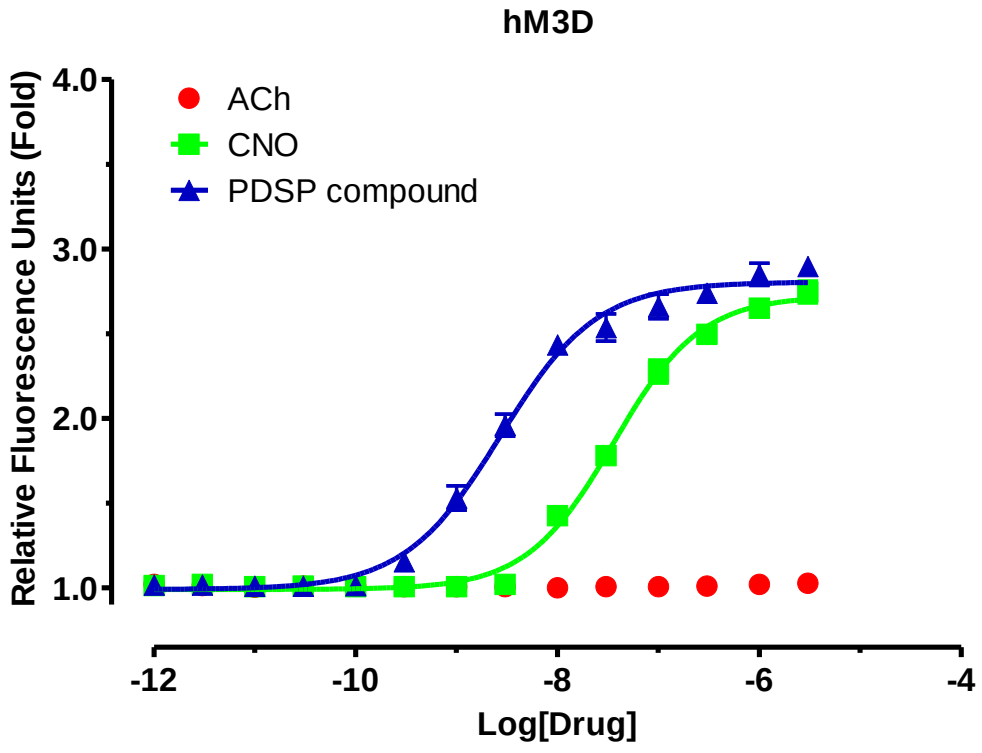
-6
-4
Log[Drug]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CNO
PDSP compound
Relative Fluorescence Units (Fold)
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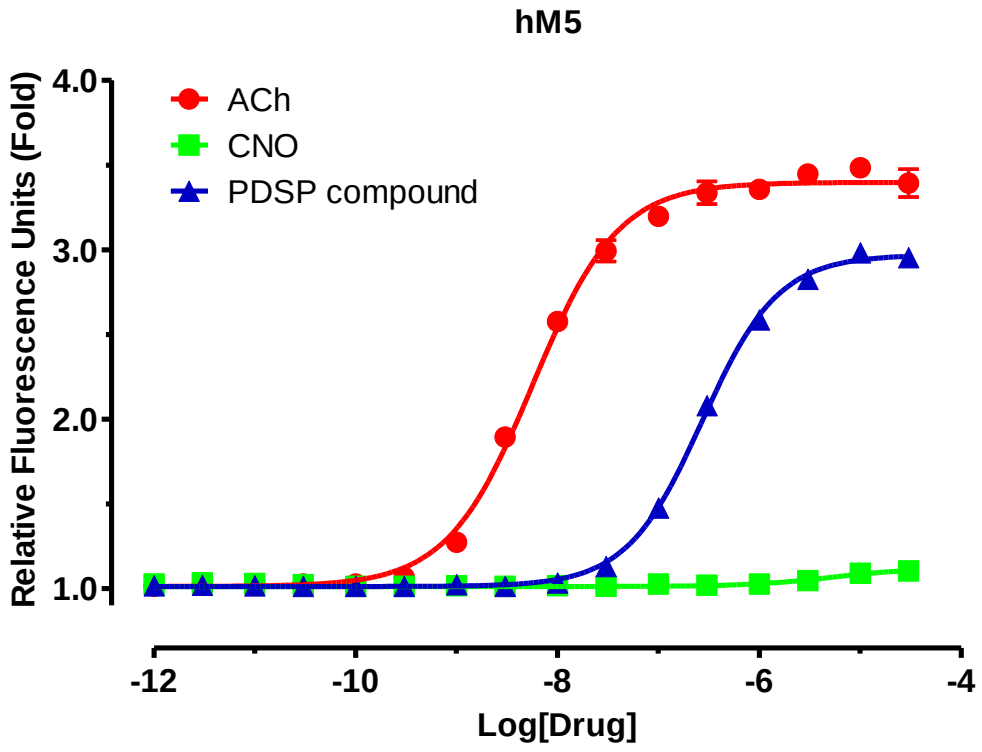
Log[Drug]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CNO
PDSP compound
Relative Fluorescence Units (Fold)
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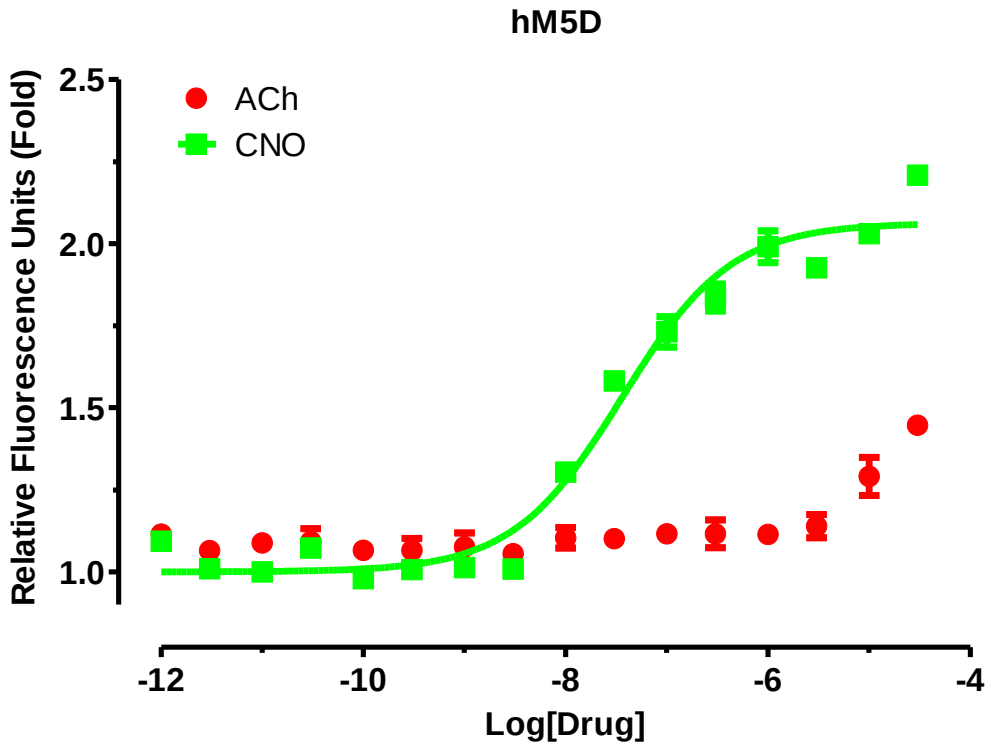
Log[Drug]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ACh
CNO
Relative Fluorescence Units (Fold)
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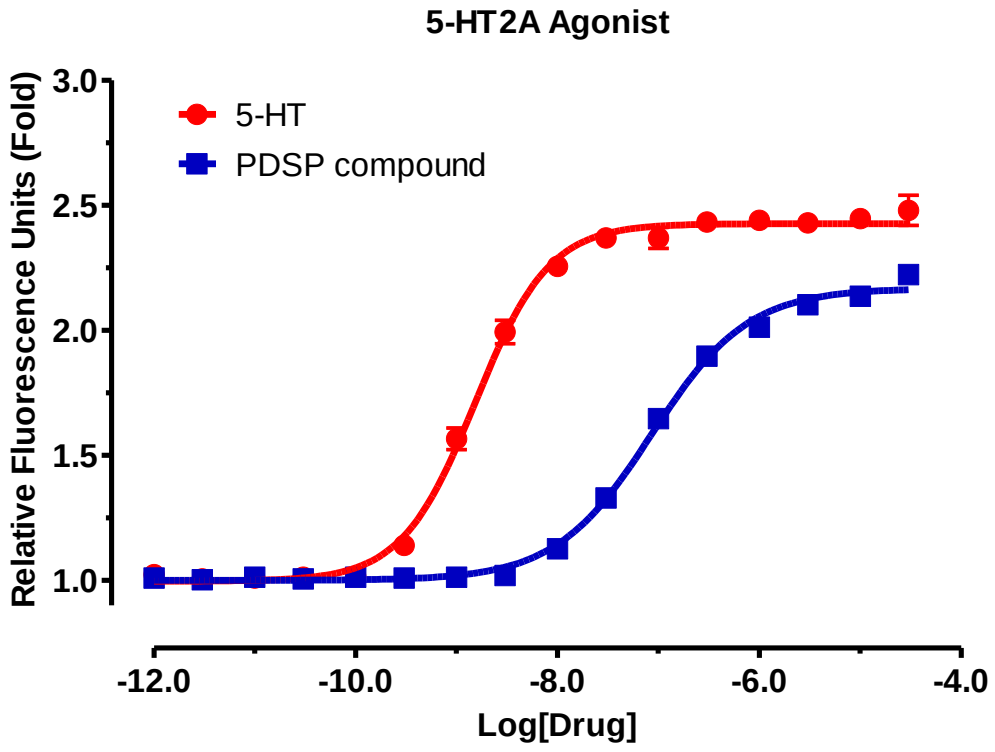
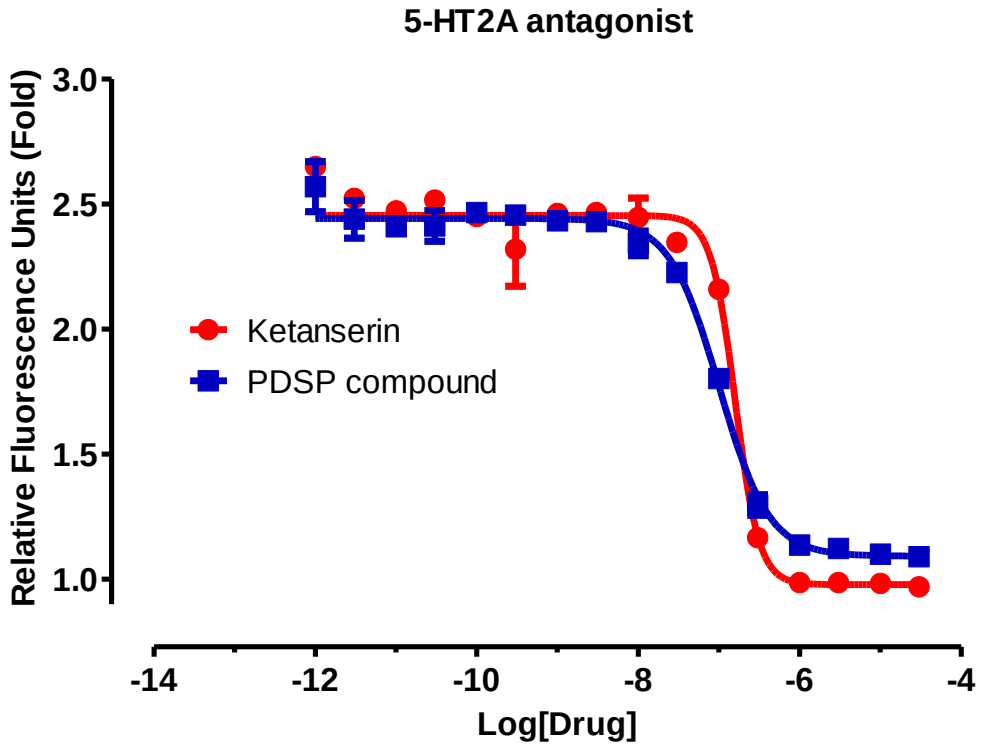
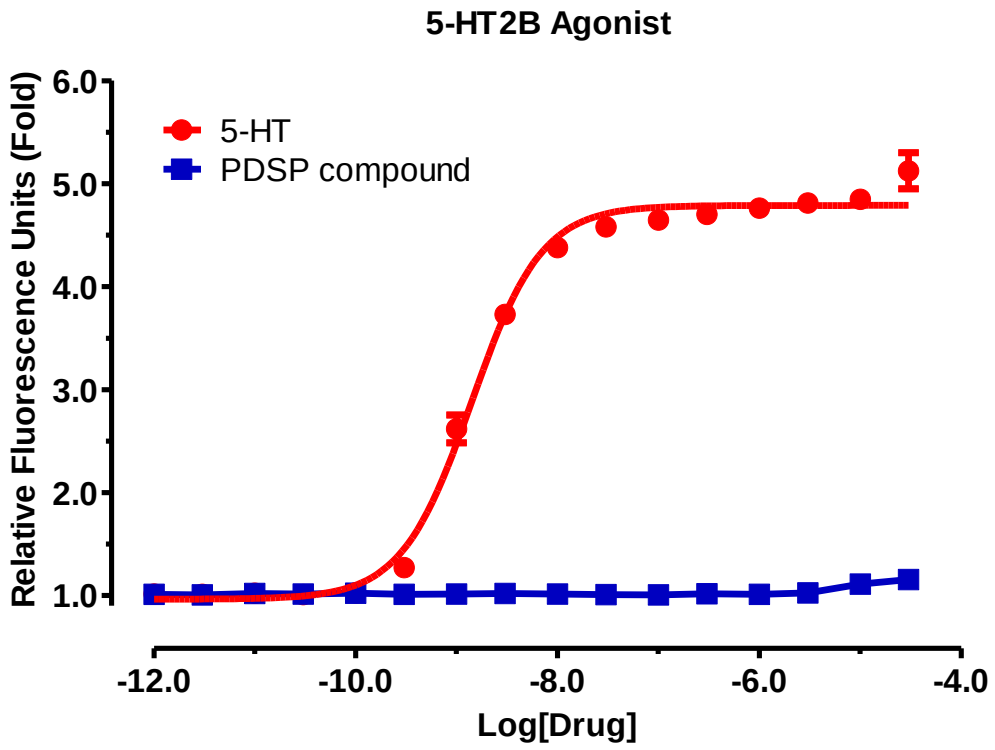
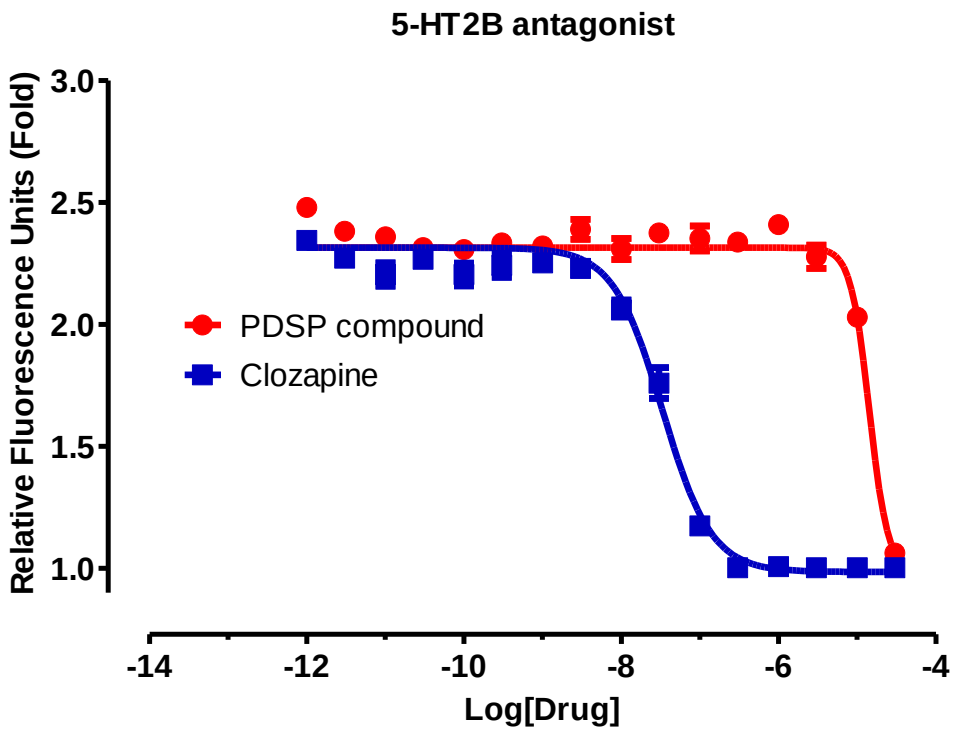
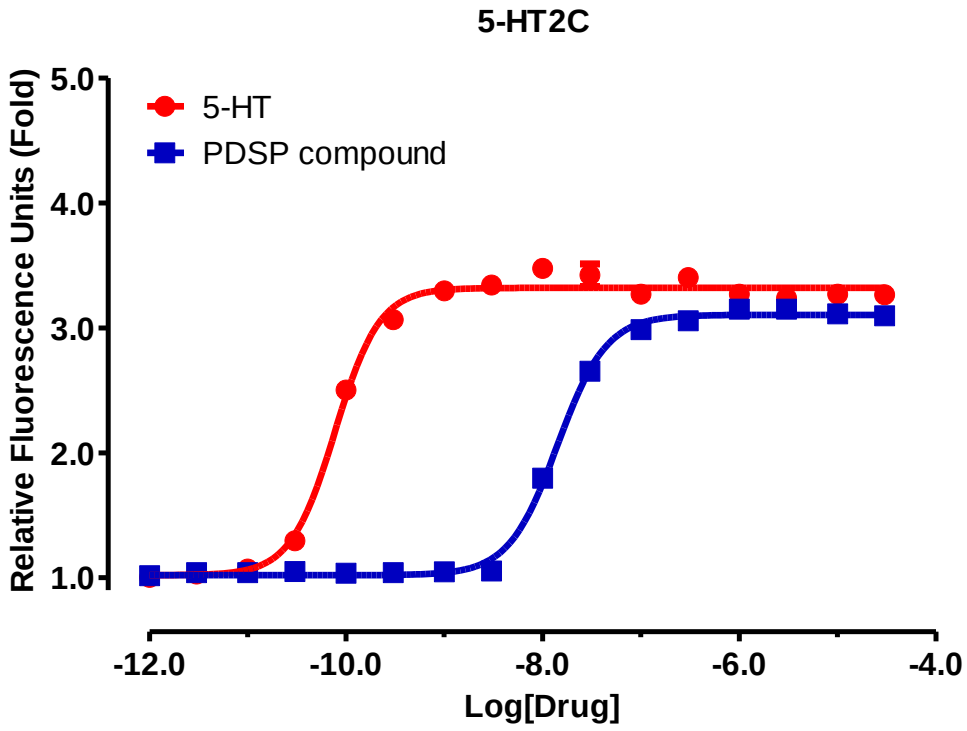
2.0
1.5
1.0
-12
-10
-8
-6
-4
Log[Drug]

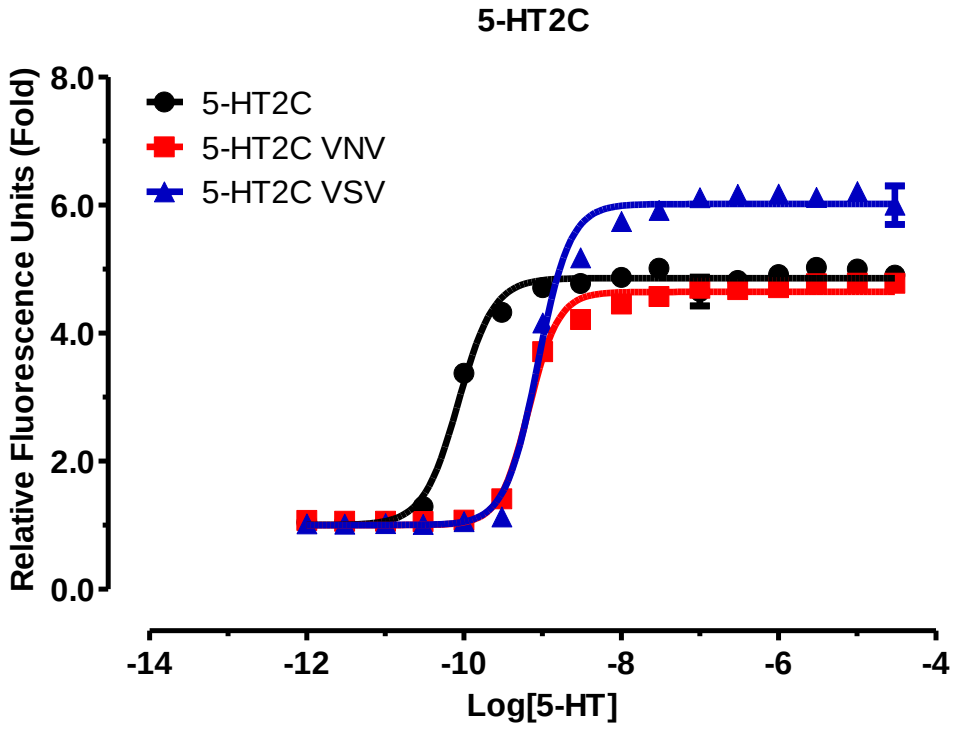

5-HT2A Agonist
Relative Fluorescence Units (Fold)
3.0
2.5
2.0
1.5
1.0
5-HT
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

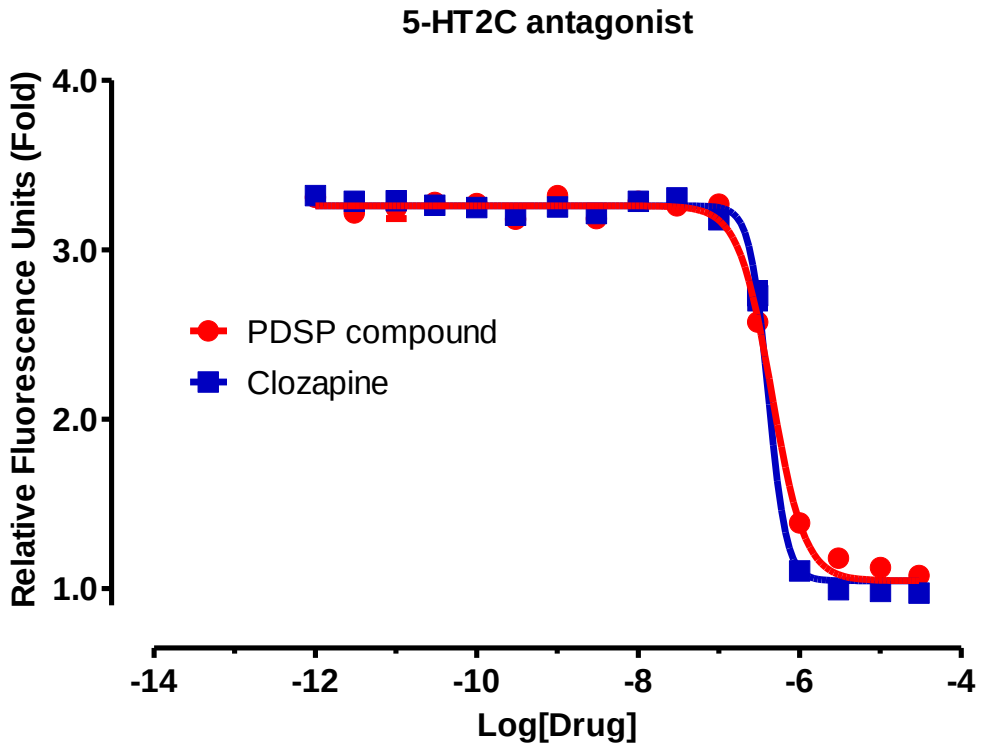

5-HT2A antagonist
Relative Fluorescence Units (Fold)
3.0
2.5
2.0
1.5
1.0
Ketanserin
PDSP compound
-14
-12
-10
-8
-6
-4
Log[Drug]

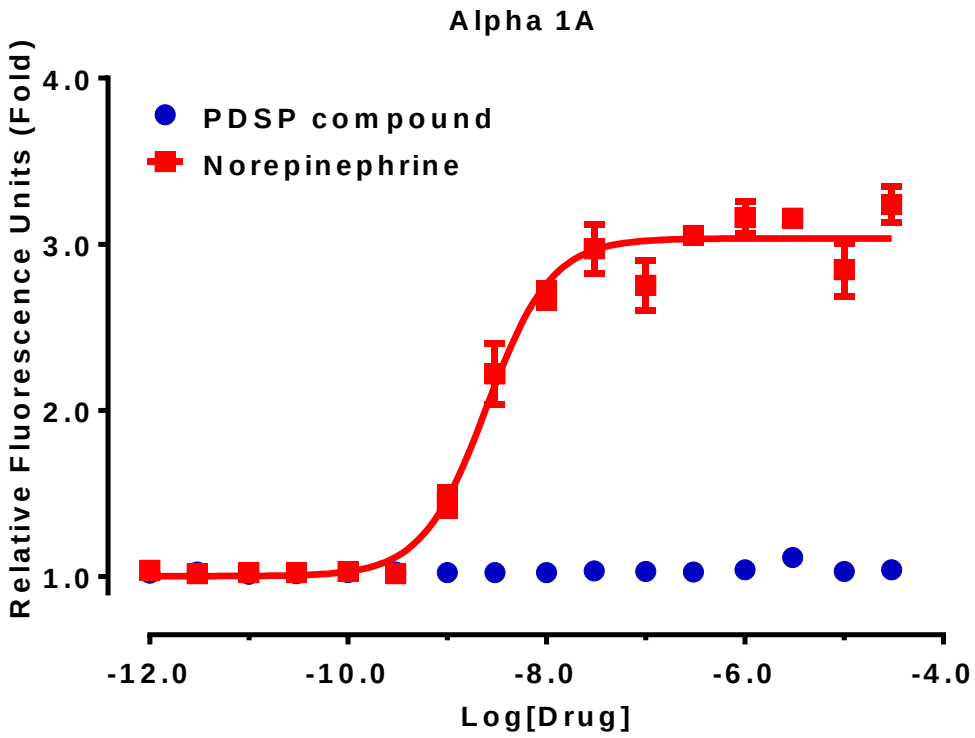

5-HT2B Agonist
Relative Fluorescence Units (Fold)
6.0
5.0
4.0
3.0
2.0
1.0
5-HT
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

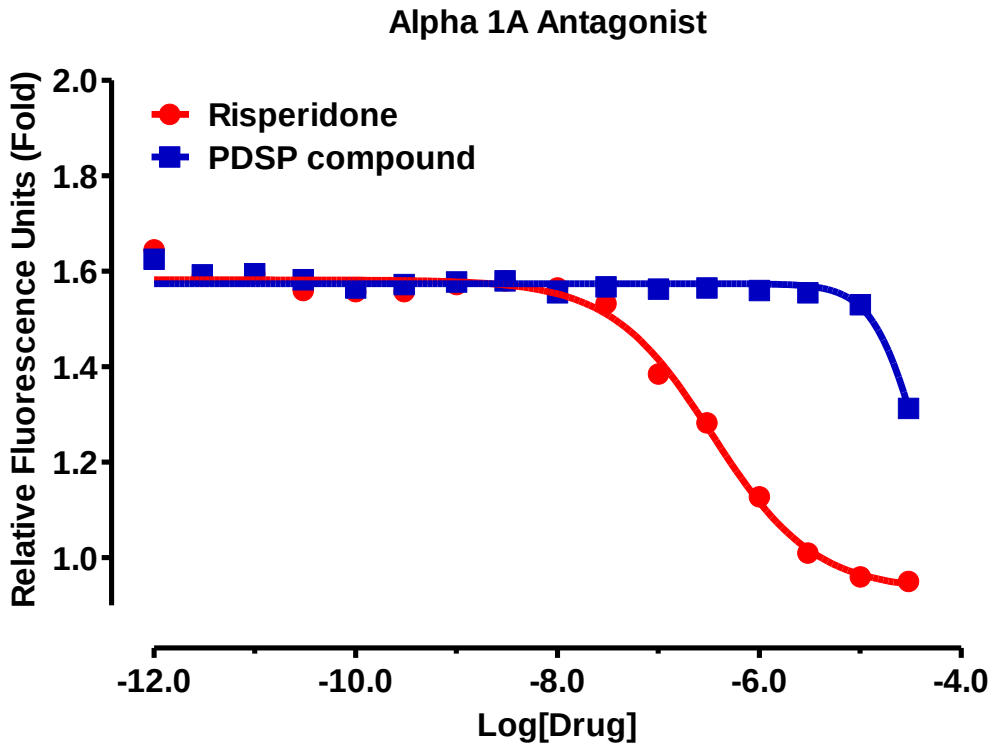

5-HT2B antagonist
Relative Fluorescence Units (Fold)
3.0
2.5
2.0
1.5
1.0
PDSP compound
Clozapine
-14
-12
-10
-8
-6
-4
Log[Drug]

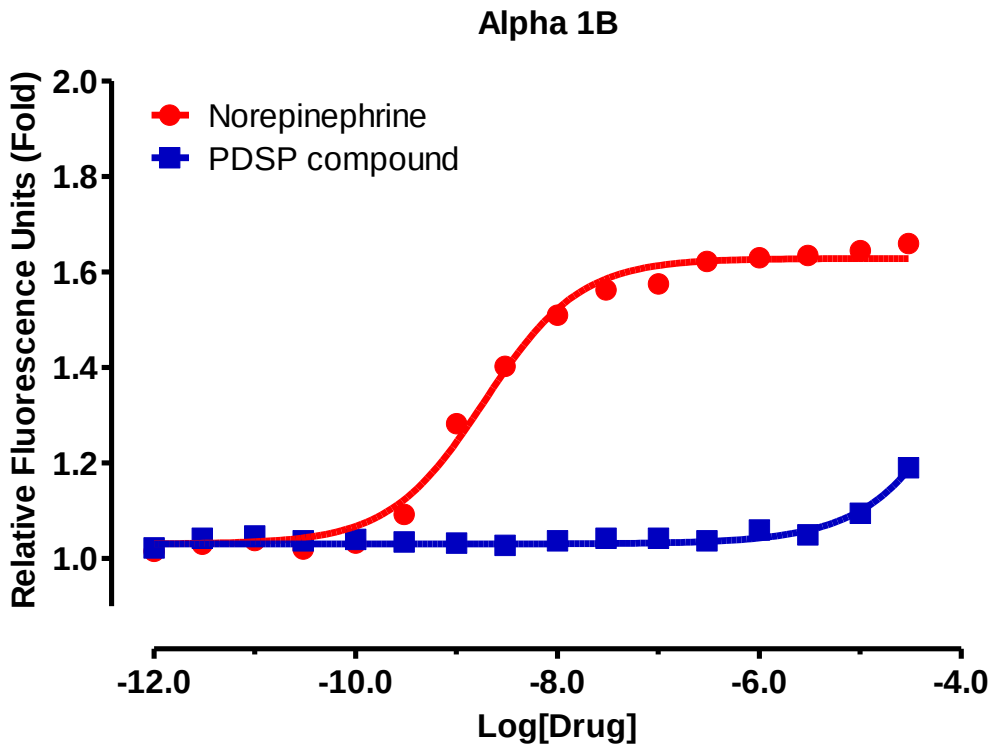

5-HT2C
5-HT
PDSP compound
Relative Fluorescence Units (Fold)
5.0
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

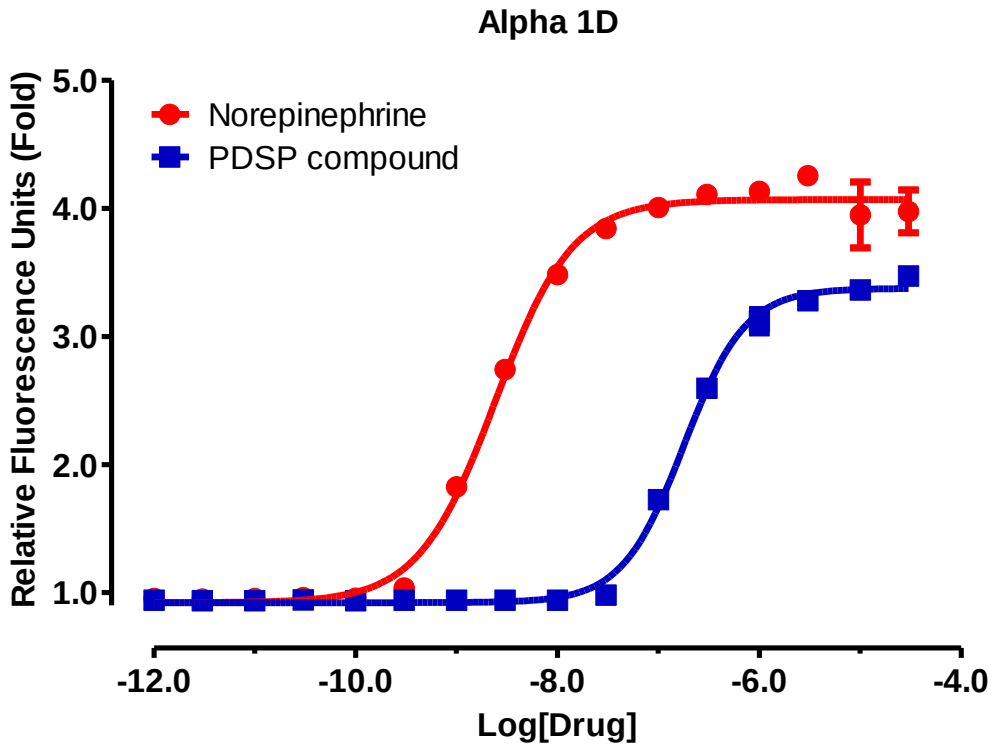

5-HT2C
5-HT2C
5-HT2C VNV
5-HT2C VSV
Relative Fluorescence Units (Fold)
8.0
6.0
4.0
2.0
0.0
-14
-12
-10
-8
-6
-4
Log[5-HT]

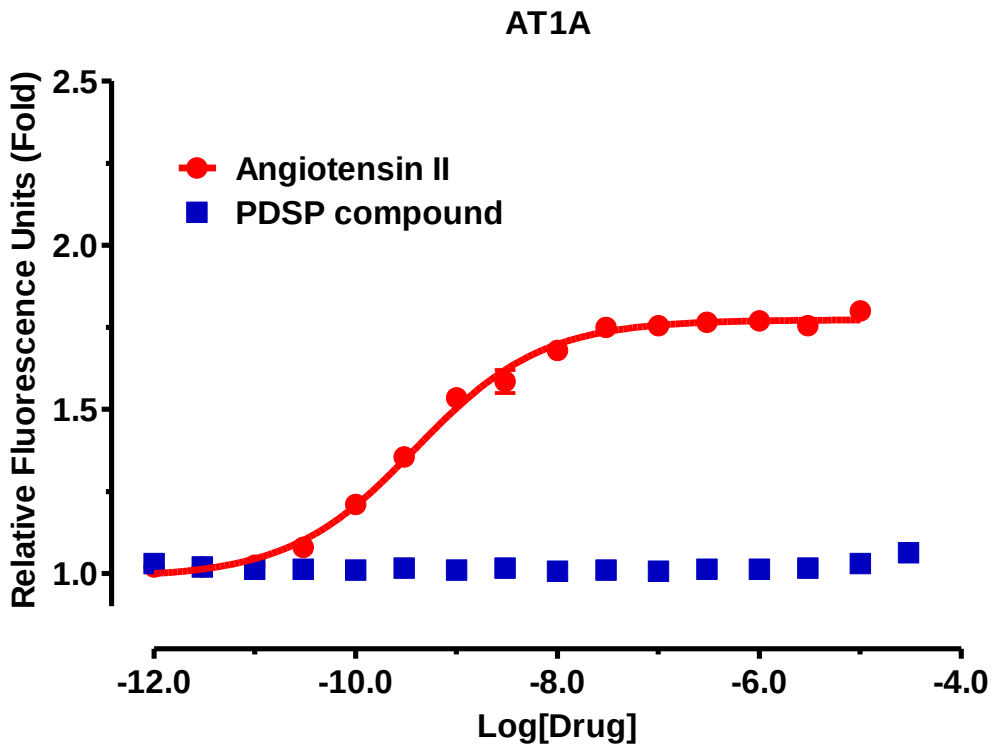

5-HT2C antagonist
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
PDSP compound
Clozapine
-14
-12
-10
-8
-6
-4
Log[Drug]

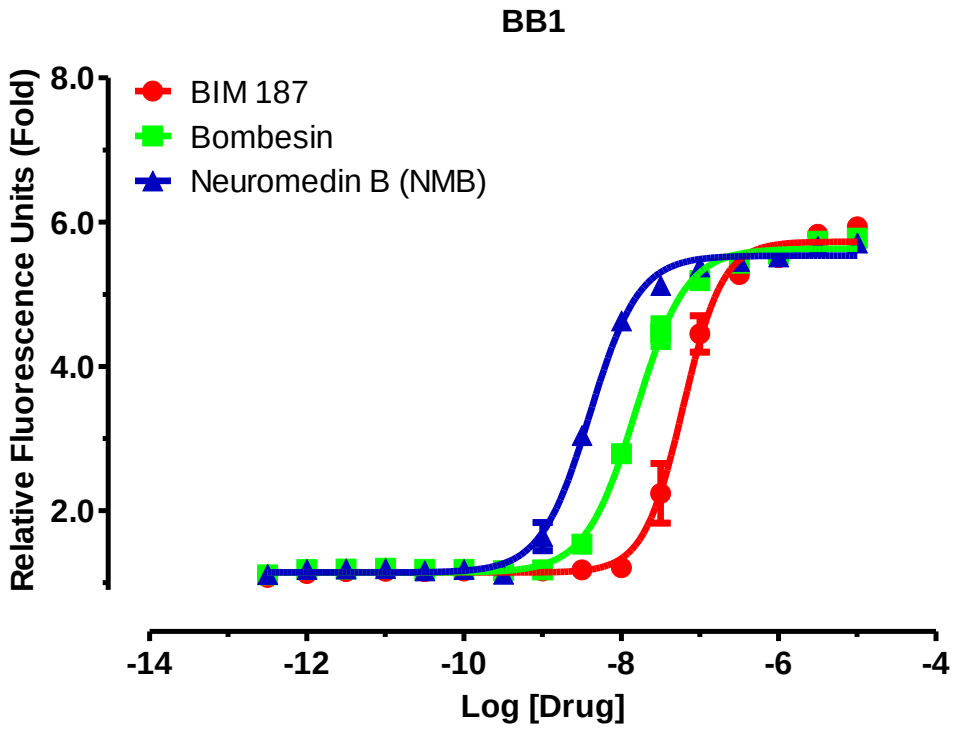

Alpha 1A
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
PDSP compound
Norepinephrine
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

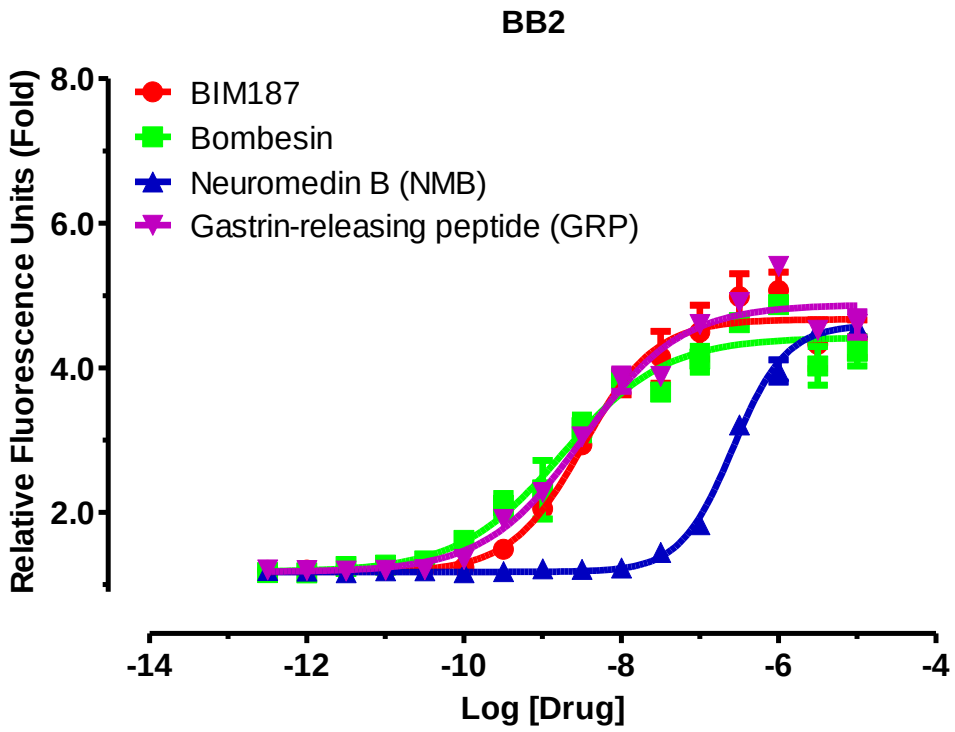

Alpha 1A Antagonist
Relative Fluorescence Units (Fold)
2.0
1.8
1.6
1.4
1.2
1.0
Risperidone
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

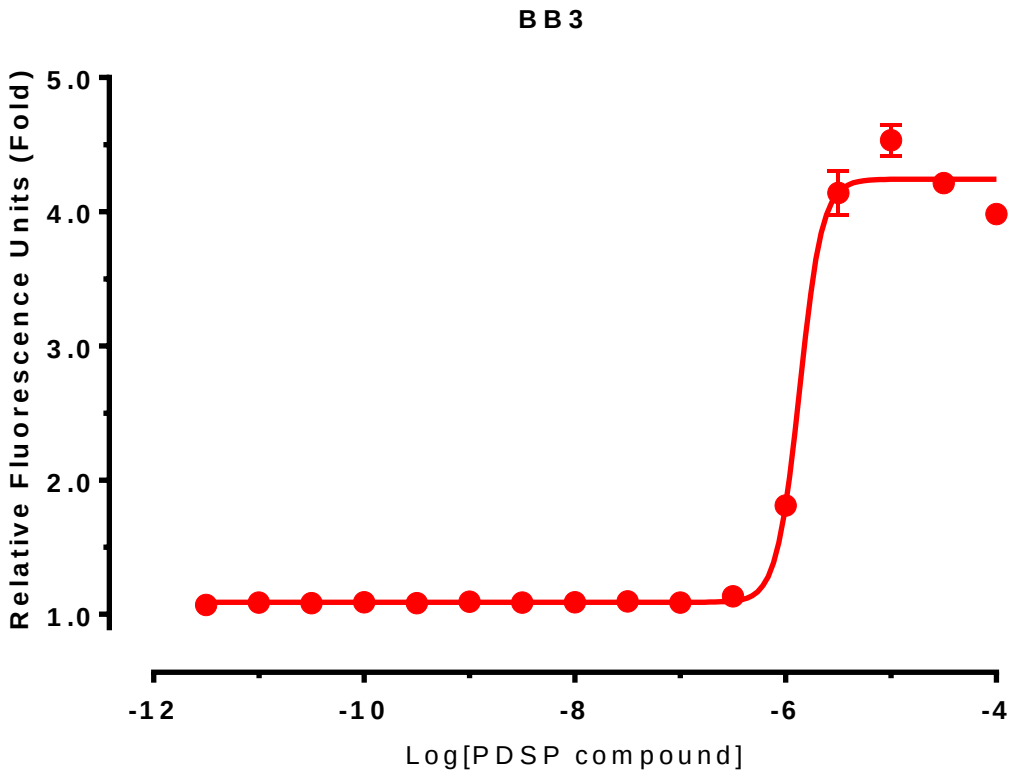

Alpha 1B
Relative Fluorescence Units (Fold)
2.0
1.8
1.6
1.4
1.2
1.0
Norepinephrine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

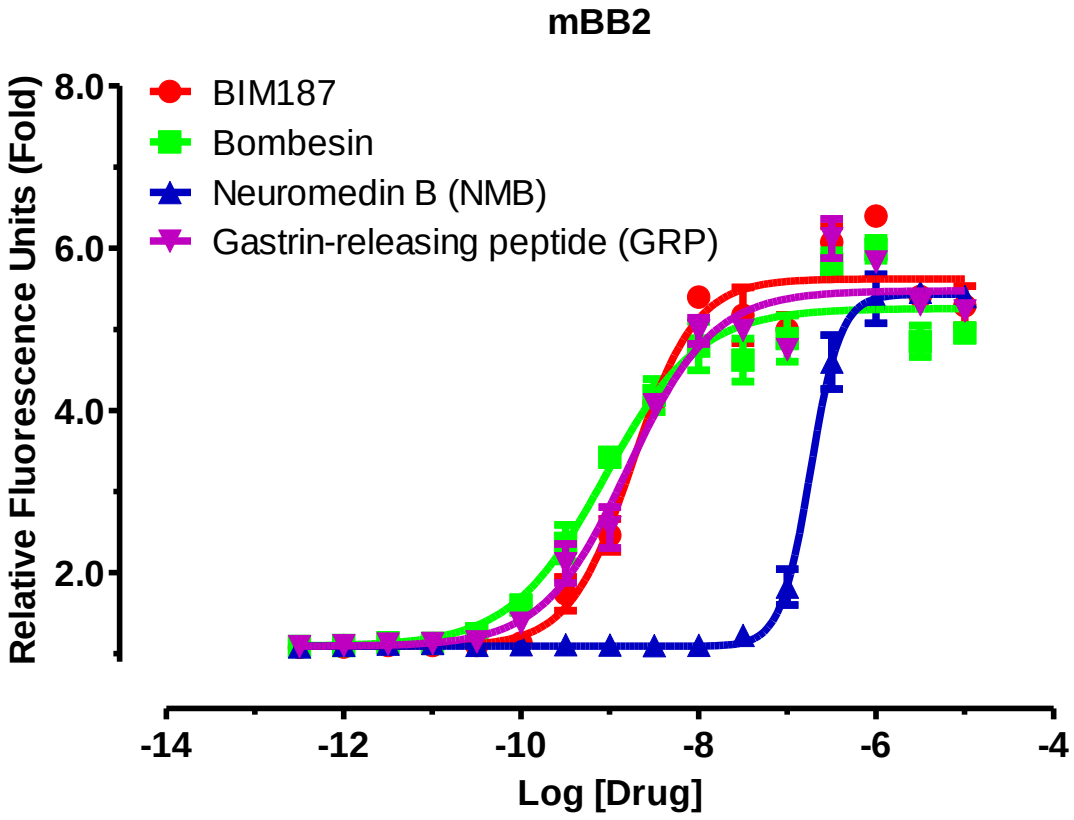

Alpha 1D
Norepinephrine
PDSP compound
Relative Fluorescence Units (Fold)
5.0
4.0
3.0
2.0
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


AT1A
Angiotensin II
PDSP compound
Relative Fluorescence Units (Fold)
2.5
2.0
1.5
1.0
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


BB1
BIM 187
Bombesin
Neuromedin B (NMB)
Relative Fluorescence Units (Fold)
8.0
6.0
4.0
2.0
-14
-12
-10
-8
-6
-4
Log [Drug]


BB2
BIM187
Bombesin
Neuromedin B (NMB)
Gastrin-releasing peptide (GRP)
Relative Fluorescence Units (Fold)
8.0
6.0
4.0
2.0
-14
-12
-10
-8
-6
-4
Log [Drug]





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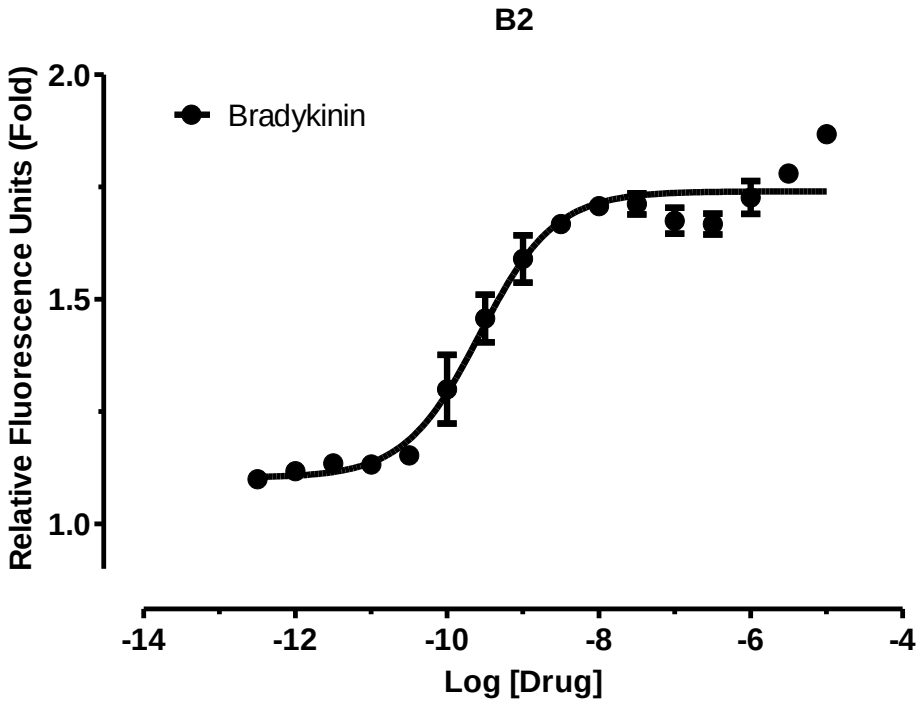
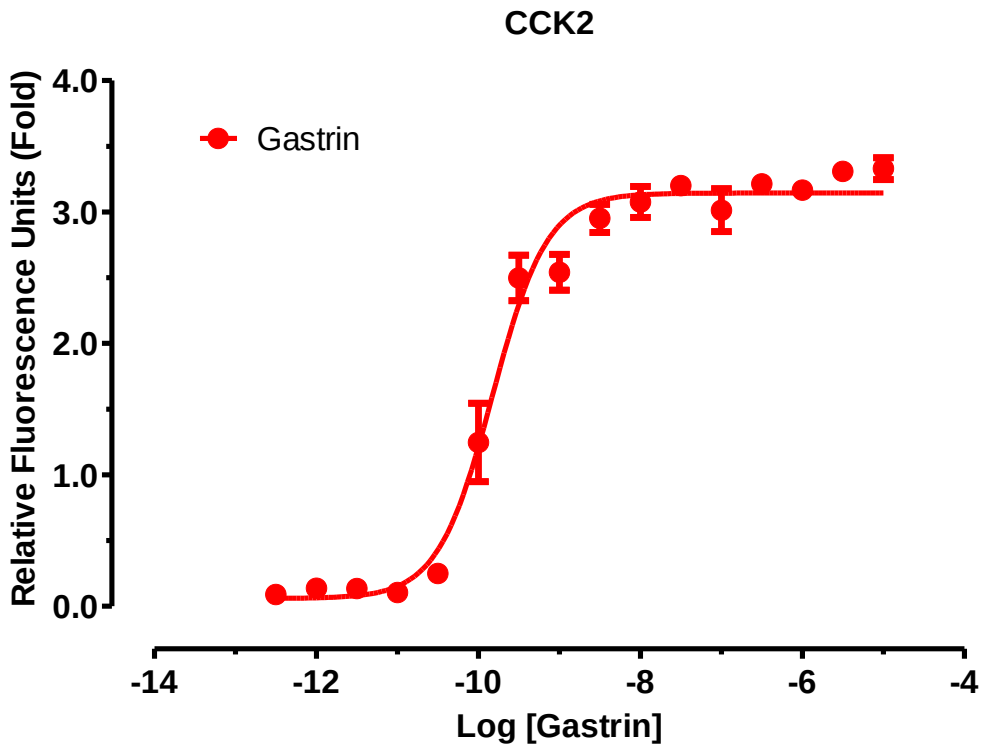
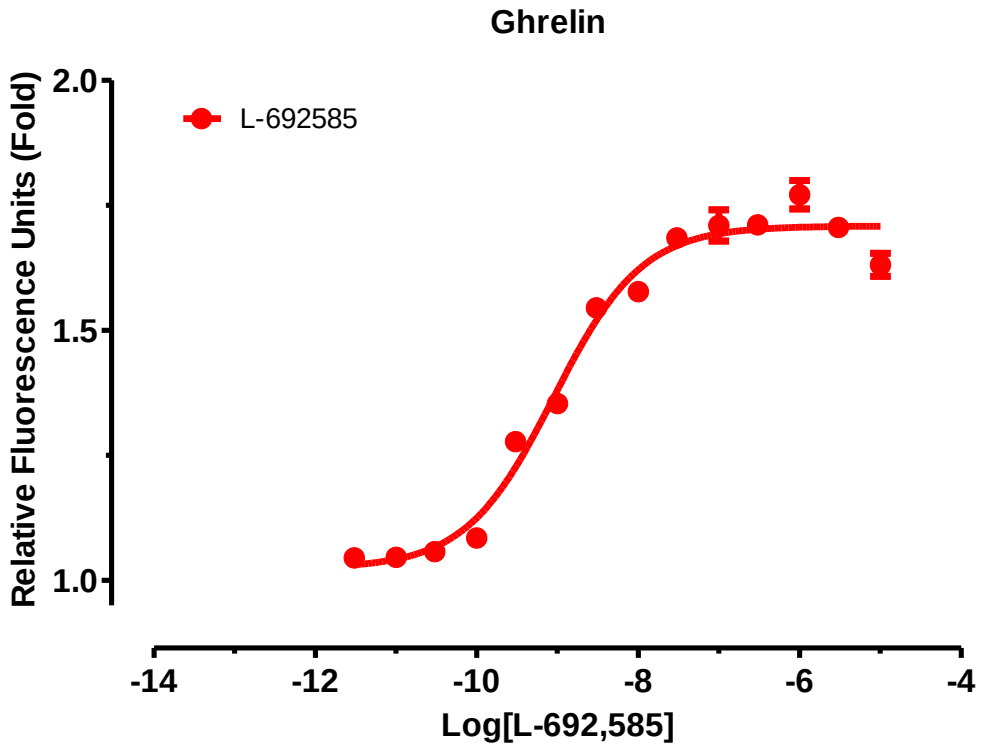
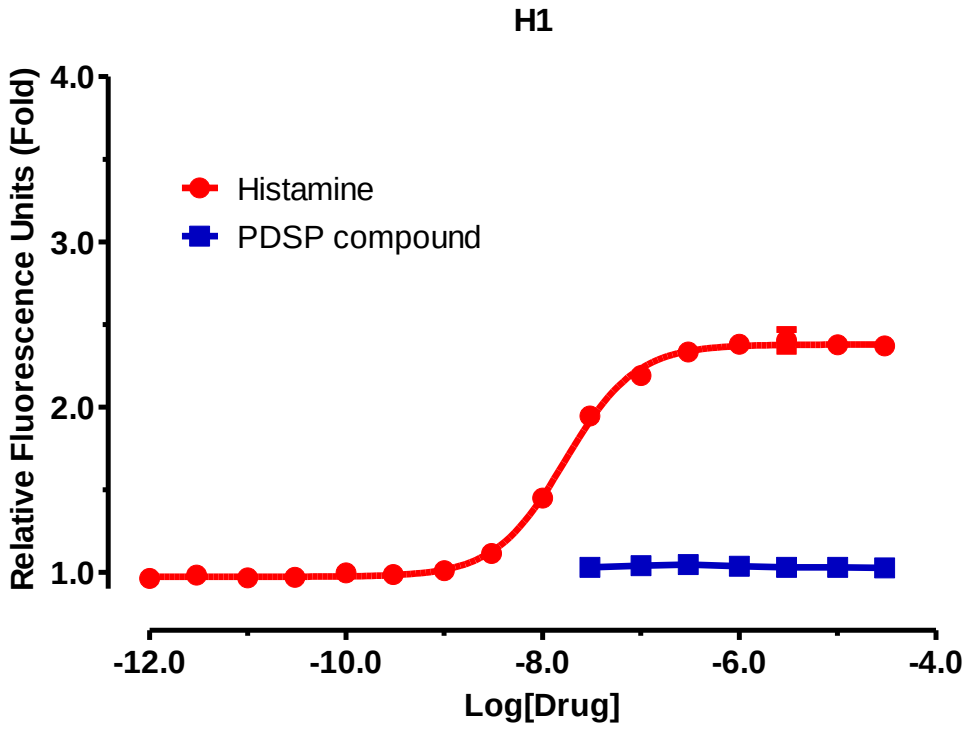
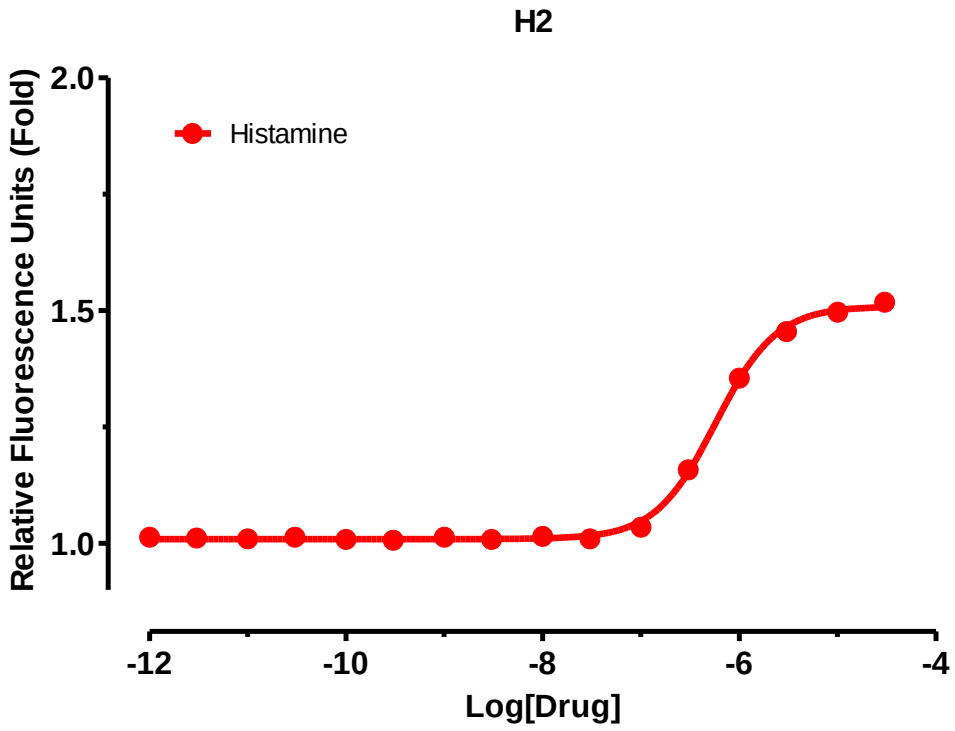
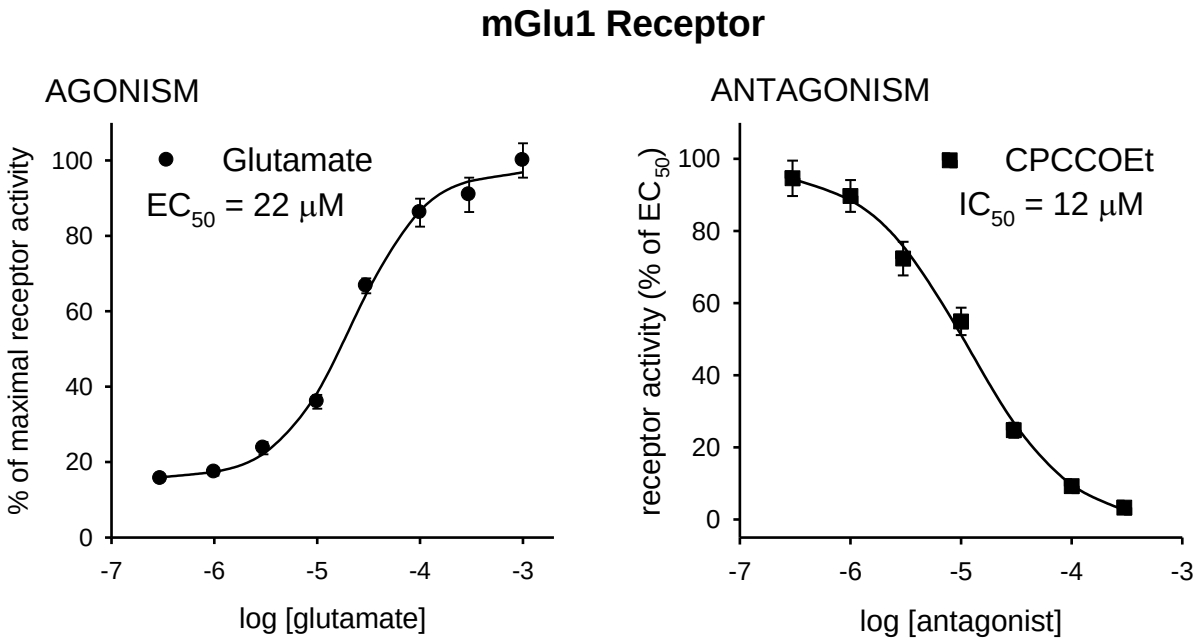

B2
Bradykinin
Relative Fluorescence Units (Fold)
2.0
1.5
1.0
-14
-12
-10
-8
-6
-4
Log [Drug]


CCK2
Gastrin
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
0.0
-14
-12
-10
-8
-6
-4
Log [Gastrin]


Ghrelin
Relative Fluorescence Units (Fold)
2.0
1.5
1.0
L-692585
-14
-12
-10
-8
-6
-4
Log[L-692,585]


H1
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
Histamine
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]





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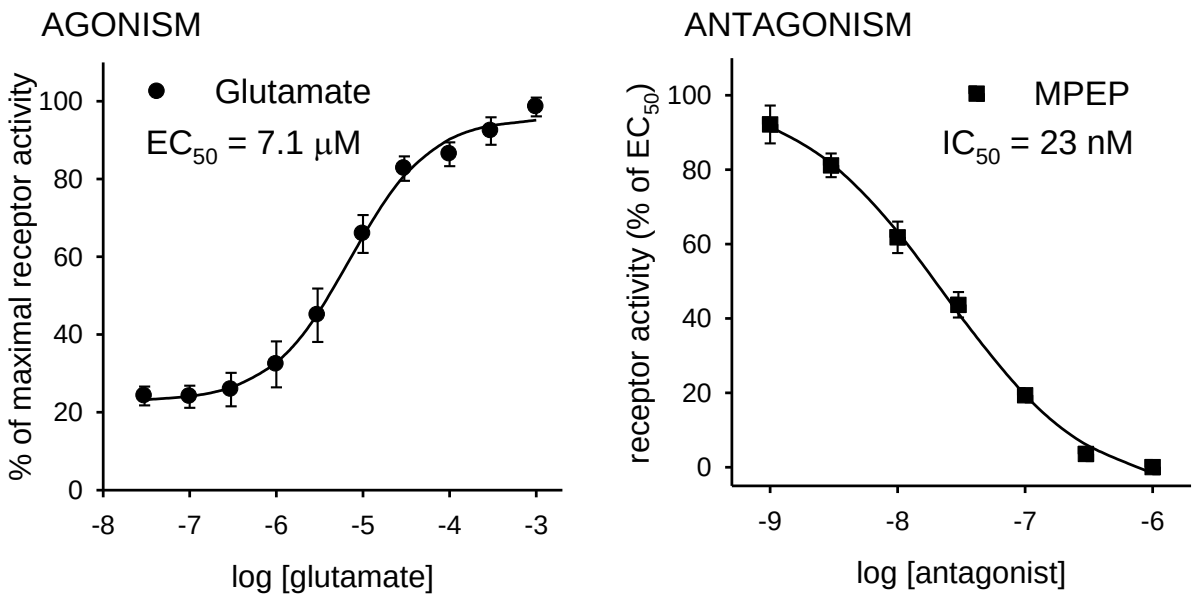
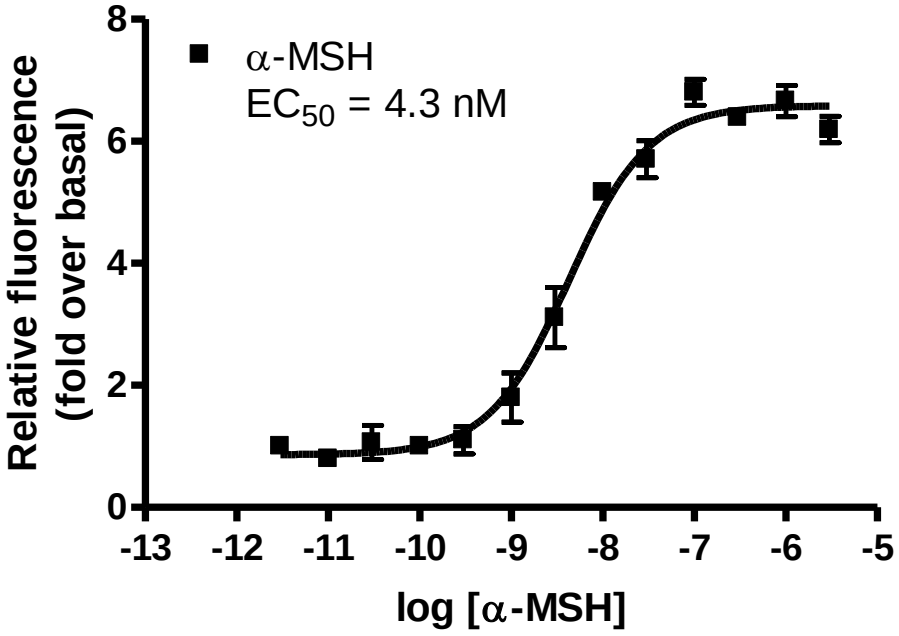
mGlu5 Receptor



MC3 Receptor
Hank's Balanced Salt Solution, 20 mM HEPES, 2.5 mM probenecid



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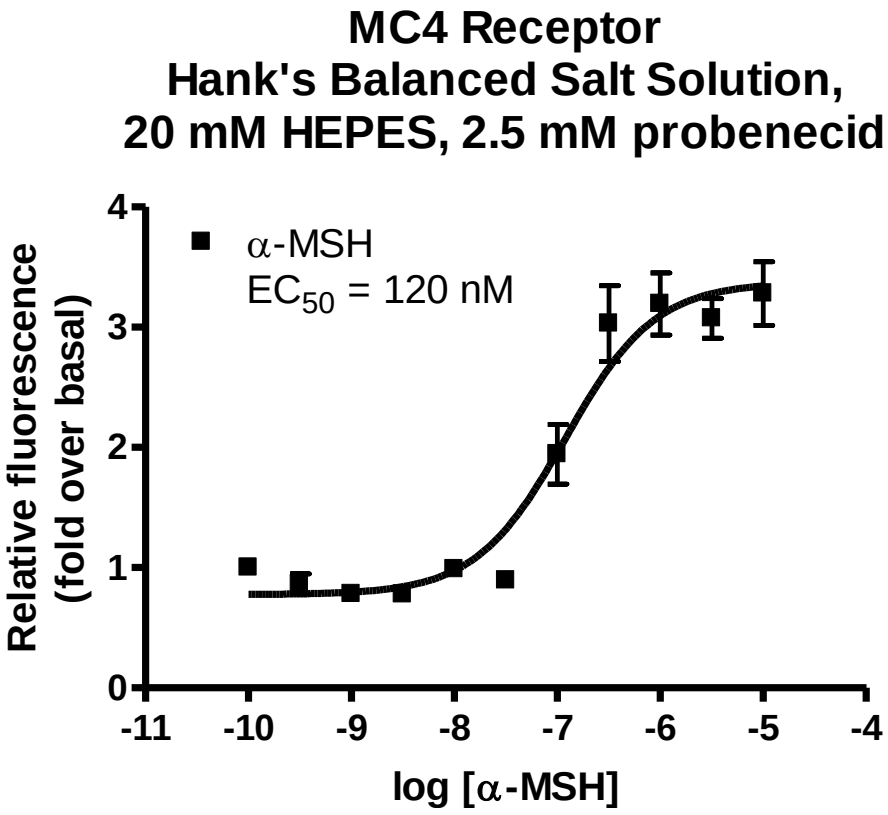
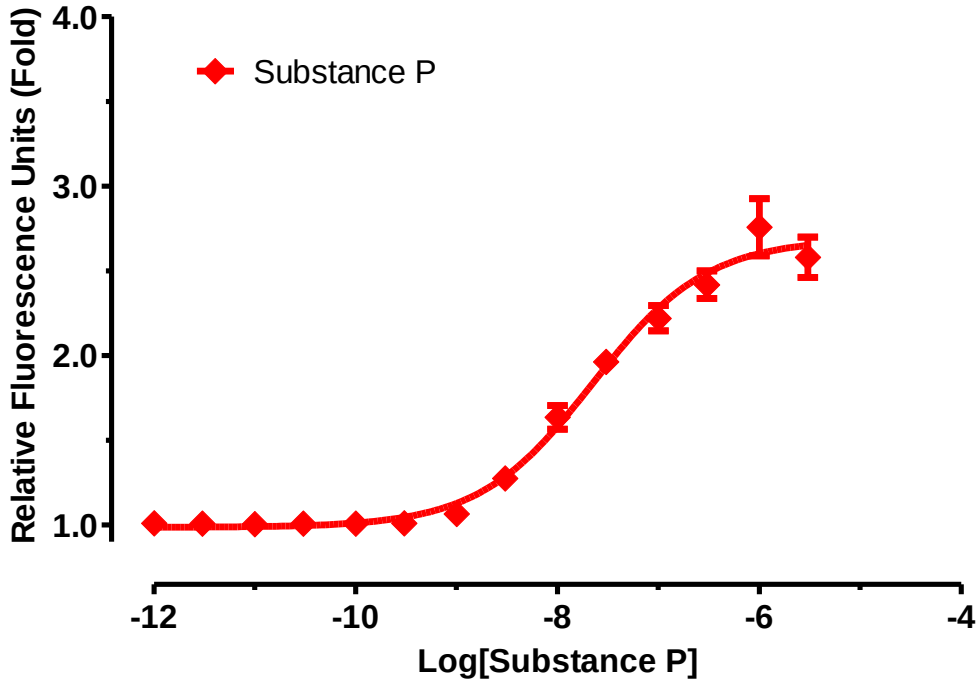
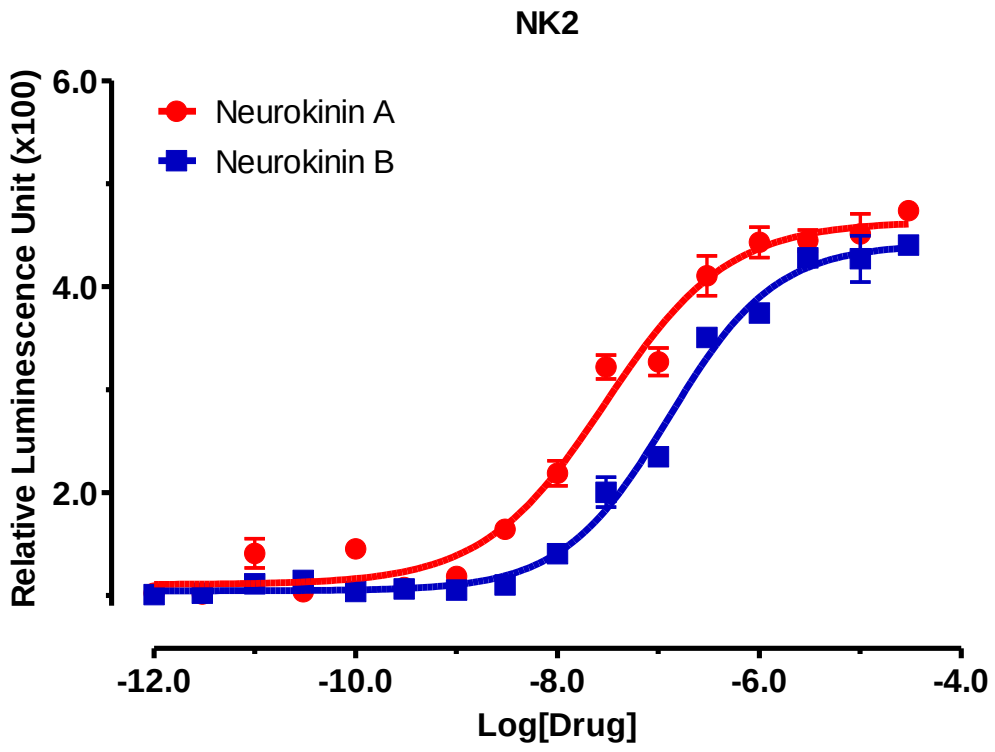
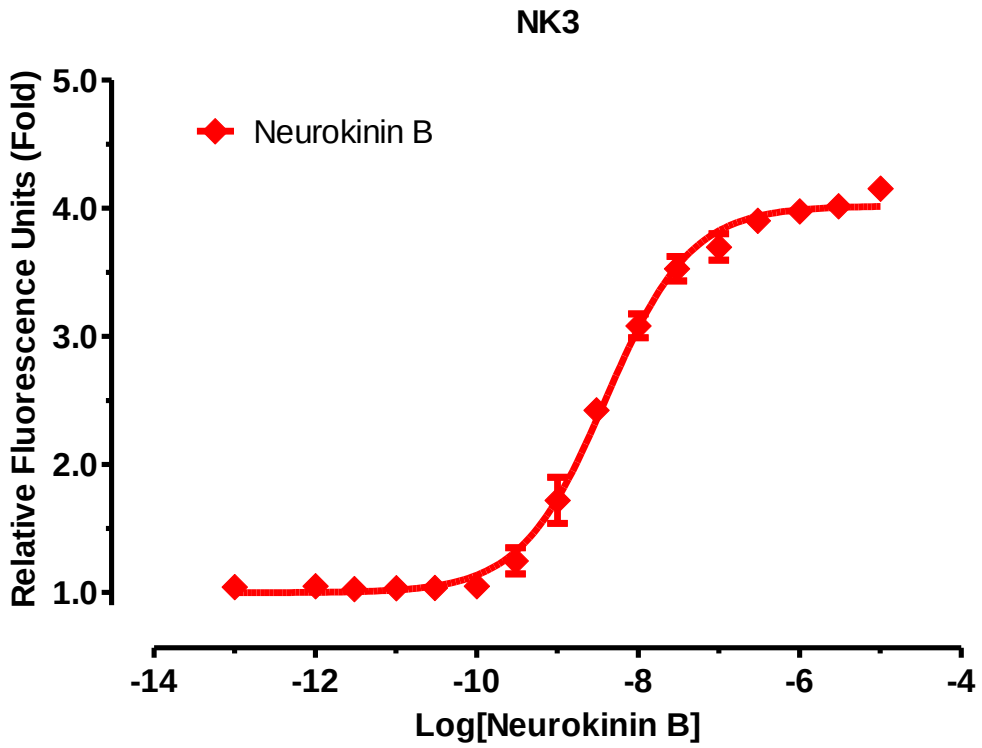
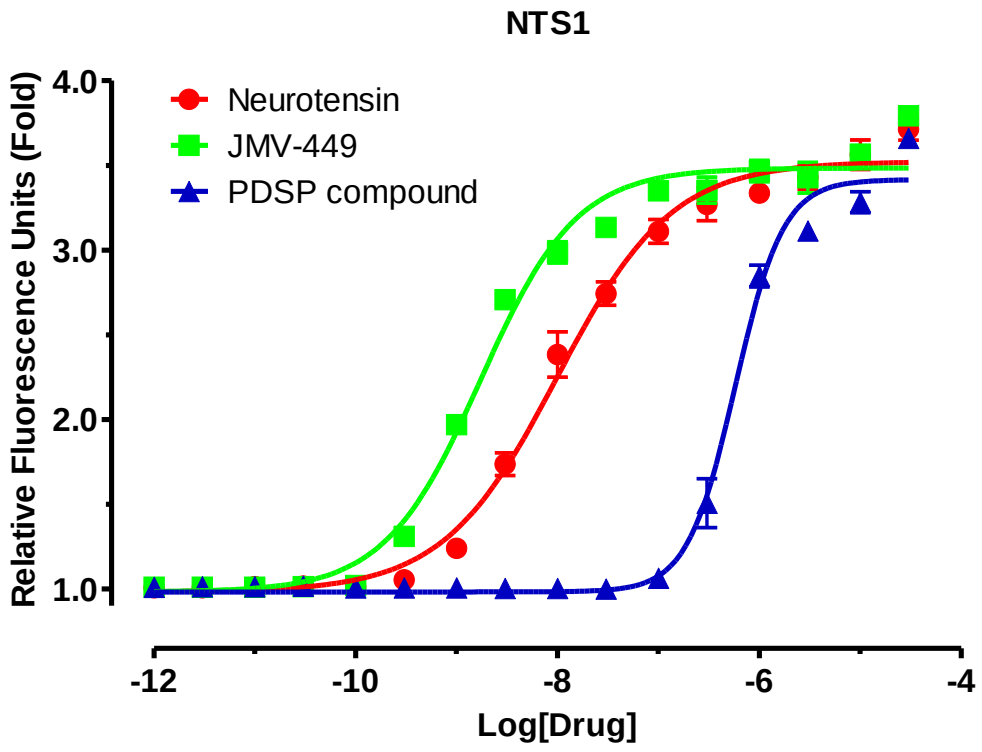
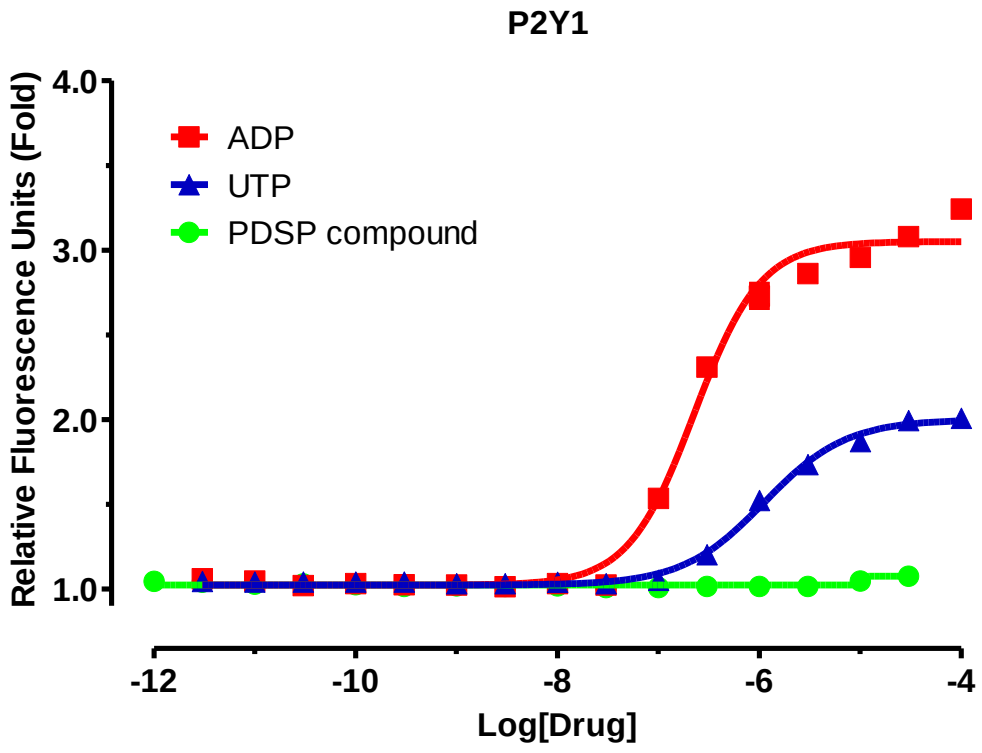

MC4 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
α-MSH
EC50 = 120 nM
Relative fluorescence
(fold over basal)
4
3
2
1
0
-11
-10
-9
-8
-7
-6
-5
-4
log [α-MSH]

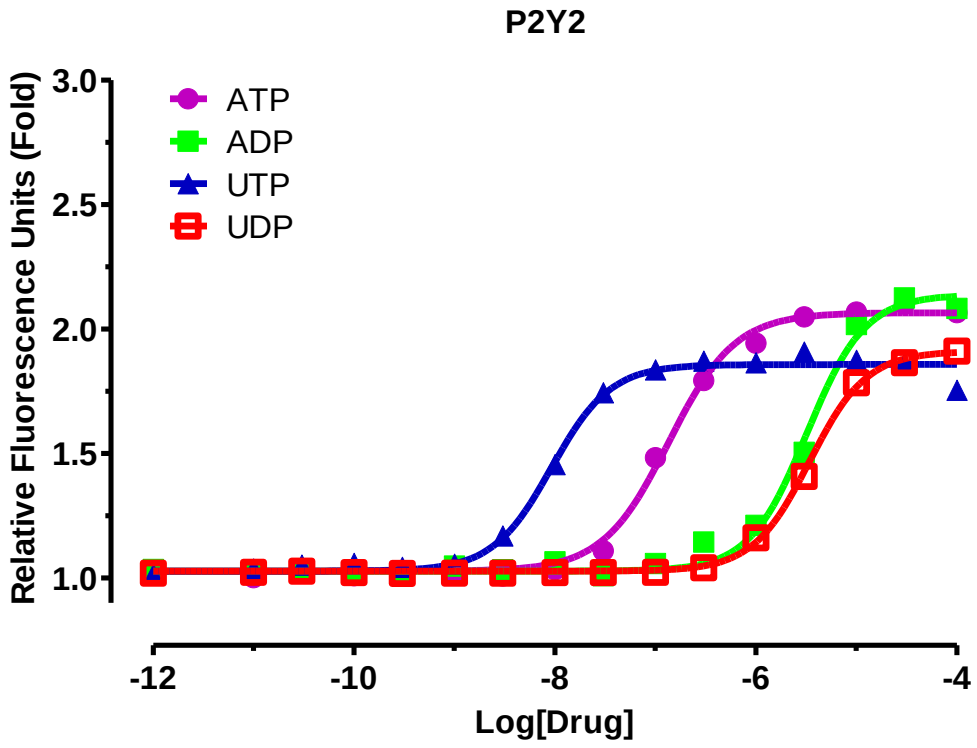

NK1
Substance P
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
-12
-10
-8
-6
-4
Log[Substance P]

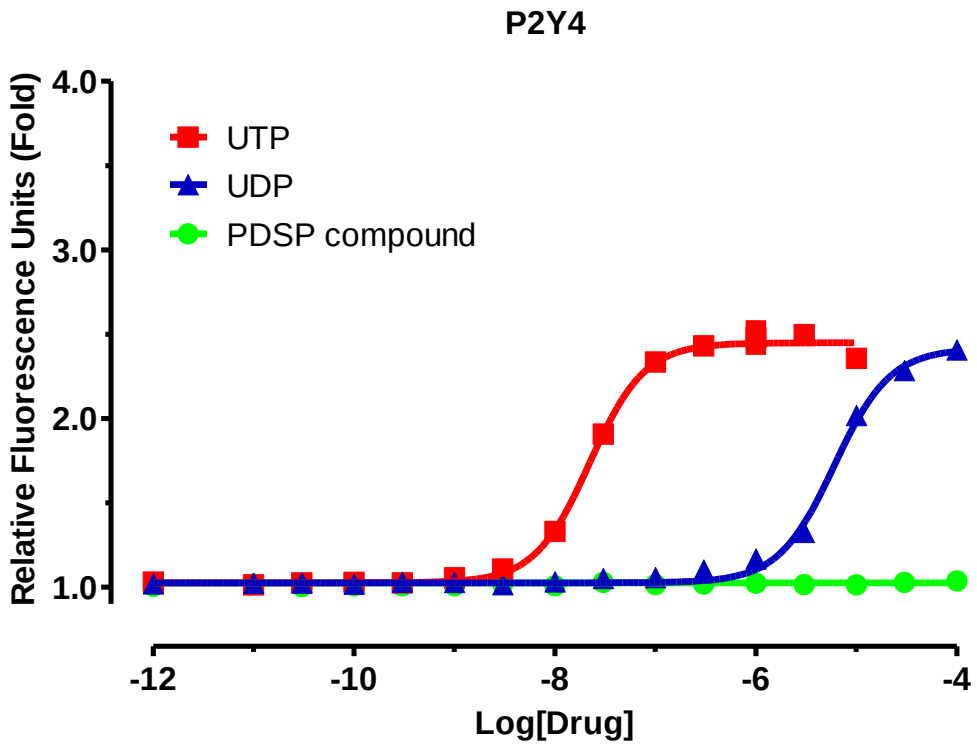

NK2
Relative Luminescence Unit (x100)
6.0
4.0
2.0
Neurokinin A
Neurokinin B
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

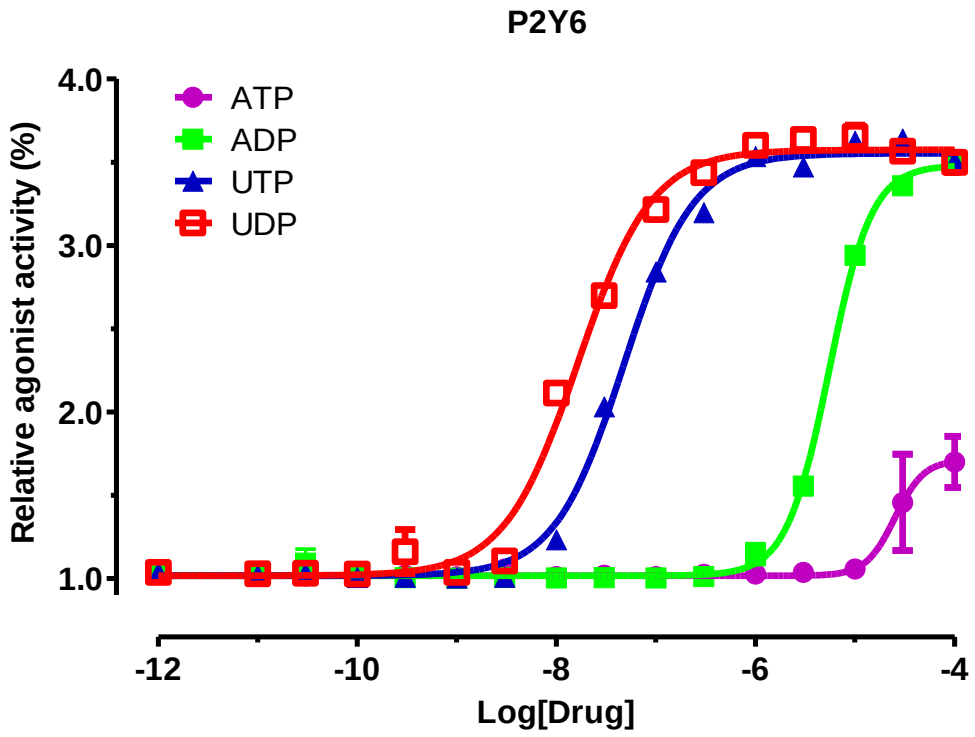

NK3
Relative Fluorescence Units (Fold)
5.0
4.0
3.0
2.0
1.0
Neurokinin B
-14
-12
-10
-8
-6
-4
Log[Neurokinin B]

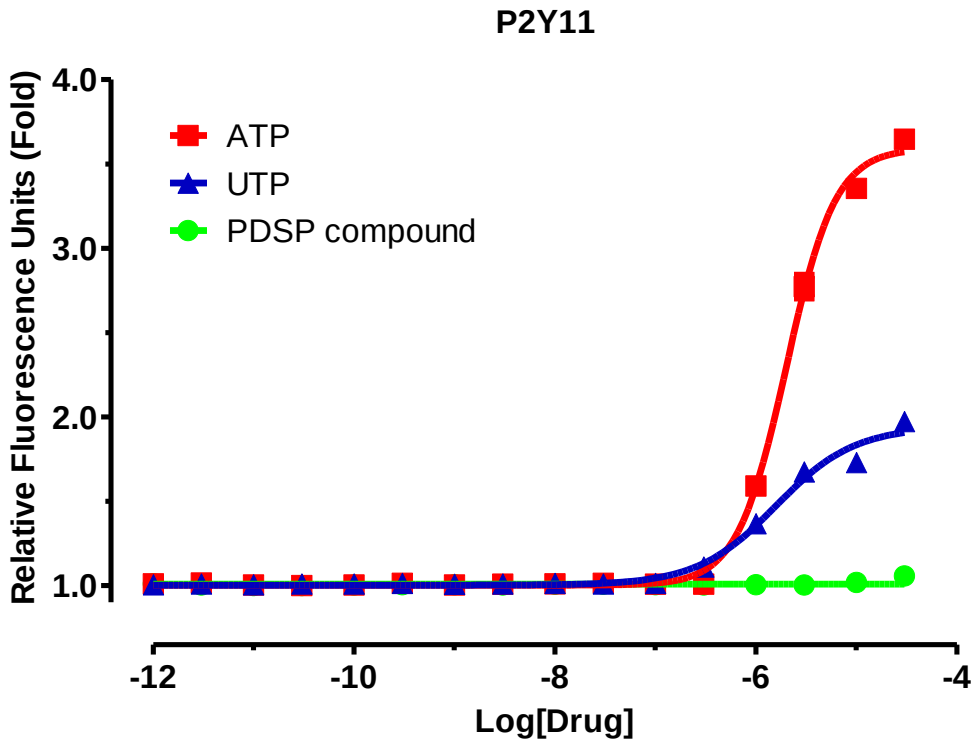

NTS1
Neurotensin
JMV-449
PDSP compound
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
-12
-10
-8
-6
-4
Log[Drug]

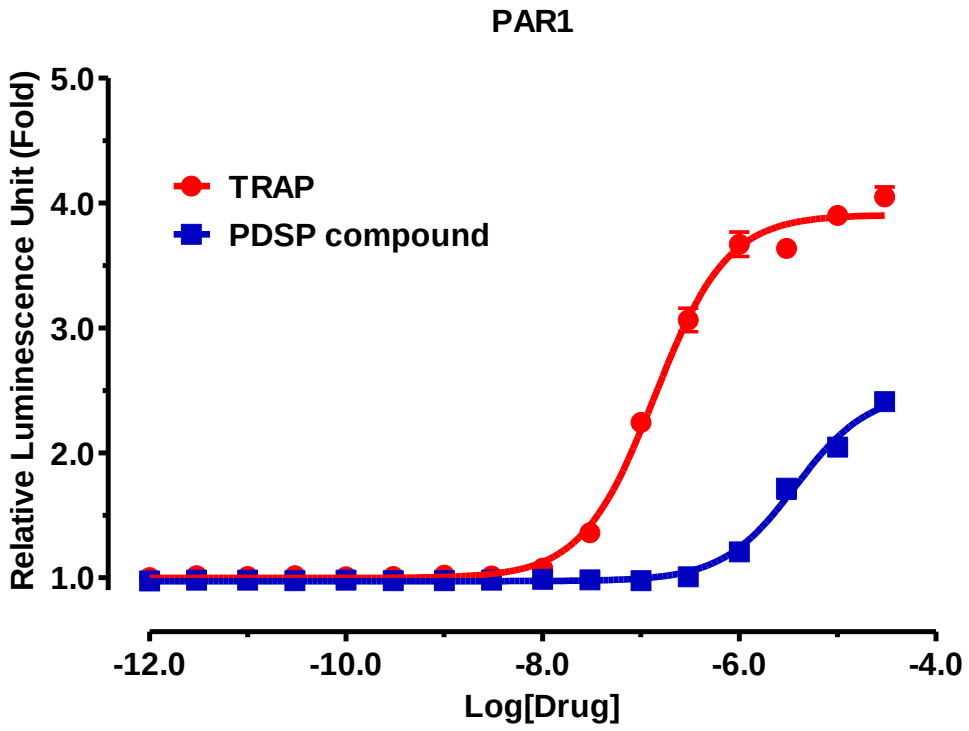

P2Y1
ADP
UTP
PDSP compound
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
-12
-10
-8
-6
-4
Log[Drug]

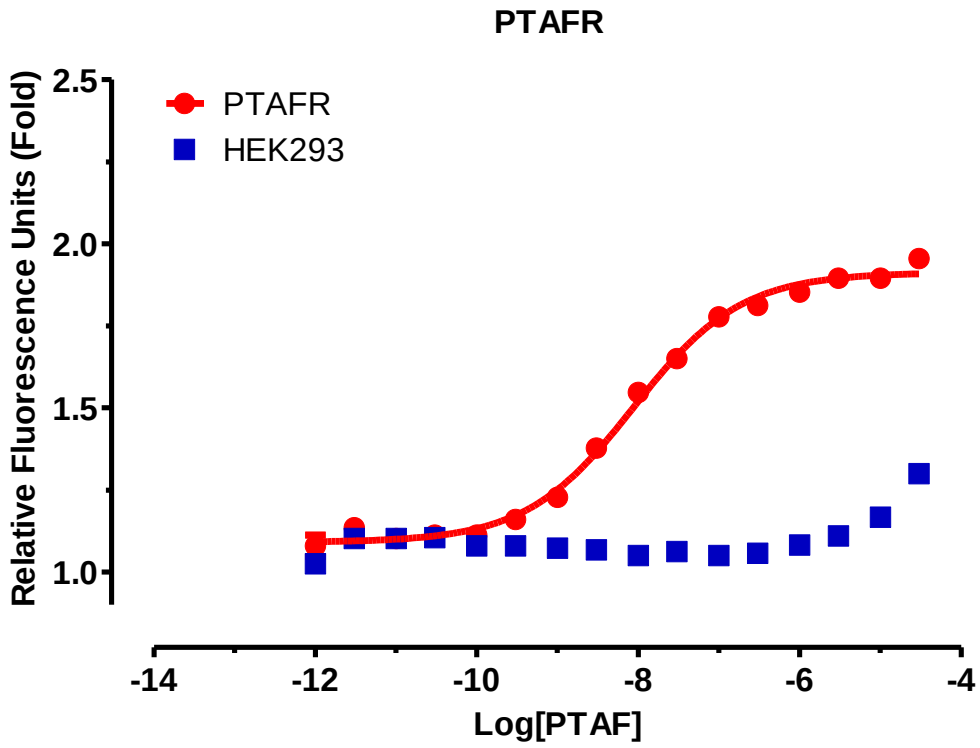

P2Y2
Relative Fluorescence Units (Fold)
3.0
2.5
2.0
1.5
1.0
ATP
ADP
UTP
UDP
-12
-10
-8
-6
-4
Log[Drug]

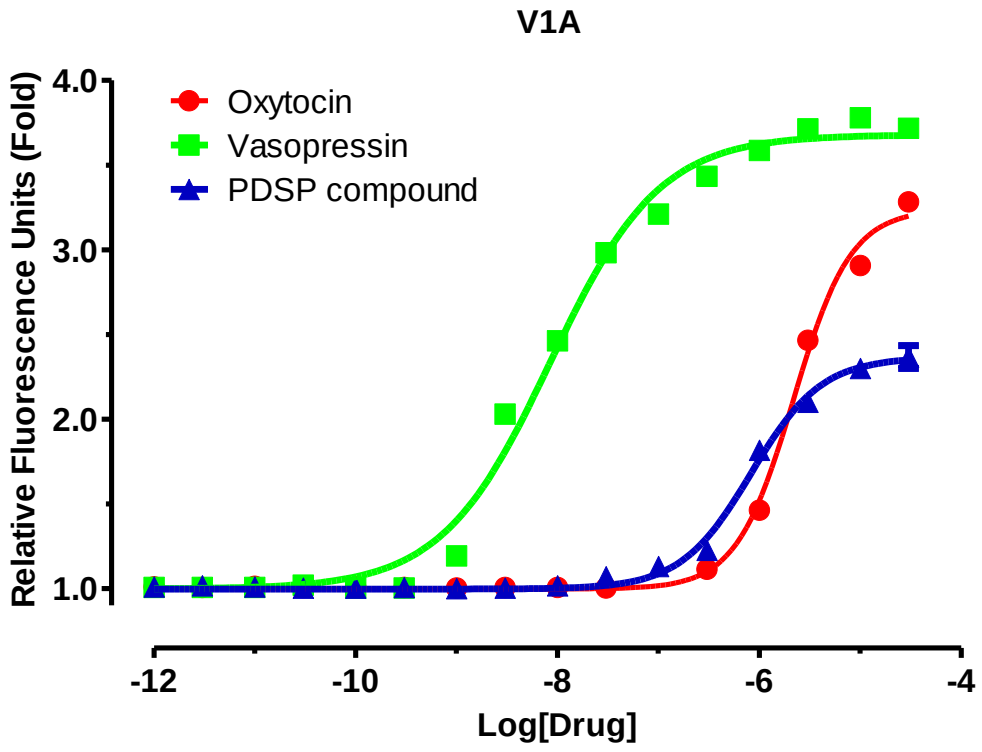

P2Y4
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
UTP
UDP
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

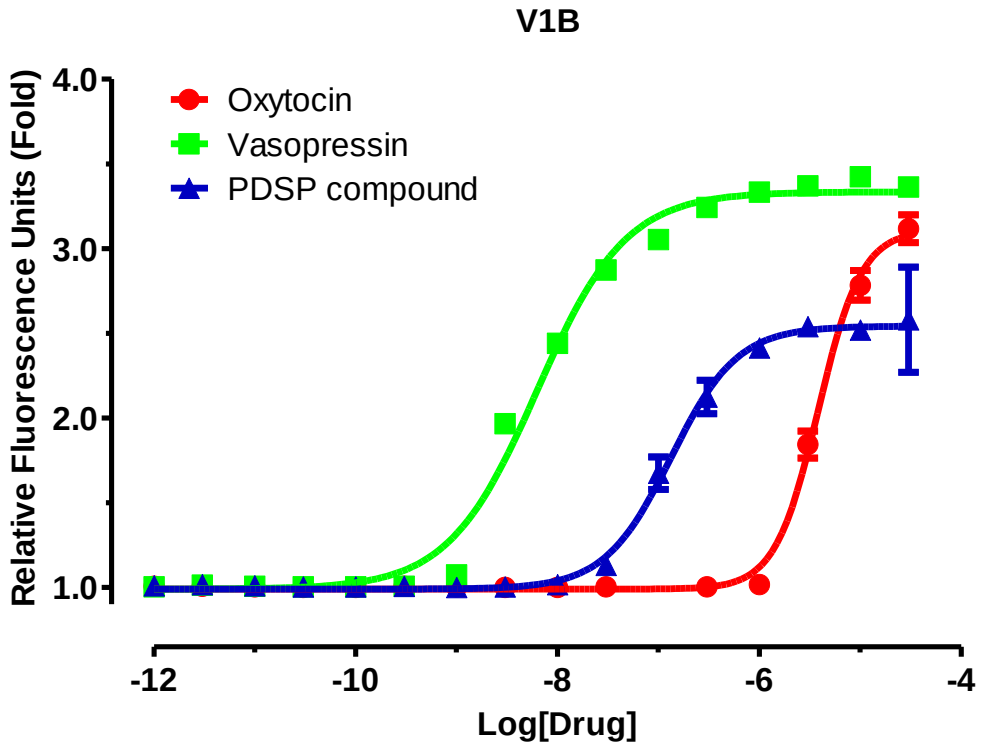

P2Y6
Relative agonist activity (%)
4.0
3.0
2.0
1.0
ATP
ADP
UTP
UDP
-12
-10
-8
-6
-4
Log[Drug]

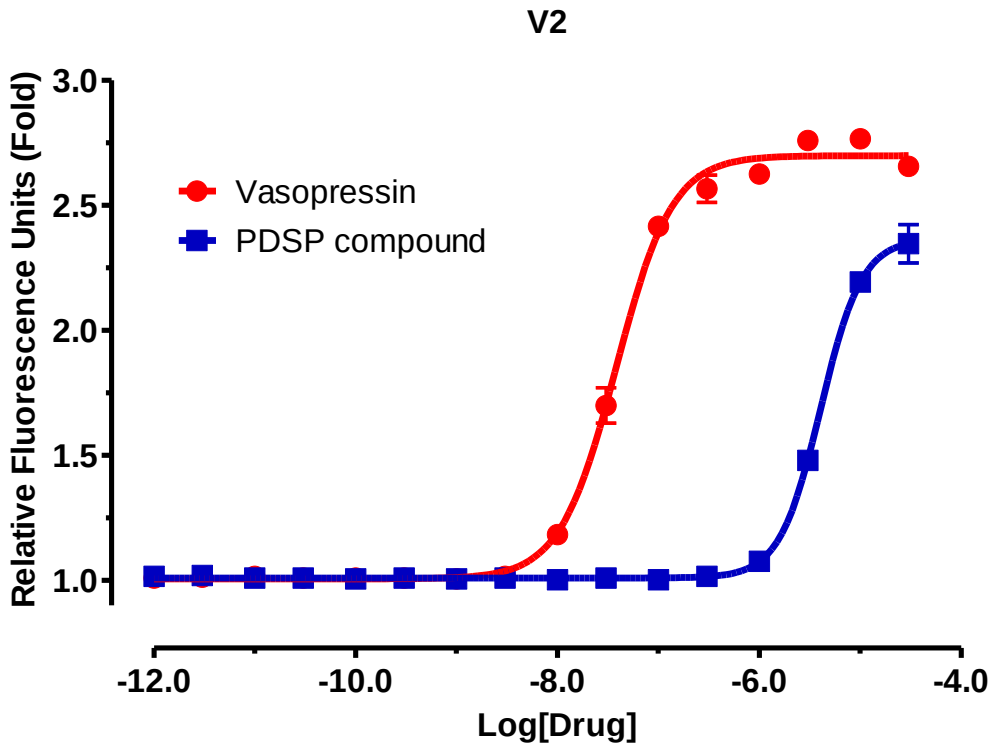

P2Y11
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
ATP
UTP
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

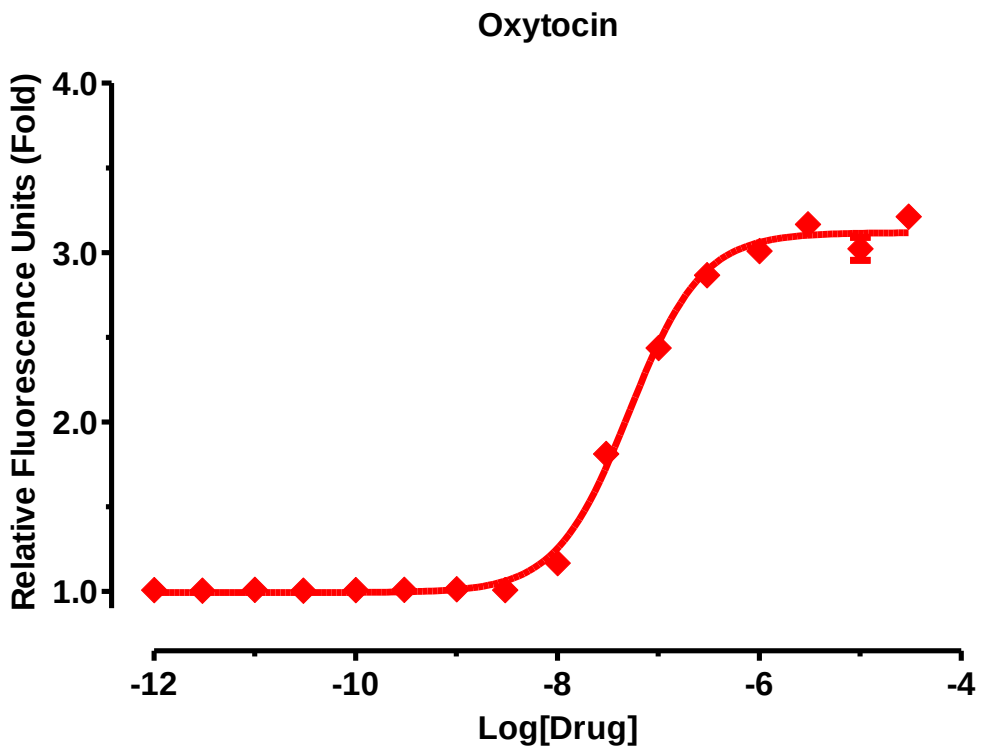

PAR1
Relative Luminescence Unit (Fold)
5.0
4.0
3.0
2.0
1.0
TRAP
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


PTAFR
Relative Fluorescence Units (Fold)
2.5
2.0
1.5
1.0
PTAFR
HEK293
-14
-12
-10
-8
-6
-4
Log[PTAF]

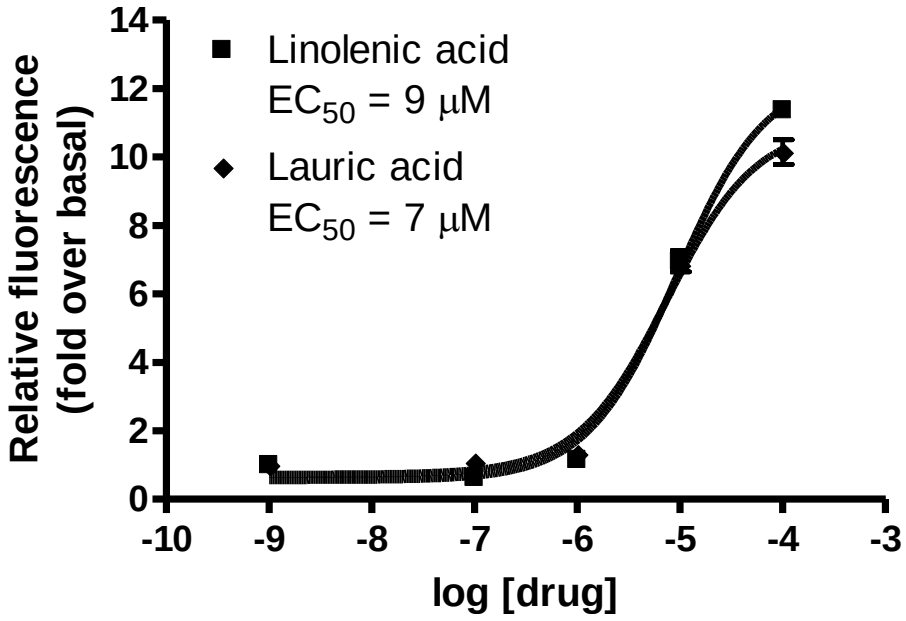

V1A
Oxytocin
Vasopressin
PDSP compound
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
-12
-10
-8
-6
-4
Log[Drug]


V1B
Oxytocin
Vasopressin
PDSP compound
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
-12
-10
-8
-6
-4
Log[Drug]

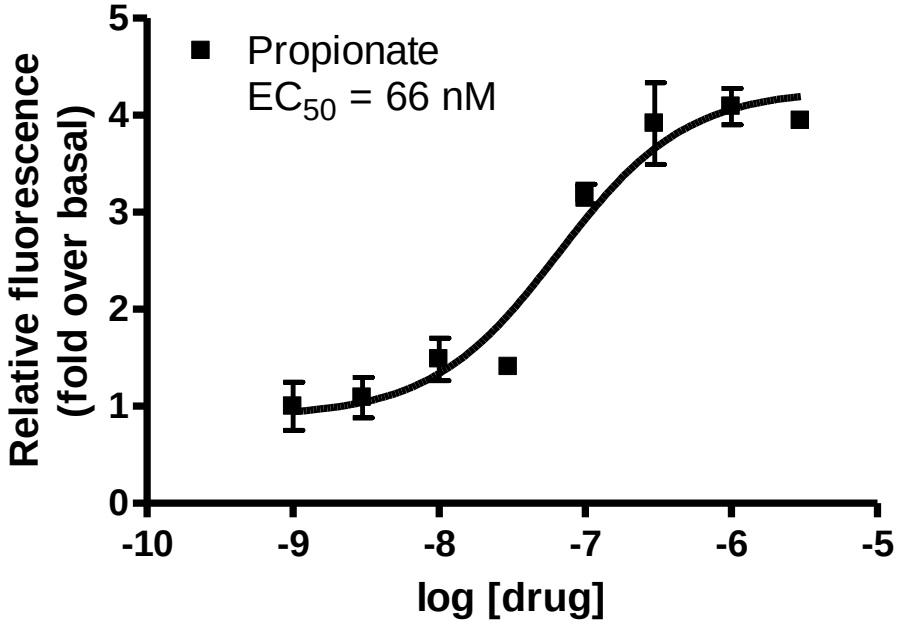

V2
Relative Fluorescence Units (Fold)
3.0
2.5
2.0
1.5
1.0
Vasopressin
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


Oxytocin
Relative Fluorescence Units (Fold)
4.0
3.0
2.0
1.0
-12
-10
-8
-6
-4
Log[Drug]

GPR40 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid



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



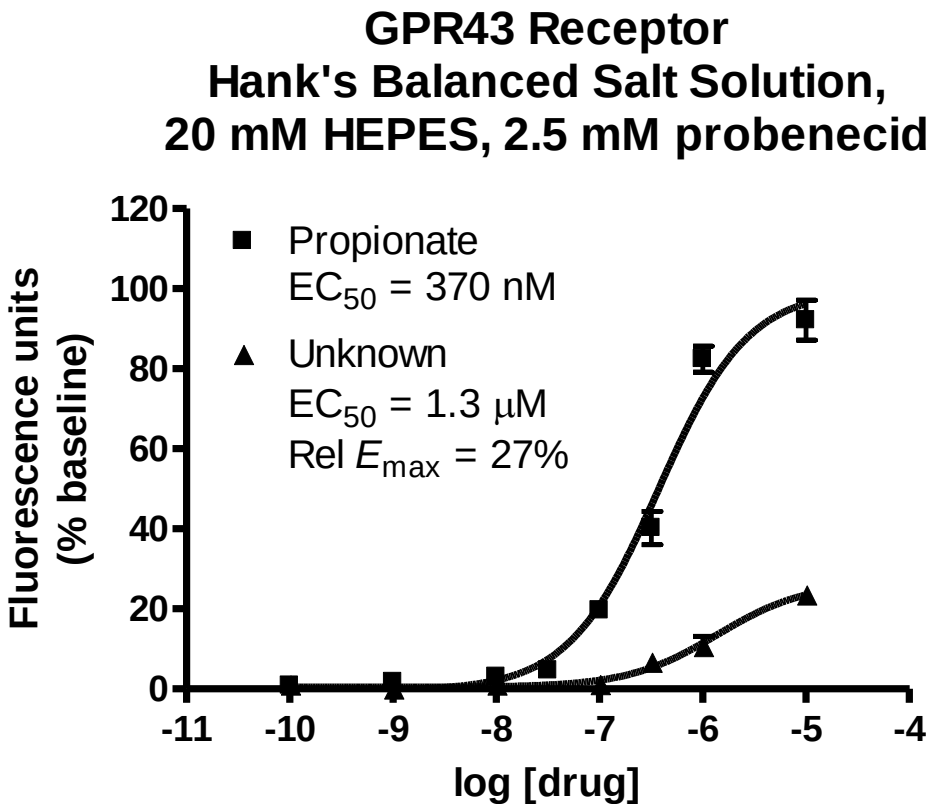

GPR43 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Fluorescence units
(% baseline)
120
100
80
60
40
20
0
Propionate
EC50 = 370 nM
Unknown
EC50 = 1.3 μM
Rel Emax = 27%
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]

Figure 36. Schild analysis with a PDSP compound at 5-HT2A receptors.

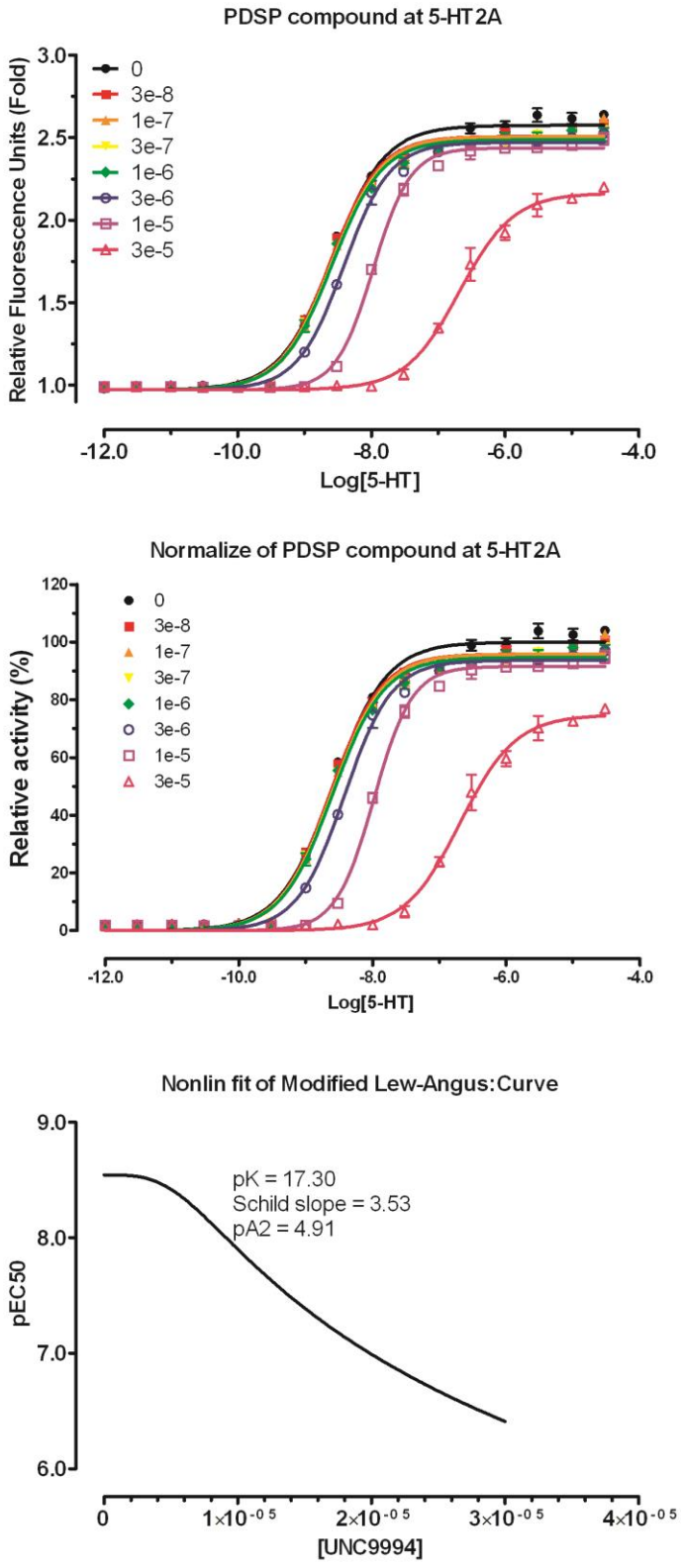


Figure 37. Schild analysis of a PDSP compound at 5-HT2B receptors

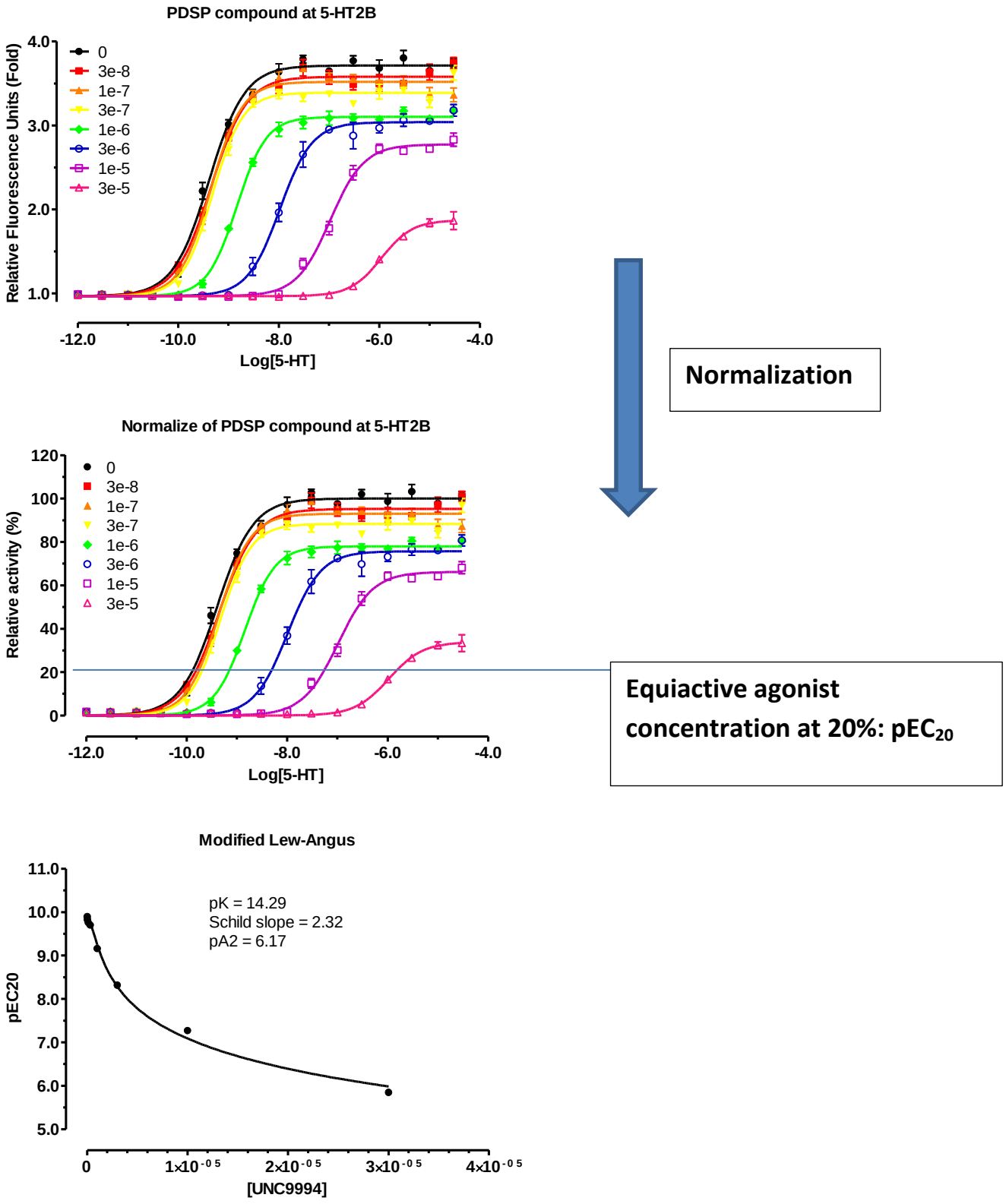


Figure 38. Schild analysis with a PDSP compound at 5-HT2C receptors.

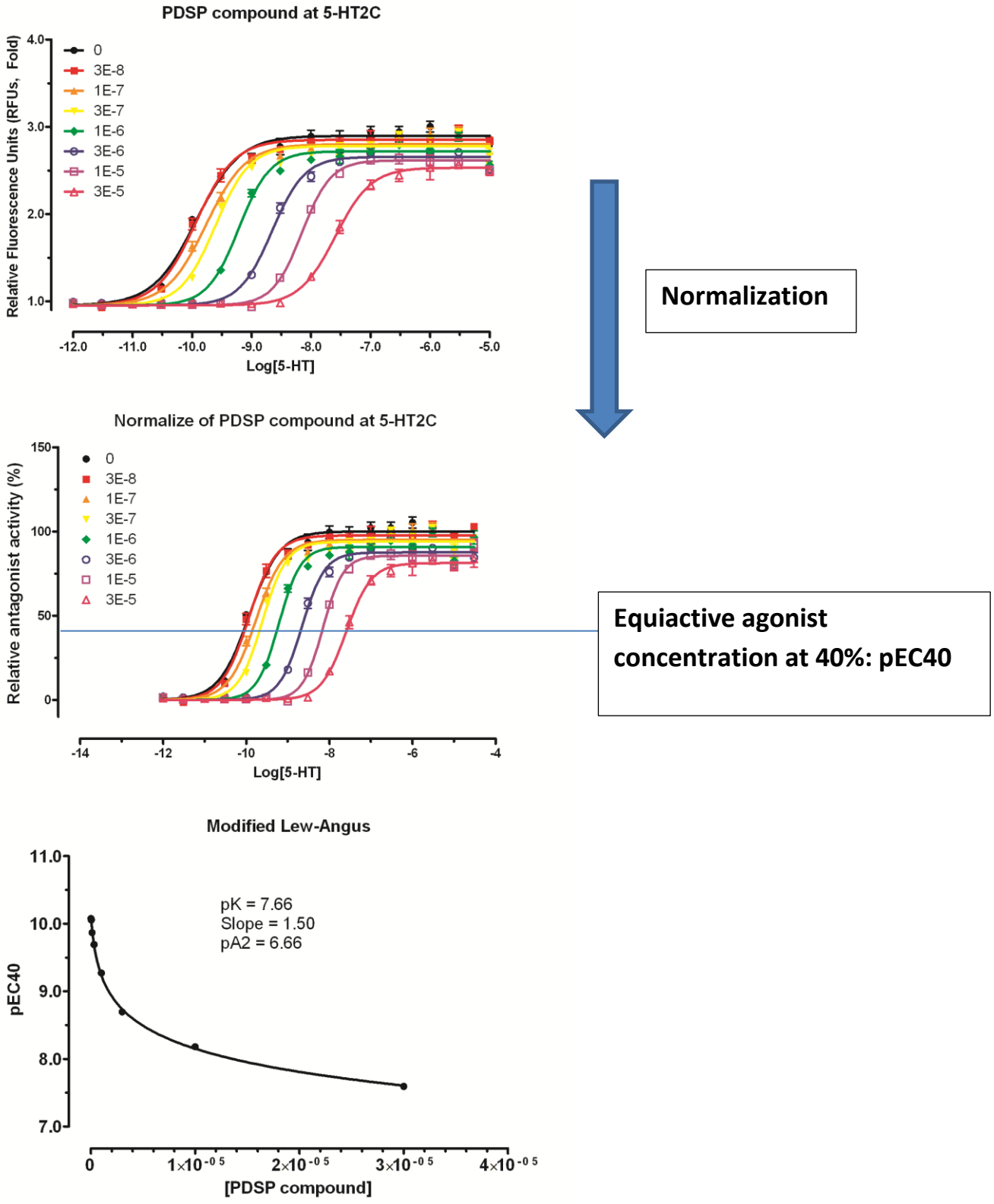
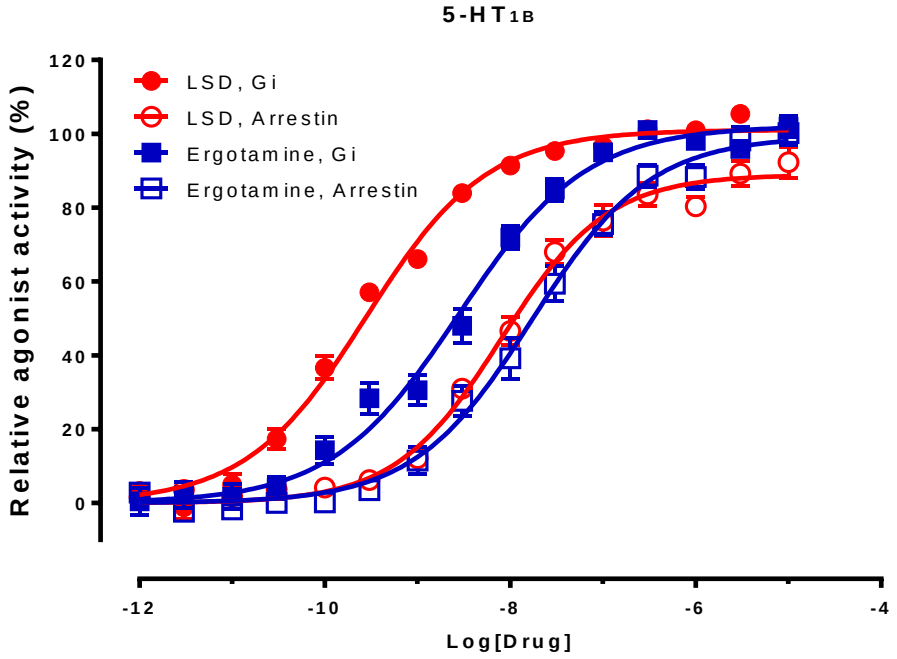


Figure 39. Representative figures for bias analysis. LSD and Ergotamine (ERG) agonist activity in G_i (for 5-HT_{1B}) or G_q (for 5-HT_{2B}) or β-arrestin signaling pathways were determined at 5-HT_{1B} and 5-HT_{2B} receptors as outlined in the functional assay section, and results were normalized to corresponding 5-HT activity and analyzed in Prism using the Black and Leff operational model to determine the transduction coefficient, Log(τ/K_A), as listed in **Tables 26 and 27**.



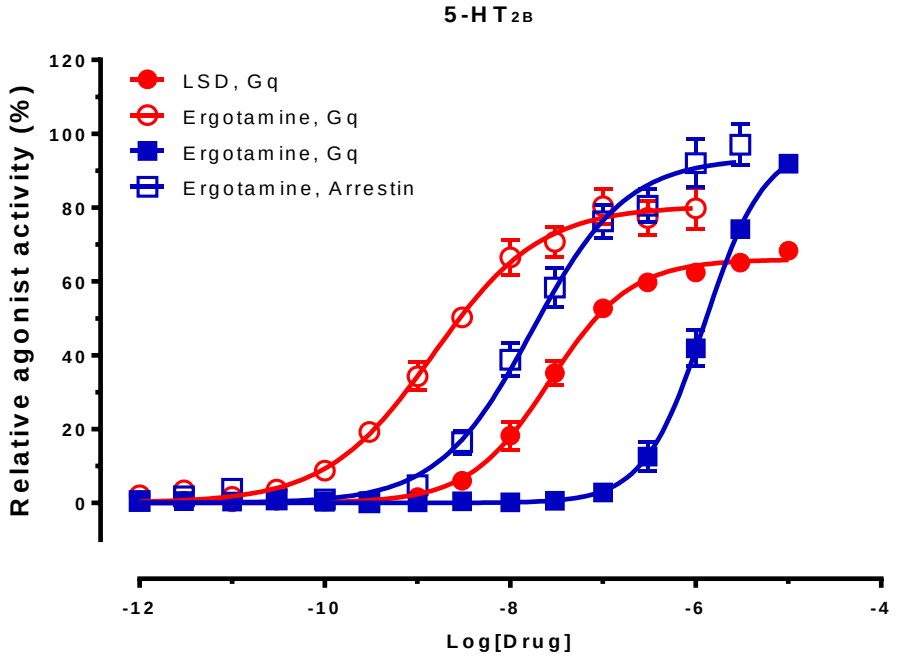


Table 26. Transduction coefficients, Log(τ/K_A), and bias factor calculation for indicated pathways of agonists at 5-HT_{1B}. See representative concentration-response curves in **Figure 39**. Bias factor = $10^{\Delta\Delta Log(\tau/KA)}$.

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

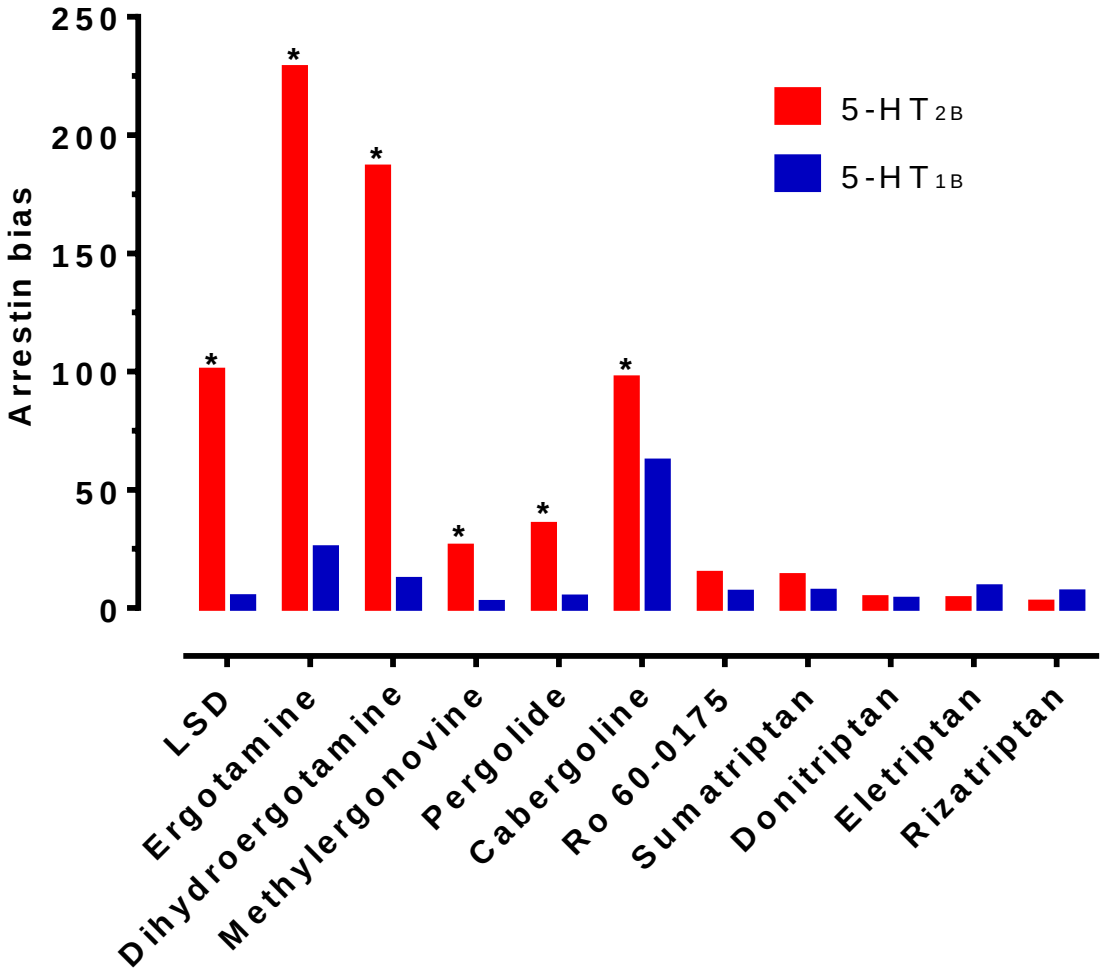
DHE = Dihydroergotamine; MTE = Methylergonovine

Table 27. Transduction coefficients, Log(τ/K_A), and bias factor calculation for indicated pathways of agonists at 5-HT_{2B}. See representative concentration-response curves in **Figure 39.**

Ligands	Bias calculation at 5-HT_{2B} receptors					
	G_q pathway	$\Delta Log(\tau/K_A)$	β-arrestin pathway	$\Delta Log(\tau/K_A)$	$\Delta\Delta Log(\tau/K_A)$	Bias Factor
5-HT	9.61 ± 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 40. Comparison of bias factors of various ligands at 5-HT_{1B} and 5-HT_{2B} receptors. Values are taken from Tables 26 and 27 and Figure 33. * indicates a significant difference ($p < 0.0001$ by two-way ANOVA, selected drugs at two pathways). Results (**Figure 39**, **Tables 26 and 27**, and **Figure 40**) are from a recent ***Science*** paper (DOI: 10.1126/science.1232808).



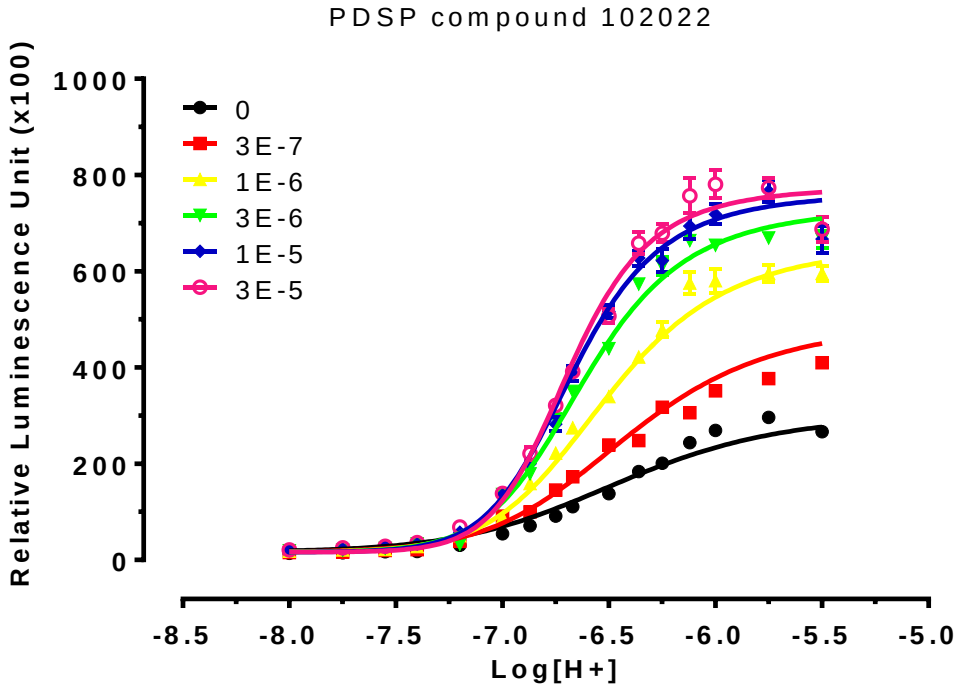


Figure 41. Representative curves for the allosteric operational model. GPR68-mediated cAMP production was determined in the absence and presence of increasing concentrations of PDSP compound 102022. Results were analyzed using the allosteric operational model and best-fit values are listed in the following table. In the curve-fitting, Log(K_A) is set at -6.50 (equivalent to the pEC_{50} of protons in the absence of 102022), while the E_{max} is constrained at 800 (which is the maximum activity of the system), and τ_B is constrained at "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 biosensor cAMP assays

Main equipment: luminescence counter

Reagents: GloSensor cAMP construct from Promega and Luciferin

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

Note: See section 2.5.5 protocols for G_i coupled mGluRs

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 cells stably expressing target receptors, we transfect with the GloSensor cAMP DNA construct overnight; otherwise, we co-transfect HEK293 T cells with target receptor DNA and GloSensor cAMP DNA construct overnight. For detailed calcium phosphate transfection protocol, see Section 2.4.1.2. To prepare plates for assays, cells are seeded into PLL coated 384-well white clear bottom cell culture plates with DMEM supplemented with 1% dFBS at a density of 15-20,000 cells in 40 µl medium 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: On the day of the assay, wells are emptied of culture medium and loaded for 90 min at 37°C with 20 µl of 4 mM Luciferin prepared in assay buffer. All the following steps are carried out at room temperature. To measure agonist activity at Gs coupled receptors, 10 µl of 3x drug solutions is added per well and plates are counted for chemiluminescence after 15 minutes. To measure antagonist activity at Gs coupled receptors, cells are preincubated with test compounds 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 Gi coupled receptors, 10 µl 3x test drug solution is added for 15 minutes before addition of 5 µl of isoproterenol at a final concentration of 200 nM, followed by counting of the plate for chemiluminescence after 15 minutes. To measure antagonist activity at Gi coupled receptors, cells are preincubated with test drug for 15 minutes

before an EC_{80} concentration of a reference agonist is added for another 15 minutes, then, 5 µl of isoproterenol at a final concentration of 200 nM is added, and counting is done after 15 minutes. In these experiments, isoproterenol is used to activate the endogenous Gs protein via the endogenous $ß_2$ adrenergic receptors expressed in HEK293 T cells. Different receptors and different cell lines may need different preincubation times; when these are done, especially before larger scale screening assays, preliminary assays are performed to optimize count windows and incubation times.

2.5.3. Split luciferase biosensor cAMP assay – Drug first protocol: On the day of the assay, wells are emptied of 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 Gs 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 Gi coupled receptors, 10 µl of 4 mM Luciferin supplemented with isoproterenol at a final concentration 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; this is followed by addition of 10 µl of 4 mM Luciferin supplemented with isoproterenol at a final concentration of 200 nM, and counting is done after 15 minutes. Different receptors and different cell lines may need different preincubation times; preliminary assays are performed to optimize incubation times and count windows before large scale screening assays.

2.5.4. Split luciferase biosensor cAMP assay protocol for G_i-coupled mGluRs: $mGluR_2$, $mGluR_3$, $mGluR_4$, $mGluR_6$, and $mGluR_8$.

Assay Buffer 1 ($mGluR_2$, $mGluR_4$, $mGluR_6$, $mGluR_8$): Modified Locke's buffer containing 125 mM NaCl, 31 mM NaGluconate, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM glucose, and 20 mM HEPES, pH 7.4)

Assay Buffer 2 ($mGluR_3$): Modified Locke's buffer containing 156 mM Na Gluconate, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM glucose, and 20 mM HEPES, pH 7.4)

2.5.4.1. General experimental procedure: The second type of glutamate receptor functional assays consists of measurements of cAMP production and is used with receptors negatively coupled to adenylyl cyclase (Gi-coupled receptors). These are $mGluR_2$, $mGluR_3$, $mGluR_4$, $mGluR_6$, and $mGluR_8$ receptors expressed in CHO cells also stably expressing the GloSensor cAMP biosensor. The activity of agonists at these receptors is determined by measurement of their ability to decrease forskolin-induced elevation of cyclic AMP formation. Cells cultured in white-walled, clear-bottom, 96-well culture plates are preincubated for 1 hour at room temperature in 100 µl of the aforementioned Locke's medium containing 6% w/v D-Luciferin, as a substrate for the GloSensor enzyme. After 1 hour, the basal RLUs are measured 5 times, every 2 min. 1 µM forskolin and 6% w/v D-Luciferin is added without or with mGluR agonists, and the incubation is continued for 16 min. After the incubation, relative luminescence units (RLUs) are measured. All of these studies are performed in the absence and in the presence of agonists appropriate for the different mGluRs, and used at concentrations equivalent to their EC_{50} values. Hence, 3 µM glutamate is used for $mGluR_2$, $mGluR_3$, 4 µM glutamate is used for $mGluR_4$, 2 µM glutamate is used for $mGluR_6$, and 0.7 µM glutamate is used for $mGluR_8$.

2.5.4.2. Primary assays - Single concentration assays. Each new compound is tested on selected 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 cell passages. In addition to the tested compounds, each 96-well plate contains points 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 percent of maximal activity (as obtained with maximal agonist concentrations), and for antagonists 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.

2.5.4.3. Secondary assays - Concentration-response assays. Compounds determined to be active as agonists or antagonists may be tested for their potency in concentration-response experiments. Eight-point concentration-response curves are performed in duplicate twice on two separate passages 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 the 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 also include the EC_{50} or IC_{50} values of a known agonist or antagonist for comparison purposes.

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

2.5.6. Table and Figures. List of GPCRs for which the PDSP provides functional assays to determine Gi or Gs activity and their corresponding concentration-response curves. The PDSP will also provide functional assays for other Gi or Gs coupled GPCRs upon request.

Table 28. List of GPCRs for which the PDSP provides cAMP measurement using GloSensor cAMP technology for Gi or Gs coupled receptors, and representative figures.

Receptor	Cell line	Reference	Emax (fold)[#]	pEC50	Hill slope
M2	HEK T	Acetylcholine	2.2	7.63	0.72
M2D	HEK T	CNO	3.2	6.27	0.86
M4	HEK T	Acetylcholine	2.6	8.16	1.07
M4D	HEK T	CNO	3.6	7.07	1.78
GsDREADD	HEK T	CNO	9.9	7.87	1.53
5-HT1A	HEK T	5-HT	5.8	8.71	1.02
5-HT1B	HEK T	5-HT	5.1	9.71	0.94
5-HT1D	HEK T	5-HT	2.0	9.44	1.77
5-HT1E	HEK T	5-HT	3.2	9.12	0.87
5-HT1F					
5-HT4	HEK T	5-HT	2.3	10.18	0.98
5-HT5A					
5-HT6	HEK T	5-HT	24.5	7.06	1.25
5-HT7A	HEK T	5-HT	60.1	8.60	1.58
A2A	HEK T	NECA	2.4	9.58	0.74
A2A	HEK T	CCPA	1.9	7.80	1.42
A2A	HEK T	CGS21680	2.2	9.67	0.72
CRF-1	HEK T	CRF	100	8.57	0.76
CRF-2	HEK T	CRF	122	7.26	0.95
D1	HEK T	Dopamine	148.1	8.18	0.94
D2	HEK T	Dopamine	2.8	8.99	1.01
D3					
D4	HEK T	Dopamine	2.0	9.19	1.67
D5	HEK T	Dopamine	37.6	9.24	0.80
H2	HEK T	Histamine	40.0	8.07	1.08
H3	HEK T	Histamine	4.6	8.90	0.70
ß2	HEK T	Isoproterenol	54.3	10.71	0.94
DOR	HEK T	DADLE	2.7	7.36	0.94
KOR	HEK T	Salvinorin A	2.4	9.43	1.33
MOR	HEK T	DAMGO	3.2	8.94	0.75
NOP	HEK T	Nociceptin	3.6	8.64	1.06
MC1	HEK T	α-MSH	31.5	7.47	1.01
MC3	HEK T	α-MSH	188.7	7.31	1.05
mGluR2	CHO	Glutamate	75.2%*	5.62	1.30
mGluR3	CHO	Glutamate	83.2%*	5.54	1.60
mGluR4	CHO	Glutamate	91.0%*	5.42	1.50
mGluR6	CHO	Glutamate	60.2%*	5.92	1.50
mGluR7	In progress				

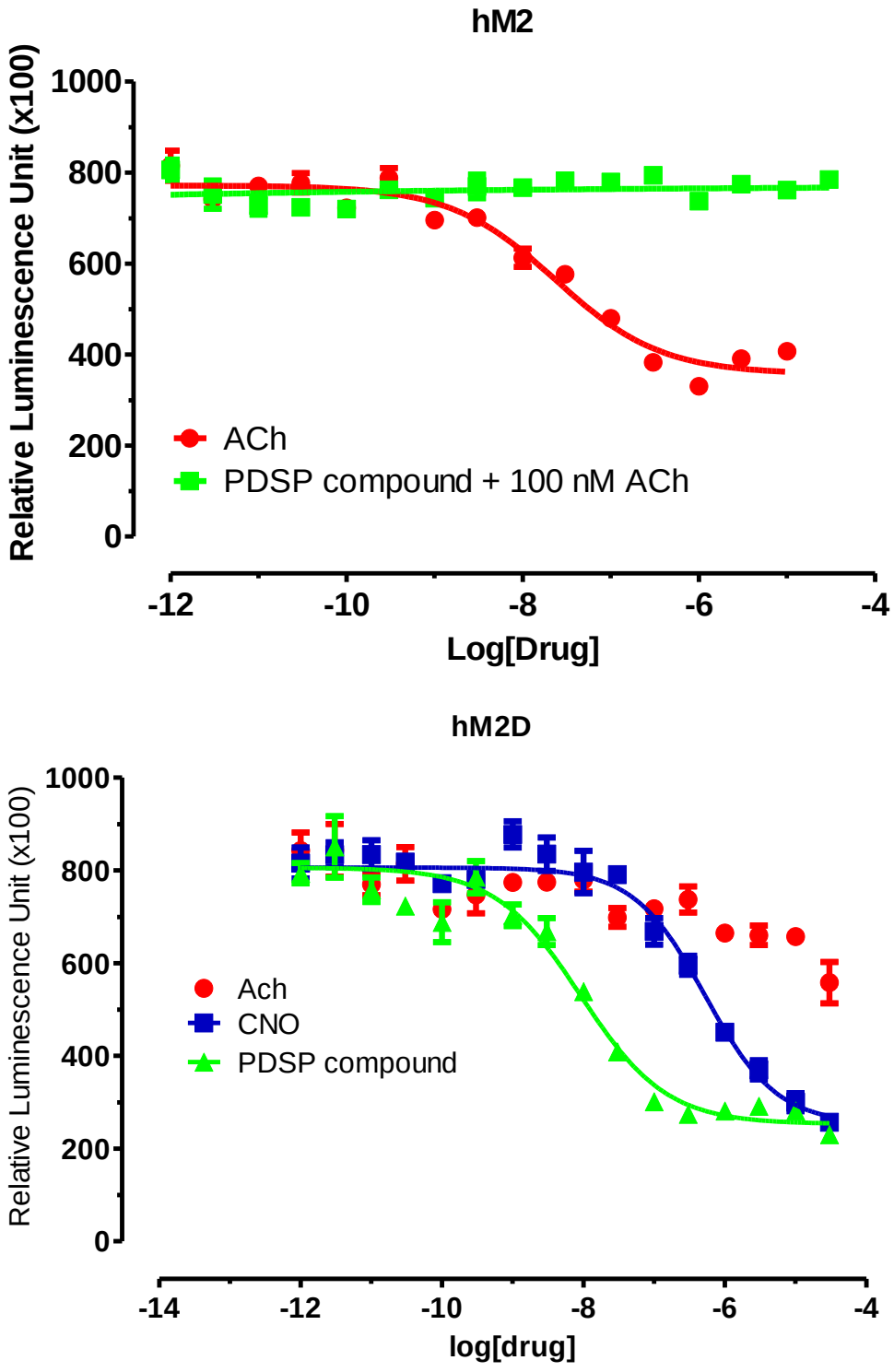
Receptor	Cell line	Reference	Emax (fold)[#]	pEC50	Hill slope
mGluR8	CHO	Glutamate	52.6%*	6.18	1.70
SSTR5	HEK T	Somatostatin	7.8	8.63	1.66
GPR88	HEK T	PDSP cmpd	6.1	6.00	0.74
GPR68	HEK T	H^+	23.2	6.50	1.64

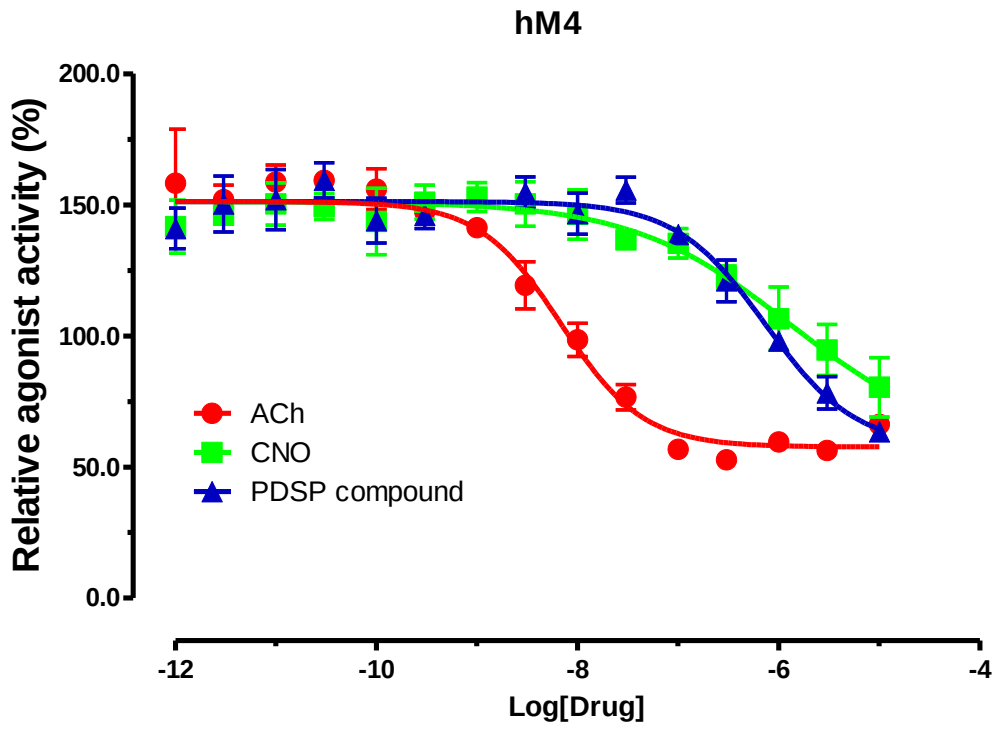
Notes

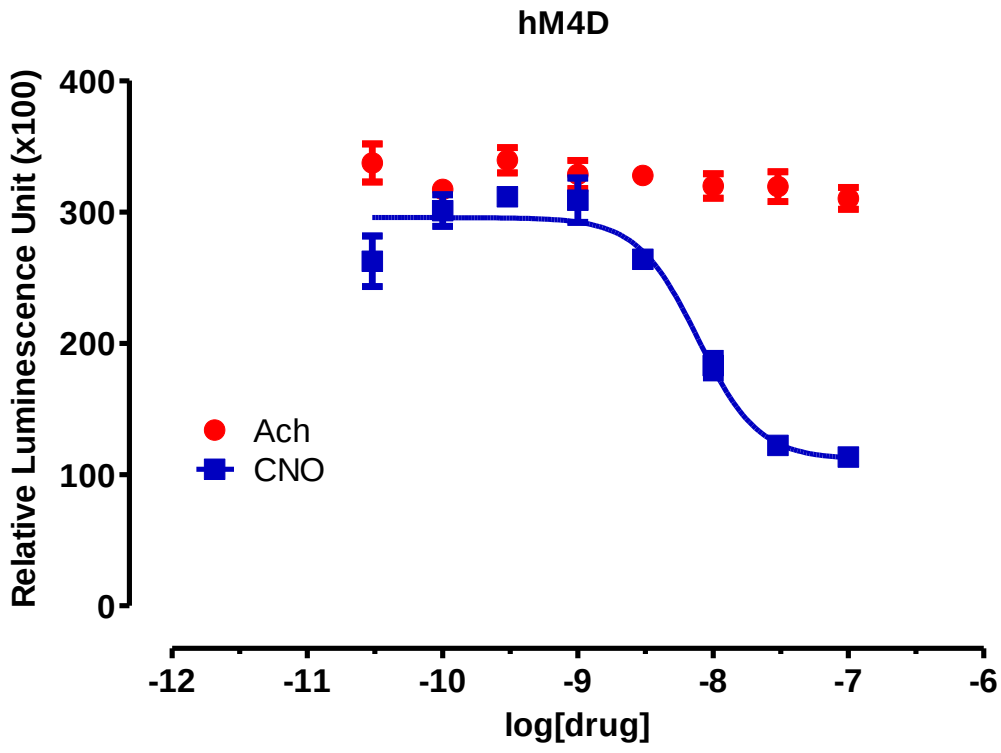
*: percentage inhibition of Forskolin-activated cAMP production

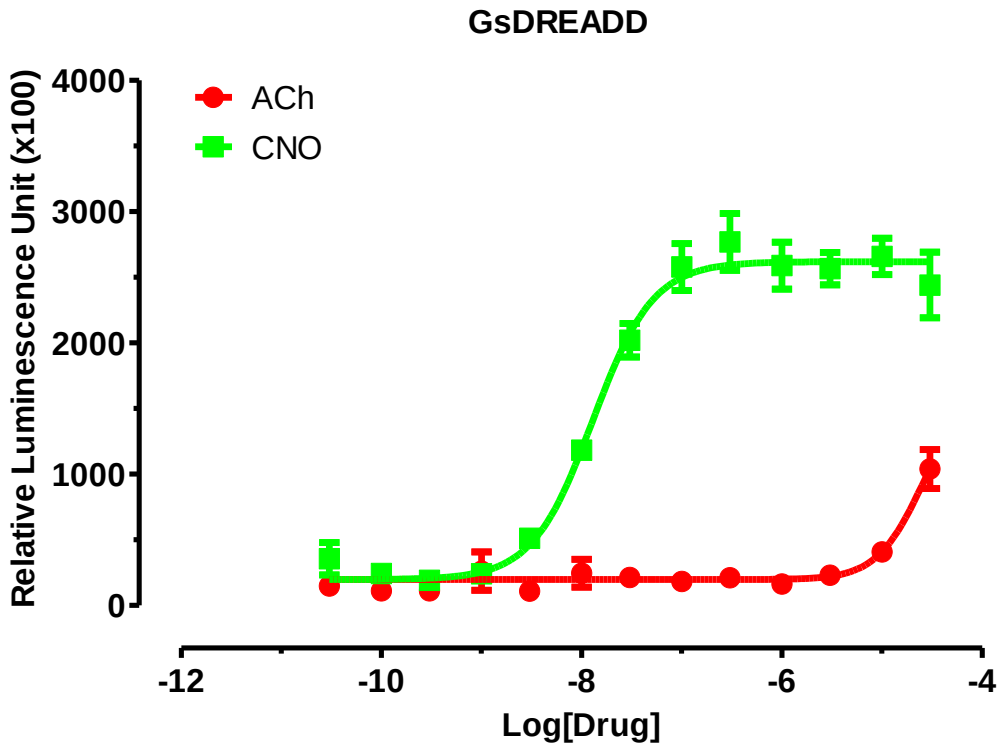
#: Emax in fold for Gi pathway represents the ratio of basal vs maximal activity; Emax in fold for Gs pathway represents the ratio of maximal vs basal activity.

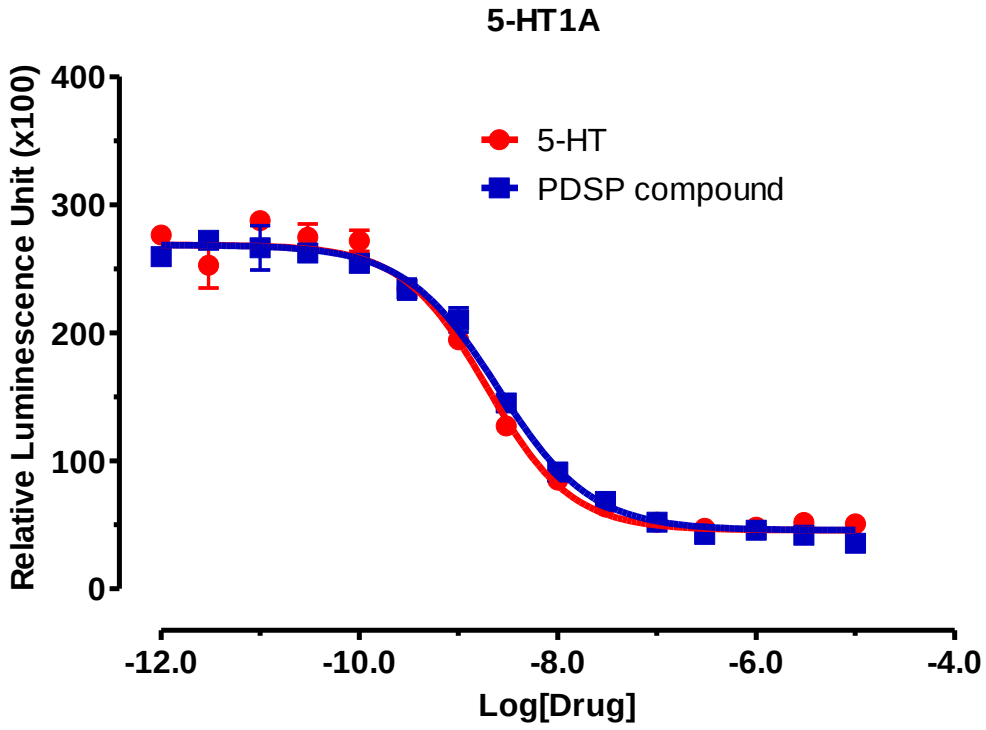
Figure 42. Representative concentration-response curves for the GloSensor cAMP assay. The assays were conducted according to the above procedure and were analyzed using GraphPad Prism 5.0.

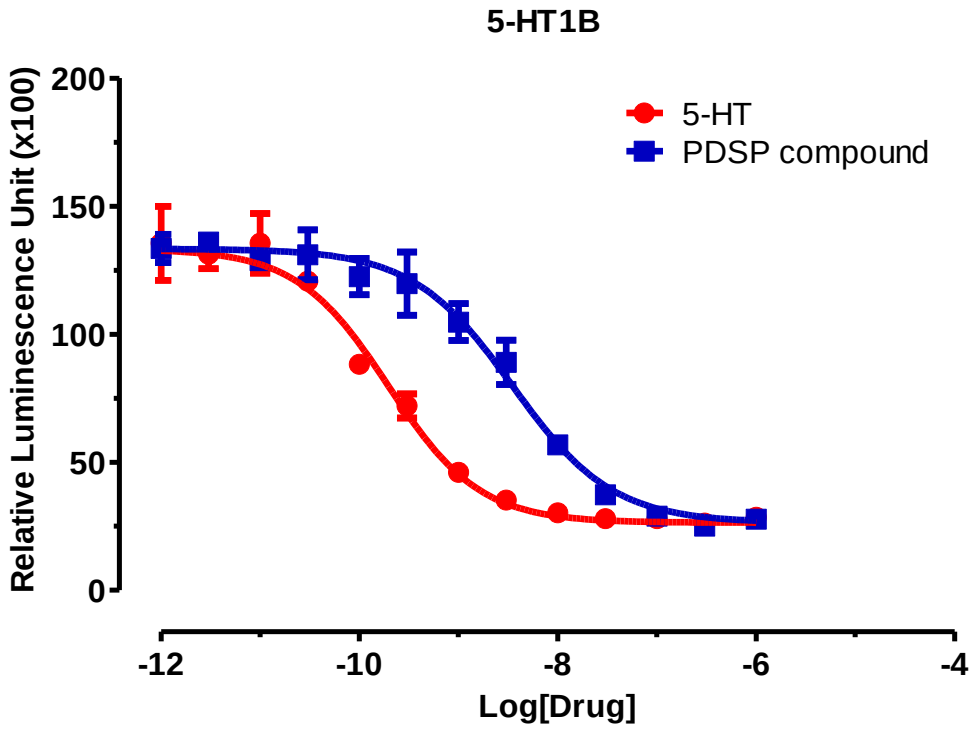


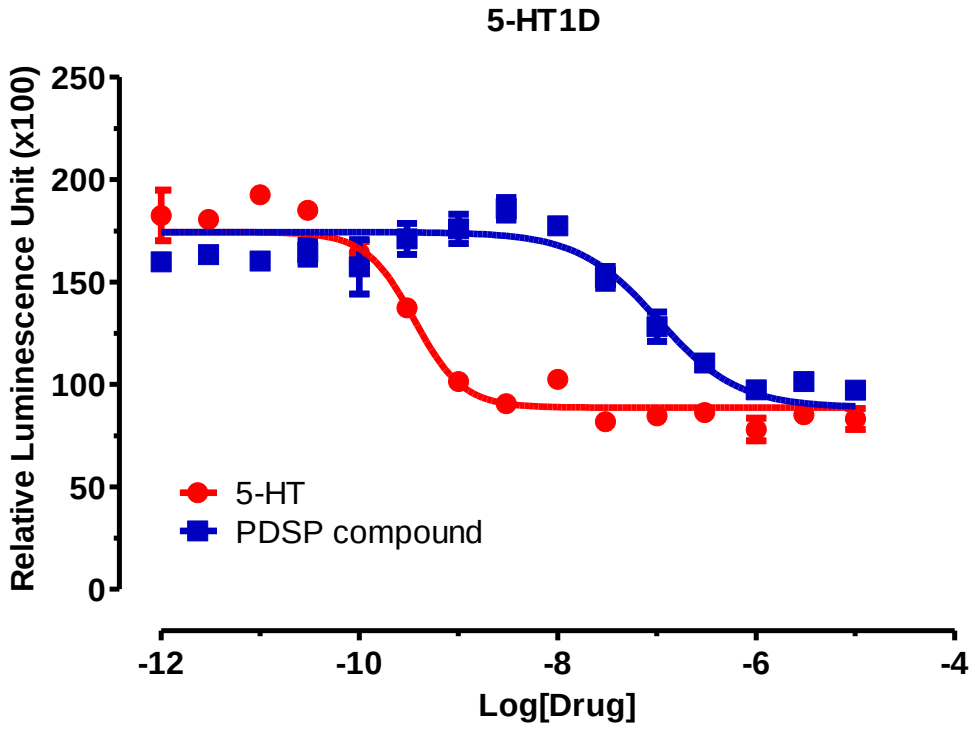

hM4
Relative agonist activity (%)
200.0
150.0
100.0
50.0
0.0
ACh
CNO
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

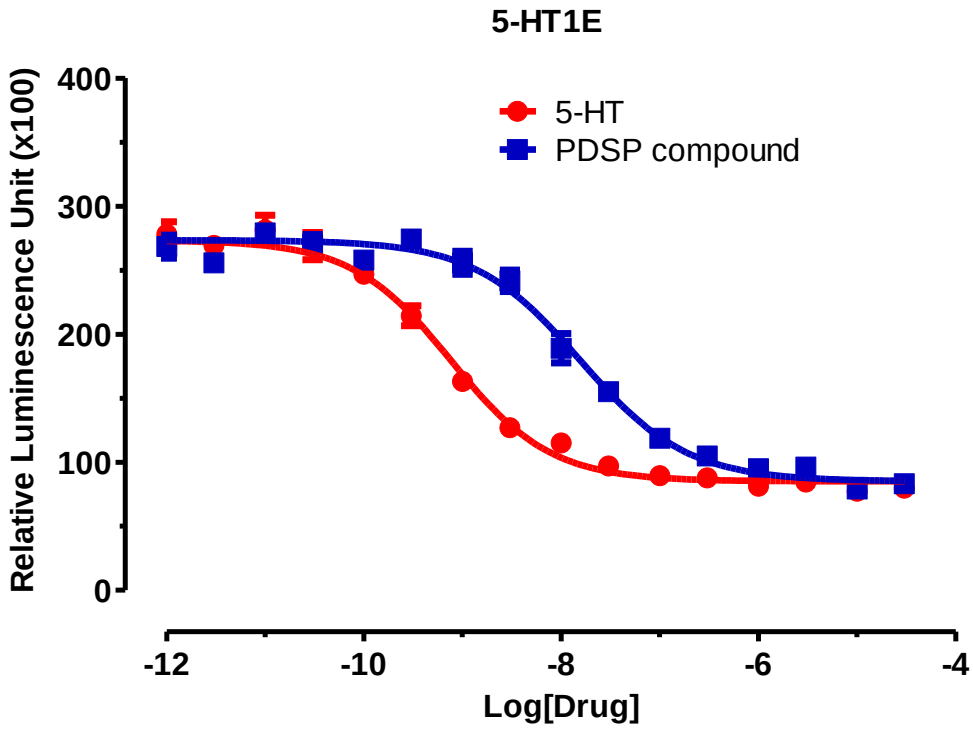

hM4D
Relative Luminescence Unit (x100)
400
300
200
100
0
Ach
CNO
-12
-11
-10
-9
-8
-7
-6
log[drug]

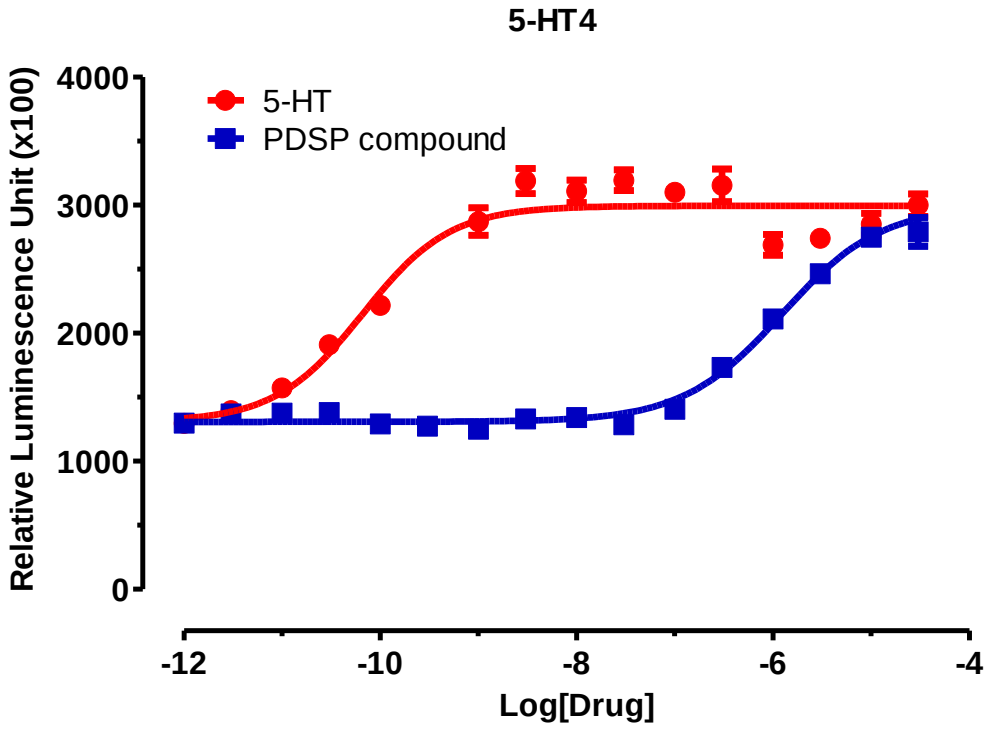

GsDREADD
Relative Luminescence Unit (x100)
4000
3000
2000
1000
0
ACh
CNO
-12
-10
-8
-6
-4
Log[Drug]

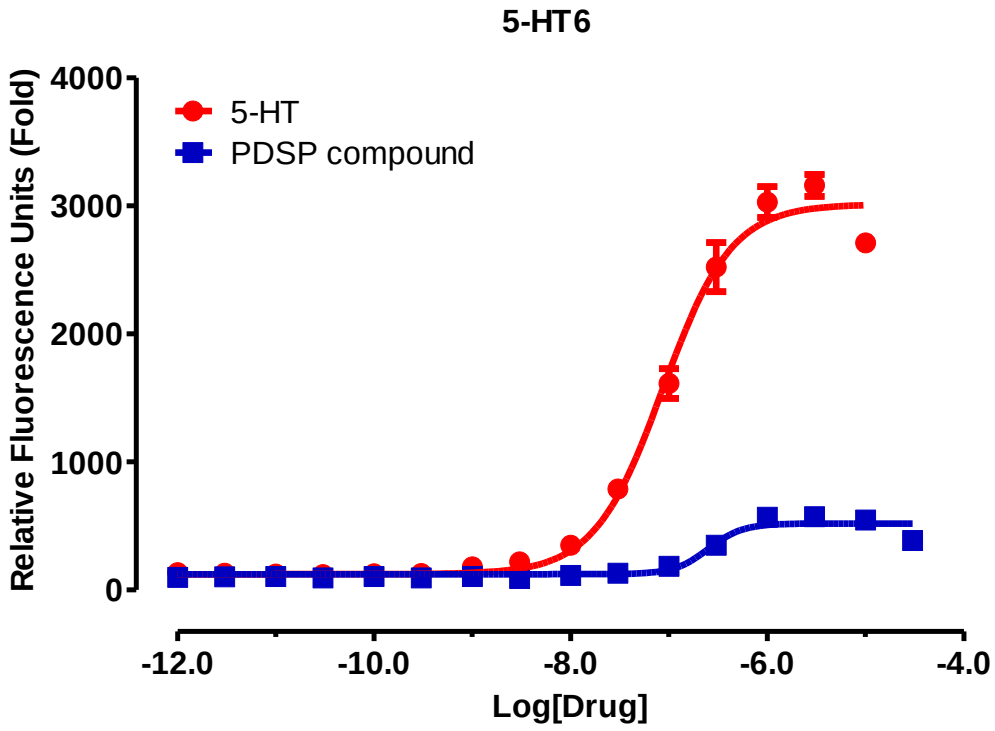

5-HT1A
Relative Luminescence Unit (x100)
400
300
200
100
0
5-HT
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

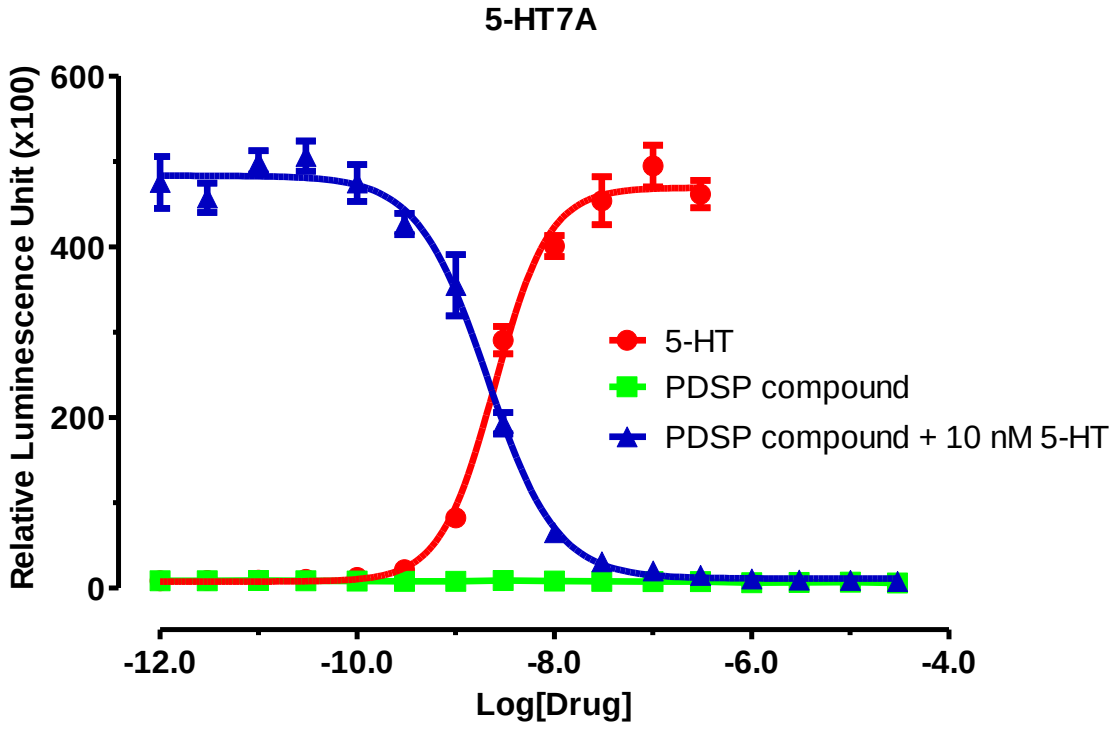

5-HT1B
Relative Luminescence Unit (x100)
200
150
100
50
0
5-HT
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

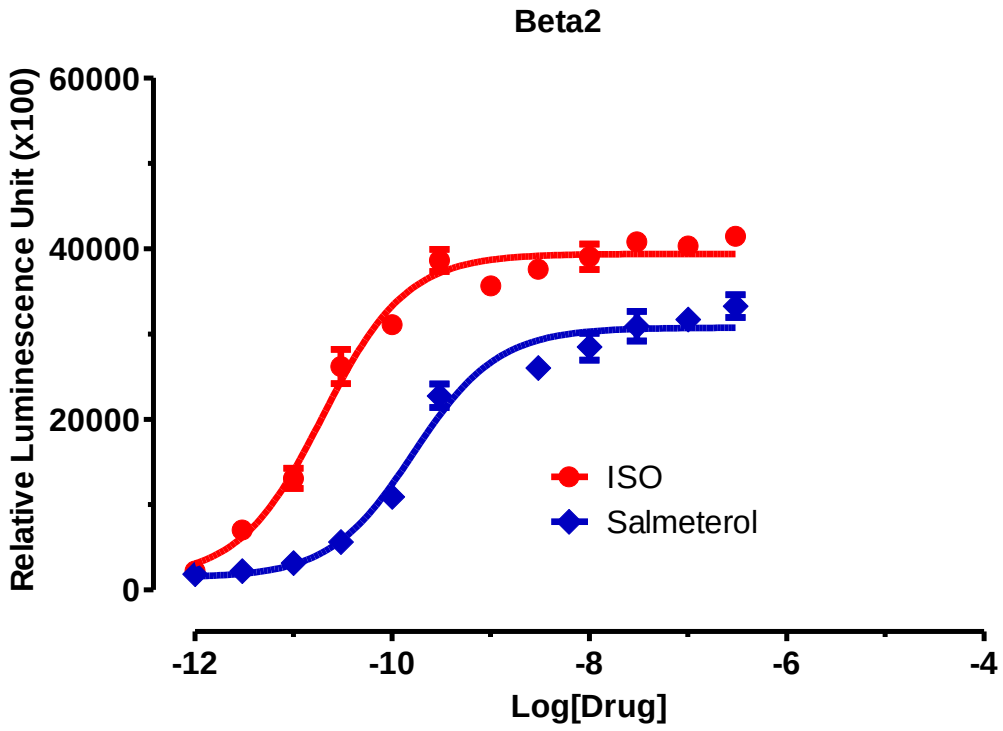

5-HT1D
Relative Luminescence Unit (x100)
250
200
150
100
50
0
5-HT
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

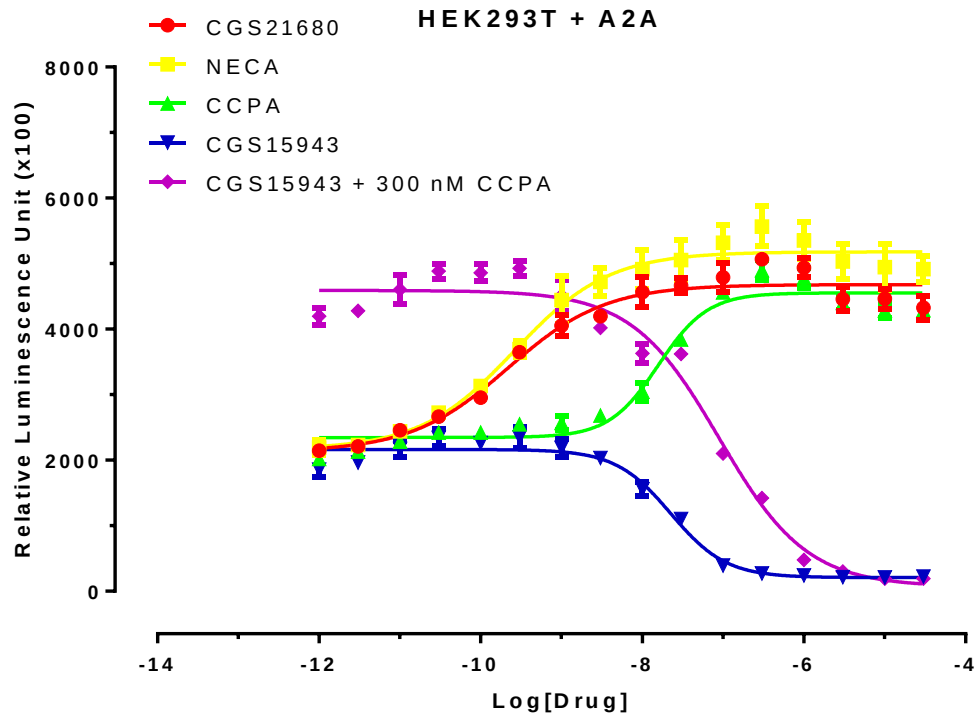

5-HT1E
Relative Luminescence Unit (x100)
400
300
200
100
0
5-HT
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

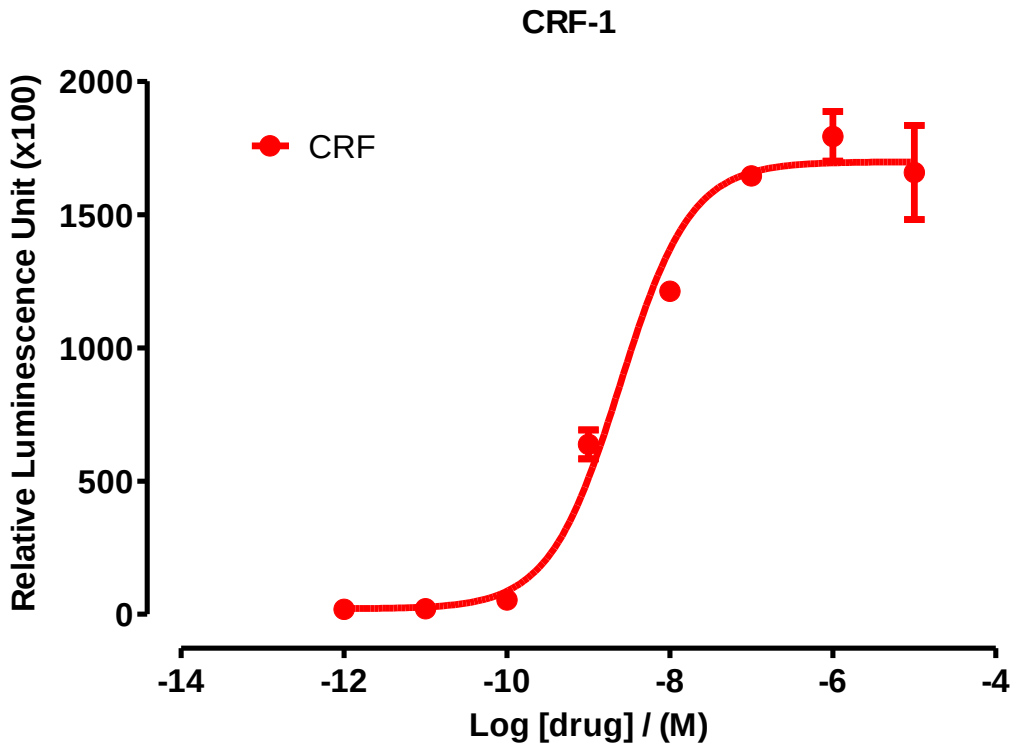

5-HT4
Relative Luminescence Unit (x100)
4000
3000
2000
1000
0
5-HT
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

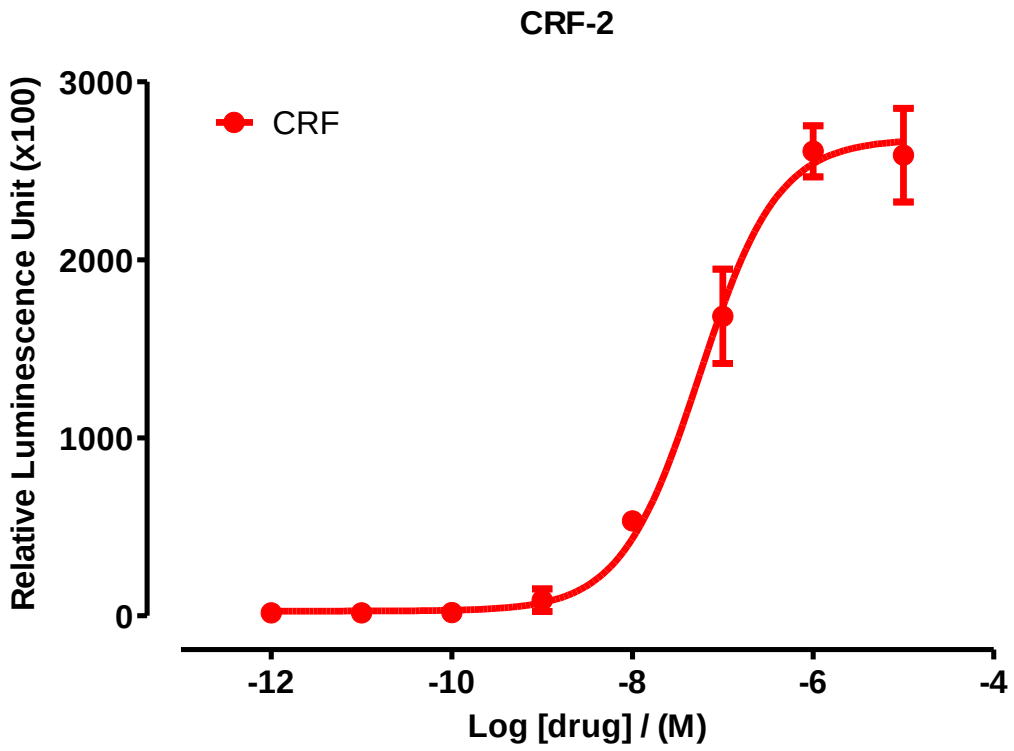

5-HT6
Relative Fluorescence Units (Fold)
4000
3000
2000
1000
0
5-HT
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


5-HT7A
Relative Luminescence Unit (x100)
600
400
200
0
5-HT
PDSP compound
PDSP compound + 10 nM 5-HT
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]


Beta2
Relative Luminescence Unit (x100)
60000
40000
20000
0
-12
-10
-8
-6
-4
Log[Drug]
ISO
Salmeterol


HEK293T + A2A
CGS21680
NECA
CCPA
CGS15943
CGS15943 + 300 nM CCPA
Relative Luminescence Unit (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Drug]





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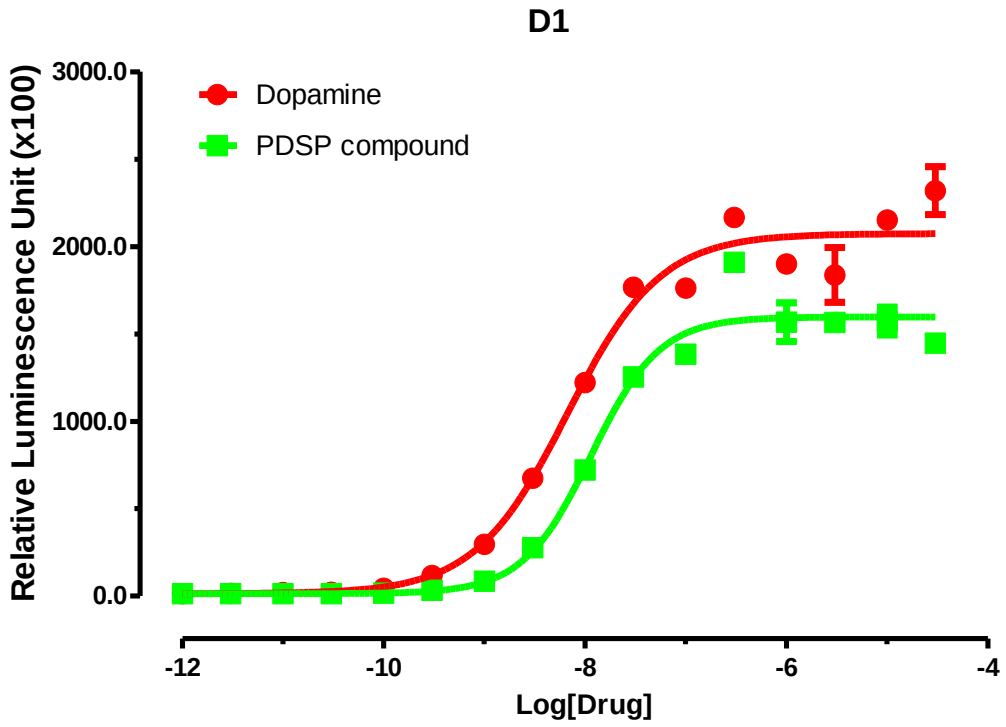

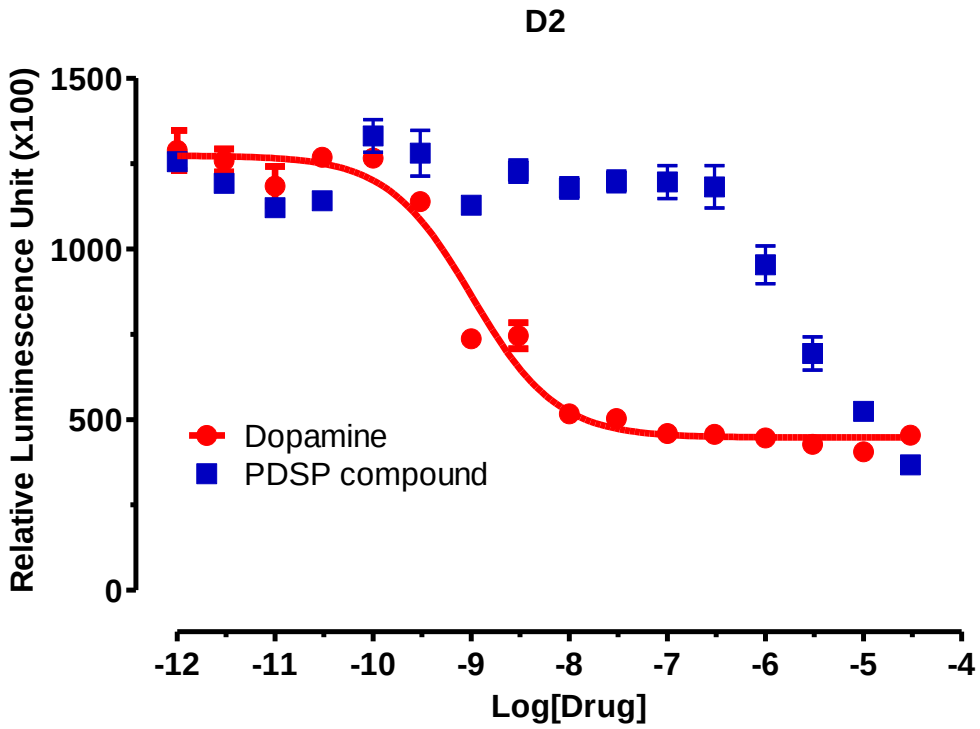

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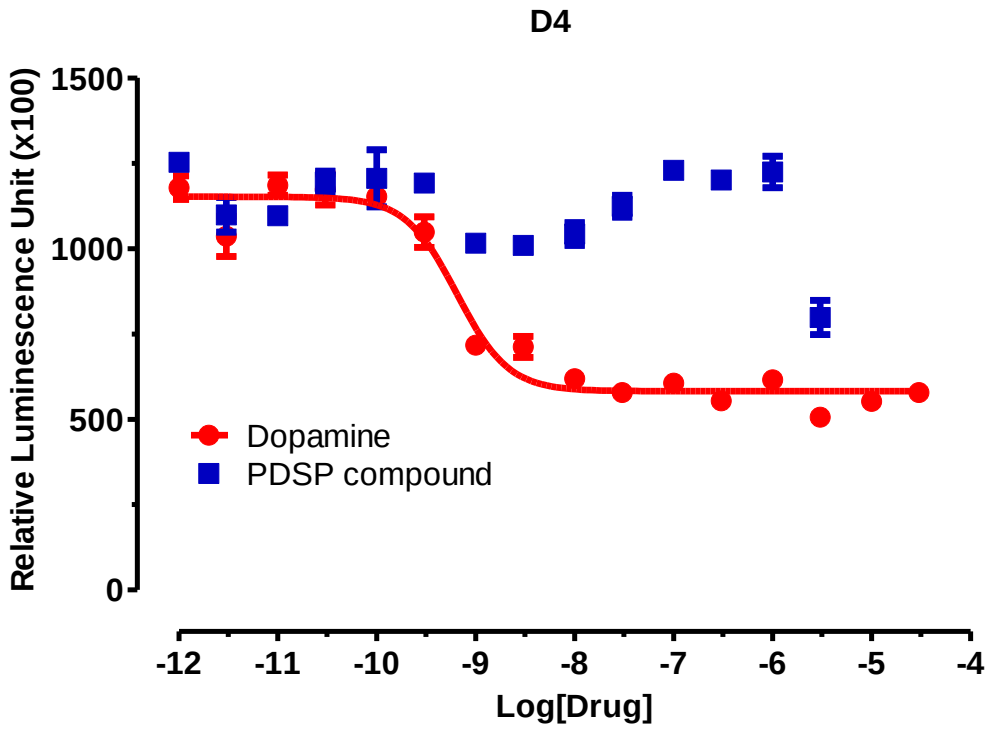
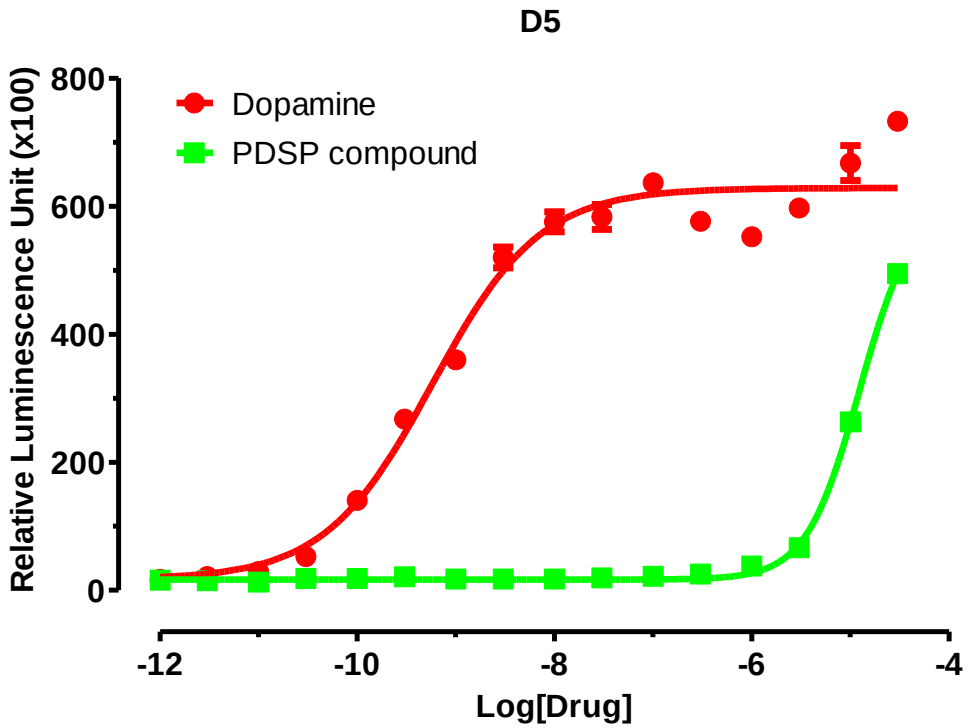
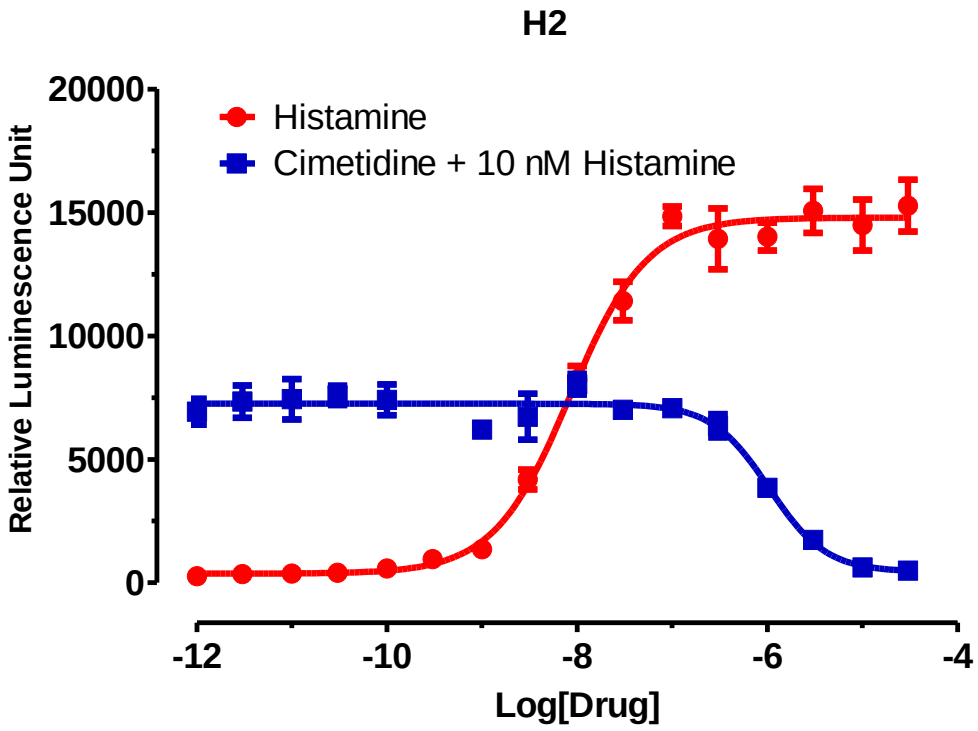
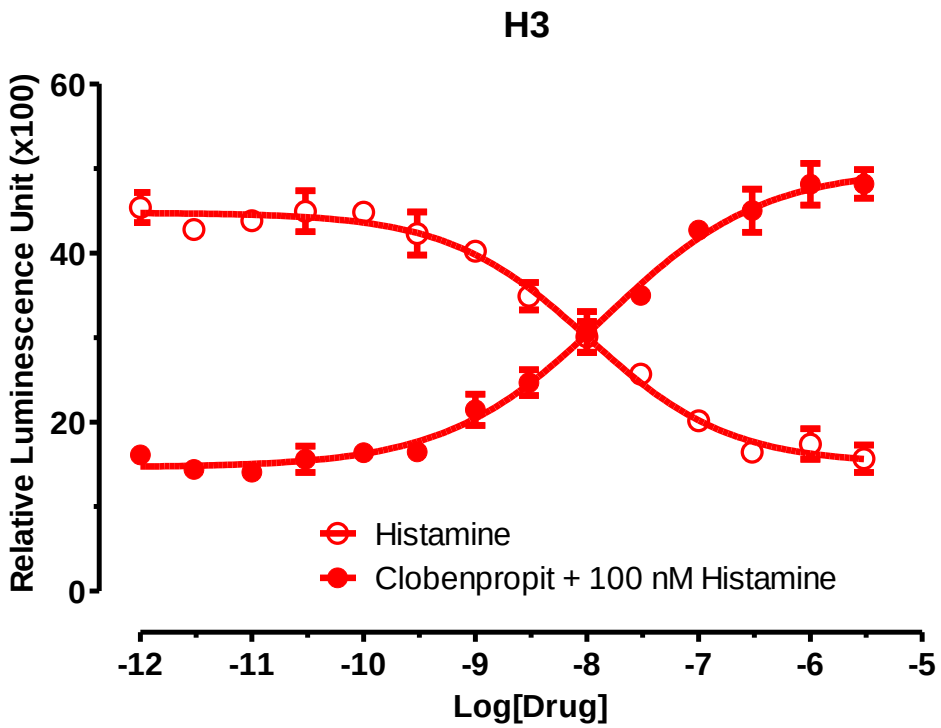
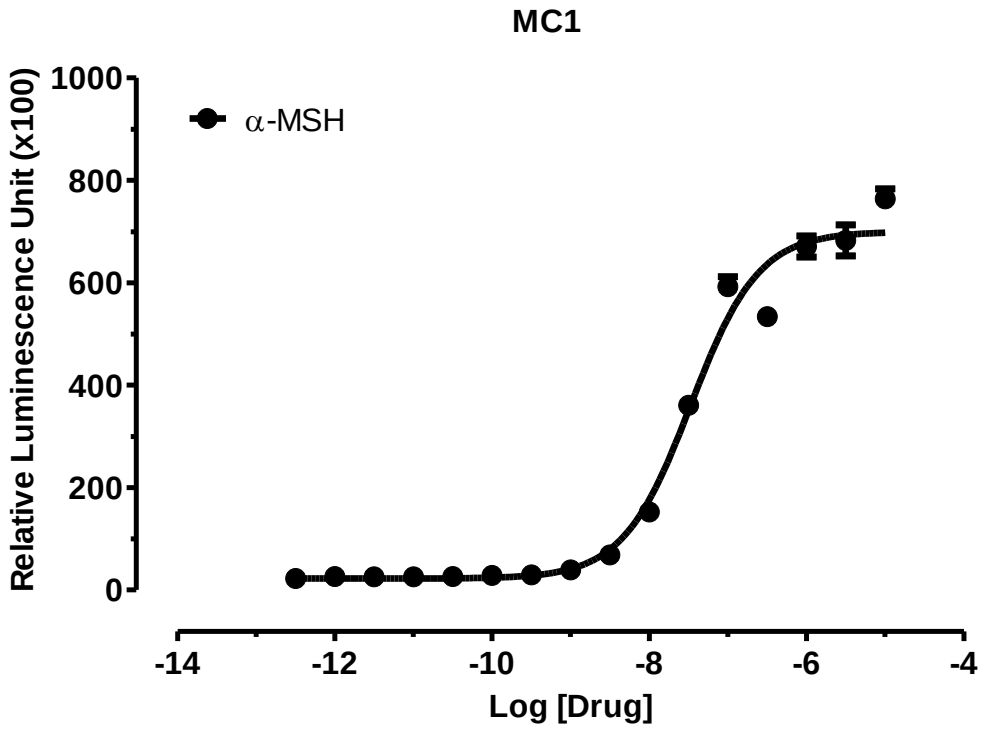
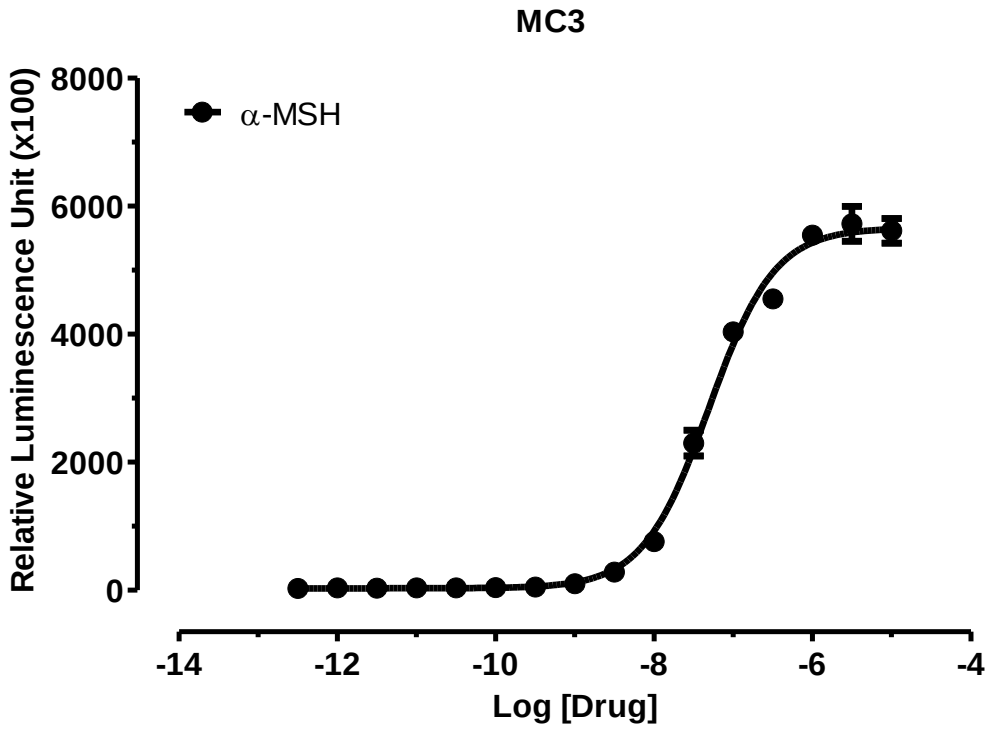

D4
Relative Luminescence Unit (x100)
1500
1000
500
0
Dopamine
PDSP compound
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[Drug]

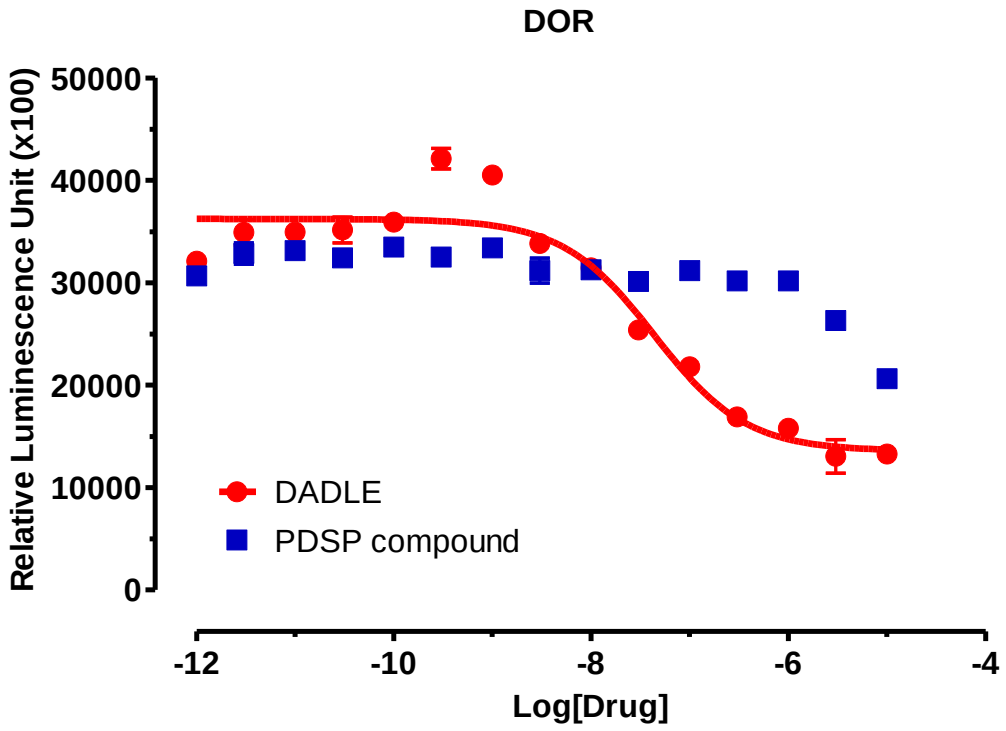

D5
Relative Luminescence Unit (x100)
800
600
400
200
0
Dopamine
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

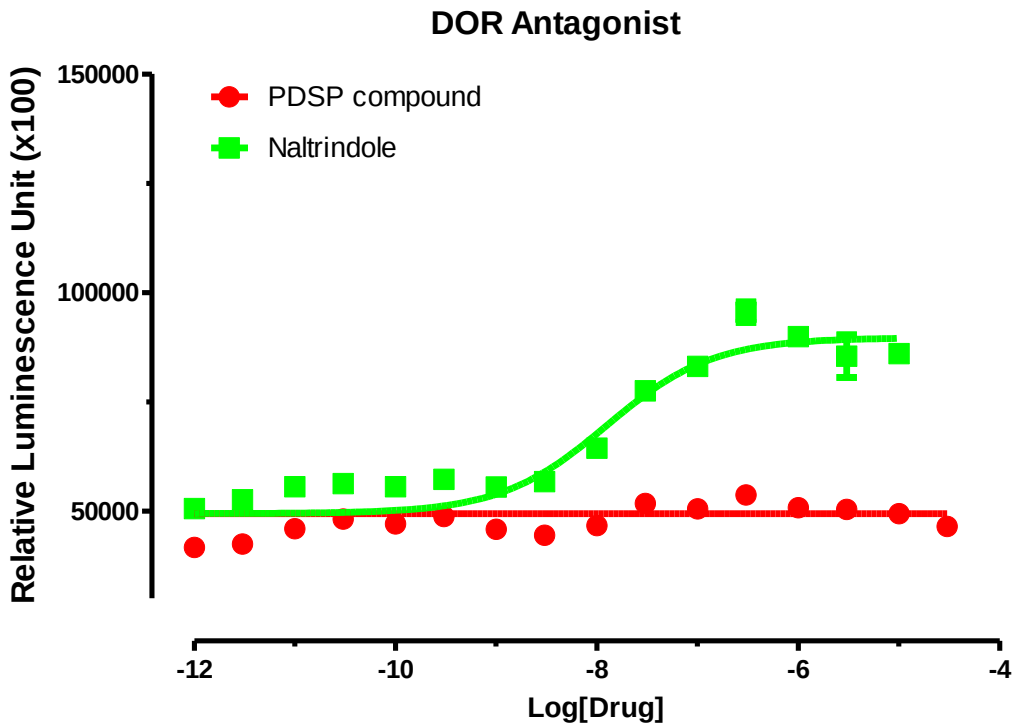

H2
Relative Luminescence Unit
20000
15000
10000
5000
0
Histamine
Cimetidine + 10 nM Histamine
-12
-10
-8
-6
-4
Log[Drug]

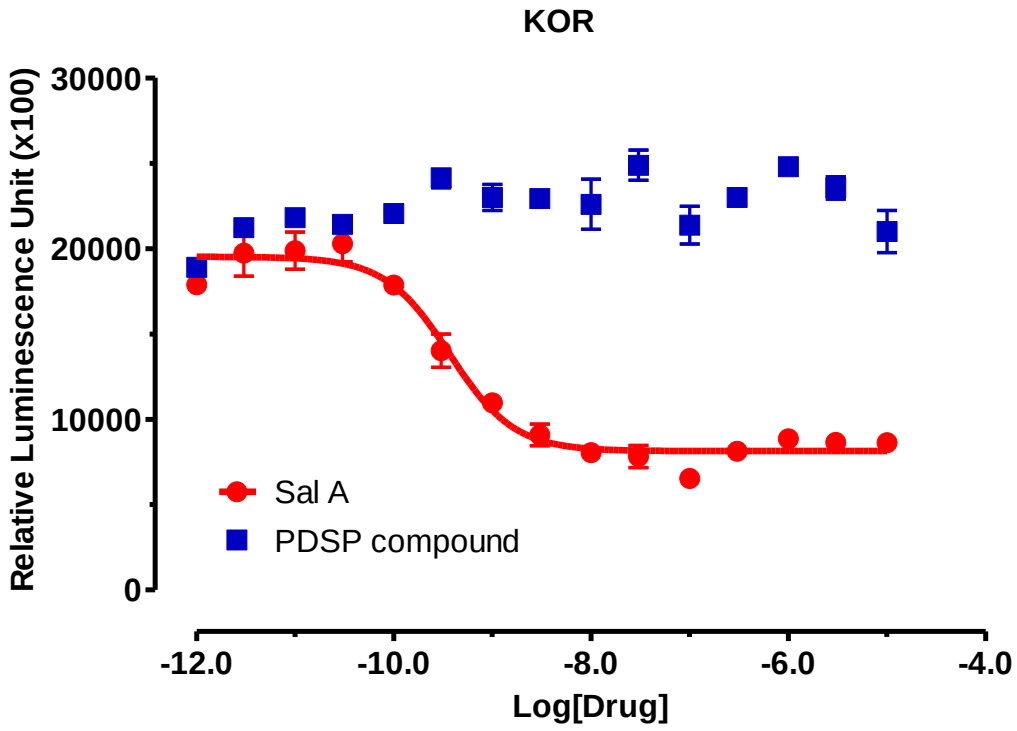

H3
Relative Luminescence Unit (x100)
60
40
20
0
Histamine
Clobenpropit + 100 nM Histamine
-12
-11
-10
-9
-8
-7
-6
-5
Log[Drug]

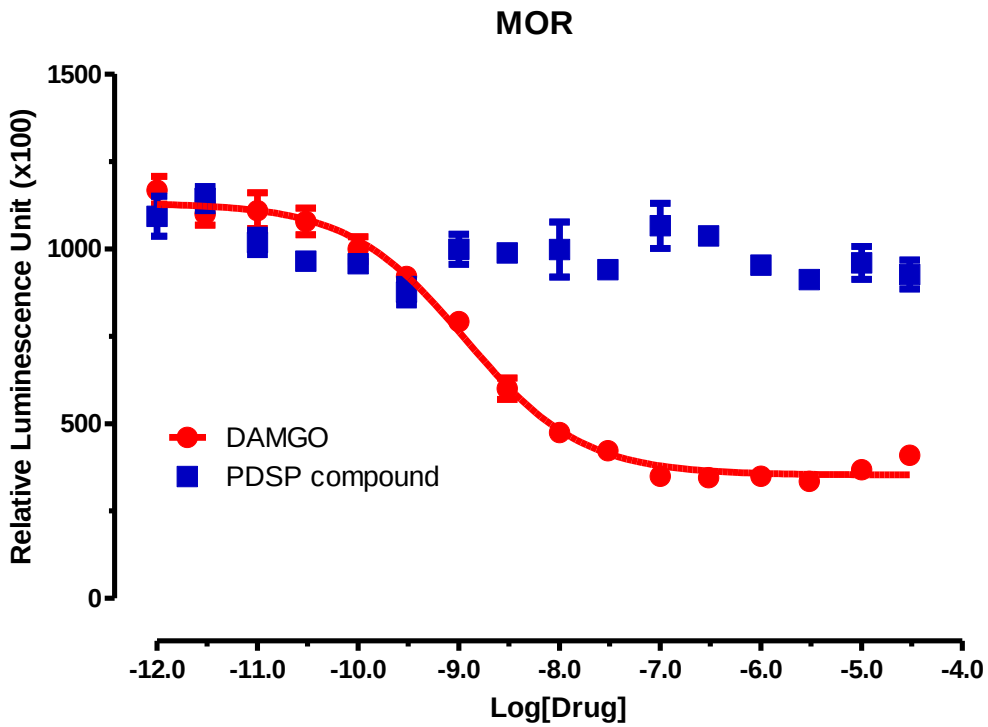

MC1
Relative Luminescence Unit (x100)
1000
800
600
400
200
0
α-MSH
-14
-12
-10
-8
-6
-4
Log [Drug]

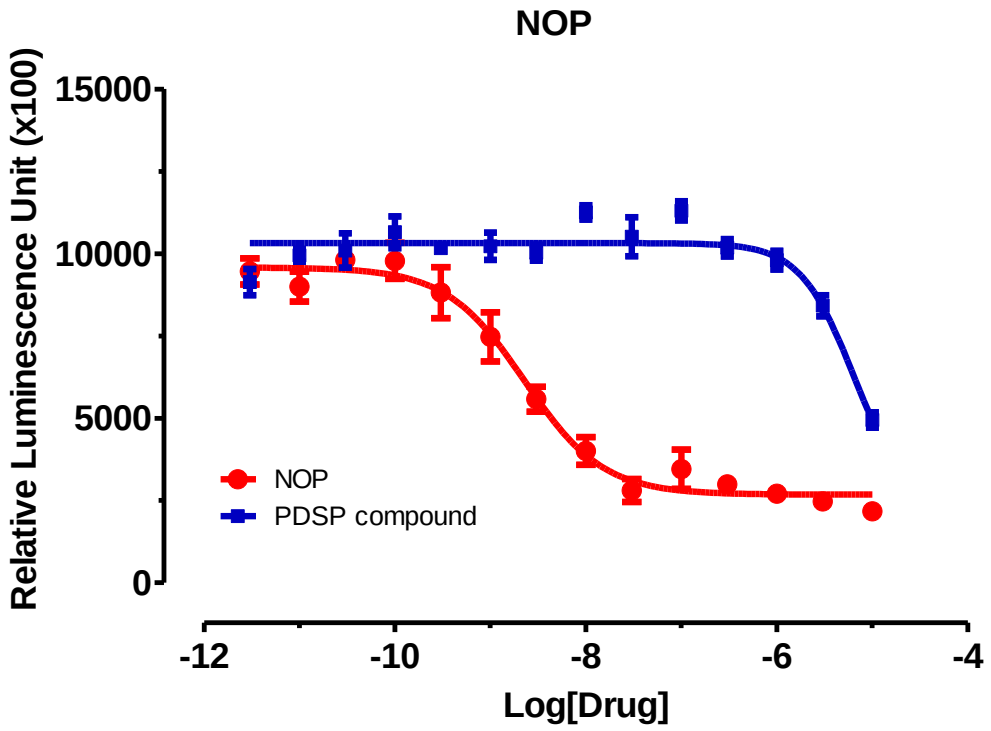

MC3
Relative Luminescence Unit (x100)
8000
6000
4000
2000
0
α-MSH
-14
-12
-10
-8
-6
-4
Log [Drug]

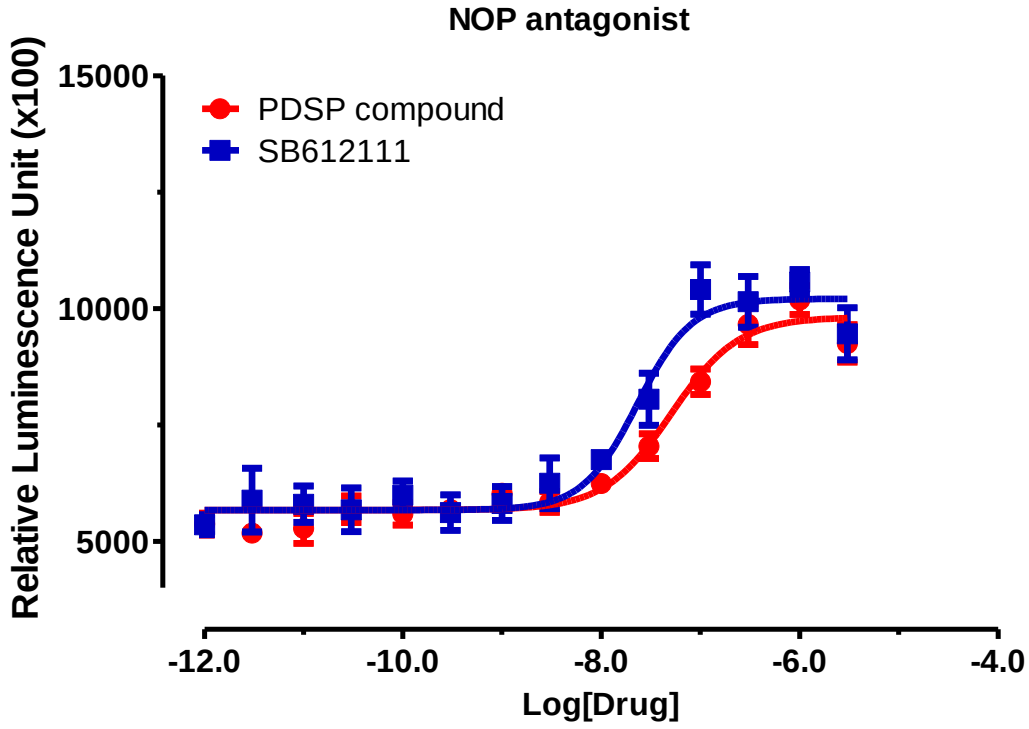

DOR
Relative Luminescence Unit (x100)
50000
40000
30000
20000
10000
0
DADLE
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

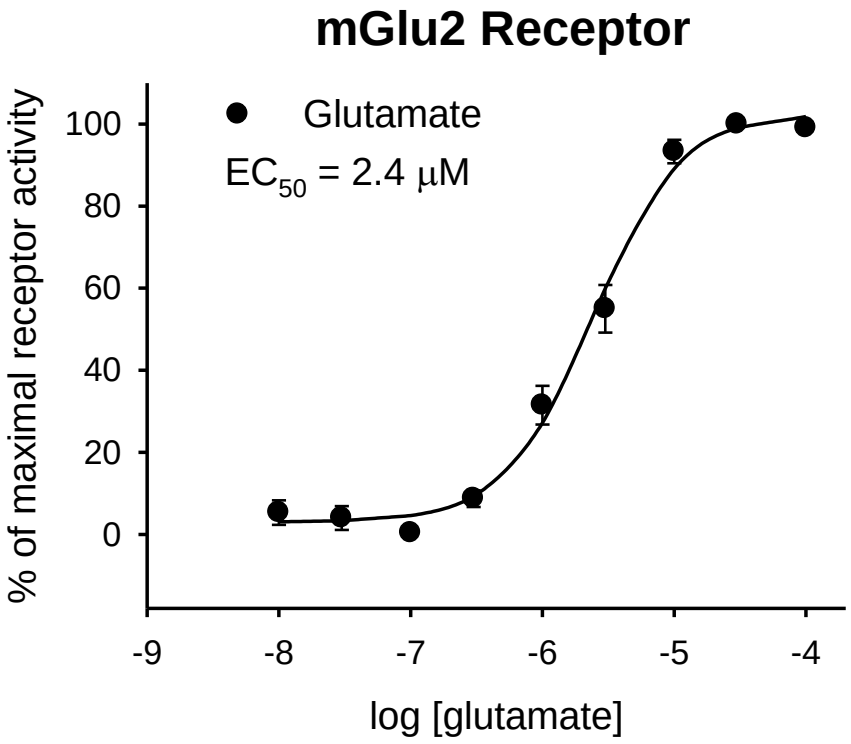

DOR Antagonist
Relative Luminescence Unit (x100)
150000
100000
50000
PDSP compound
Naltrindole
-12
-10
-8
-6
-4
Log[Drug]

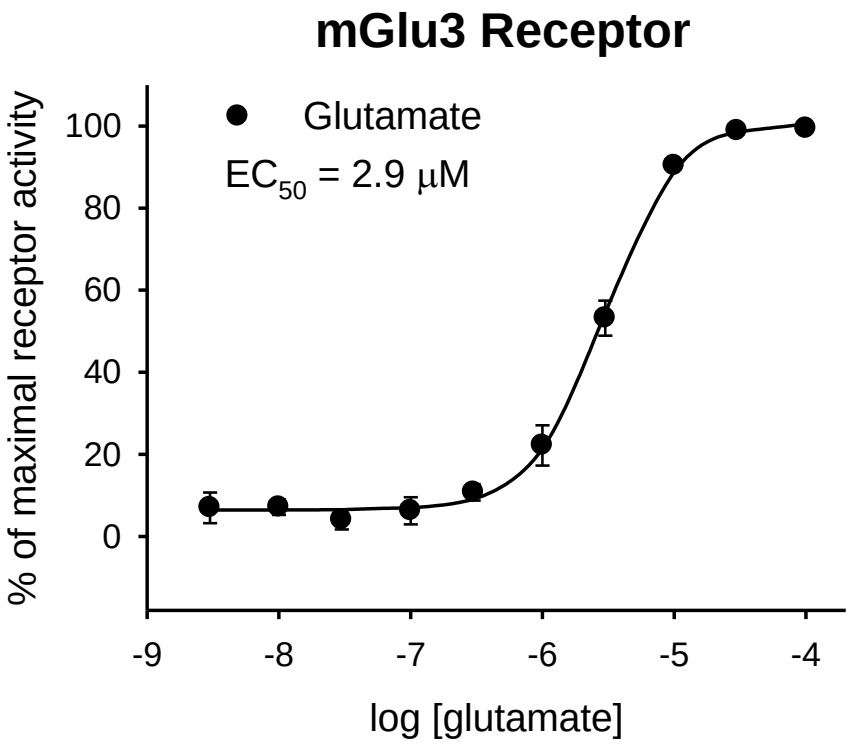

KOR
Relative Luminescence Unit (x100)
30000
20000
10000
0
Sal A
PDSP compound
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

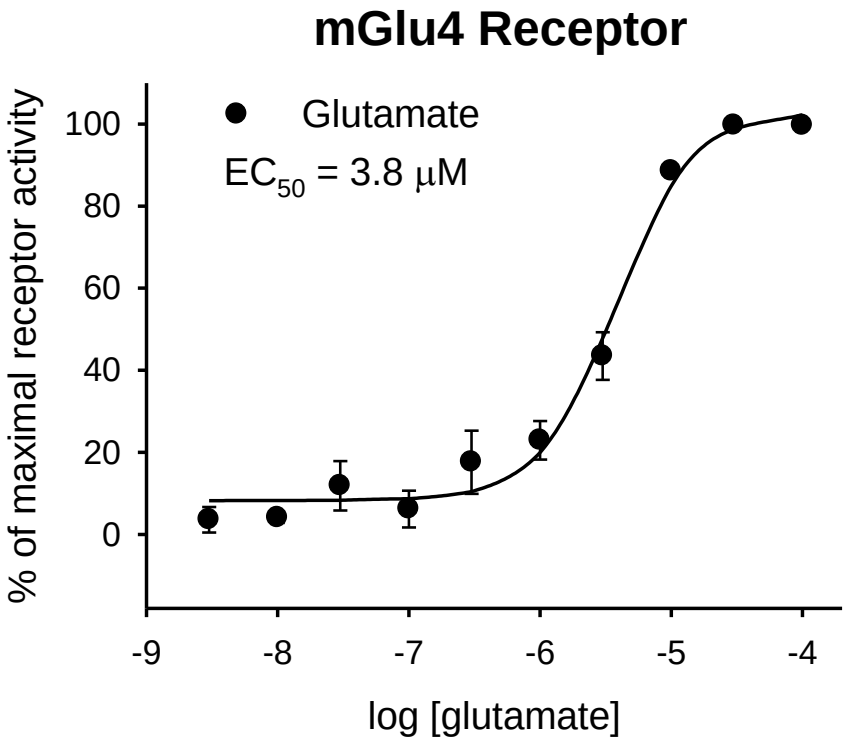

MOR
Relative Luminescence Unit (x100)
1500
1000
500
0
DAMGO
PDSP compound
-12.0
-11.0
-10.0
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
Log[Drug]

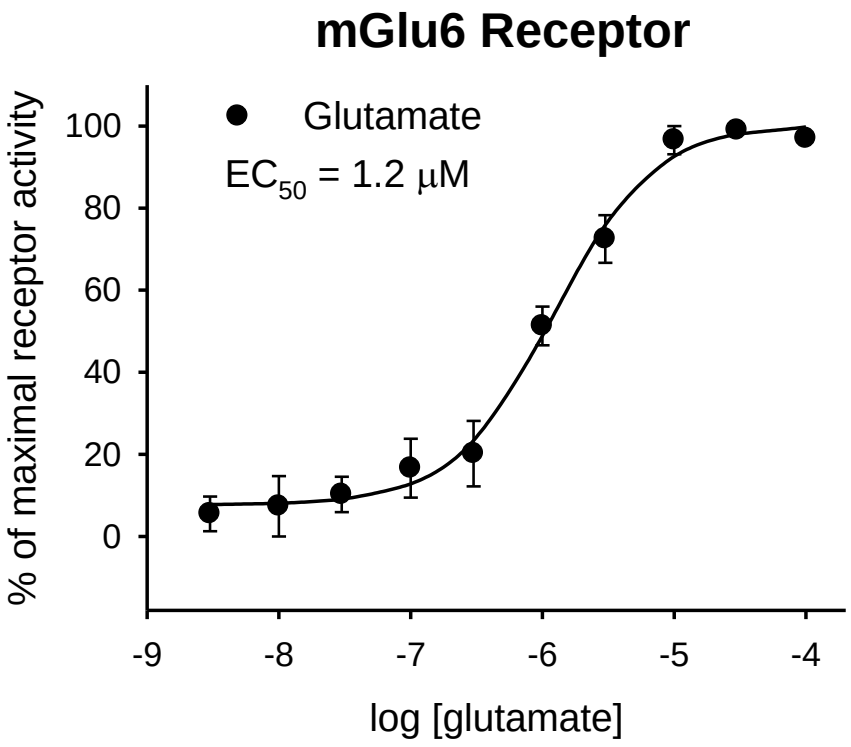

NOP
Relative Luminescence Unit (x100)
15000
10000
5000
0
NOP
PDSP compound
-12
-10
-8
-6
-4
Log[Drug]

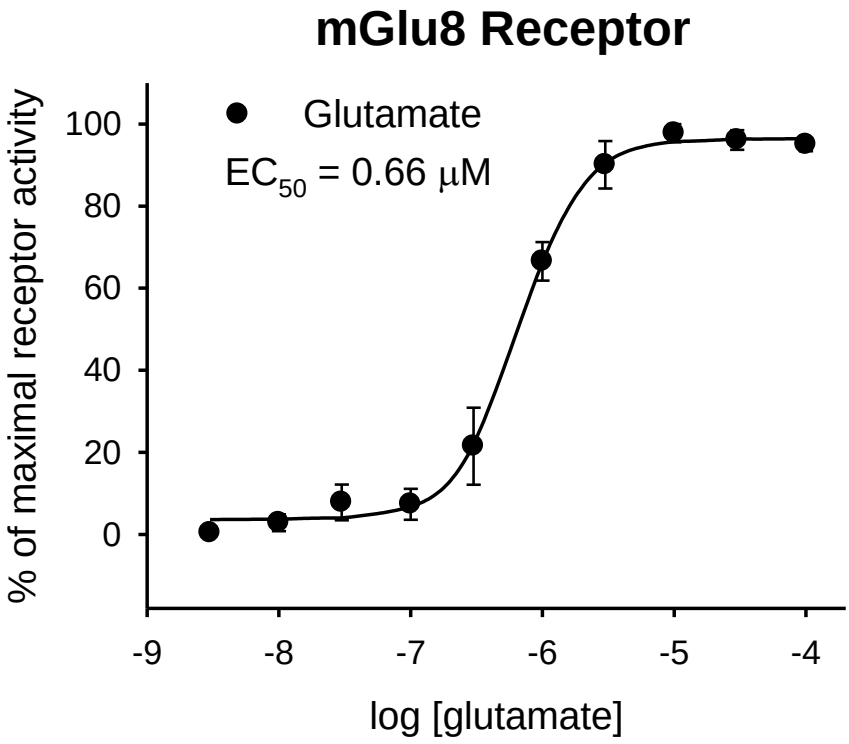

NOP antagonist
Relative Luminescence Unit (x100)
15000
10000
5000
PDSP compound
SB612111
-12.0
-10.0
-8.0
-6.0
-4.0
Log[Drug]

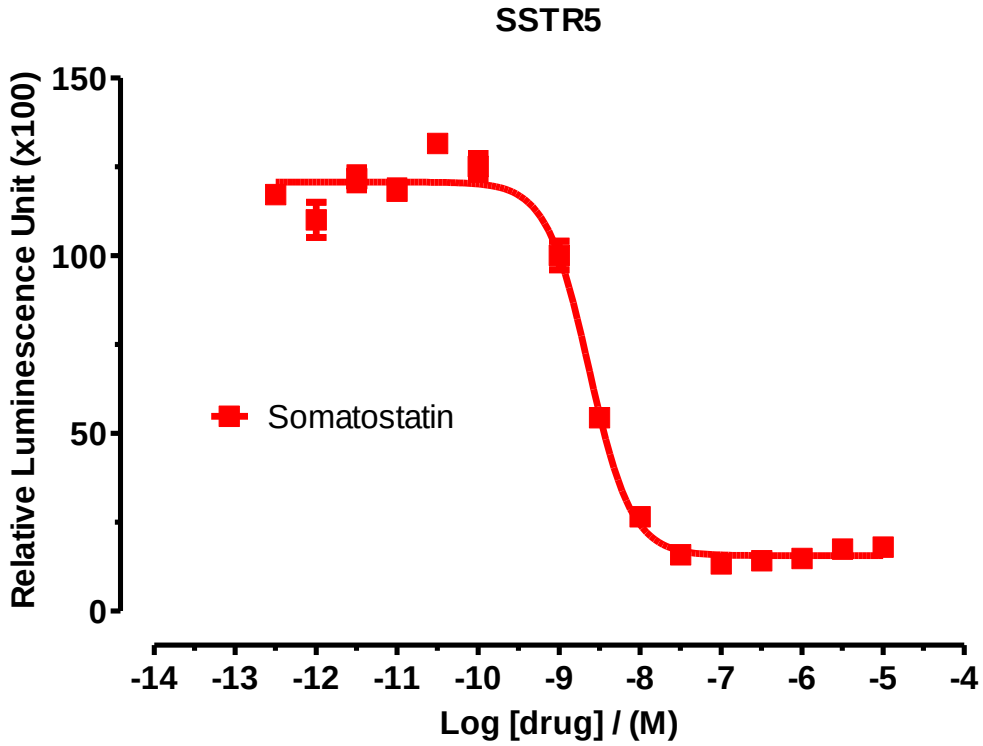

mGlu2 Receptor
Glutamate
EC50 = 2.4 μM
% of maximal receptor activity
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
log [glutamate]


mGlu3 Receptor
Glutamate
EC50 = 2.9 μM
% of maximal receptor activity
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
log [glutamate]


mGlu4 Receptor
Glutamate
EC50 = 3.8 μM
% of maximal receptor activity
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
log [glutamate]


mGlu6 Receptor
Glutamate
EC50 = 1.2 μM
% of maximal receptor activity
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
log [glutamate]





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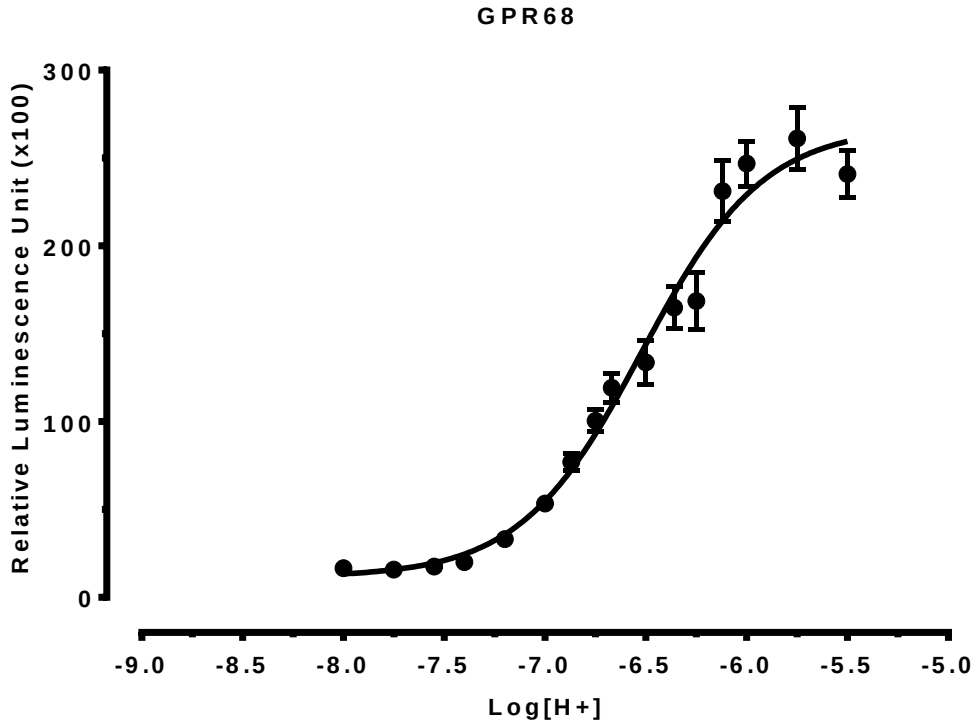
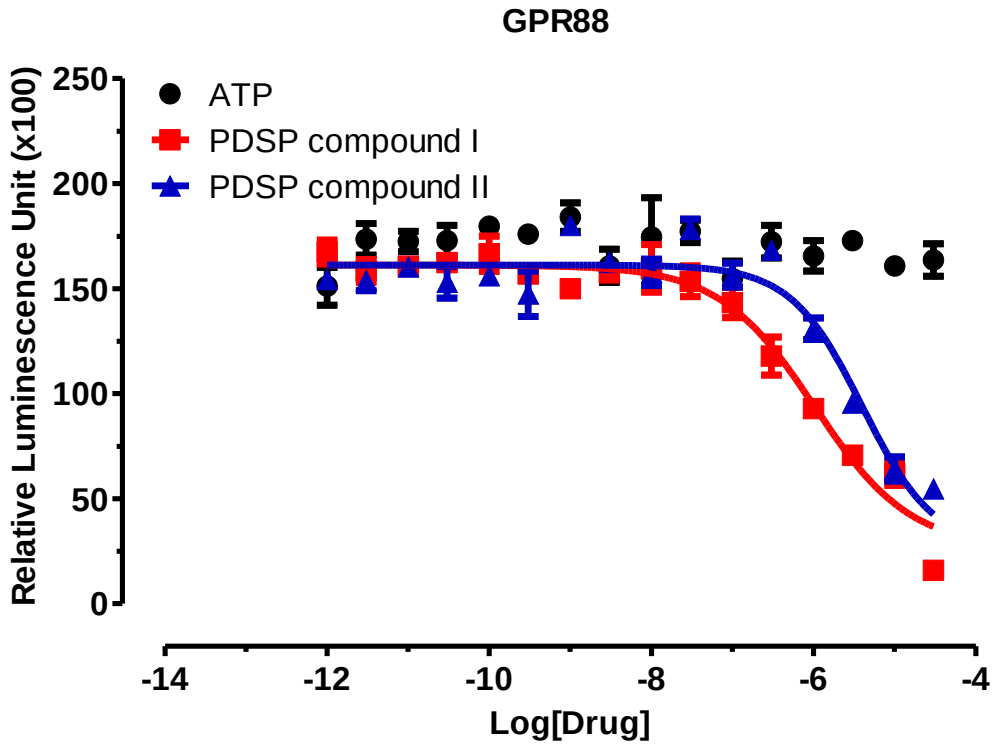

GPR68
Relative Luminescence Unit (x100)
300
200
100
0
-9.0
-8.5
-8.0
-7.5
-7.0
-6.5
-6.0
-5.5
-5.0
Log[H+]


GPR88
Relative Luminescence Unit (x100)
250
200
150
100
50
0
ATP
PDSP compound I
PDSP compound II
-14
-12
-10
-8
-6
-4
Log[Drug]

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

Main equipment: Luminescence counter

Main reagents: BrightGlo® reagents from Promega

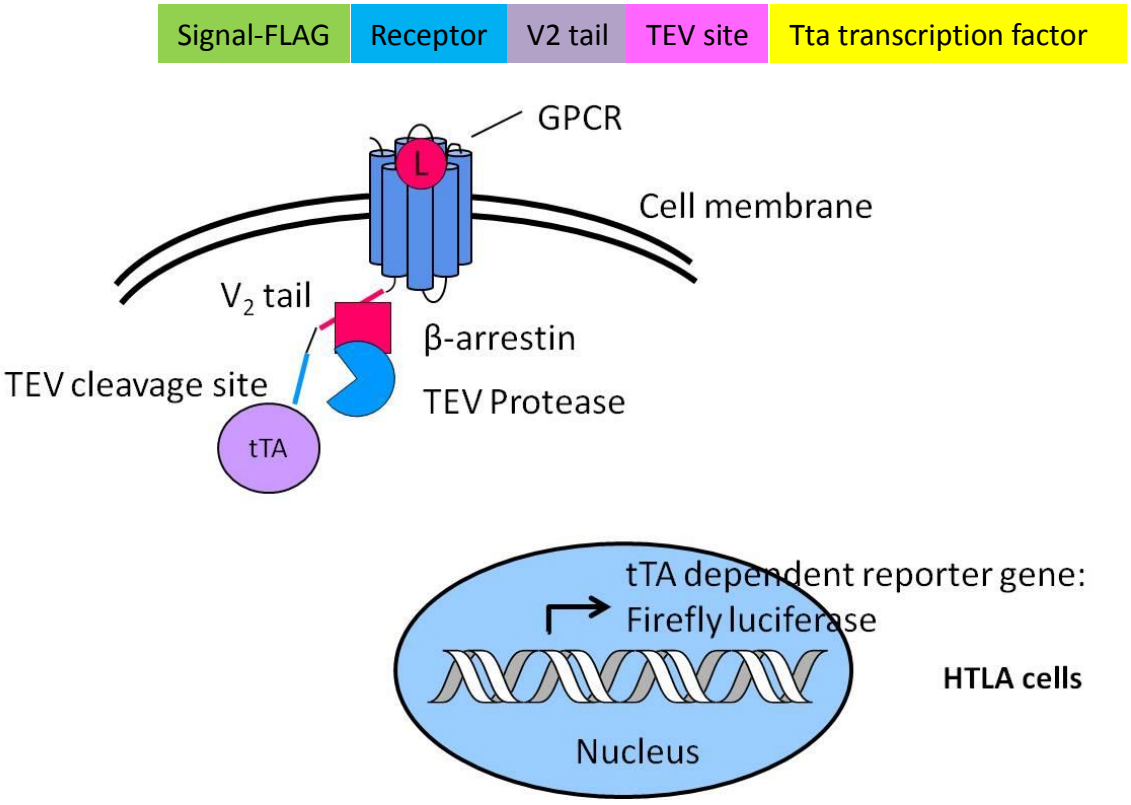
Tango assay buffer: 20 mM HEPES, 1x HBSS, pH 7.40

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 [Barnea et al., PNAS (2008) 105:64-69]. GPCR tango constructs (**Figure 43**, below) were codon-optimized for better expression in mammalian cell lines, synthesized by Blue Heron Biotech (Bothell, WA), and finally confirmed by sequencing. The HTLA cells (an HEK293 cell line stably expressing a tTA-dependent luciferase reporter and a ß-arrestin2-TEV fusion gene) were 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 B. An N-terminal Flag epitope tag was designed into the GPCR tango constructs for confirmation of surface expression (**Figure 44**) and comparison of expression levels.

2.6.2. GPCR β-arrestin recruitment assay: HTLA cells are transfected with GPCR tango constructs overnight (see Section 2.4.1.2 for detailed transfection protocol) and are plated in Poly-L-Lys (PLL) coated 384-well white clear-bottom cell culture plates at a density of 15,000 cells in 40 µl per well of DMEM with 1% dFBS. The cells are incubated for at least 6 hours (usually overnight) for them to recover before receiving drug stimulation. Drug stimulation solutions are prepared in filtered Tango assay buffer at 5x and added to cells (10 µl per well) for 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 and EC_{80} concentration of a 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 by 20-fold with Tango assay buffer) is added. The plate is

incubated for 20 minutes at room temperature in the dark before being counted on a luminescence counter.

Figure 43. Design of Tango constructs (**upper panel**) and GPCR Tango assay principle (**lower panel**). GPCR Tango constructs are designed as indicated in the top panel. Each construct contains the following elements in thisorder: (1) signal/FLAG tag; (2) receptor gene of interest; (3) Vasopressin 2 receptor C-tail; (4) TEV protease cleavage site; (5) Tta transcription factor. The GPCR Tango assay is carried out in transiently transfected HEK293 T cells genetically modified to express β-arrestin 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 factors then is transported to the cell nucleus, where it activates the luciferase reporter gene. The principle diagram is adapted from Barnea et al., (2008**)** ***The genetic design of signaling cascades to record receptor activation. PNAS 105:4-69.***



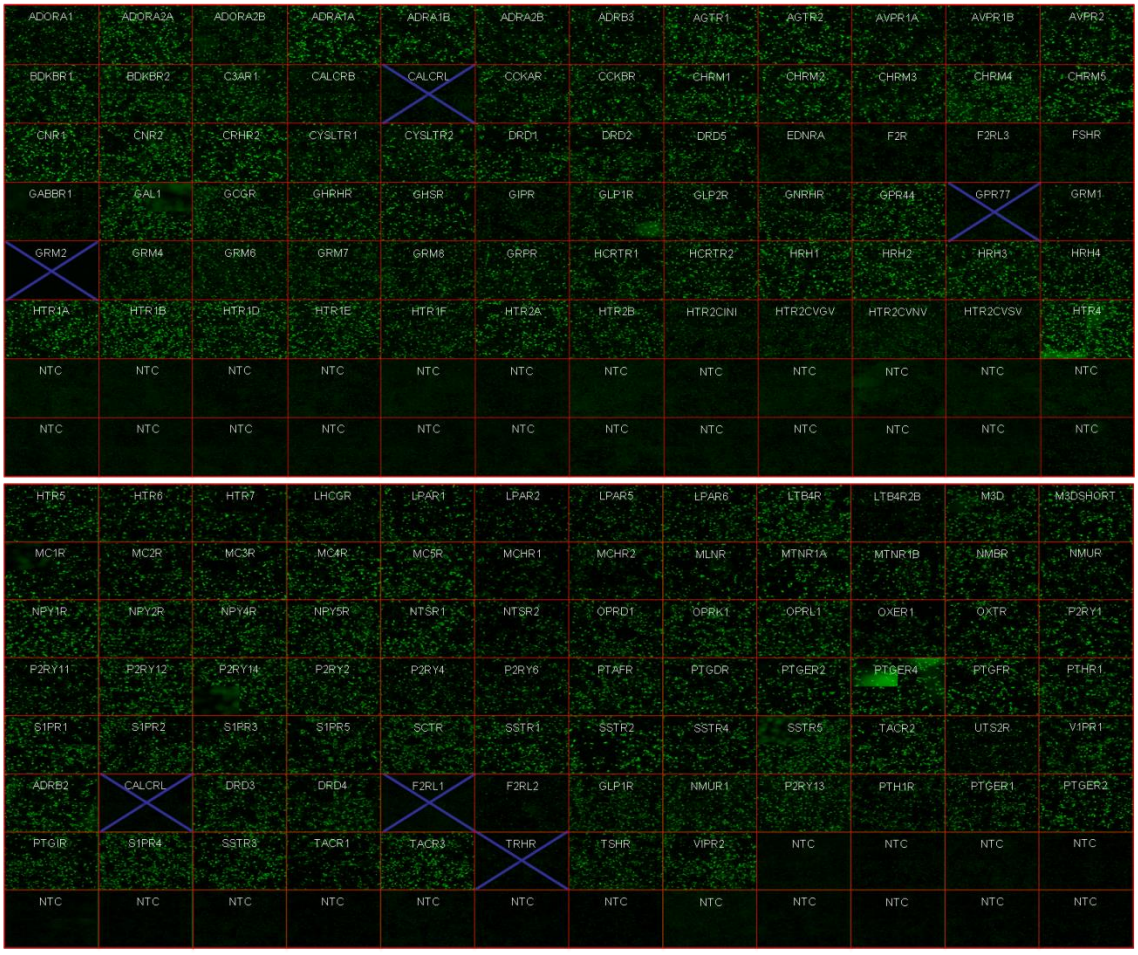


Figure 44. Confirmation of surface expression of FLAG-tagged non-orphan non-olfactory GPCRs used for the β-arrestin recruitment assay. Cells were pre-fixed with paraformaldeyhyde (PFA) for 30 min, blocked, probed using rabbit anti-FLAG antibody (primary)incubated for 1h at room temperature and then overnight at 4°C. On day 2, the plate was incubated with Alexa Fluor 594-conjugated 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 Pathway High Throughput Bioimager. Blue crosses indicated constructs that were not expressed on the surface of cells; 146 of 152 receptors (96%) were expressed on the cell surface.

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

Table 29. List of GPCR Tango constructs and validation. All DNA constructs are human clones, designed according to Barnea et al., (2008), codon optimized, synthesized by Blue Heron Biotech, and confirmed by sequencing. The β-arrestin recruitment assays using GPCR Tango constructs were conducted according to the above β-arrestin recruitment assay procedures and results were analyzed in GraphPad Prism 5.0.

* "Yes" for those assays are available in the PDSP; "No" for those not yet available; "In Progress" for those being synthesized or validated; "TBO" for those being optimized and ready soon.

Target Gene	IUPHAR Name	Reference Ligand(s)	E_{max} (fold)	pEC_{50}	Figure on page	Status*
HTR1A	5-HT1A	5-HT	15	6.35	P198	Yes
HTR1A	5-HT1A	LSD	13	7.21	P198	Yes
HTR1B	5-HT1B	5-HT	3.5	7.48	P198	Yes
HTR1B	5-HT1B	LSD	2.8	8.21	P198	Yes
HTR1D	5-HT1D	5-HT	3.5	7.16	P198	Yes
HTR1D	5-HT1D	LSD	2.9	8.45	P198	Yes
HTR1E	5-HT1E	5-HT	3.9	8.42	P199	Yes
HTR1E	5-HT1E	LSD	3.6	8.07	P199	Yes
HTR1F	5-HT1F	5-HT	100	6.96	P199	Yes
HTR1F	5-HT1F	LSD	83	7.42	P199	Yes
HTR2A	5-HT2A	5-HT	20	6.60	P199	Yes
HTR2A	5-HT2A	LSD	10	8.72	P199	Yes
HTR2B	5-HT2B	5-HT	3.3	8.75	P200	Yes
HTR2B	5-HT2B	LSD	2.9	8.93	P200	Yes
HTR2C-INI	5-HT2C	5-HT	2.0	7.42	P200	Yes
HTR2C-INI	5-HT2C	LSD	3.1	7.41	P200	Yes
HTR2C-VGV		LSD				NO
HTR2C-VNV		LSD	1.8	8.30	P200	TBO
HTR2C-VSV		LSD	1.6	8.82	P200	TBO
HTR4	5-HT4	5-HT	47	7.69	P201	Yes
HTR4	5-HT4	LSD	3.8	<5	P201	Yes
HTR5A	5-HT5A	5-HT	12	8.07	P201	Yes
HTR5A	5-HT5A	LSD	16	9.83	P201	Yes
HTR6	5-HT6	5-HT	136	6.53	P201	Yes
HTR6	5-HT6	LSD	23	7.85	P201	Yes
HTR7D	5-HT7D	5-HT				TBO
HTR7	5-HT7D	LSD				NO
HTR7A	5-HT7A	5-HT, LSD				In Progress

Target Gene	IUPHAR Name	Reference Ligand(s)	E_{max} (fold)	pEC_{50}	Figure on page	Status*
ADORA1	A1	NECA	4.9	7.98	P202	Yes
ADORA2A	A2A	NECA				No
ADORA2B	A2B	NECA				No
ADORA3	A3	NECA				No
ADRA1A	α1A	epinephrine				No
ADRA1B	α1B	epinephrine	10.4	6.64	P202	Yes
ADRA1D	α1D	norepinephrine	2.4	5.82	P202	Yes
ADRA2A	α2A	clonidine	5.4	8.36	P202	No
ADRA2B	α2B	norepinephrine	8.2	7.28	P202	Yes
ADRA2C	α2C	clonidine	152.9	7.18	P202	Yes
ADRB1	ß1	epinephrine	38.7	5.49	P203	Yes
ADRB2	ß2	epinephrine	21.4	6.24	P203	Yes
ADRB3	ß3	epinephrine				No
AGTR1	AT1	angiotensin II	7.8	7.97	P203	Yes
AGTR2	AT2	angiotensin II				No
APJ	Apelin	apelin-17	5.3	8.80	P203	Yes
AVPR1A	V1a	vasopressin	14.2	8.58	P222	Yes
AVPR1B	V1b	vasopressin	6.3	8.63	P222	Yes
AVPR2	V2	vasopressin	9.7	8.18	P222	Yes
BDKRB1	B1	bradykinin	8.0	5.41	P203	Yes
BDKRB2	B2	bradykinin	4.8	8.84	P203	Yes
NMBR	BB1	Bombesin	3.3	8.16	P204	Yes
NMBR	BB1	Gastrin-releasing peptide (GRP)	4.6	6.98	P204 P215	Yes
NMBR	BB1	Neuromedin B	6.0	8.68	P204	Yes
GRPR	BB2	Bombesin	6.2	8.50	P204	Yes
GRPR	BB2	Gastrin-releasing peptide (GRP)	6.1	8.32	P204	Yes
GPRP	BB2	Neuromedin B	5.3	7.46	P204	Yes
BRS3	BB3	PDSP compound	13.2	6.15	P204	Yes
C3AR1	C3a	C3a (70-77)	38.3	6.15	P204	Yes
CALCRb	CT	calcitonin				No
CALCRL	CT-like	calcitonin				No
CASR	CaS	spermine				No
CCKAR	CCK1	[Thr28, Nle31]CCK(25-33)	3.7	7.45	P204	Yes
CCR4	CCR4	CCL22			P206	Yes
CCR6	CCR6	CCL20 (Exodus-1)	2.4	7.79	P206	Yes
CMKLR1	GPCR27	Chemerin	15.7	7.96	P205	Yes

Target Gene	IUPHAR Name	Reference Ligand(s)	E_{max} (fold)	pEC_{50}	Figure on page	Status*
CNR1	CB1	WIN 55212-2	3.1	6.25	P207	Yes
CNR2	CB2	CP55940	10.1	6.57	P207	Yes
CXCR1	CXCR1	IL-8	38.1	<5.0	P205	Yes
CXCR1	CXCR1	CXCL6	3.5	6.60	P205	Yes
CXCR1	CXCR1	CXCL8	4.1	6.95	P205	Yes
CXCR2	CXCR2	IL-8	2.0	8.53	P205	Yes
CXCR2	CXCR2	CXCL6	1.8	7.30	P205	Yes
CXCR2	CXCR2	CXCL8	1.9	6.88	P205	Yes
CXCR4	CXCR4	SDF1-α	2.0	8.62	P206	Yes
CXCR6	CXCR6	CXCL16 (SRPOX)	10.3	<5.0	P206	Yes
CX3CR1	CX3CR1	SDF-1α	5.8	9.52	P206	Yes
CYSLTR1	CysLT1	leukotriene D4	4.5	7.42	P206	Yes
DRD1	D1	cabergoline	12.7	5.09	P207	Yes
DRD2	D2	LSD	149.9	9.30	P207	Yes
DRD3	D3	quinpirole	23.4	7.44	P207	Yes
DRD4	D4	LSD				No
DRD5	D5	LSD	40.3	6.59	P207	Yes
EDNRA	ET_A	endothelin-1	77.5	9.21	P208	Yes
EDNRB	ET_B	endothelin-1				No
F2R	PAR1	TRAP				No
F2RL1	PAR2	TRAP				No
F2RL2	PAR3	TRAP				No
F2RL3	PAR4	TRAP				No
FPR1	FPR1	fMLP	150.5	8.64	P208	Yes
FPR2	FPR2/ALX	fMLP	18.3	<5.0	P208	Yes
FPR3	FPR3	fMLP	1.2	6.64	P208	Yes
FSHR	FSH	FSH				No
GAL1	GAL1	galanin	8.9	6.36	P208	Yes
GAL2	GAL2	galnon	3.6	8.29	P205	Yes
GAL3	GAL3	galanin	3.6	9.99	P208	Yes
GHRH	GHRH	GRF (1-29)				No
GHSR	Ghrelin	ghrelin	1.7	7.30	P210	Yes
GIPR	GIP	GIP (1-42)				No
GLP1R	GLP-1	glucagon	6.7	<5.0	P209	Yes
GLP2R	GLP-2	glucagon				No
GNRHR	GnRH	leuprolide	2.6	8.47	P209	Yes
GPBA	GPBA	sodium taurodeoxycholate	2.3	<5.0	P209	Yes

Target Gene	IUPHAR Name	Reference Ligand(s)	E_{max} (fold)	pEC_{50}	Figure on page	Status*
GPR44	GPR44	prostaglandin D2	4.0	7.22	P209	Yes
GPR75	GPR75	RANTES				No
GRM1	mGluR1	D-homocysteic acid, L-Glu				No
GRM2	mGluR2	D-homocysteic acid, L-Glu				No
GRM3	mGluR3	D-homocysteic acid, L-Glu				No
GRM4	mGluR4	D-homocysteic acid, L-Glu				No
GRM5	mGluR5	D-homocysteic acid, L-Glu				No
GRM6	mGluR6	D-homocysteic acid, L-Glu				No
GRM7	mGluR7	D-homocysteic acid, L-Glu				No
GRM8	mGluR8	D-homocysteic acid, L-Glu				No
HRH1	Histamine H1	N-methylhistaprodifen	1.3	7.21	P210	Yes
HRH2	H2	Histamine	6.9	5.40	P210	Yes
HRH3	H3	n-methylhistamine	15.8	7.75	P210	Yes
HRH4	H4	histamine	3.1	6.88	P210	Yes
HCRTR1	OX1	orexin-A	109.3	7.78	P209	Yes
HCRTR2	OX2	orexin-A	92.8	8.16	P209	Yes
LHCGR	LH	LH luteinizing hormone				No
LPAR1	LPA1	1-oleoyl LPA	6.4	5.29	P223	Yes
LPAR2	LPA2	1-oleoyl LPA	291.0	<5.0	P223	Yes
LPAR3	LPA3	LPA				No
LPAR4	LPA4	LPA				No
LPAR5	LPA5	1-oleoyl LPA	14.2	<5.0	P223	Yes
LPAR5	LPA5	LPA				No
LTB4R	BLT1	leukotriene D4	33.2	7.31	P210	Yes
LTB4R	BLT1	Leukotriene B4	27.7	7.55	P211	Yes
LTB4R2b	BLT2	leukotriene D4				No
CHRM1	M1	Acetylcholine	2.1	4.58	P213	Yes
CHRM1	M1	Carbachol	1.5	6.74	P212 P213	Yes

Target Gene	IUPHAR Name	Reference Ligand(s)	E_{max} (fold)	pEC_{50}	Figure on page	Status*
CHRM1	M1	Arecoline	3.7	<5.0	P213	Yes
CHRM2	M2	Acetylcholine	8.1	6.41	P213	Yes
CHRM2	M2	Carbachol	3.6	6.60	P212 P213	Yes
CHRM2	M2	Arecoline	22.7	5.67	P213	Yes
CHRM3	M3	Acetylcholine	>10	<5.0	P213	Yes
CHRM3	M3	Carbachol	12.2	<5.0	P212 P213	Yes
CHRM3	M3	Arecoline	>10	<5.0	P213	Yes
M3D	M3DREADD	CNO	6.0	6.57	P213	Yes
CHRM4	M4	Acetylcholine	11.5	5.61	P213	Yes
CHRM4	M4	Carbachol	34.4	5.67	P212 P213	Yes
CHRM4	M4	Arecoline	33.8	5.62	P213	Yes
CHRM5	M5	Acetylcholine	<2.0	<5.0	P213	Yes
CHRM5	M5	Carbachol	1.6	6.91	P212 P213	Yes
CHRM5	M5	Arecoline	8.9	<5.0	P213	Yes
MC1R	MC1	α-MSH	2.7	7.39	P214	Yes
MC2R	MC2	ACTH				No
MC3Rb	MC3	α-MSH				No
MC4R	MC4	ACTH	7.0	6.53	P214	Yes
MC5R	MC5	α-MSH	2.0	6.38	P214	Yes
MCHR1	MCH1	[Ala-17]-MCH	7.0	7.66	P214	Yes
MCHR2	MCH2	[Ala-17]-MCH	17.2	8.42	P214	Yes
MLNR	Motilin	motilin	158.7	8.08	P214	Yes
MRGRPX1	MRGPRX1	BAM-22				No
MRGRPX2	MRGPRX2	BAM-22				No
MRGRPX3	MRGPRX3	BAM-22	4.4	10.14	P215	Yes
MRGRPX4	MRGPRX4	BAM-22	4.4	8.62	P215	Yes
MTNR1A	MT1	melatonin	3.8	10.07	P215	Yes
MTNR1B	MT2	melatonin	93.1	8.18	P215	Yes
NMUR1	NMU1	neuromedin S	48.1	8.02	P216	Yes
NMUR2	NMU2	neuromedin S	14.2	7.92	P215	Yes
NPS	NPS	neuropeptide S	58.2	6.07	P216	Yes
NPY1R	NPY1	pancreatic polypeptide	3.8	9.27	P216	Yes
NPY2R	NPY2	pancreatic polypeptide	17.0	8.75	P216	Yes

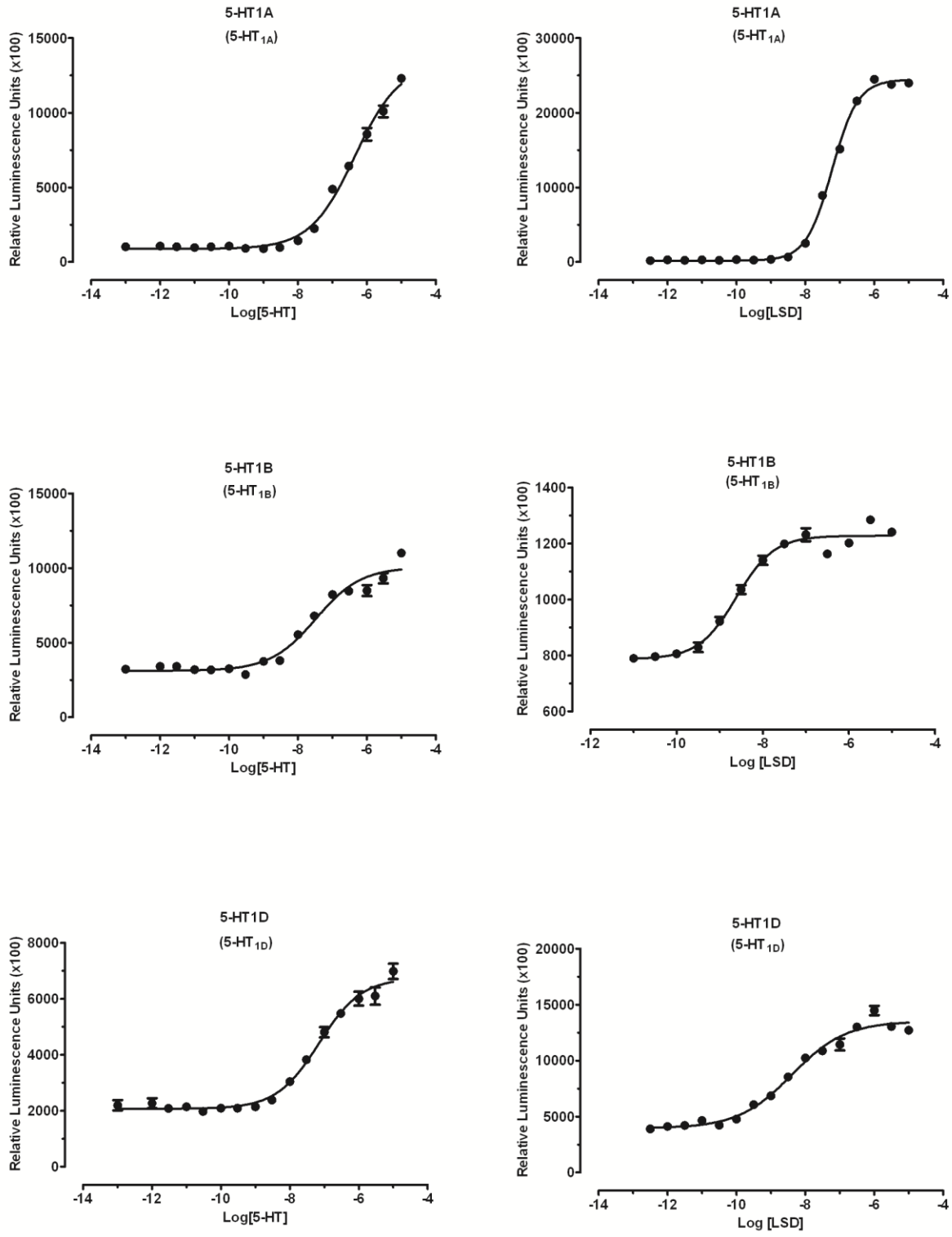
Target Gene	IUPHAR Name	Reference Ligand(s)	E_{max} (fold)	pEC_{50}	Figure on page	Status*
NPY4R	NPY4	neuropeptide Y	2.3	6.89	P216	Yes
NPY5R	NPY5	neuropeptide Y				No
NTSR1	NTS1	neurotensin				No
NTSR2	NTS2	SR48692	10.6	6.23	P216	Yes
OPRD1	δ	DADLE	7.1	9.16	P211	Yes
KOR	κ	salvinorin A	8.7	7.74	P211	Yes
OPRM1	μ	methadone	10.3	<5.0	P211	Yes
OPRL1	NOP	orphanin	151.5	7.92	P211	Yes
OXER1	OXE	5-oxo-ETE				No
OXTR	Oxytocin	oxytocin	26.9	7.87	P211 P222	Yes
P2RY1	P2Y1	2-MeSADP				Yes
P2RY12	P2Y12	2-MeSADP	46.3	7.34	P217	Yes
P2RY13	P2Y13	2-MeSADP	1.9	7.54	P217	Yes
P2RY14	P2Y14	UDP-glucose	1.5	6.21	P218	Yes
P2RY2	P2Y2	UTP	67.1	<5.0	P217	Yes
P2RY4	P2Y4	UTP	6.0	5.12	P217	Yes
P2RY6	P2Y6	UDP	1.8	5.52	P217	Yes
P2YR11	P2Y11	ATP	4.7	<5.0	P217	Yes
PTAFR	PAF	PAF (C16)	1.4	6.30	P218	Yes
PTGDR	DP1	prostaglandin D2				No
PTGER1	EP1	prostaglandin E2	8.0	7.35	P218	Yes
PTGER2	EP2	prostaglandin E2	66.0	5.69	P218	Yes
PTGER3	EP3	prostaglandin E2	3.5	8.14	P218	Yes
PTGER4	EP4	prostaglandin E2	38.7	8.91	P218	Yes
PTGFR	FP	prostaglandin F2α	21.8	7.02	P219	Yes
PTGIR	IP1	iloprost	10.9	<5.0	P219	Yes
PTHR1	PTH1	PTH (1-42)	9.1	6.41	P219	Yes
PTHR2	PTH2	PTH (1-42)				No
S1PR1	S1P1	sphingosine-1-phosphate	9.0	5.97	P219	Yes
S1PR2	S1P2	sphingosine-1-phosphate	15.5	5.77	P219	Yes
S1PR3	S1P3	sphingosine-1-phosphate	3.8	5.68	P219	Yes
S1PR4	S1P4	sphingosine-1-phosphate				No
S1PR5	S1P5	sphingosine-1-phosphate				No

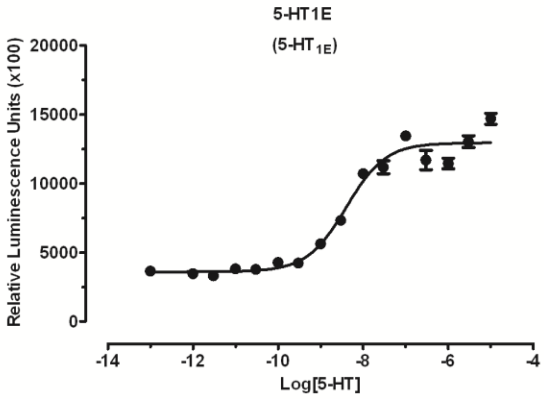
Target Gene	IUPHAR Name	Reference Ligand(s)	E_{max} (fold)	pEC_{50}	Figure on page	Status*
SCTR	Secretin	secretin	5.9	5.29	P220	Yes
SSTR1	SST1	somatostatin	6.7	5.89	P220	Yes
SSTR2	SST2	somatostatin	92.1	5.69	P220	Yes
SSTR3	SST3	somatostatin	119.2	5.32	P220	Yes
SSTR4	SST4	somatostatin	54.1	6.06	P220	Yes
SSTR5	SST5	somatostatin	254.2	5.81	P220	Yes
TA1	TA1	β-phenylethylamine	2.6	<5.0	P221	Yes
TACR1	NK1	substance P	3.3	6.71	P221	Yes
TACR2	NK2	substance P	31.4	8.80	P221	Yes
TACR3	NK3	substance P	3.1	5.76	P221	Yes
TBXA2R	TP	thromboxane B2				No
TRHR	TRH1	TRH				No
TSHR	TSH	thyrotropic hormone				No
UTSR	UT	urotensin II	9.5	8.72	P221	Yes
VIPR1	VPAC1	VIP	7.7	<5.0	P222	Yes
VIPR2	VPAC2	VIP	12.9	<5.0	P222	Yes

Table 29a. List of available Tango constructs for peptide and orphan GPCRs at NIMH-PDSP. Some of them have been verified and are also listed in Table 29. Many others are in the process of being verified and/or optimized. IUPHAR target gene names are used.

APJ-Tango	GPR115-Tango	GPR21-Tango	GPRC6A-Tango
BB3-Tango	GPR116-Tango	GPR22-Tango	HCA1-Tango
C5A-Tango	GPR119-Tango	GPR25-Tango	HCA2-Tango
CCR1-Tango	GPR120-Tango	GPR26-Tango	HCA3-Tango
CCR2-Tango	GRP123-Tango	GPR27-Tango	KISS-Tango
CCR3-Tango	GPR124-Tango	GPR31-Tango	LPA4-Tango
CCR4-Tango	GPR125-Tango	GPR32-Tango	MAS1L-Tango
CCR5-Tango	GPR126-Tango	GPR34-Tango	MAS1-Tango
CCR6-Tango	GPR12-Tango	GPR35-TangO	MRGPRD-Tango
CCR7-Tango	GPR132-Tango	GPR37L1-Tango	MRGPRE-Tango
CCR8-Tango	GPR133-Tango	GPR37-Tango	MRGPRF-Tango
CCR10-Tango	GPR135-Tango	GPR39-Tango	MRGPRG-Tango
CCRL2-Tango	GPR141-Tango	GPR3-Tango	MRGPRX1-Tango
CD97-Tango	GPR142-Tango	GPR45-Tango	MRGPRX2-Tango
CMKLR1-Tango	GPR143-Tango	GPR4-Tango	MRGPRX3-Tango
CX3C1-Tango	GPR144-Tango	GPR50-Tango	MRGPRX4-Tango
CXCR1-Tango	GPR146-Tango	GPR52-Tango	NPBW1-Tango
CXCR2-Tango	GPR148-Tango	GPR55-Tango	NPBW2-Tango
CXCR3-Tango	GPR148-Tango	GPR56-Tango	NPFF1-Tango
CXCR4-Tango	GPR150-Tango	GPR61-Tango	NPFF2-Tango
CXCR5-Tango	GPR151-Tango	GPR62-Tango	NPS-Tango
CXCR6-Tango	GPR152-Tango	GPR63-Tango	OPN3-Tango
CXCR7-Tango	GPR153-Tango	GPR64-Tango	OPN5-Tango
ELTD1-Tango	GPR156-Tango	GPR65-Tango	OXGR1-Tango
FFA1-Tango	GPR157-Tango	GPR68-Tango	PK1-Tango
FFA2-Tango	GPR158-Tango	GPR6-Tango	PK2-Tango
FFA3-Tango	GPR15-Tango	GPR75-Tango	PRRP-Tango
FPR1-Tango	GPR160-Tango	GPR78-Tango	QRFP-Tango
FPR2-Tango	GPR161-Tango	GPR82-Tango	RXFP1-Tango
FPR3-Tango	GPR162-Tango	GPR83-Tango	RXFP2-Tango
GAL1-Tango	GPR171-Tango	GPR84-Tango	RXFP3-Tango
GAL2-Tango	GPR173-Tango	GPR85-Tango	RXFP4-Tango
GAL3-Tango	GPR174-Tango	GPR87-Tango	SUCNR1-Tango
GPBA-Tango	GPR17-Tango	GPR88-Tango	TA1-Tango
GPER-Tango	GPR182-Tango	GPR92-Tango	TAAR2-Tango
GPR101-Tango	GPR183-Tango	GPR97-Tango	TAAR5-Tango
GPR110-Tango	GPR18-Tango	GPRC5A-Tango	TAAR6-Tango
GPR111-Tango	GPR19-Tango	GPRC5B-Tango	TAAR8-Tango
GPR113-Tango	GPR1-Tango	GPRC5C-Tango	TAAR9-Tango
GPR114-Tango	GPR20-Tango	GPRC5D-Tango	

Figure 45. Representative concentration-response curves for GPCR mediated ß-arrestin translocation. Curves are in essentially the same order as in **Table 29**.




5-HT1E
(5-HT1E)
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15000
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5000
0
-14
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-10
-8
-6
-4
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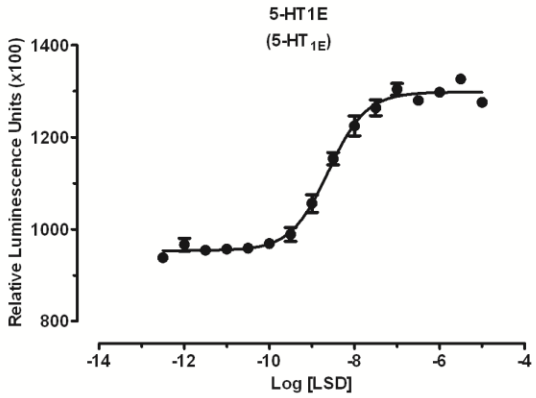
5-HT1E
(5-HT1E)
Relative Luminescence Units (x100)
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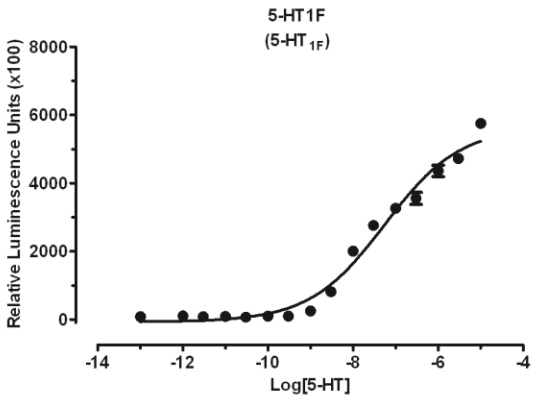
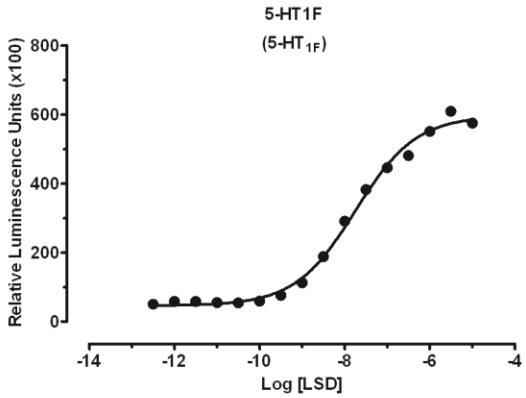
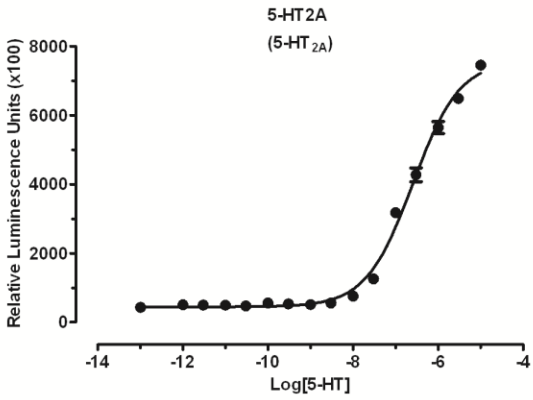
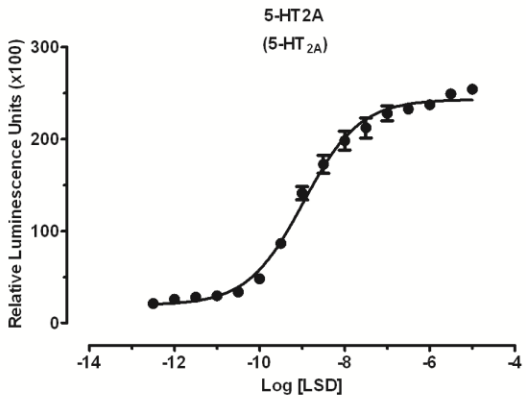
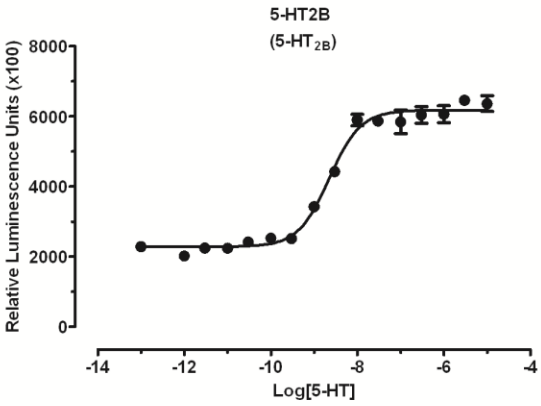
1200
1000
800
-14
-12
-10
-8
-6
-4
Log [LSD]

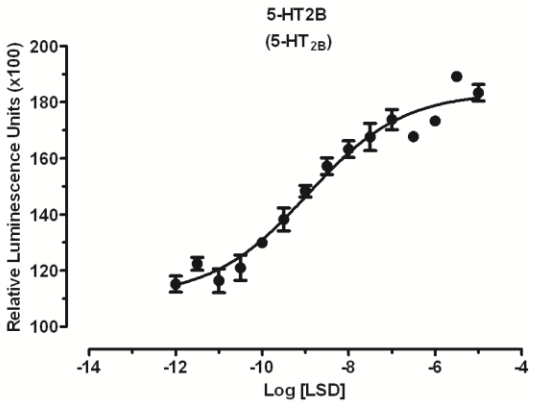

5-HT1F
(5-HT1F)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[5-HT]

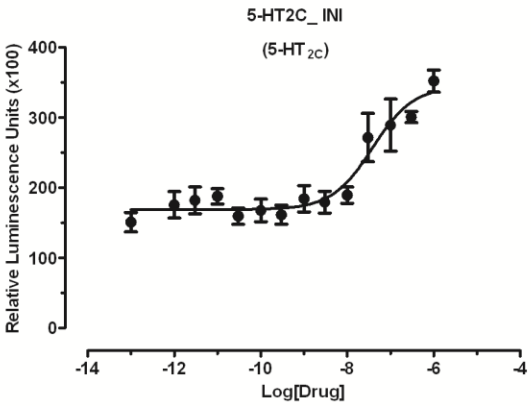

5-HT1F
(5-HT1F)
Relative Luminescence Units (x100)
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log [LSD]

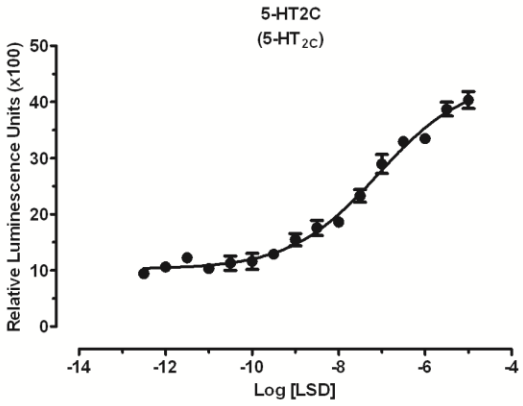

5-HT2A
(5-HT2A)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[5-HT]

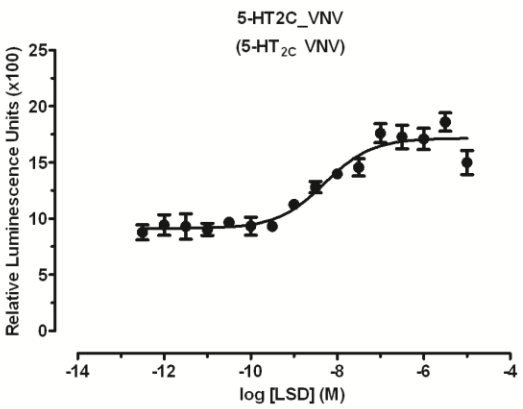

5-HT2A
(5-HT2A)
Relative Luminescence Units (x100)
300
200
100
0
-14
-12
-10
-8
-6
-4
Log [LSD]

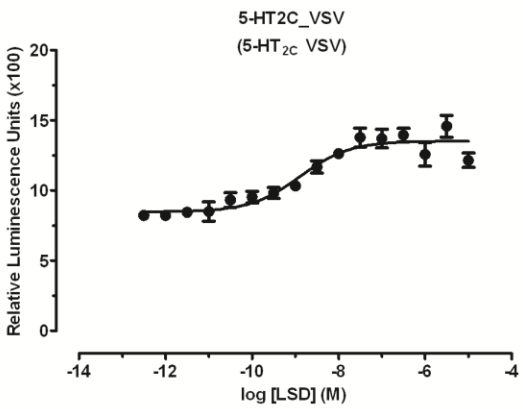

5-HT2B
(5-HT2B)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[5-HT]

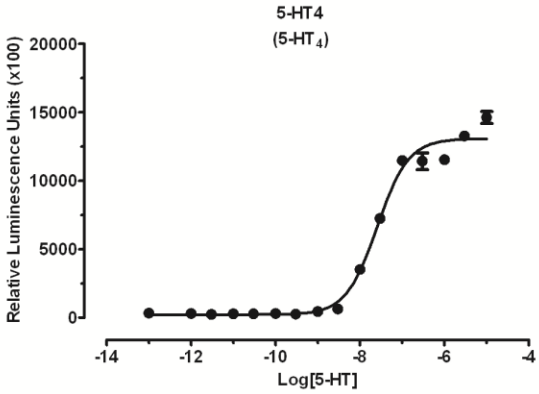

5-HT2B
(5-HT2B)
Relative Luminescence Units (x100)
200
180
160
140
120
100
-14
-12
-10
-8
-6
-4
Log [LSD]

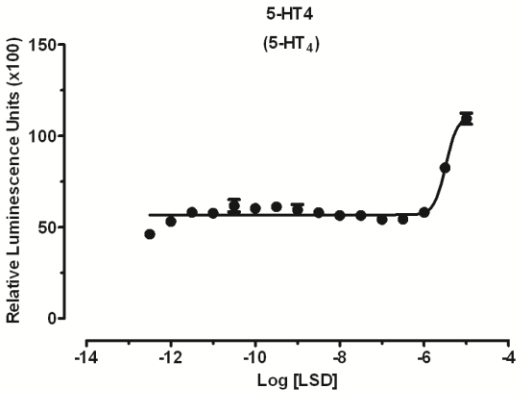

5-HT2C_ INI
(5-HT2C)
Relative Luminescence Units (x100)
400
300
200
100
0
-14
-12
-10
-8
-6
-4
Log[Drug]

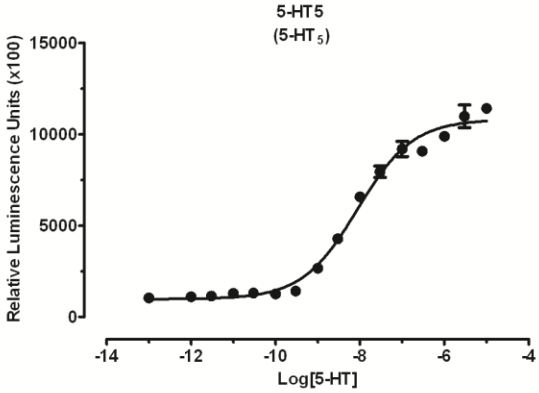

5-HT2C
(5-HT2C)
Relative Luminescence Units (x100)
50
40
30
20
10
0
-14
-12
-10
-8
-6
-4
Log [LSD]

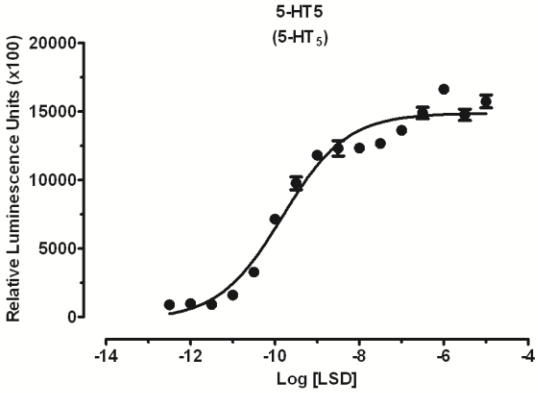

5-HT2C_VNV
(5-HT2C VNV)
Relative Luminescence Units (x100)
25
20
15
10
5
0
-14
-12
-10
-8
-6
-4
log [LSD] (M)

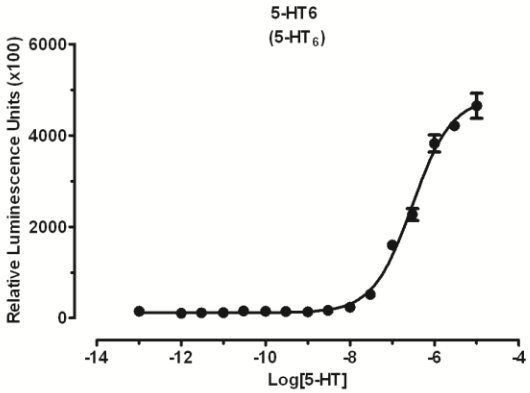

5-HT2C_VSV
(5-HT2C VSV)
Relative Luminescence Units (x100)
20
15
10
5
0
-14
-12
-10
-8
-6
-4
log [LSD] (M)

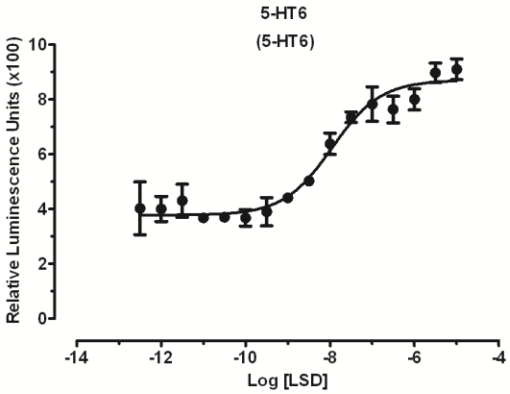

5-HT4
(5-HT4)
Relative Luminescence Units (x100)
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[5-HT]

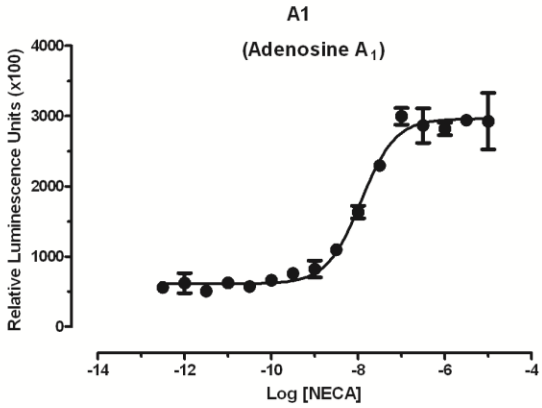

5-HT4
(5-HT4)
Relative Luminescence Units (x100)
150
100
50
0
-14
-12
-10
-8
-6
-4
Log [LSD]

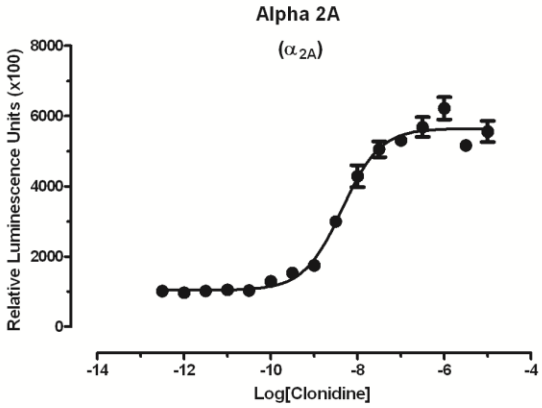

5-HT5
(5-HT5)
Relative Luminescence Units (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[5-HT]

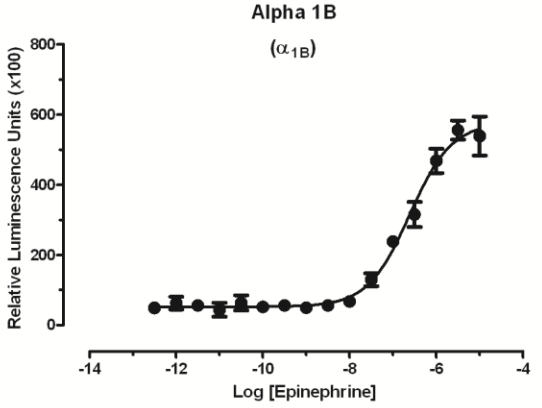

5-HT5
(5-HT5)
Relative Luminescence Units (x100)
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log [LSD]

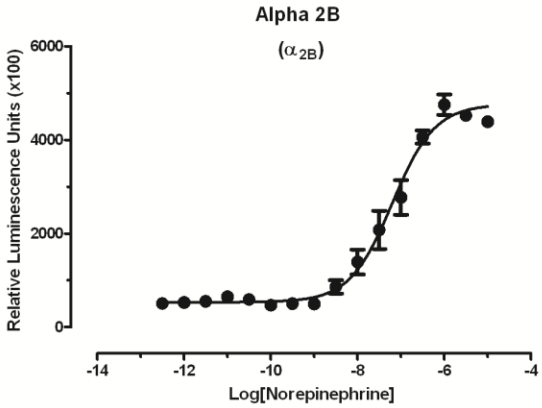

5-HT6
(5-HT6)
Relative Luminescence Units (x100)
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[5-HT]

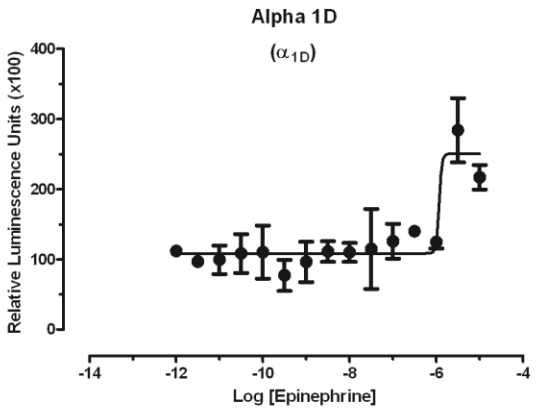

5-HT6
(5-HT6)
Relative Luminescence Units (x100)
10
8
6
4
2
0
-14
-12
-10
-8
-6
-4
Log [LSD]

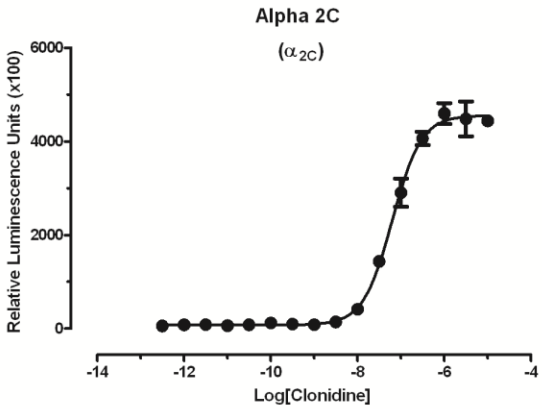

A1
(Adenosine A1)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log [NECA]

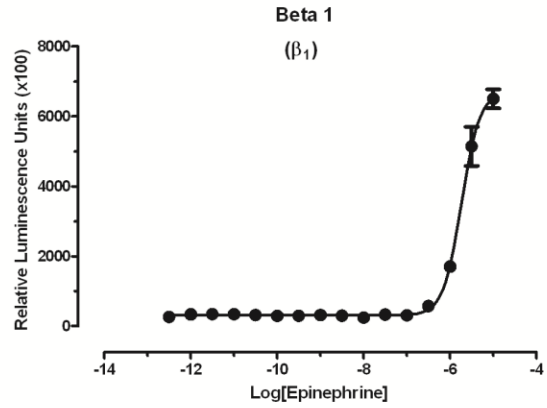

Alpha 2A
(α2A)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Clonidine]

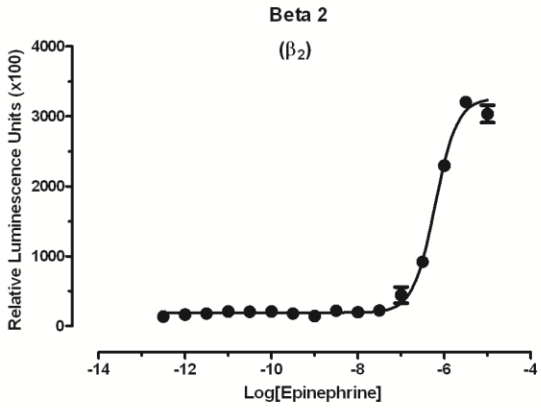

Alpha 1B
(α1B)
Relative Luminescence Units (x100)
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log [Epinephrine]

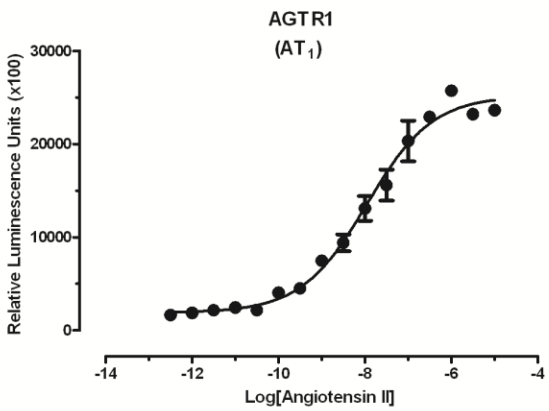

Alpha 2B
(α2B)
Relative Luminescence Units (x100)
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Norepinephrine]

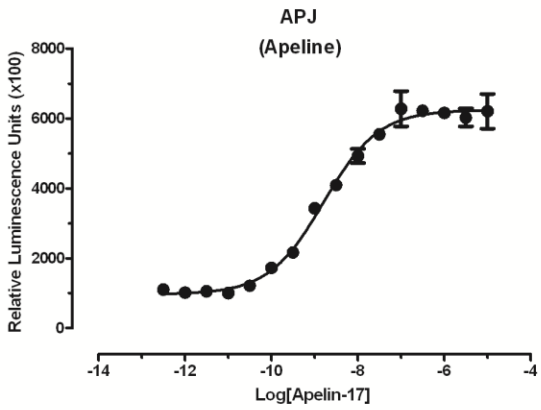

Alpha 1D
(α1D)
Relative Luminescence Units (x100)
400
300
200
100
0
-14
-12
-10
-8
-6
-4
Log [Epinephrine]

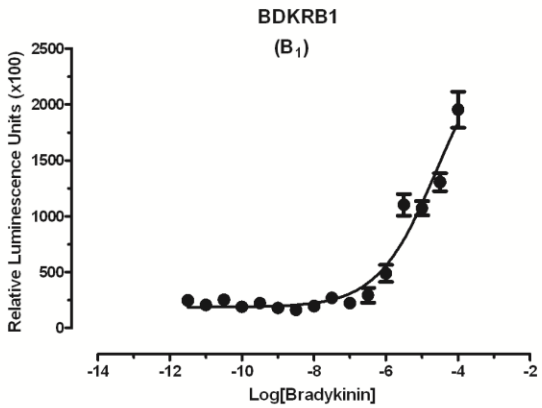

Alpha 2C
(α2C)
Relative Luminescence Units (x100)
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Clonidine]

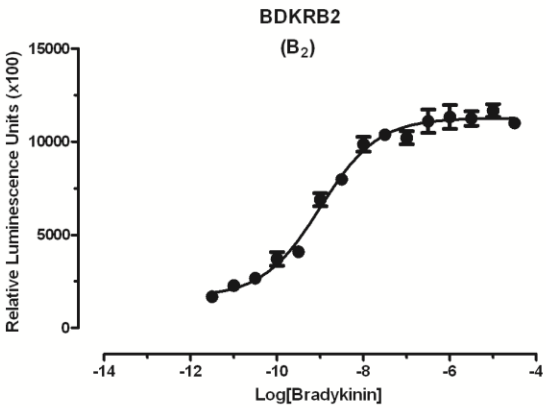

Beta 1
(β1)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Epinephrine]

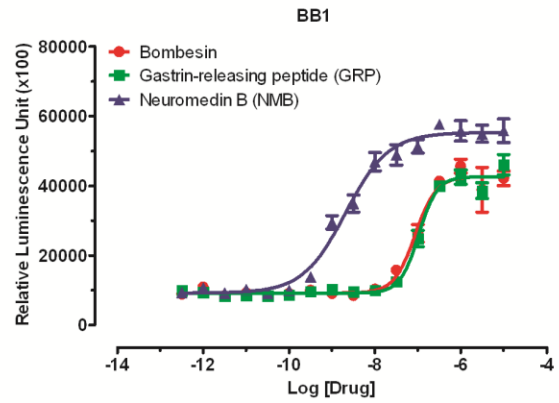

Beta 2
(β2)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Epinephrine]

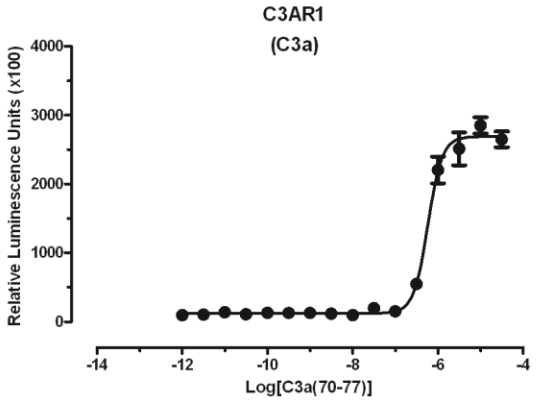

AGTR1
(AT1)
Relative Luminescence Units (x100)
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[Angiotensin II]

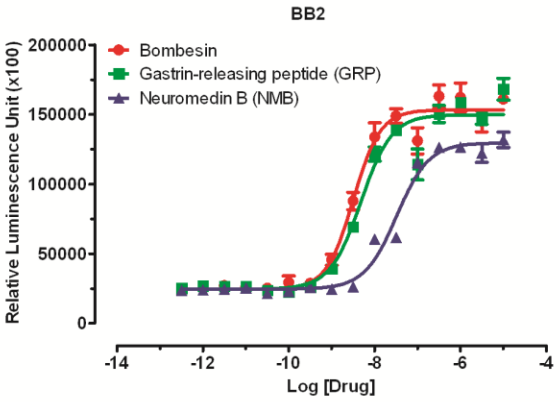

APJ
(Apeline)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Apelin-17]

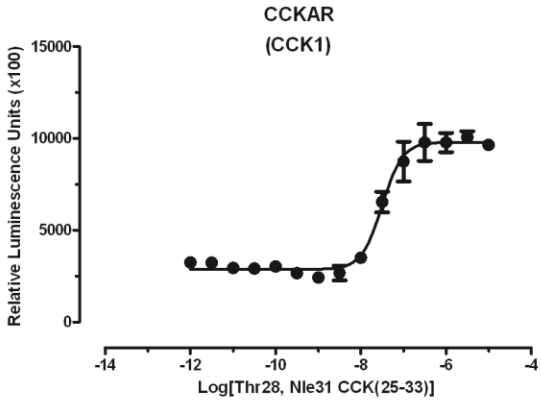

BDKRB1
(B1)
Relative Luminescence Units (x100)
2500
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
-2
Log[Bradykinin]

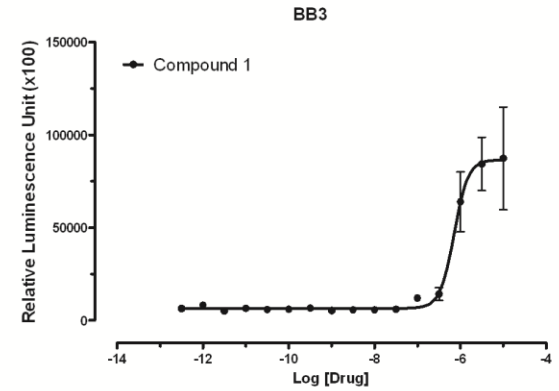

BDKRB2
(B2)
Relative Luminescence Units (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Bradykinin]

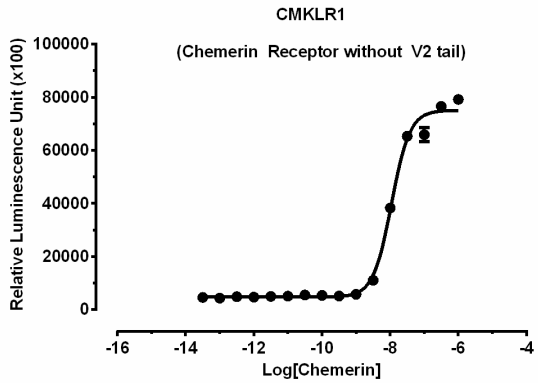

BB1
Bombesin
Gastrin-releasing peptide (GRP)
Neuromedin B (NMB)
Relative Luminescence Unit (x100)
80000
60000
40000
20000
0
-14
-12
-10
-8
-6
-4
Log [Drug]

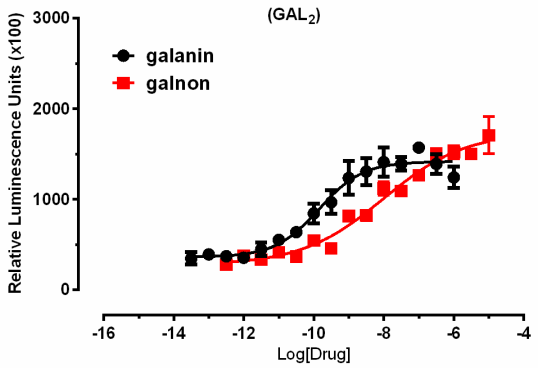

C3AR1
(C3a)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[C3a(70-77)]

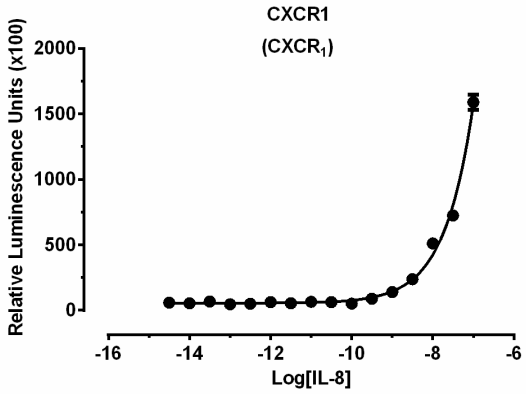

BB2
Bombesin
Gastrin-releasing peptide (GRP)
Neuromedin B (NMB)
Relative Luminescence Unit (x100)
200000
150000
100000
50000
0
-14
-12
-10
-8
-6
-4
Log [Drug]

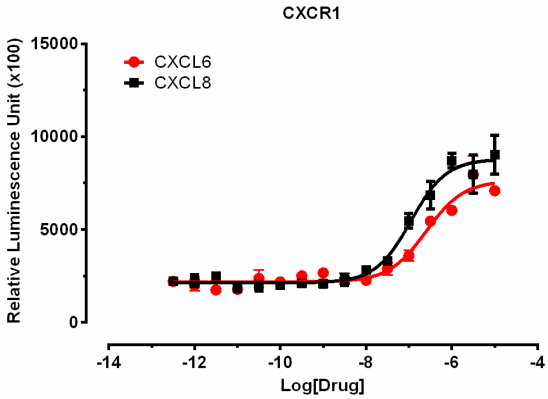

CCKAR
(CCK1)
Relative Luminescence Units (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Thr28, Nle31 CCK(25-33)]

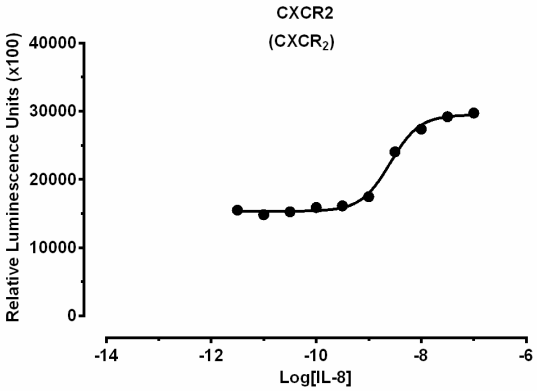

BB3
Compound 1
Relative Luminescence Unit (x100)
150000
100000
50000
0
-14
-12
-10
-8
-6
-4
Log [Drug]

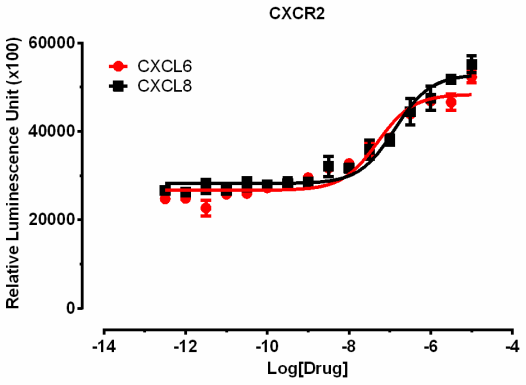

CMKLR1
(Chemerin Receptor without V2 tail)
Relative Luminescence Unit (x100)
100000
80000
60000
40000
20000
0
-16
-14
-12
-10
-8
-6
-4
Log[Chemerin]

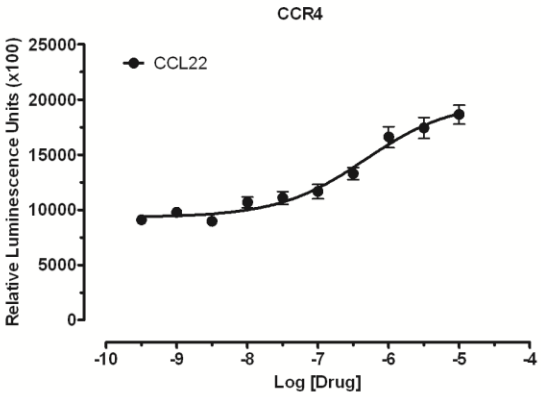

(GAL2)
galanin
galnon
Relative Luminescence Units (x100)
3000
2000
1000
0
-16
-14
-12
-10
-8
-6
-4
Log[Drug]

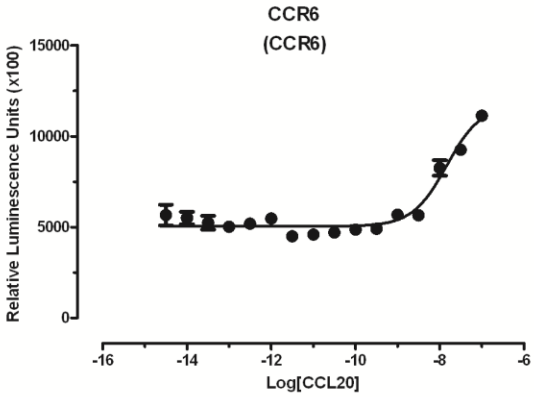

CXCR1
(CXCR1)
Relative Luminescence Units (x100)
2000
1500
1000
500
0
-16
-14
-12
-10
-8
-6
Log[IL-8]

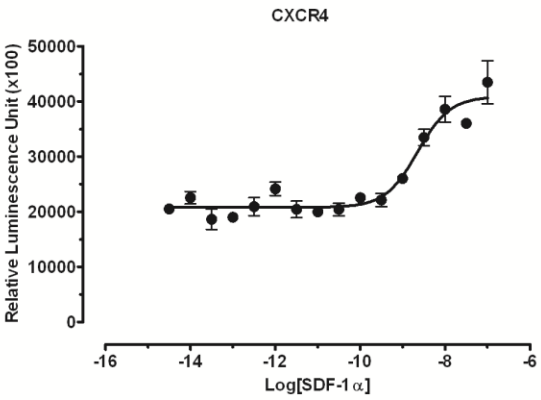

CXCR1
CXCL6
CXCL8
Relative Luminescence Unit (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Drug]

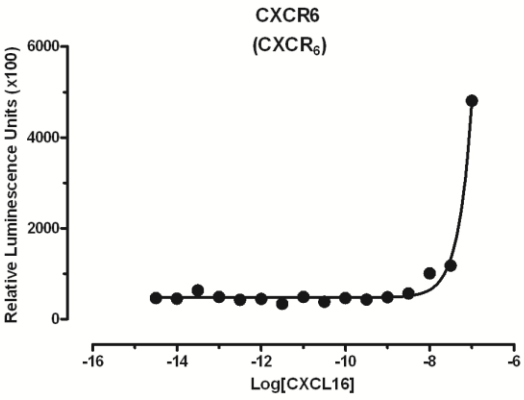

CXCR2
(CXCR2)
Relative Luminescence Units (x100)
40000
30000
20000
10000
0
-14
-12
-10
-8
-6
Log[IL-8]

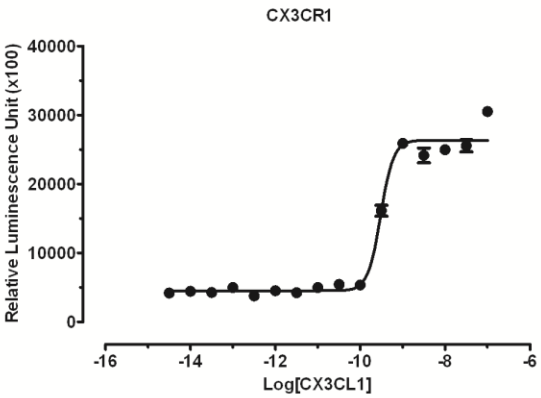

CXCR2
CXCL6
CXCL8
Relative Luminescence Unit (x100)
60000
40000
20000
0
-14
-12
-10
-8
-6
-4
Log[Drug]

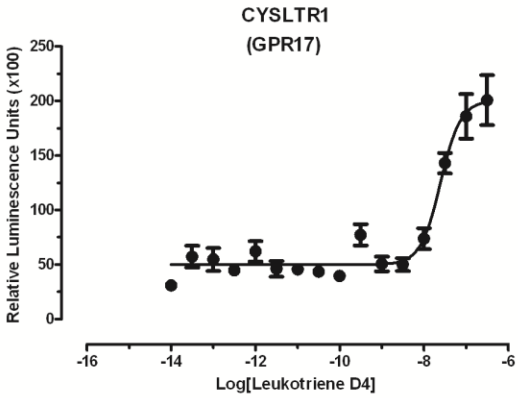

CCR4
CCL22
Relative Luminescence Units (x100)
25000
20000
15000
10000
5000
0
-10
-9
-8
-7
-6
-5
-4
Log [Drug]

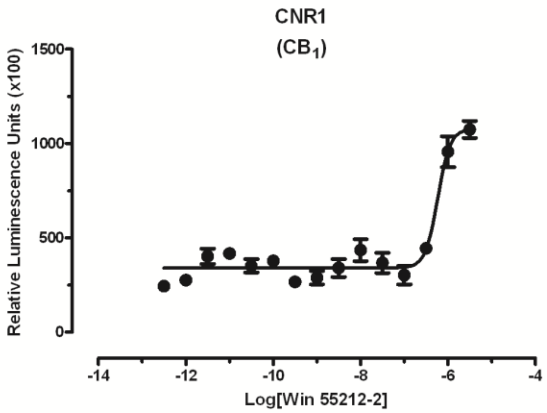

CCR6
(CCR6)
Relative Luminescence Units (x100)
15000
10000
5000
0
-16
-14
-12
-10
-8
-6
Log[CCL20]

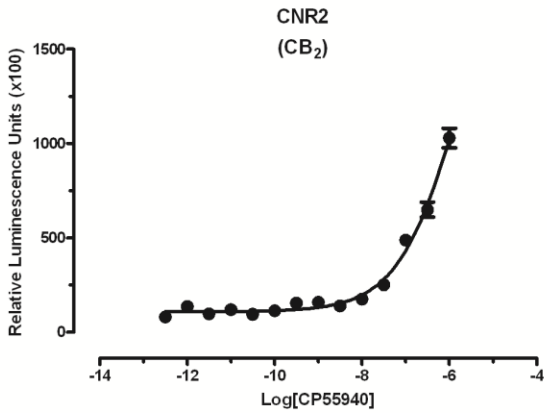

CXCR4
Relative Luminescence Unit (x100)
50000
40000
30000
20000
10000
0
-16
-14
-12
-10
-8
-6
Log[SDF-1α]

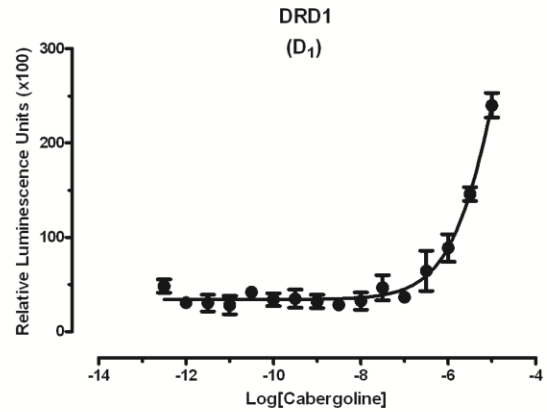

CXCR6
(CXCR6)
Relative Luminescence Units (x100)
6000
4000
2000
0
-16
-14
-12
-10
-8
-6
Log[CXCL16]

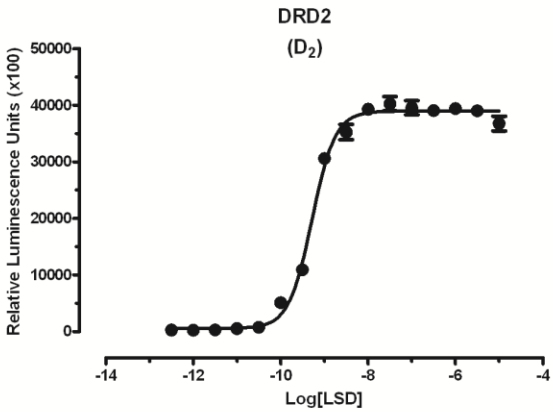

CX3CR1
Relative Luminescence Unit (x100)
40000
30000
20000
10000
0
-16
-14
-12
-10
-8
-6
Log[CX3CL1]

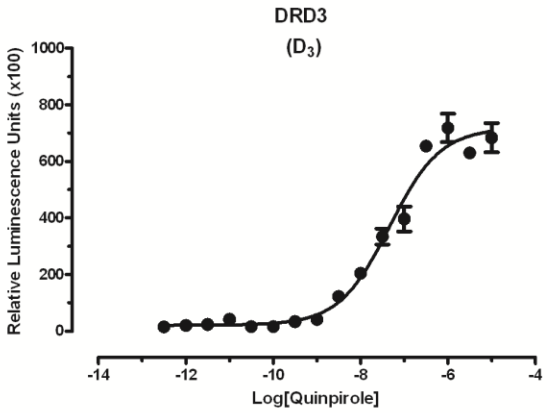

CYSLTR1
(GPR17)
Relative Luminescence Units (x100)
250
200
150
100
50
0
-16
-14
-12
-10
-8
-6
Log[Leukotriene D4]

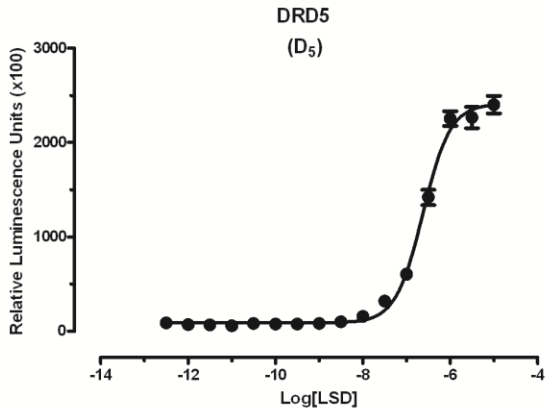

CNR1
(CB1)
Relative Luminescence Units (x100)
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Win 55212-2]

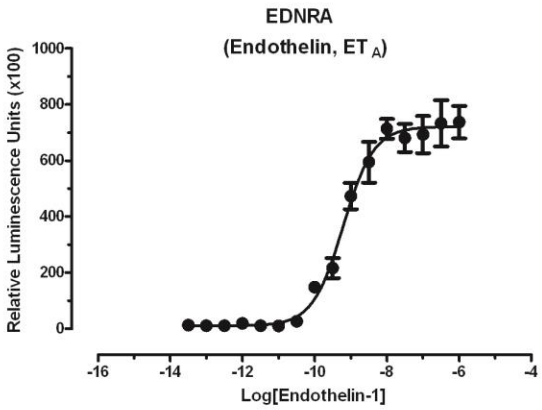

CNR2
(CB2)
Relative Luminescence Units (x100)
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[CP55940]

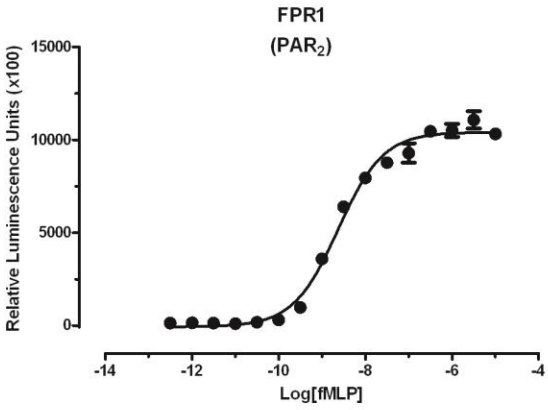

DRD1
(D1)
Relative Luminescence Units (x100)
300
200
100
0
-14
-12
-10
-8
-6
-4
Log[Cabergoline]

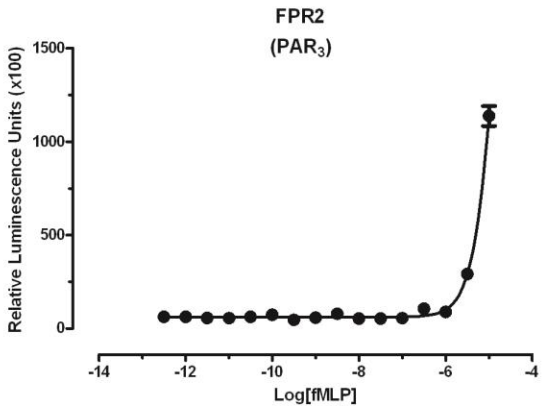

DRD2
(D2)
Relative Luminescence Units (x100)
50000
40000
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[LSD]

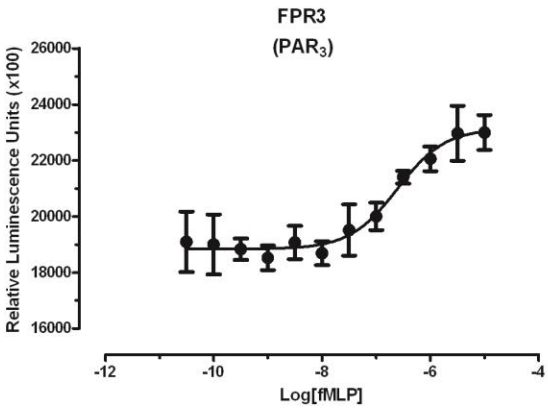

DRD3
(D3)
Relative Luminescence Units (x100)
1000
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Quinpirole]

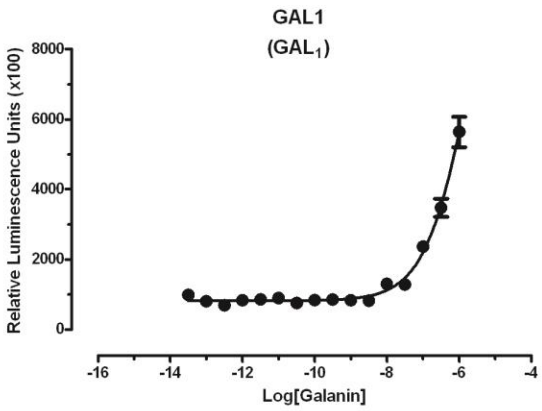

DRD5
(D5)
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[LSD]

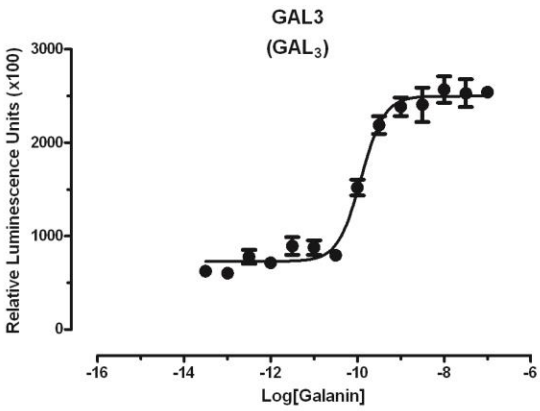

EDNRA
(Endothelin, ET A)
Relative Luminescence Units (x100)
1000
800
600
400
200
0
-16
-14
-12
-10
-8
-6
-4
Log[Endothelin-1]

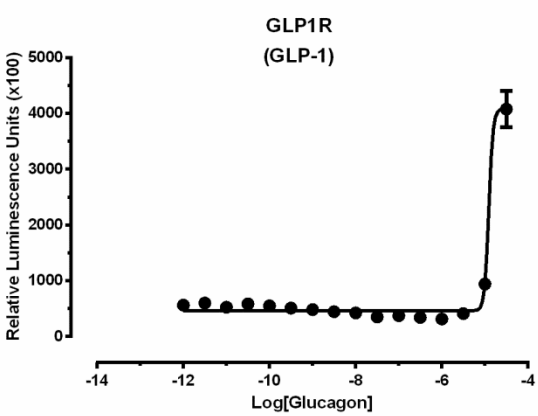

FPR1
(PAR2)
Relative Luminescence Units (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[fMLP]

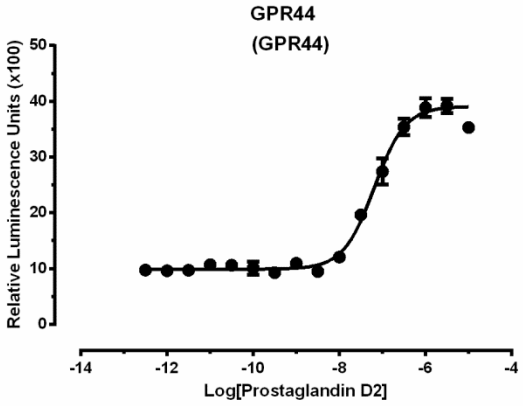

FPR2
(PAR3)
Relative Luminescence Units (x100)
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[fMLP]

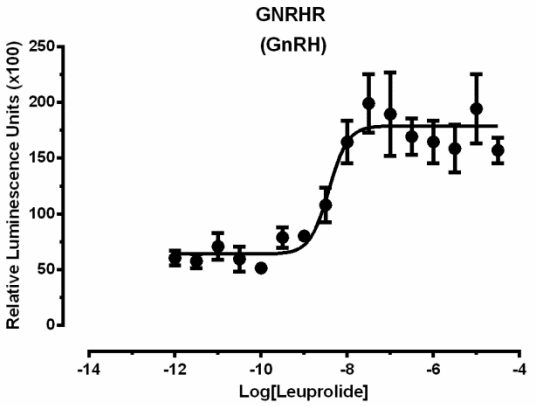

FPR3
(PAR3)
Relative Luminescence Units (x100)
26000
24000
22000
20000
18000
16000
-12
-10
-8
-6
-4
Log[fMLP]

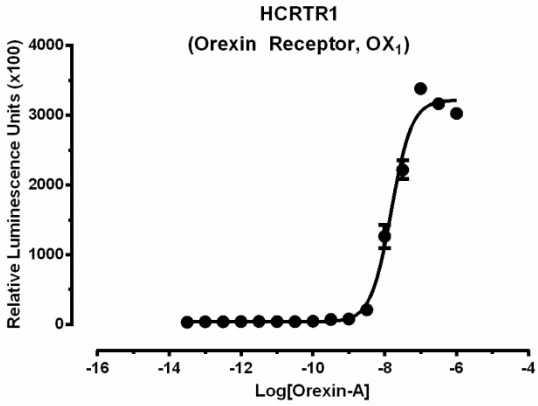

GAL1
(GAL1)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-16
-14
-12
-10
-8
-6
-4
Log[Galanin]

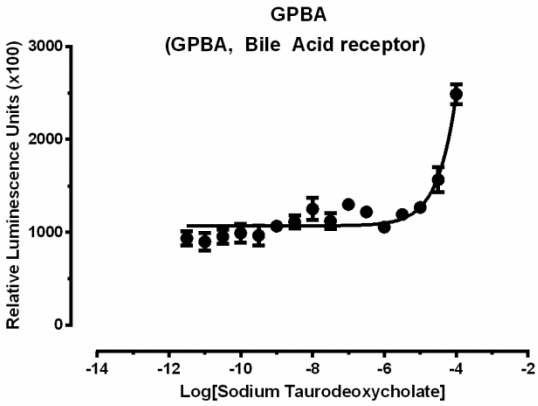

GAL3
(GAL3)
Relative Luminescence Units (x100)
3000
2000
1000
0
-16
-14
-12
-10
-8
-6
Log[Galanin]

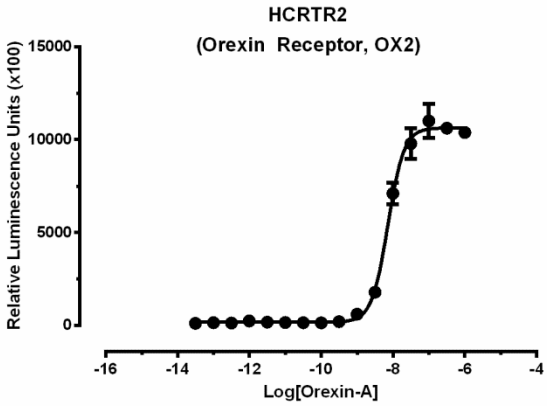

GLP1R
(GLP-1)
Relative Luminescence Units (x100)
5000
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Glucagon]

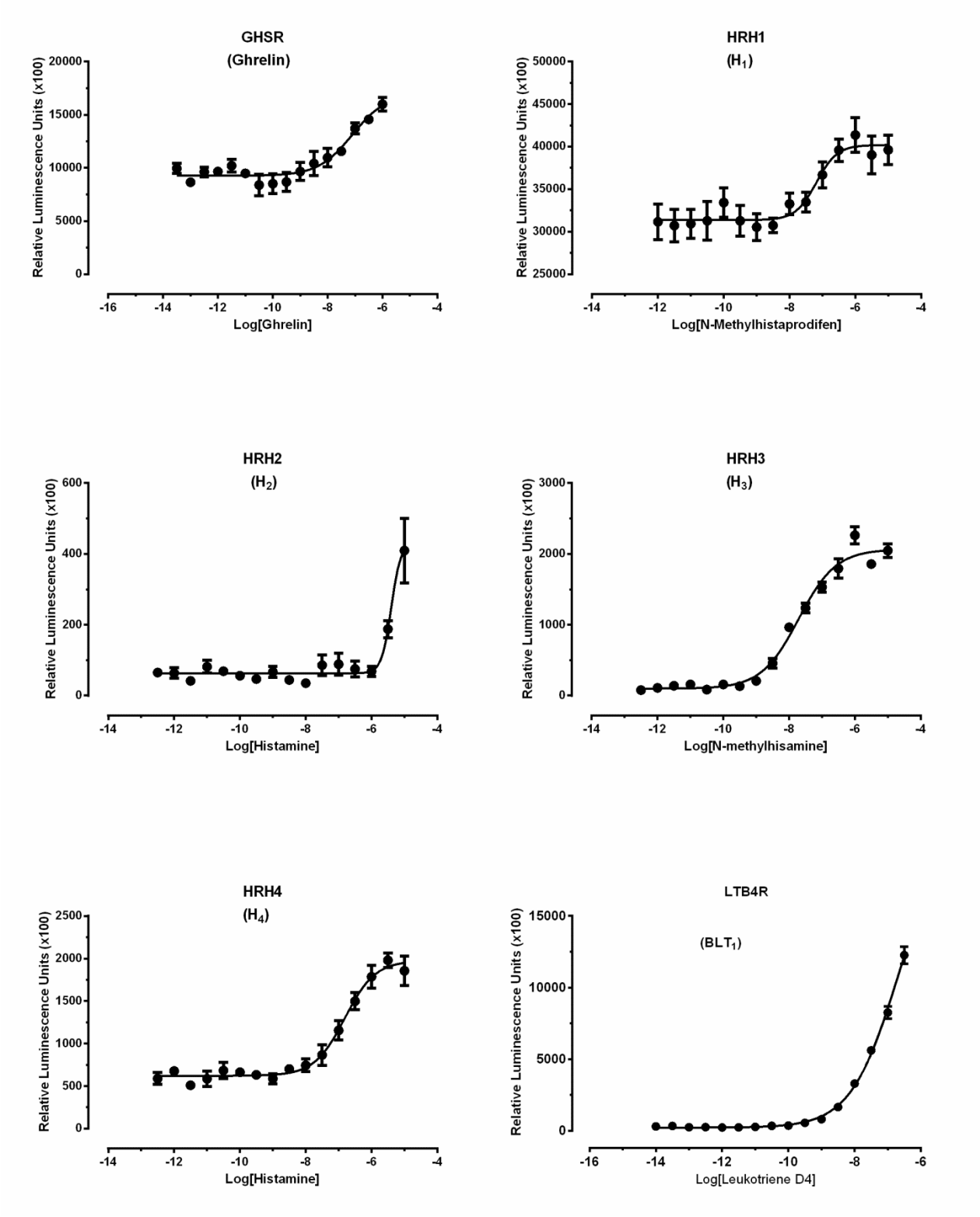

GPR44
(GPR44)
Relative Luminescence Units (x100)
50
40
30
20
10
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandin D2]

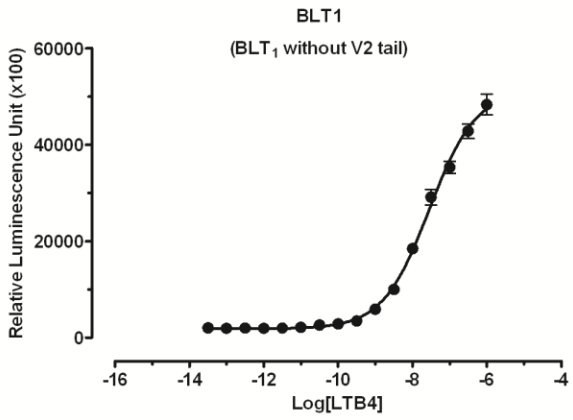

GNRHR
(GnRH)
Relative Luminescence Units (x100)
250
200
150
100
50
0
-14
-12
-10
-8
-6
-4
Log[Leuprolide]

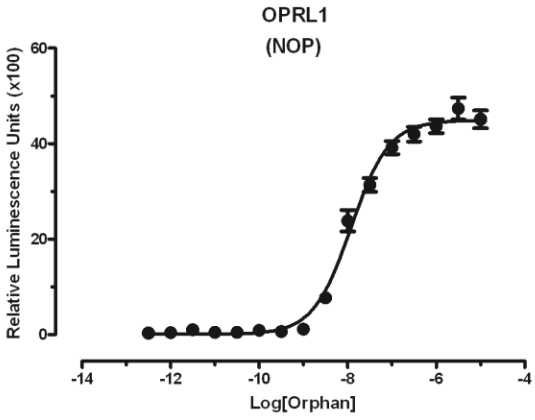

HCRTR1
(Orexin Receptor, OX1)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-16
-14
-12
-10
-8
-6
-4
Log[Orexin-A]

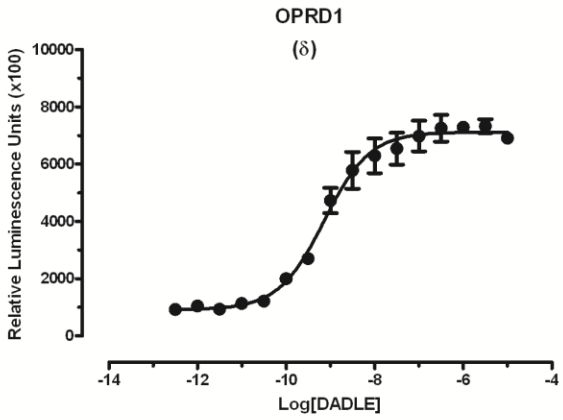

GPBA
(GPBA, Bile Acid receptor)
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
-2
Log[Sodium Taurodeoxycholate]

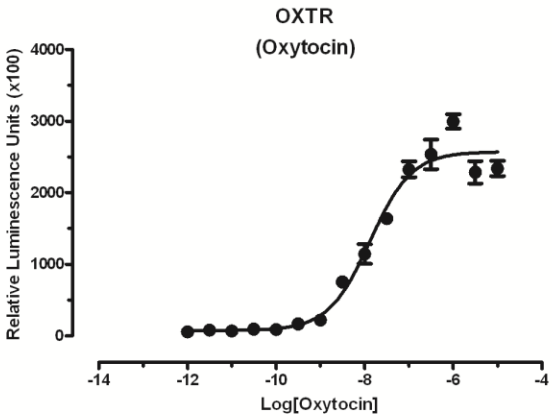

HCRTR2
(Orexin Receptor, OX2)
Relative Luminescence Units (x100)
15000
10000
5000
0
-16
-14
-12
-10
-8
-6
-4
Log[Orexin-A]

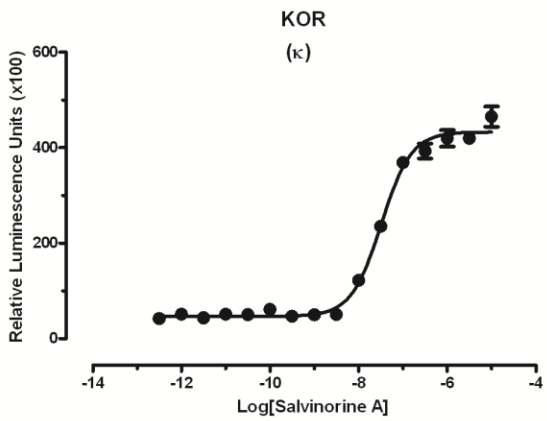

GHSR
(Ghrelin)
Relative Luminescence Units (x100)
Log[Ghrelin]
HRH1
(H1)
Relative Luminescence Units (x100)
Log[N-Methylhistaprodifen]
HRH2
(H2)
Relative Luminescence Units (x100)
Log[Histamine]
HRH3
(H3)
Relative Luminescence Units (x100)
Log[N-methylhisamine]
HRH4
(H4)
Relative Luminescence Units (x100)
Log[Histamine]
LTB4R
(BLT1)
Relative Luminescence Units (x100)
Log[Leukotriene D4]

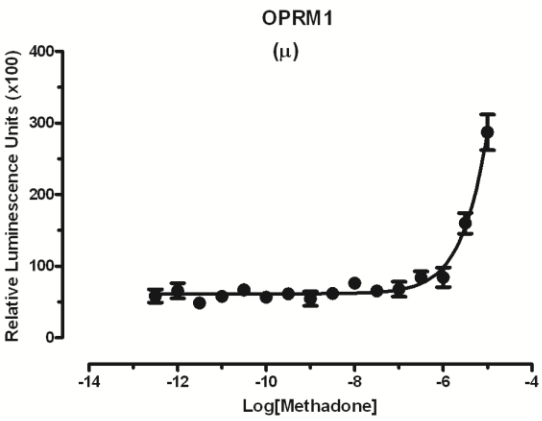

BLT1
(BLT1 without V2 tail)
Relative Luminescence Unit (x100)
60000
40000
20000
0
-16
-14
-12
-10
-8
-6
-4
Log[LTB4]

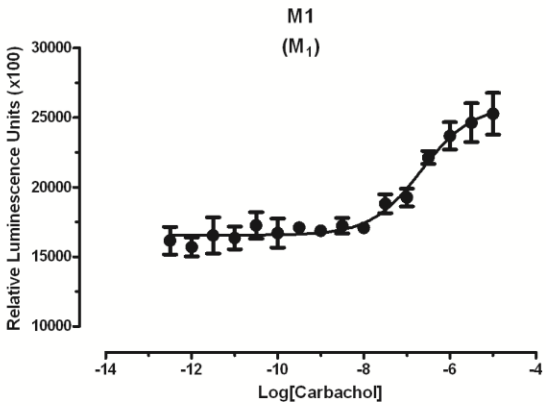

OPRL1
(NOP)
Relative Luminescence Units (x100)
60
40
20
0
-14
-12
-10
-8
-6
-4
Log[Orphan]

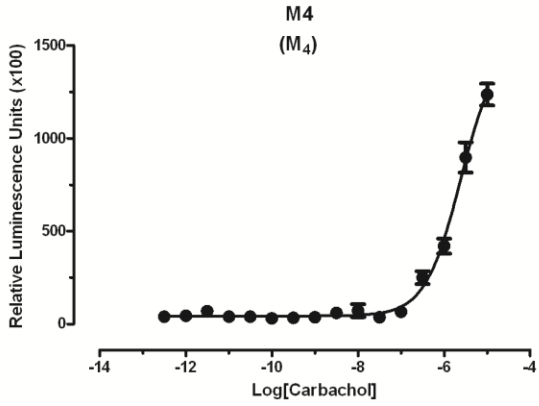

OPRD1
(δ)
Relative Luminescence Units (x100)
10000
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[DADLE]

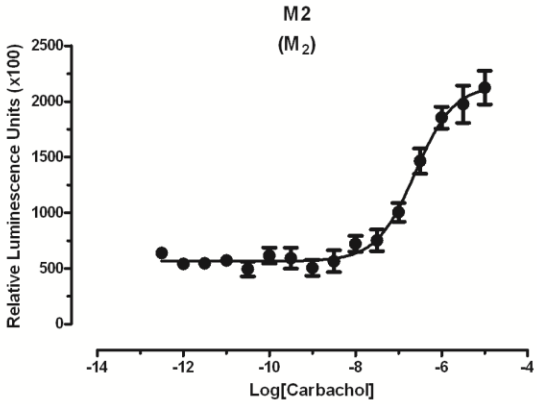

OXTR
(Oxytocin)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Oxytocin]

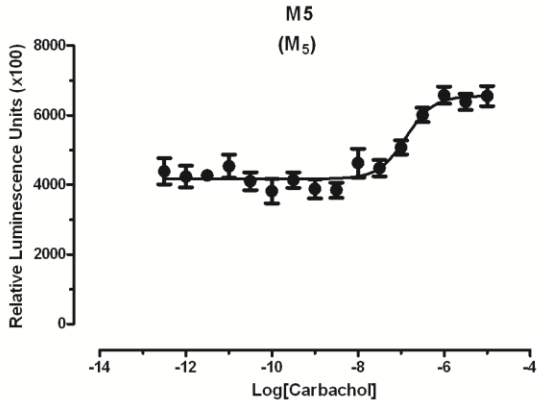

KOR
(κ)
Relative Luminescence Units (x100)
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Salvinorine A]

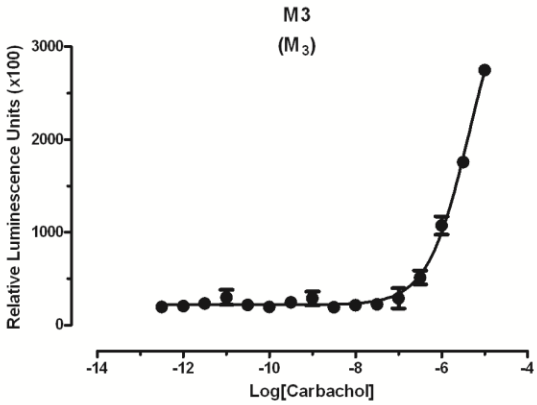

OPRM1
(μ)
Relative Luminescence Units (x100)
400
300
200
100
0
-14
-12
-10
-8
-6
-4
Log[Methadone]

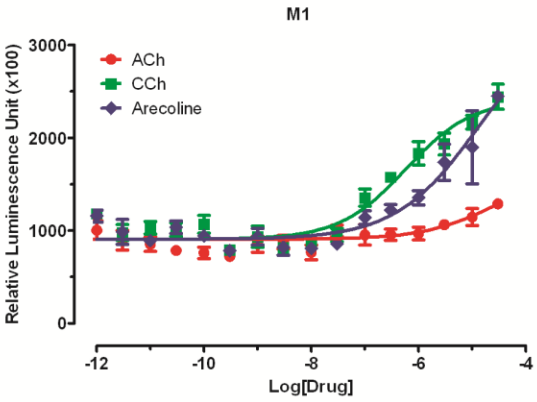

M1
(M1)
Relative Luminescence Units (x100)
30000
25000
20000
15000
10000
-14
-12
-10
-8
-6
-4
Log[Carbachol]

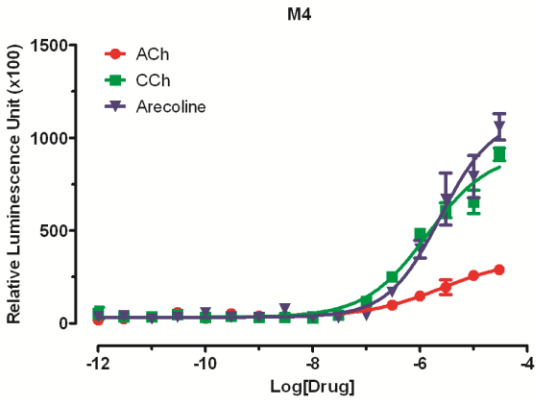

M4
(M4)
Relative Luminescence Units (x100)
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Carbachol]

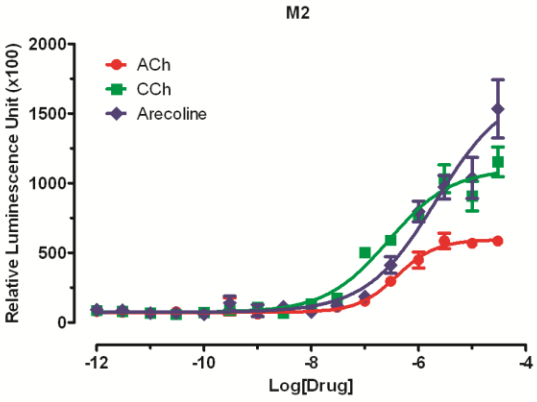

M2
(M2)
Relative Luminescence Units (x100)
2500
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Carbachol]

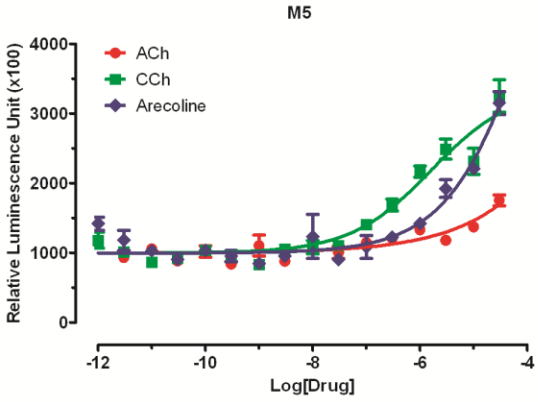

M5
(M5)
Relative Luminescence Units (x100)
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Carbachol]

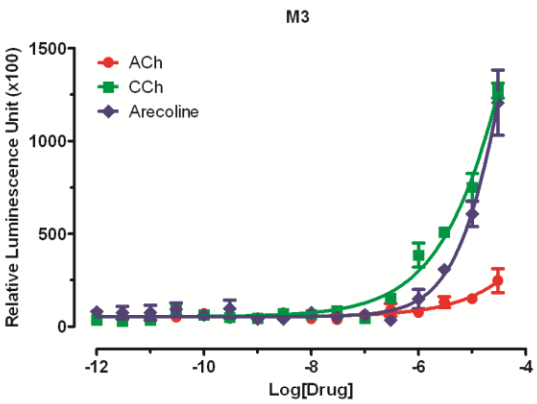

M3
(M3)
Relative Luminescence Units (x100)
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Carbachol]

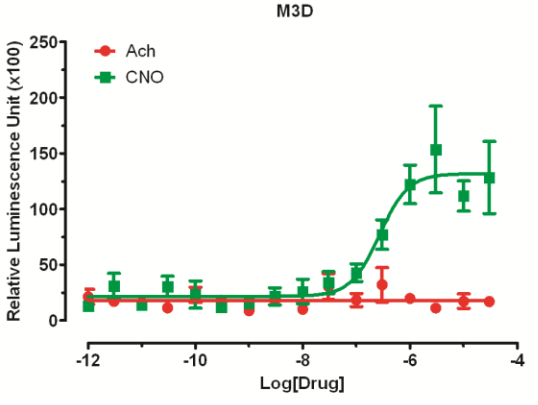

M1
ACh
CCh
Arecoline
Relative Luminescence Unit (x100)
3000
2000
1000
0
-12
-10
-8
-6
-4
Log[Drug]

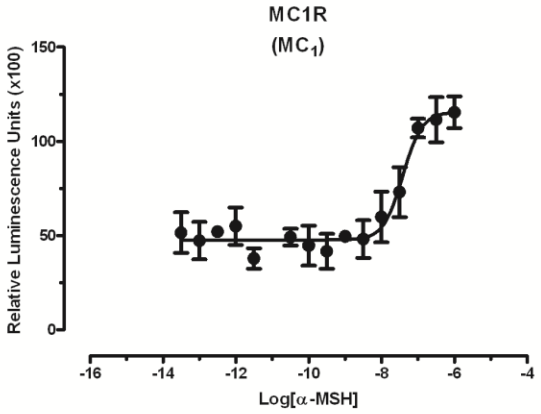

M4
ACh
CCh
Arecoline
Relative Luminescence Unit (x100)
1500
1000
500
0
-12
-10
-8
-6
-4
Log[Drug]

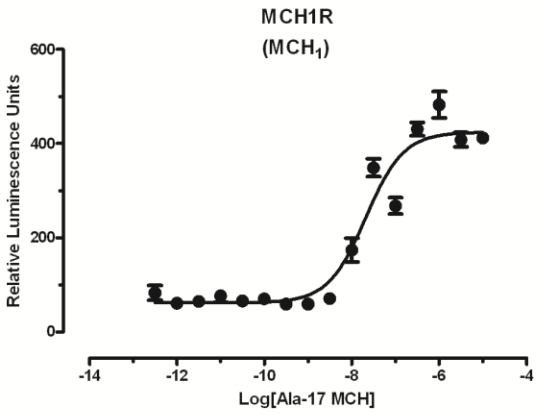

M2
ACh
CCh
Arecoline
Relative Luminescence Unit (x100)
2000
1500
1000
500
0
-12
-10
-8
-6
-4
Log[Drug]

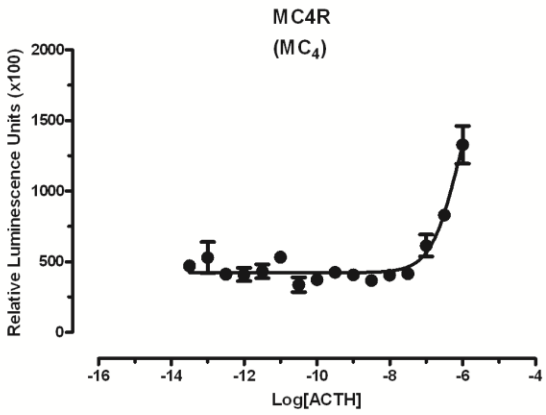

M5
ACh
CCh
Arecoline
Relative Luminescence Unit (x100)
4000
3000
2000
1000
0
-12
-10
-8
-6
-4
Log[Drug]

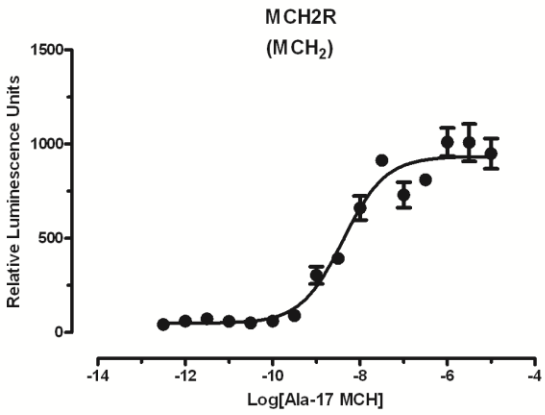

M3
ACh
CCh
Arecoline
Relative Luminescence Unit (x100)
1500
1000
500
0
-12
-10
-8
-6
-4
Log[Drug]

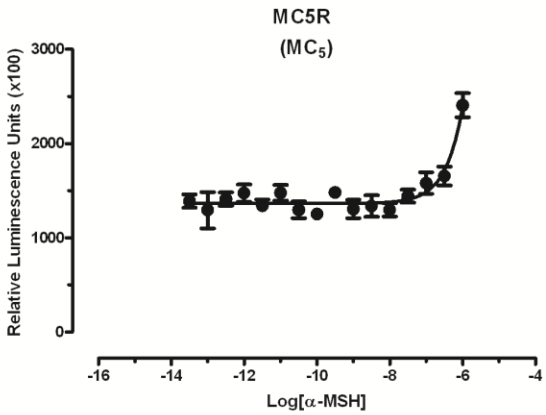

M3D
Ach
CNO
Relative Luminescence Unit (x100)
250
200
150
100
50
0
-12
-10
-8
-6
-4
Log[Drug]

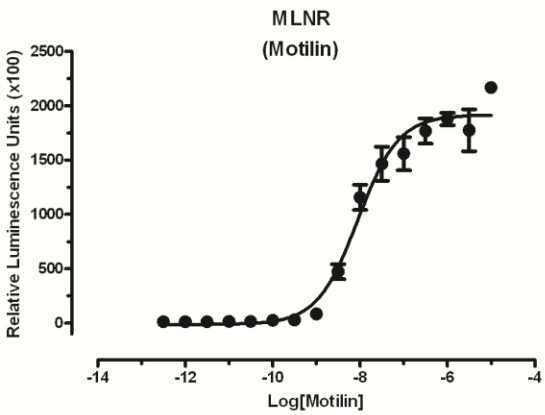

MC1R
(MC1)
Relative Luminescence Units (x100)
150
100
50
0
-16
-14
-12
-10
-8
-6
-4
Log[α-MSH]

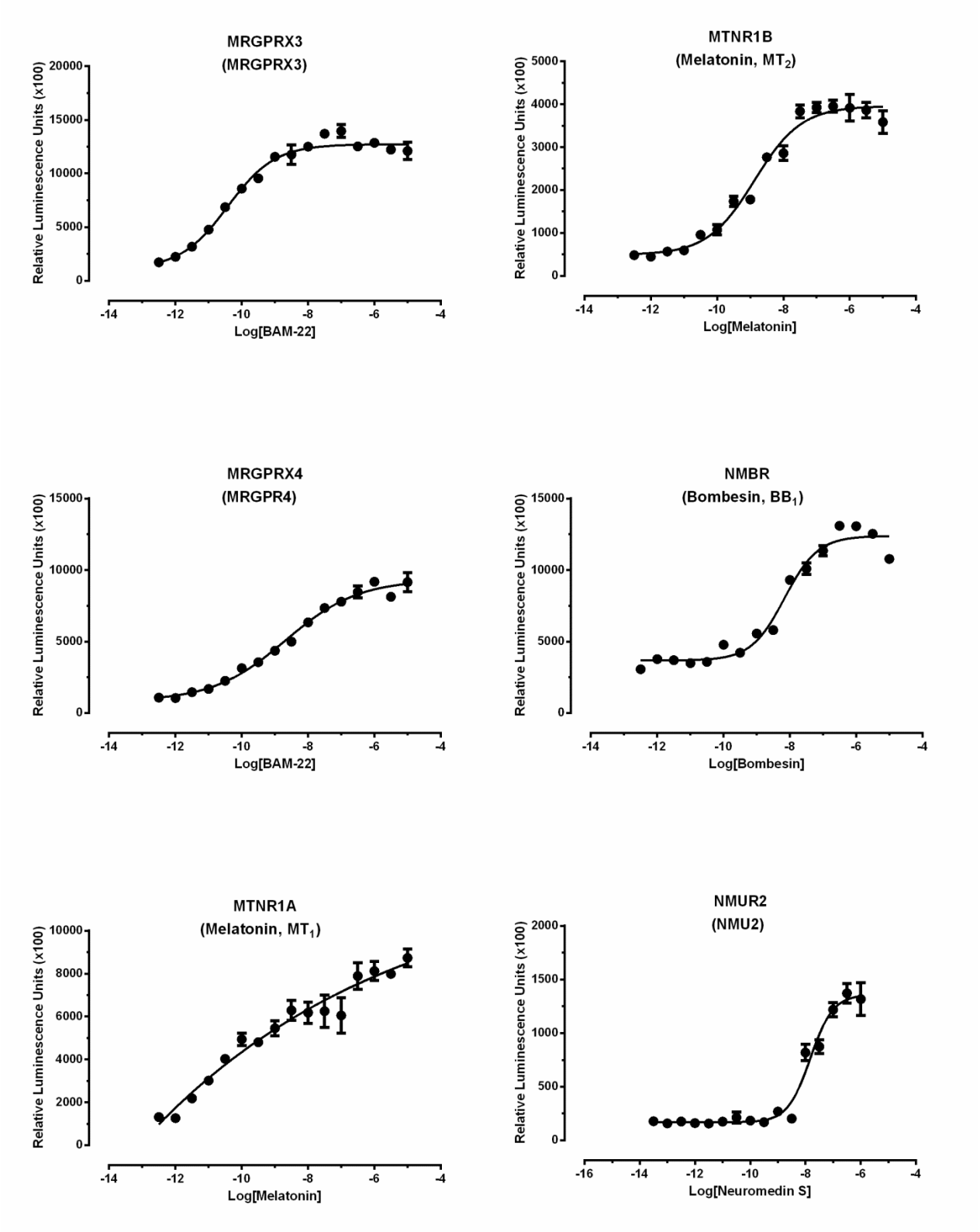

MCH1R
(MCH1)
Relative Luminescence Units
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Ala-17 MCH]

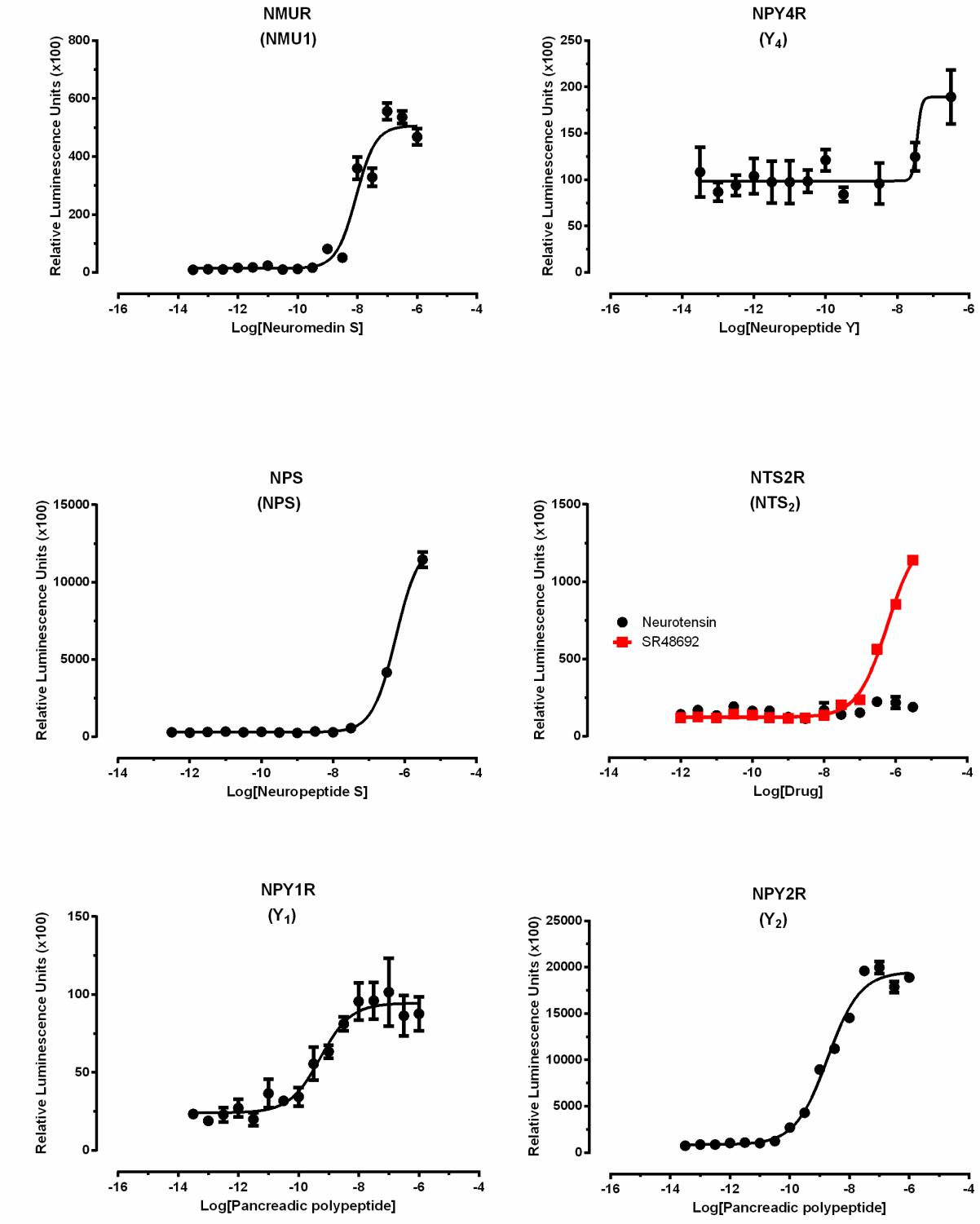

MC4R
(MC4)
Relative Luminescence Units (x100)
2000
1500
1000
500
0
-16
-14
-12
-10
-8
-6
-4
Log[ACTH]

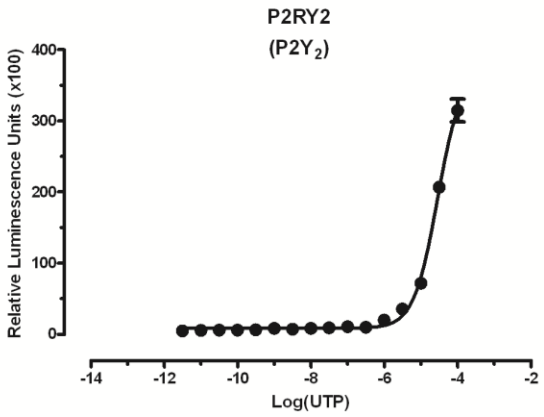

MCH2R
(MCH2)
Relative Luminescence Units
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Ala-17 MCH]

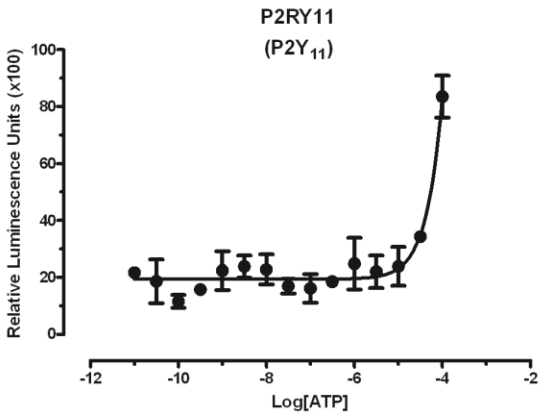

MC5R
(MC5)
Relative Luminescence Units (x100)
3000
2000
1000
0
-16
-14
-12
-10
-8
-6
-4
Log[α-MSH]

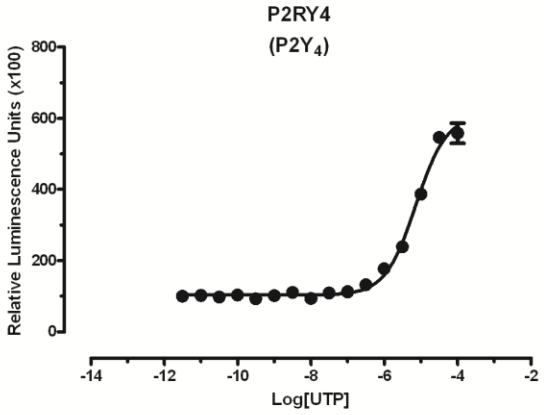

MLNR
(Motilin)
Relative Luminescence Units (x100)
2500
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[Motilin]

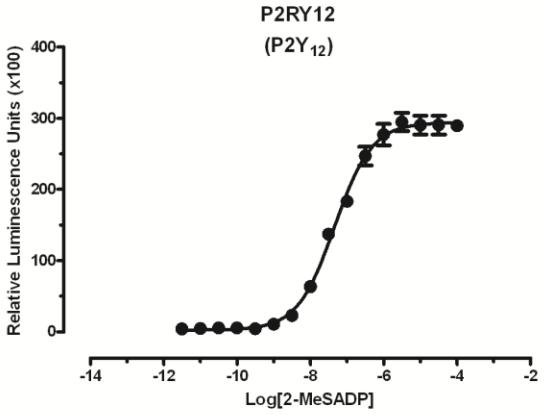

MRGPRX3
(MRGPRX3)
Relative Luminescence Units (x100)
Log[BAM-22]
MTNR1B
(Melatonin, MT2)
Relative Luminescence Units (x100)
Log[Melatonin]
MRGPRX4
(MRGPR4)
Relative Luminescence Units (x100)
Log[BAM-22]
NMBR
(Bombesin, BB1)
Relative Luminescence Units (x100)
Log[Bombesin]
MTNR1A
(Melatonin, MT1)
Relative Luminescence Units (x100)
Log[Melatonin]
NMUR2
(NMU2)
Relative Luminescence Units (x100)
Log[Neuromedin S]

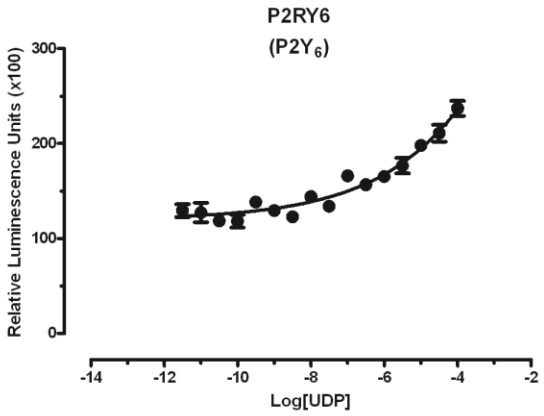

NMUR
(NMU1)
Relative Luminescence Units (x100)
Log[Neuromedin S]
NPY4R
(Y4)
Relative Luminescence Units (x100)
Log[Neuropeptide Y]
NPS
(NPS)
Relative Luminescence Units (x100)
Log[Neuropeptide S]
NTS2R
(NTS2)
Neurotensin
SR48692
Relative Luminescence Units (x100)
Log[Drug]
NPY1R
(Y1)
Relative Luminescence Units (x100)
Log[Pancreadic polypeptide]
NPY2R
(Y2)
Relative Luminescence Units (x100)
Log[Pancreadic polypeptide]

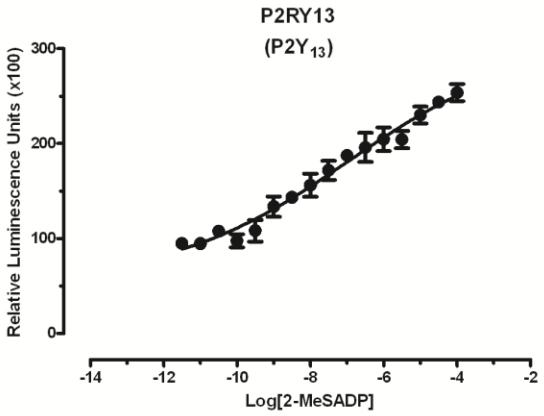

P2RY2
(P2Y2)
Relative Luminescence Units (x100)
Log(UTP)

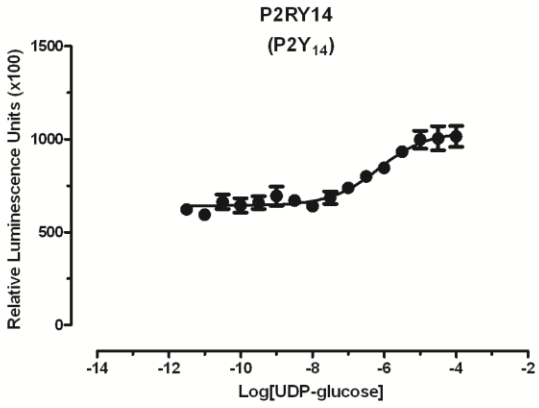

P2RY11
(P2Y11)
Relative Luminescence Units (x100)
Log[ATP]

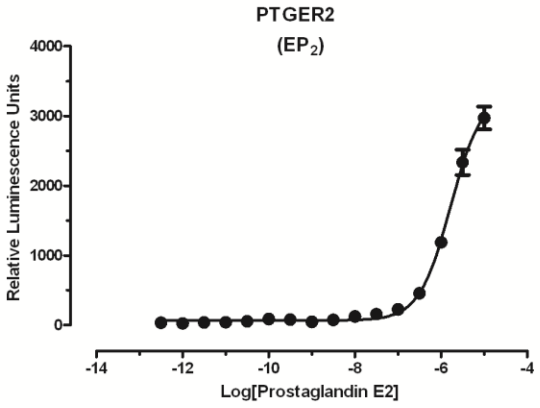

P2RY4
(P2Y4)
Relative Luminescence Units (x100)
Log[UTP]

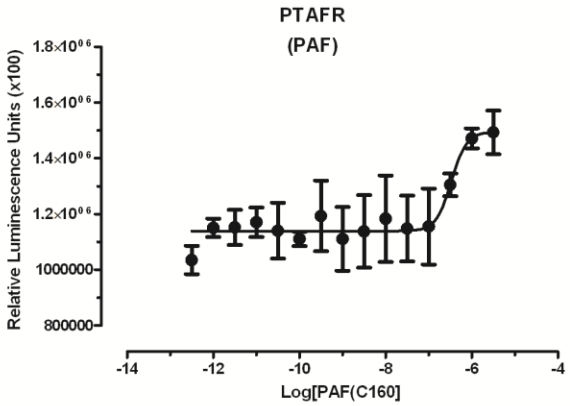

P2RY12
(P2Y12)
Relative Luminescence Units (x100)
Log[2-MeSADP]

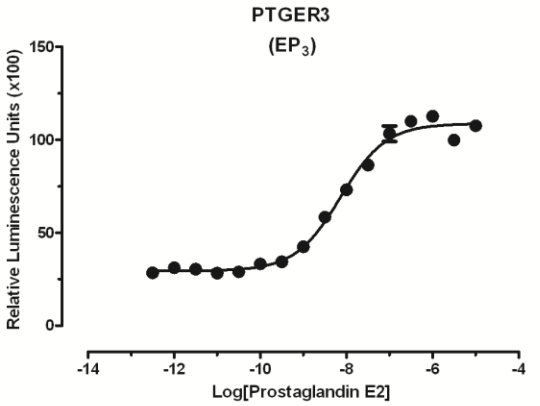

P2RY6
(P2Y6)
Relative Luminescence Units (x100)
Log[UDP]

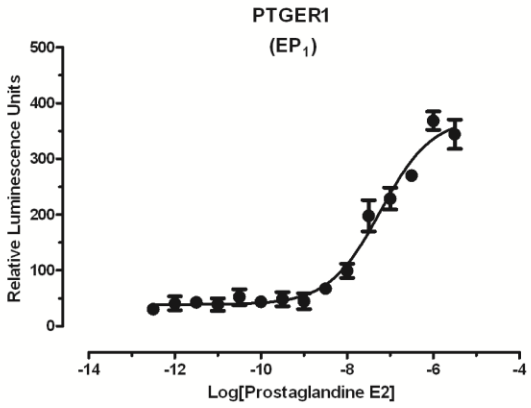

P2RY13
(P2Y13)
Relative Luminescence Units (x100)
Log[2-MeSADP]

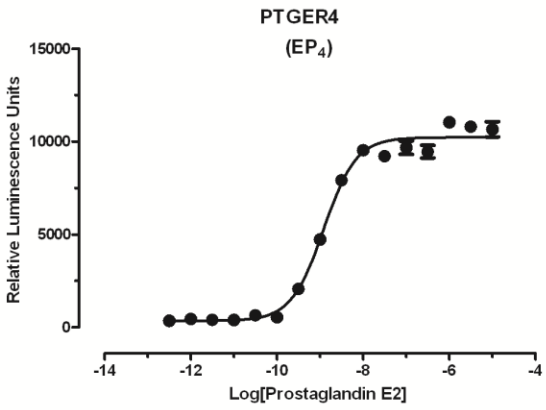

P2RY14
(P2Y14)
Relative Luminescence Units (x100)
1500
1000
500
0
-14
-12
-10
-8
-6
-4
-2
Log[UDP-glucose]

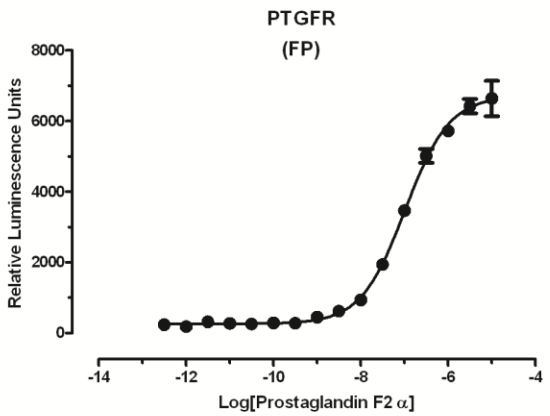

PTGER2
(EP2)
Relative Luminescence Units
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandin E2]

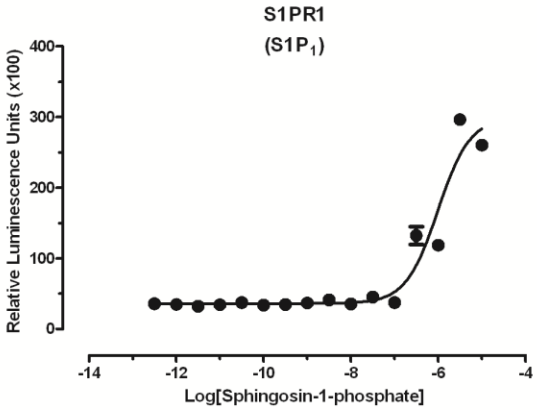

PTAFR
(PAF)
Relative Luminescence Units (x100)
1.8×10^06
1.6×10^06
1.4×10^06
1.2×10^06
1000000
800000
-14
-12
-10
-8
-6
-4
Log[PAF(C160]

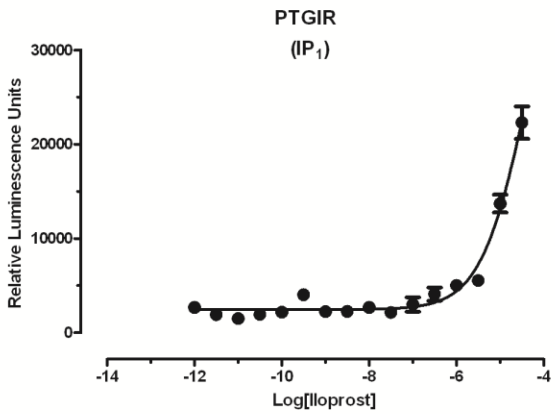

PTGER3
(EP3)
Relative Luminescence Units (x100)
150
100
50
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandin E2]

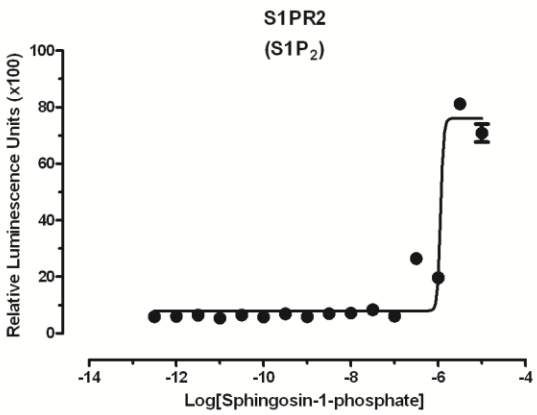

PTGER1
(EP1)
Relative Luminescence Units
500
400
300
200
100
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandine E2]

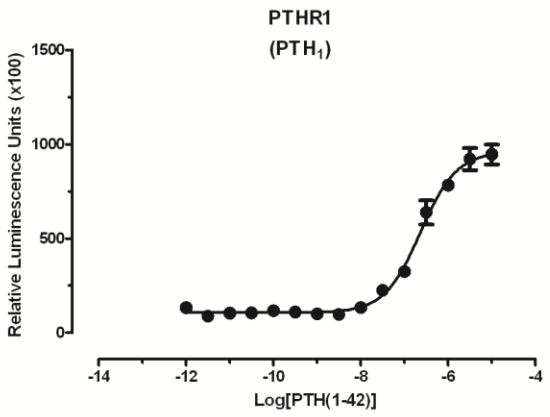

PTGER4
(EP4)
Relative Luminescence Units
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandin E2]

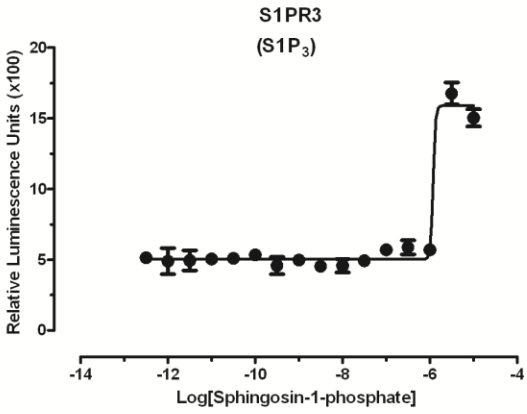

PTGFR
(FP)
Relative Luminescence Units
8000
6000
4000
2000
0
-14
-12
-10
-8
-6
-4
Log[Prostaglandin F2 α]

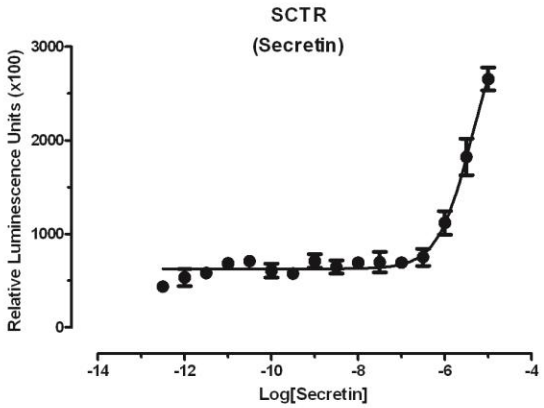

S1PR1
(S1P1)
Relative Luminescence Units (x100)
400
300
200
100
0
-14
-12
-10
-8
-6
-4
Log[Sphingosin-1-phosphate]

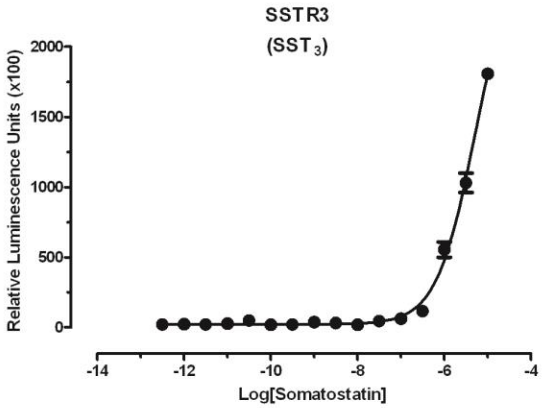

PTGIR
(IP1)
Relative Luminescence Units
30000
20000
10000
0
-14
-12
-10
-8
-6
-4
Log[Iloprost]

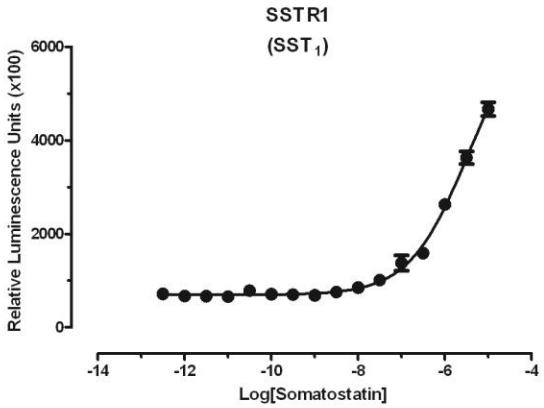

S1PR2
(S1P2)
Relative Luminescence Units (x100)
100
80
60
40
20
0
-14
-12
-10
-8
-6
-4
Log[Sphingosin-1-phosphate]

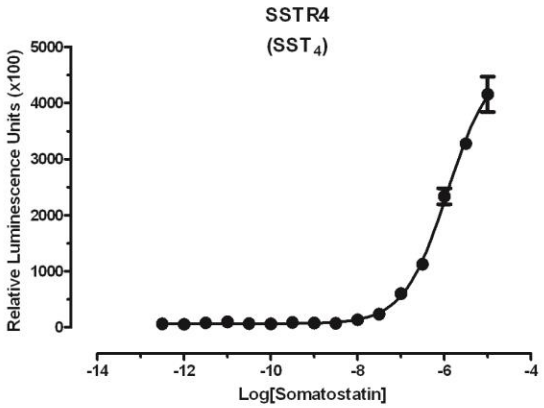

PTHR1
(PTH1)
Relative Luminescence Units (x100)
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[PTH(1-42)]


S1PR3
(S1P3)
Relative Luminescence Units (x100)
20
15
10
5
0
-14
-12
-10
-8
-6
-4
Log[Sphingosin-1-phosphate]


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


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


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


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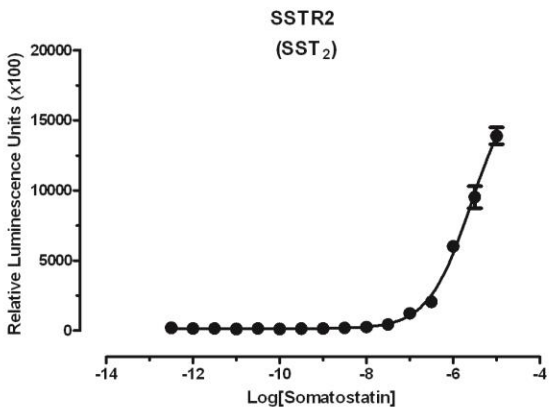
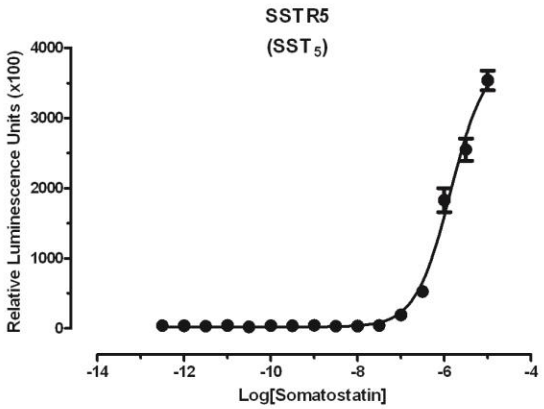
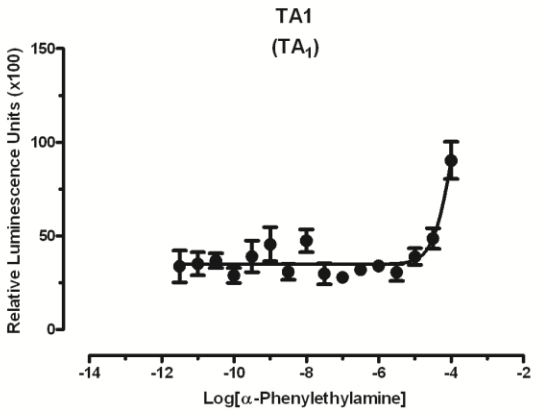
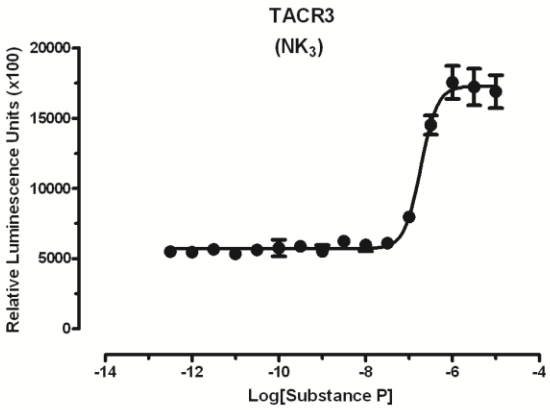
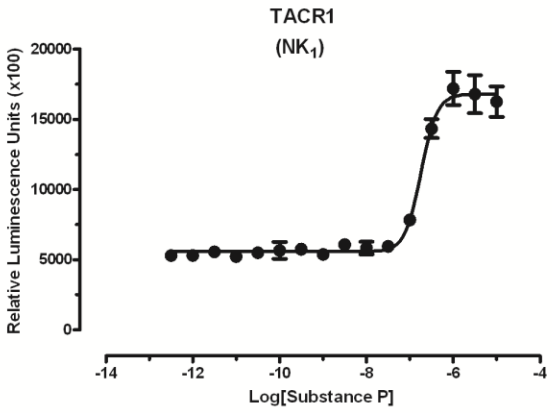
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Somatostatin]

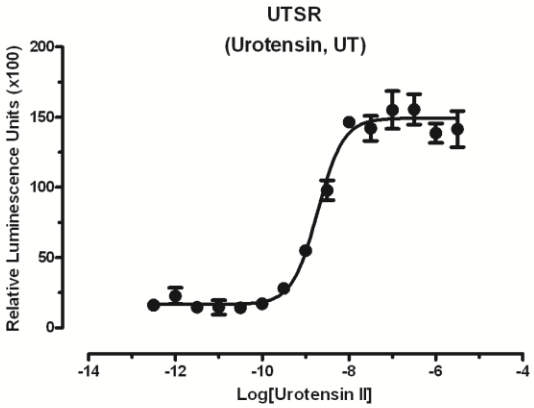

SSTR2
(SST2)
Relative Luminescence Units (x100)
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Somatostatin]

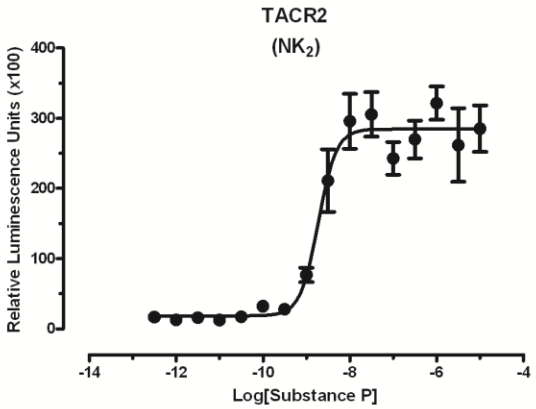

SSTR5
(SST5)
Relative Luminescence Units (x100)
4000
3000
2000
1000
0
-14
-12
-10
-8
-6
-4
Log[Somatostatin]

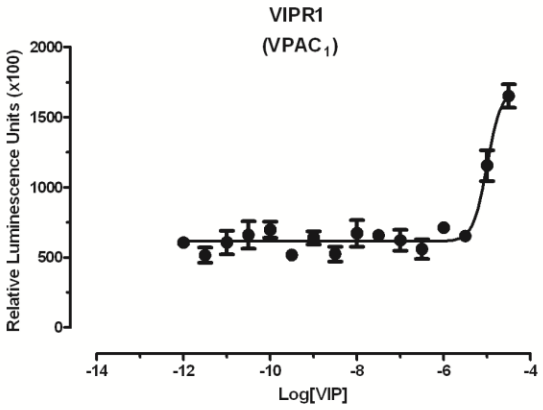

TA1
(TA1)
Relative Luminescence Units (x100)
150
100
50
0
-14
-12
-10
-8
-6
-4
-2
Log[α-Phenylethylamine]

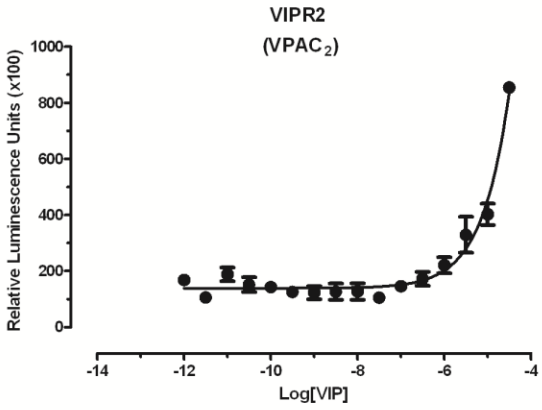

TACR3
(NK3)
Relative Luminescence Units (x100)
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Substance P]

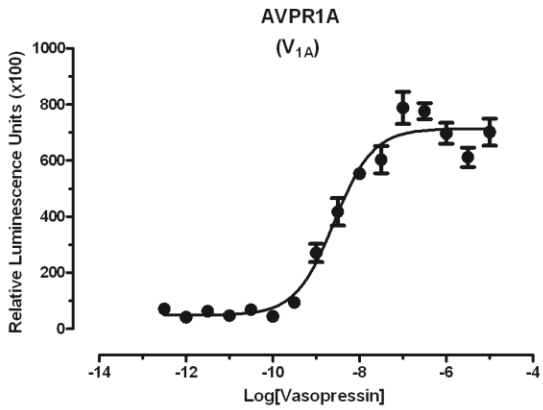

TACR1
(NK1)
Relative Luminescence Units (x100)
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
Log[Substance P]

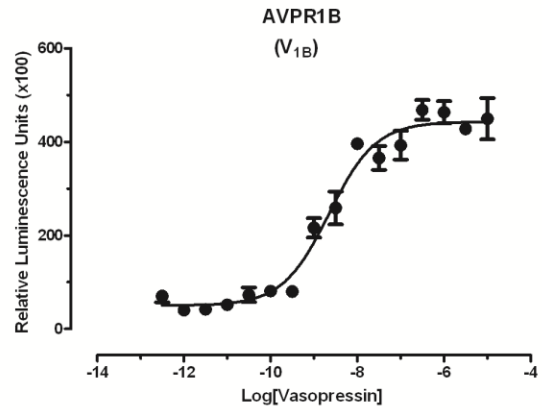

UTSR
(Urotensin, UT)
Relative Luminescence Units (x100)
200
150
100
50
0
-14
-12
-10
-8
-6
-4
Log[Urotensin II]

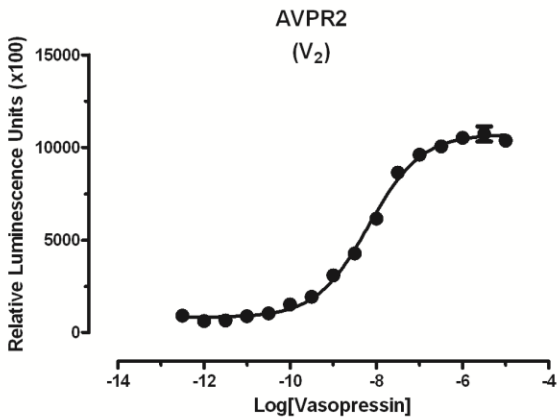

TACR2
(NK2)
Relative Luminescence Units (x100)
400
300
200
100
0
-14
-12
-10
-8
-6
-4
Log[Substance P]

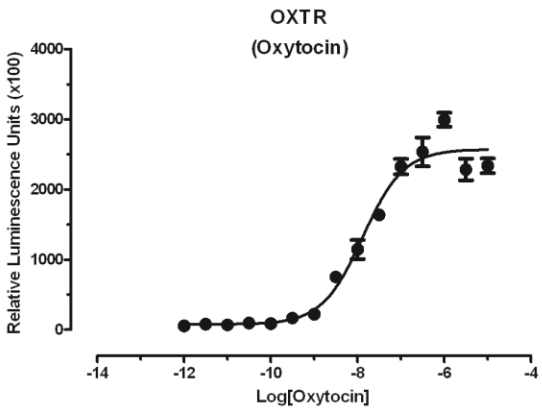

VIPR1
(VPAC1)
Relative Luminescence Units (x100)
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
Log[VIP]

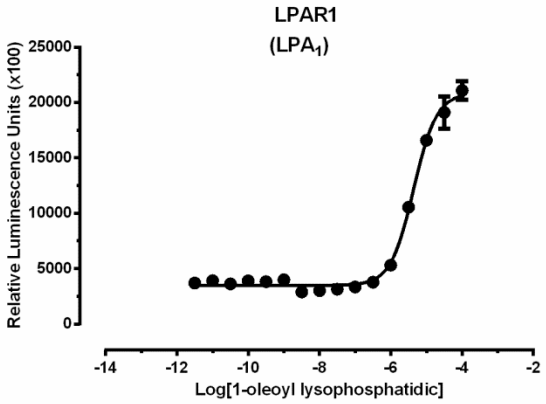

VIPR2
(VPAC2)
Relative Luminescence Units (x100)
1000
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[VIP]

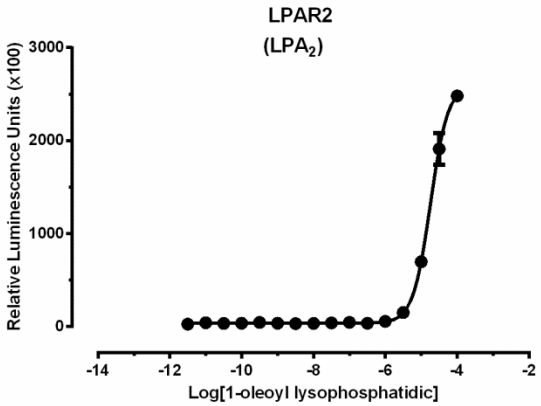

AVPR1A
(V1A)
Relative Luminescence Units (x100)
1000
800
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Vasopressin]

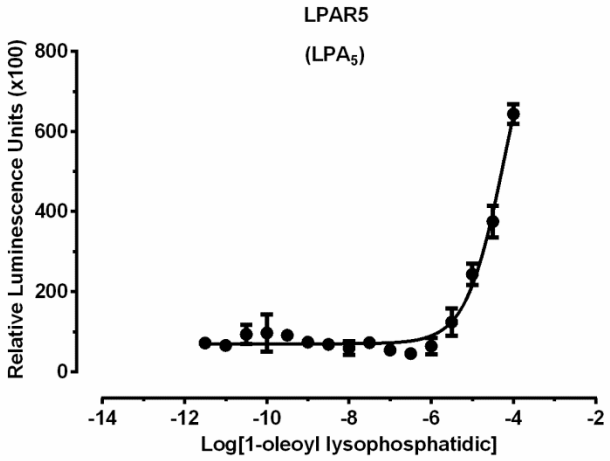

AVPR1B
(V1B)
Relative Luminescence Units (x100)
600
400
200
0
-14
-12
-10
-8
-6
-4
Log[Vasopressin]


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


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


LPAR1
(LPA1)
Relative Luminescence Units (x100)
25000
20000
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
-2
Log[1-oleoyl lysophosphatidic]


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


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

2.7. Parallel receptorome screening using β-arrestin recruitment assay

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-arrestin-TEV protease fusion gene, are maintained in DMEM supplemented with 10% FBS 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 to 20,000 cells in 50 μl per well and incubated overnight.

2.7.2. DNA plate. Each single DNA plasmid is plated using the liquid handling workstation into one well of a 96-well plate at 0.5 μg/well (enough for 8x 384-well plates). Each plate includes 80 receptor DNA samples, positive controls in wells A12 and B12 (DNA plasmids for receptors with cognate ligands), transfection controls in wells A1, B1, G12, and H12 (DNA plasmid for YFP), and negative controls with buffer only. We use D_2-Tango (quinpirole as agonist) and V_2-Tango (Vasopressin as agonist) constructs as positive assay controls. These plates are kept in the freezer until ready for assay. Immediately before transfection (see below), two DNA plates are manually combined in a cell culture hood into one 384-well plate with duplicate wells for every DNA (see **Figures 46 and 47** for DNA maps in 96-and 384-well plate formats); immediately followed by calcium phosphate transfection (see below).

2.7.3. Transfection using Calcium phosphate precipitation protocol. HTLA cells are set up as indicated above overnight before transfection. Plated DNA in 384-well plate (**Figure 47**) is first diluted to final volume of 100 μl with 0.25 M $CaCl_2$ in TE buffer (1 mM Tris–HCl, 0.1 mM EDTA, pH 7.6). An equal volume of 2x HBS solution (50 mM HEPES, 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, pH 7.00) is added to the DNA/$CaCl_2$ solution and dispensed onto the plated cells in duplicate (adjacent wells) as shown in **Figure 47** (i.e. A1 and A2, A3 and A4, …, P23 and P24). Thus, the DNAs from two 96-well plates (p1 and p2) are transfected in one 384-well plate (P1),

and therefore each DNA plasmid is transfected in duplicate. DNA plate 1 (p1) is transfected in rows A to H and DNA plate 2 (p2) is transfected in rows I to P. Plates are incubated overnight at 37°C.

2.7.4. Assay procedure. Each 384-well plate is designed to test one drug at multiple (up to 160) target receptors simultaneously. Odd-numbered columns contain Tango assay buffer to serve as a basal control and even-numbered columns contain drug stimulation solution prepared in filtered Tango assay buffer at 6 µM (final concentration is 1 µM). The drug plate design is shown in **Figure 48**. On the day of the assay, growth medium is replaced by serum-free medium supplemented with penicillin/streptomycin 4 hours before stimulation. Cells are then stimulated by addition of drugs (10 µl per well) and incubated overnight at 37°C. The following day, medium and drug solutions are removed and 20 µl per well of BrightGlo reagent (diluted by 20-fold with Tango assay buffer) are added. The plate is incubated for at least 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 save files in Excel spreadsheets for easy processing. For receptors that have positive controls (non-orphan receptors), Activation relative to positive control (%) is calculated according to the following formula:

$$Activation\ (relative\ to\ positive\ control, \%) = \frac{(\text{Test compound RLU}) - (\text{ave. negative control RLU})}{(\text{ave. positive control RLU}) - (\text{ave. negative control RLU})} * 100$$

For receptors that lack positive controls (orphan receptors), Activation relative to baseline (%) is calculated according to the formula below:

$$Activation\ (relative\ to\ baseline, \%) = \frac{(\text{Test compound RLU})}{(\text{ave.baseline RLU})} * 100$$

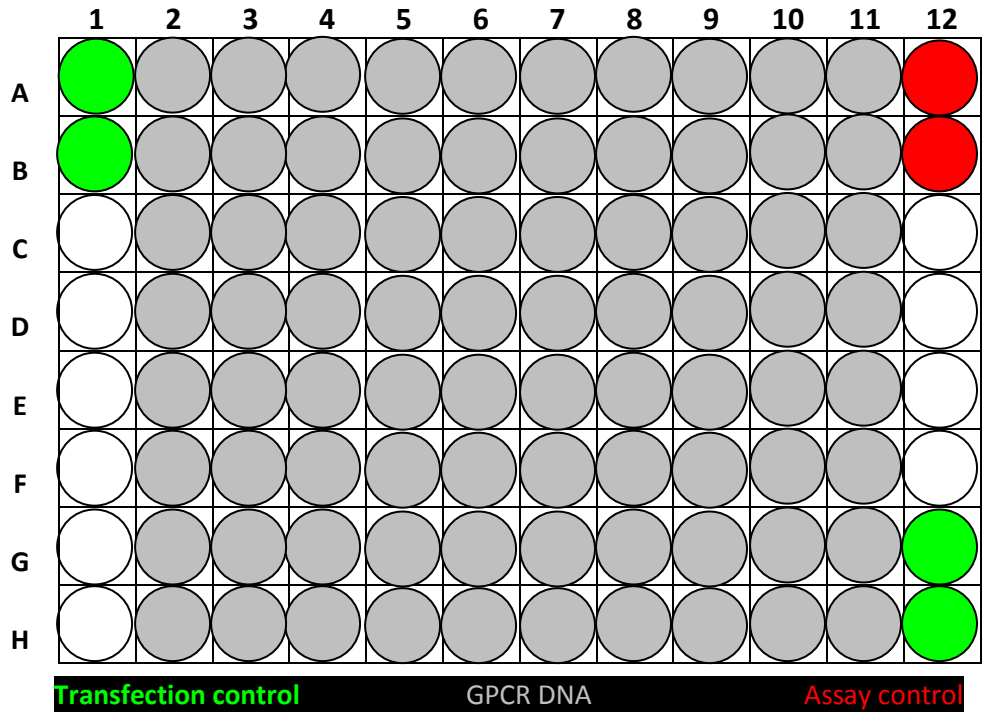


Figure 46. 96-well DNA map**.** Green wells contain a YFP plasmid for transfection control. Red wells contain non-orphan receptors for use as positive assay controls (D2-Tango and V2-tango constructs). Grey wells have DNA plasmids. White wells contain assay buffer only for use as a negative control.

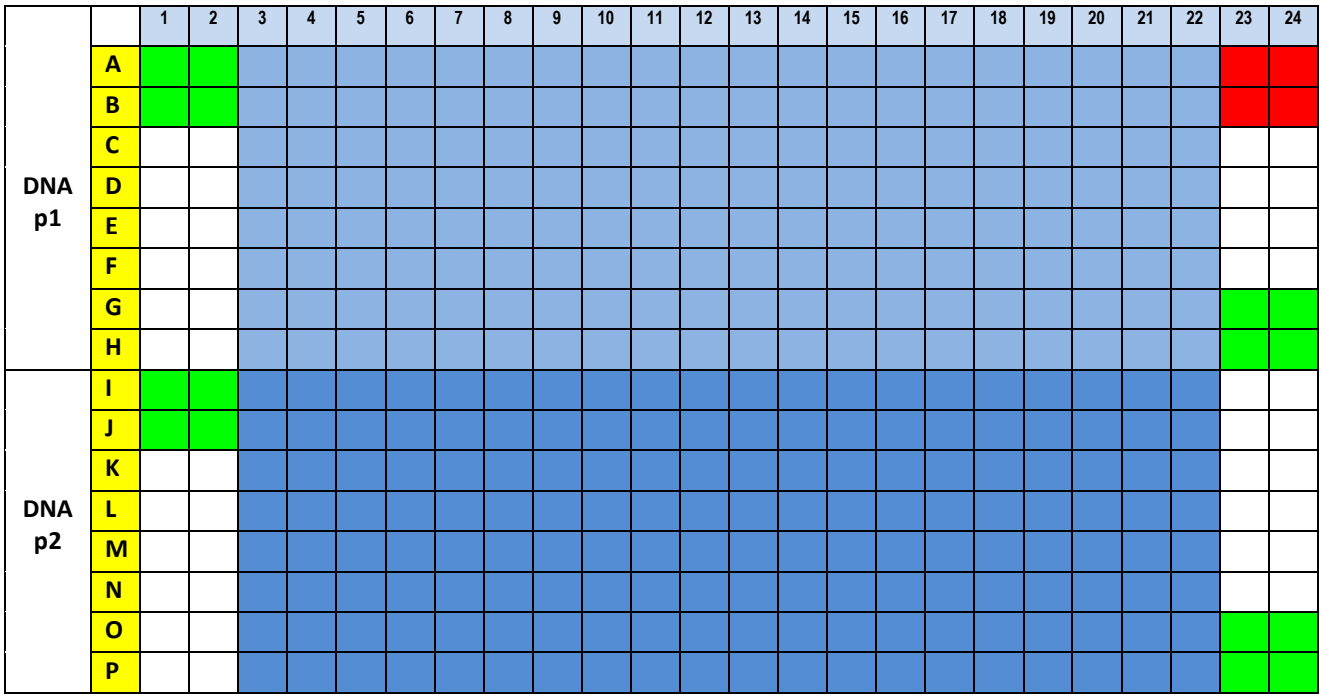


Figure 47. 384-well DNA plate for calcium precipitation and transfection (P1). DNA constructs are transfected in neighboring wells. Green wells are YFP-transfected wells for transfection controls. Red wells are assay controls with cells transfected with non-orphan receptors and stimulated with their cognate ligand. DNA from the 96-well plate (p1) is used to transfect rows A to H. DNA from a second plate (p2) is used to transfect rows I to P.

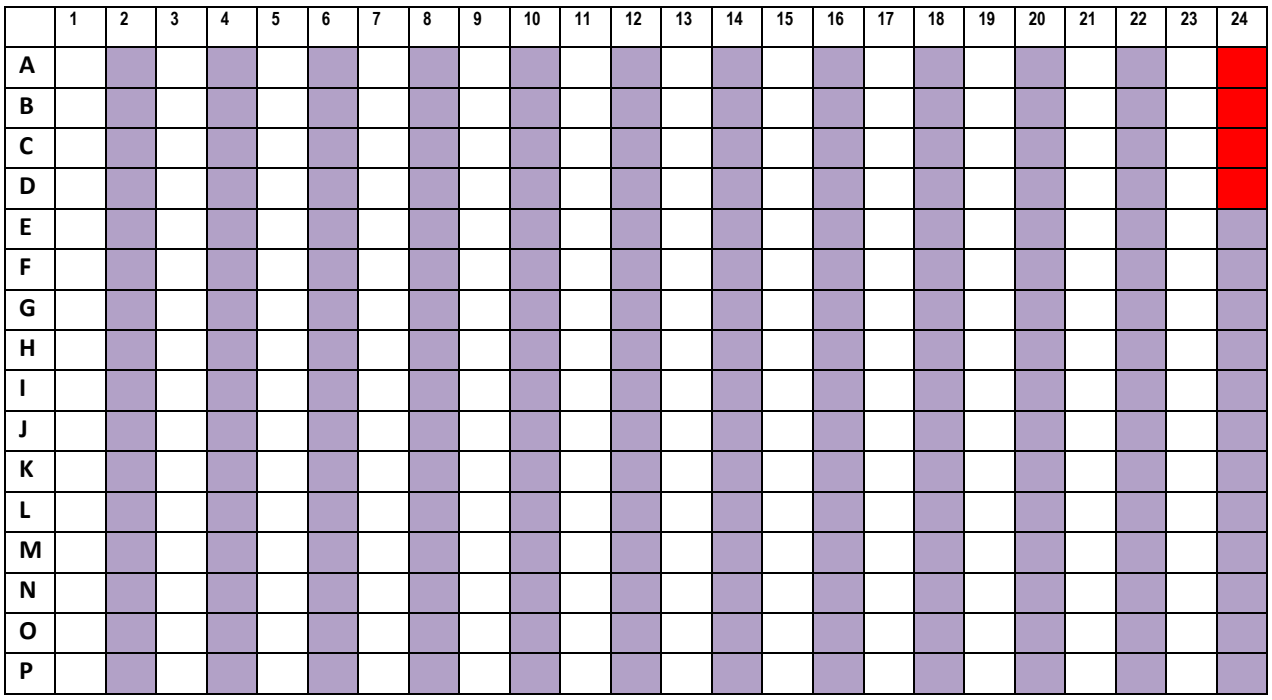


Figure 48. 384-well drug plate design for stimulation. White wells are baseline control with assay buffer; purple wells contain drug solution (6 µM). Columns 1 and 2 serve as negative controls to determine drug effects on non-transfected cells. Red wells are assay controls and are thus stimulated with cognate ligands (Quinpirole for D_2 and Vasopressin for V_2).

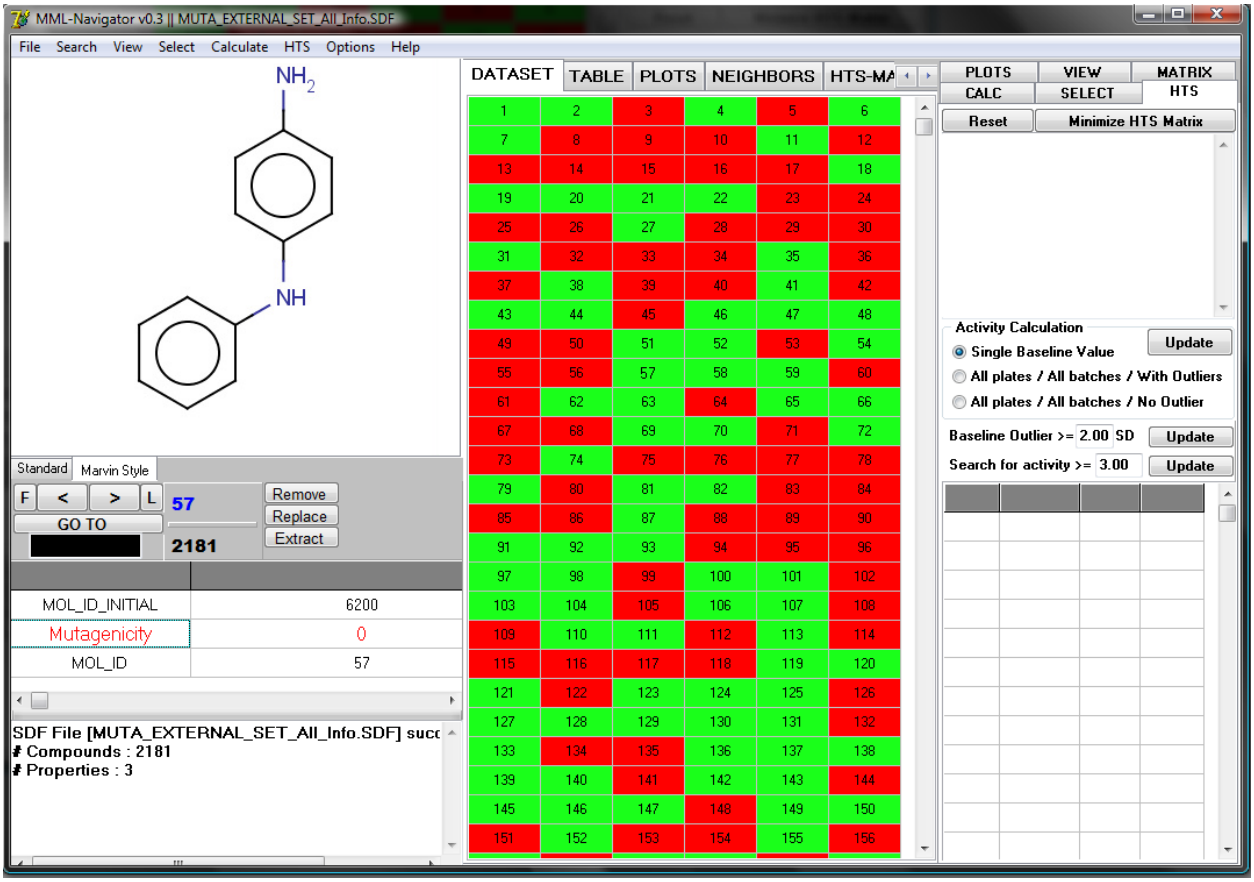


Figure 49. A screen capture of the Navigator interface**.** This software calculates Activation (%) relative to a positive control as well as relative to baseline. Compound structures, numbers of plates and compounds tested per assay, and calculated values for every receptor/compound pair can be viewed on the main screen.

To calculate and report these values, we 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 spreadsheets and calculates relative activation as indicated above. A screen shot of the Navigator interface is shown in **Figure 49**.

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 a final concentration of 1 μM. Results (fold of baseline) are reported in **Figure 50**.

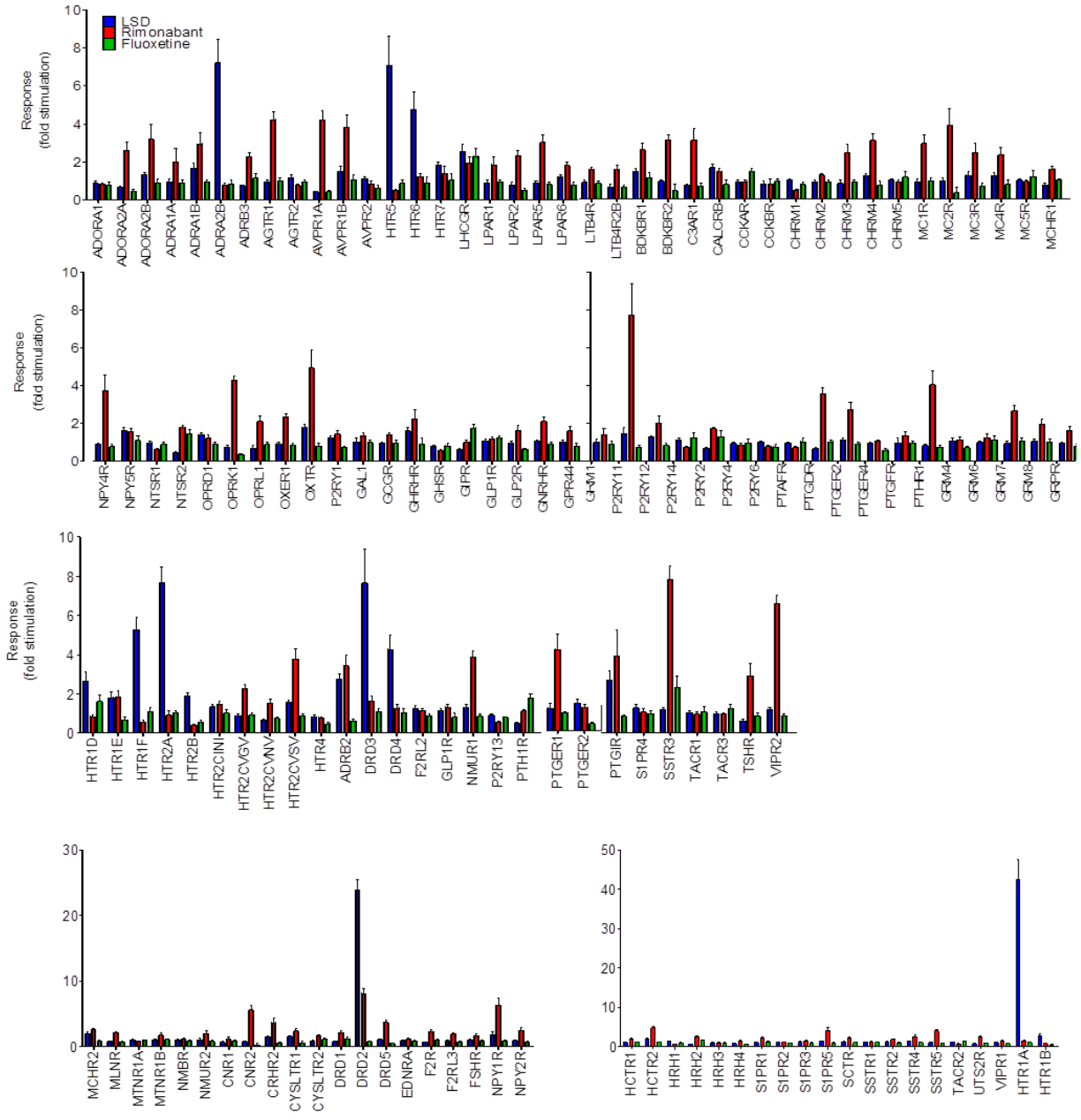


Figure 50. Sample parallel primary screening at non-orphan non-olfactory GPCRs using the β-arrestin recruitment assay. Activity is reported as Response (fold stimulation over baseline) for LSD (blue), rimonabant (red), and fluoxetine (green).

2.8. Thallium flux (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 HBSS, 2.5 mM Probenecid, pH 7.40

The following protocol was adapted from Huang et al., (2010) ASSAY and Drug Development Technologies, 6:727-742.

2.8.1. Cell culture: HEK293 cells stably expressing hERG channels are 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 reached 80-90% confluency.

2.8.2. FluxOR assays for hERG modulators: Thallium (Tl^+) Flux assays are carried out using the FluxOR Potassium Ion Channel Assay kit (Invitrogen) according to the manufacturer's instructions with modifications. In detail, HERG HEK293 cells are plated into PLL coated 384-well black clear bottom cell culture plates with 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 use. On the day of the assay, FluxOR dye reagents are reconstituted by mixing Component A (1/1000 dilution) and PowerLoad (1/100 dilution) using the FluxOR assay buffer and loaded onto cells with 20 µl of FluxOR reagent for 90 min in the dark. During the incubation, drug and stimulation solutions are prepared. The stimulation solution is composed 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, the dye is removed, and the FLIPR^{TETRA} is programmed to transfer drugs from drug plates into cell plates (25 µl per well). Following transfer, cells are incubated with the drugs for 15 min at room temperature in the dark. Then, stimulation solution (6.3 µl per well) is added with FLIPR^{TETRA}. Before the drug addition, the fluorescence intensity (excitation at 490 nm and emission at 525 nm) in each well is measured every second for 10 seconds to establish a baseline, and after addition, for additional 90 seconds. Alternatively, the FluxOR assays can be performed with

cryopreserved hERG HEK293 cells. In brief, frozen cells are washed with growth media once to remove the DMSO in the freezing medium, 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.

2.8.3. Assay procedure for hERG trafficking modulators: The above protocol is designed to measure the acute effect of drugs on hERG channel activity (such as hERG channel inhibitors or activators). For drugs without acute inhibitory effects in the Tl^+ flux assays, we have modified the standard protocol to conduct a longer "chronic" study to identify compounds that might be acting through indirect mechanism(s) (e.g., hERG trafficking inhibition, hERG internalization, and Na^+-K^+ ATPase inhibition). Briefly, cells are first treated with drugs for the desired time (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 as in the standard protocol.

2.8.4. Data processing and analysis: The fluorescence intensity time course of each well in the 384-well assay plate is processed using ScreenWorks 2.0 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**. To normalize, the average initial slope with buffer is set as 0% inhibition and the average initial slope with 10 μM Cisapride is set as 100% inhibition.

Figure 51. Raw fluorescence signal traces recorded on the FLIPR with HEK293 cells stably expressing hERG channels. Recording started 10 seconds before addition of stimulation solution and continued for another 90 seconds. Cisapride reduced signals in a concentration-depedent manner (error bars not shown). Not all concentrations are shown.

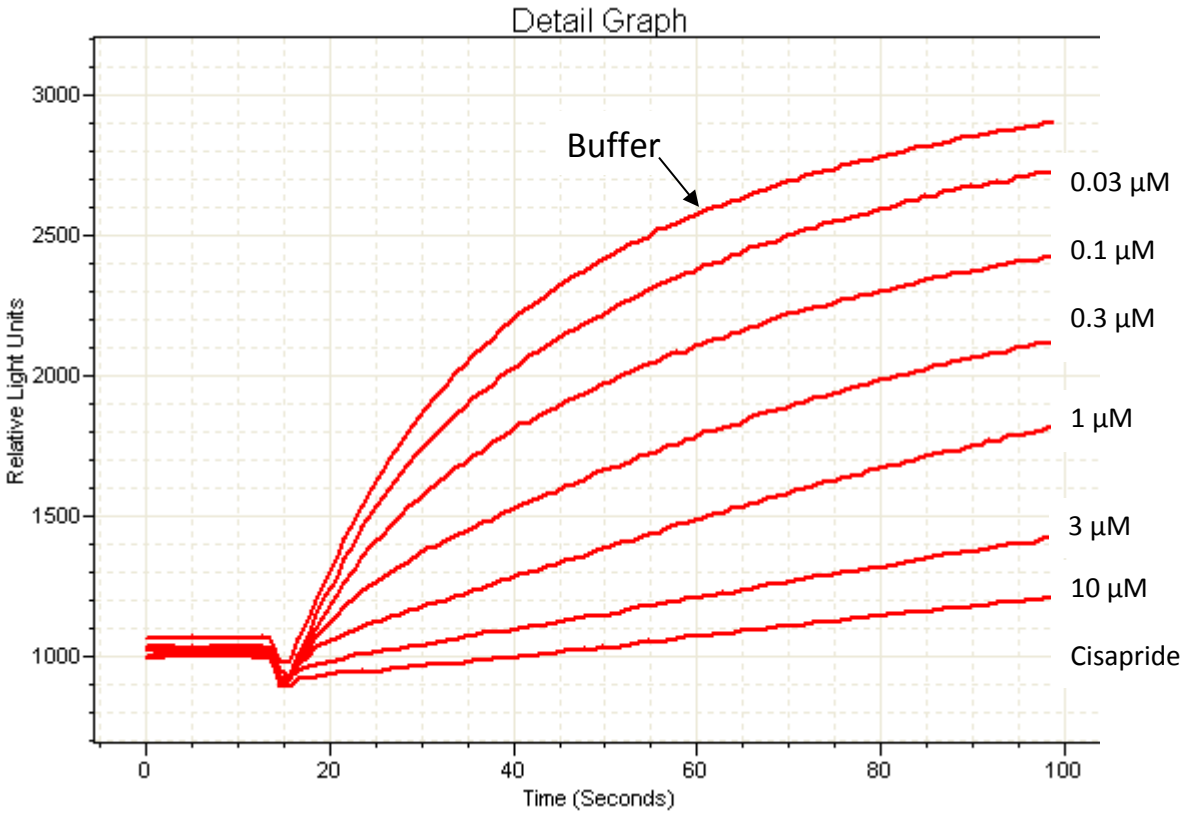


Figure 52. The Initial slope of curves from **Figure 51** was exported from FLIPR ScreenWorks, plotted against corresponding concentrations of cisapride, and fitted to a four-parameter logistic function using GraphPad Prism 5.0.

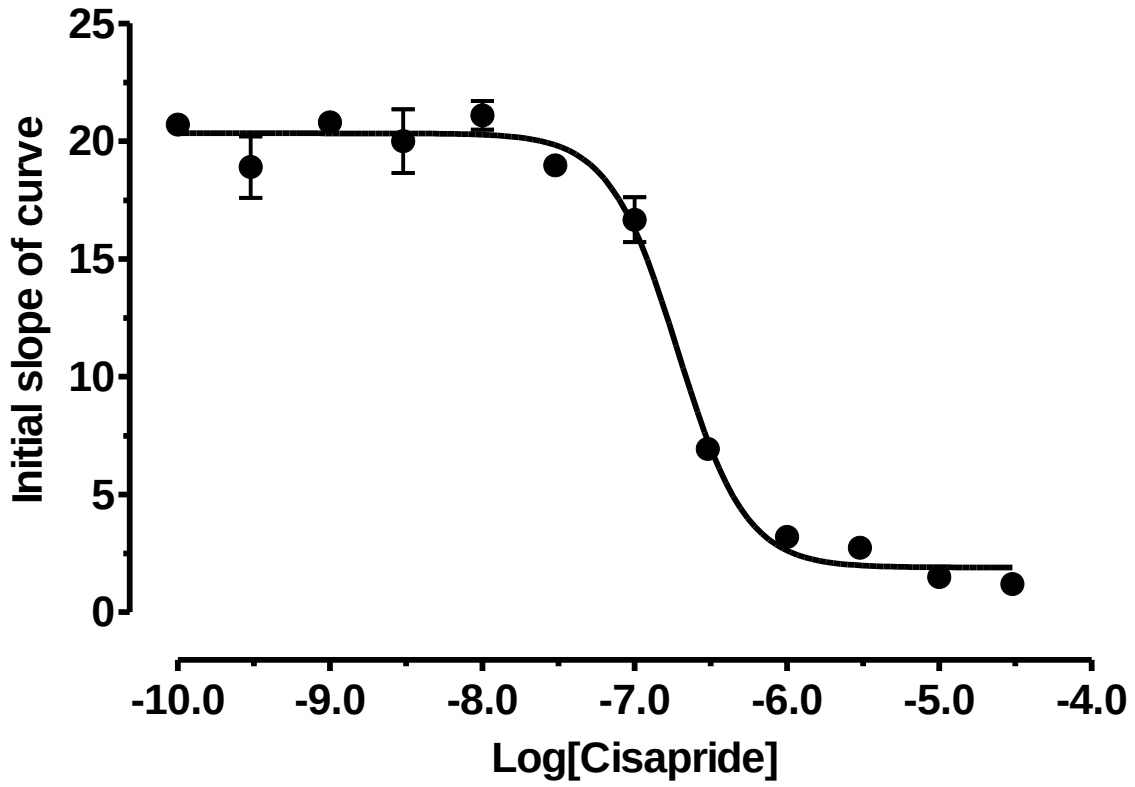


Figure 53. Acute and chronic effects of the hERG trafficking inhibitor ouabain on hERG channel activity. The activity of ouabain was determined with acute Tl^+ flux assay (upper) and chronic Tl^+ assay (lower). HEK293 cells stably expressing hERG channels were first incubated with ouabain for desired times at 37°C; the remaining hERG activity was determined.

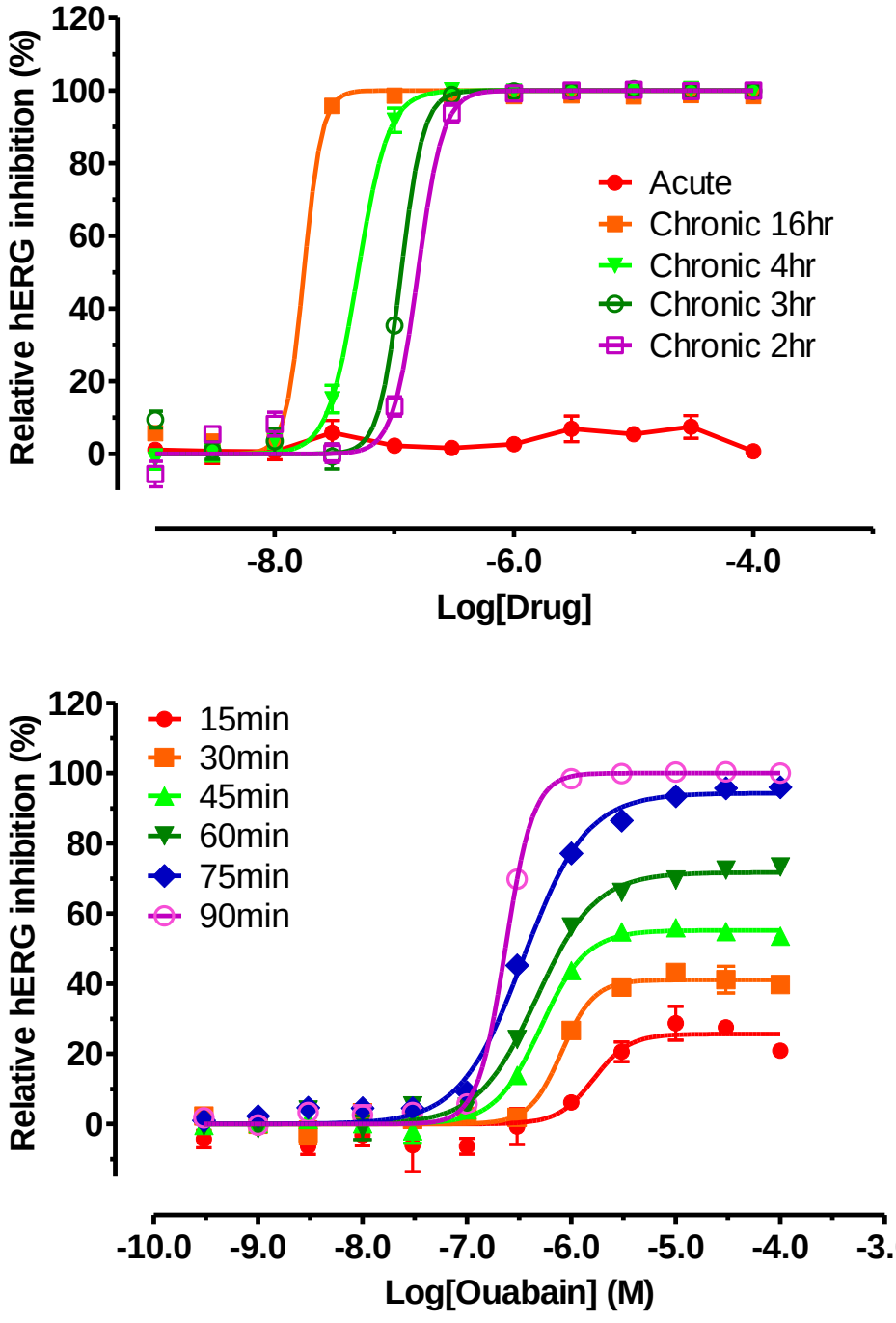
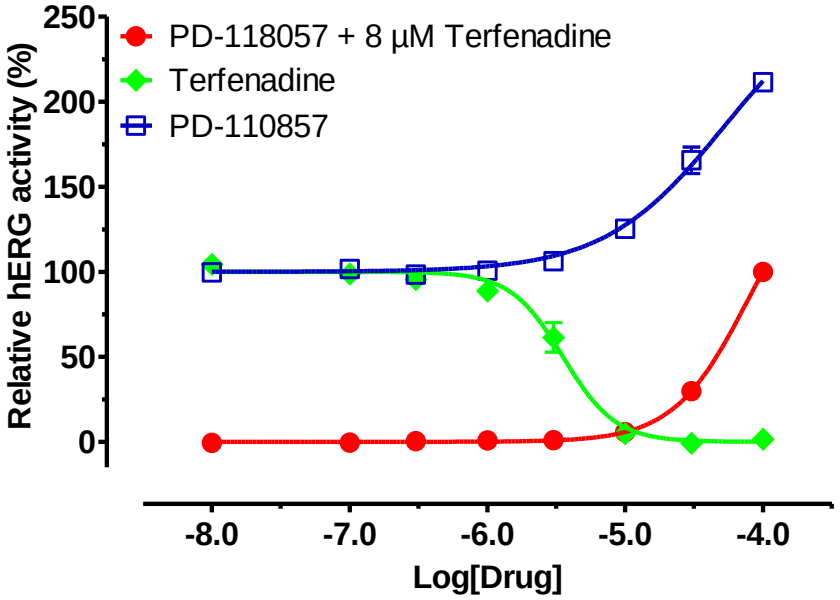
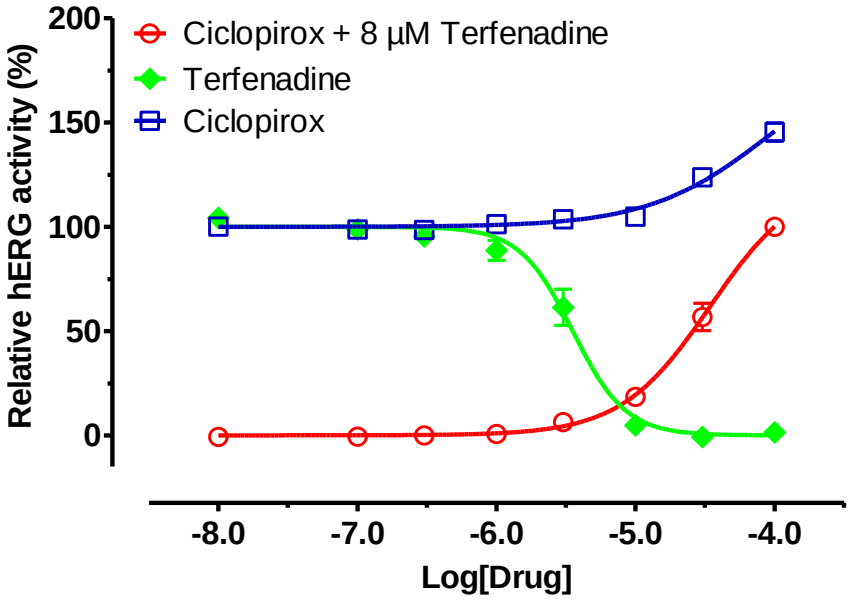


Figure 54. Activation of hERG channels in Tl^+ flux assays. Terfenadine completely inhibited hERG channel activity, whereas PD-118057 (upper panel) and Ciclopirox (lower panel) activated the hERG channel. For assays done in the presence of terfenadine, terfenadine was added 5 min before PD-118057 or ciclopirox. For hERG activator activity, data 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.





2.9. 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 by 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 by KOH.

Automated planar patch clamp (APPC): PatchXpress electrophysiology

The following protocol is adapted from Huang et al., (2010) ASSAY and Drug Development Technologies, 6:727-742.

2.9.1. External and Internal solutions: Fresh external and internal solutions are prepared at room temperature on the day of patch clamp assays. The KF-based internal buffer was adopted from a report by Zeng et al., (2008), in which they reported a greater overall success rate with a KF-based internal buffer than with a traditional KCl-internal solution. We added ATP, GTP, and Mg^{2+} to the KF-based internal solution to prevent potential current run-down. The osmolality of the buffers was determined with a VAPRO 5520 Vapor Pressure Osmometer (Wescor, INC, Logan UT). The osmolality 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.

2.9.2. Cell preparation: For patch clamp assays, hERG-expressing HEK 293 cells are maintained as described above and subcultured into 10-cm dishes two days before the 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 follow 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. 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 microcentrifuge 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 immediately after 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 then reached the 'waiting mode' for receiving the cells.

2.9.3. 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 plates (Fisher Scientific). Drugs in 10 mM DMSO stocks are diluted in external buffer at a final volume of 360 µl each well, a sufficient quantity for the drug to be tested in two cells (triple addition of 50 µl for one cell at each concentration). The final concentration of DMSO is 0.3% (v/v) for all dilutions (except for occasional drugs that require 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 is 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 usually started within 15 minutes after drug plate is prepared, and the highest concentration is tested within 90 minutes. A new drug plate is prepared for each new SealChip.

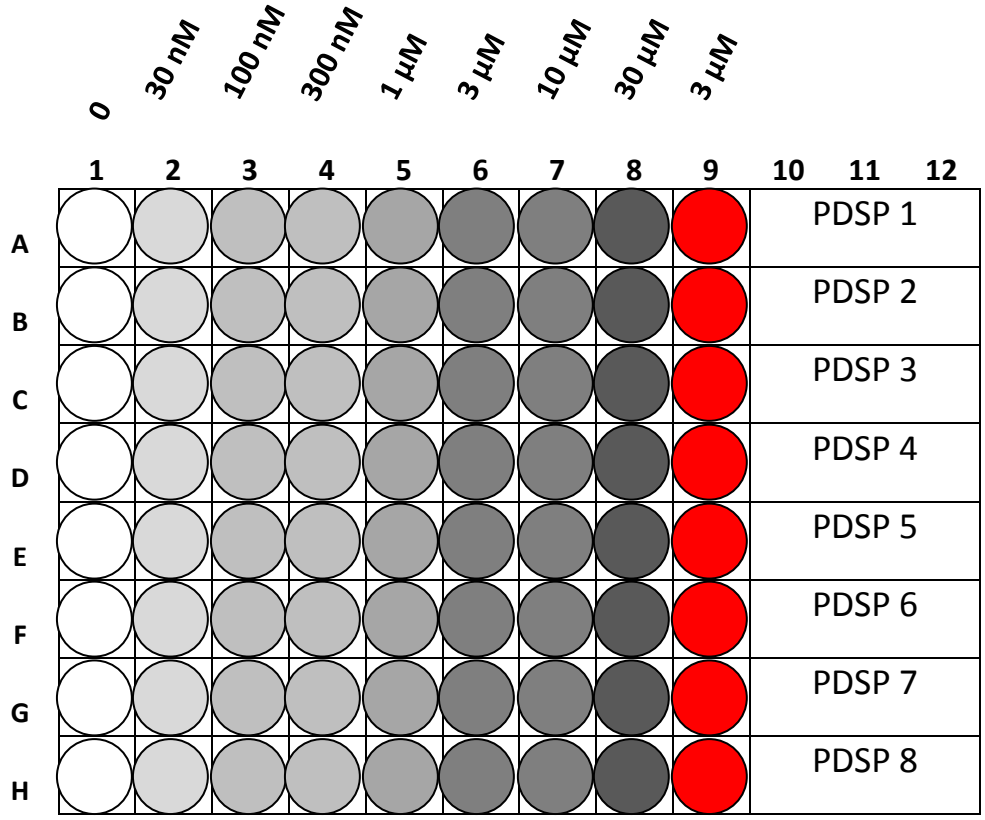


Figure 55. A typical APPC drug plate map (minimum of 360 µl per well). Each drug has eight serial dilutions 0.5 log units apart as indicated in the plate map, starting with a buffer control on the left and ending at 30 µM, followed by a positive control (**red**) on the right, usually Cisapride at 3 µM.

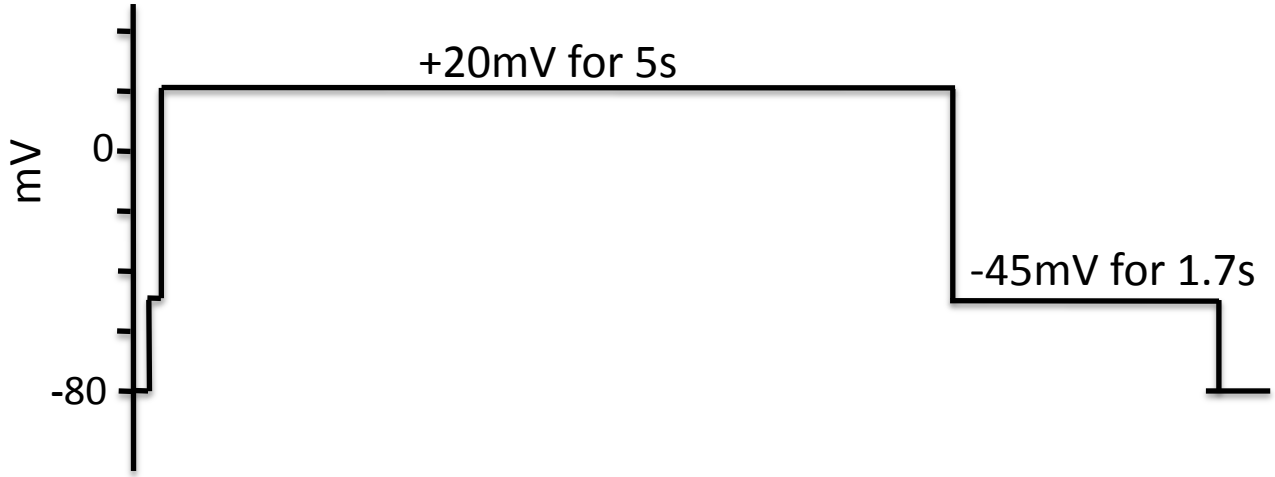
2.9.4. APPC procedures: The APPC procedure starts with manual loading of a SealChip$_{16}$™ (AVIVA Biosciences, San Diego, CA) and is executed automatically at room temperature. The system requires approximately 7 min to (1) dry and load the SealChip onto the recording station; (2) add external and internal solutions; (3) check the quality of each of the 16 chambers; (4) enter waiting mode and prompt 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 whole cell configuration. Chambers with low seal resistances (i.e. no cell detection) are terminated within 90 seconds; chambers that do not form seals within 5 minutes are terminated by either a built-in script or by user intervention. After achieving whole cell configuration, the voltage protocol is activated, beginning with a 2-minute wash-out anda 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 a 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 the inactivation and elicit the outward hERG tail current; (4) repolarization to the holding potential of -80mV to keep hERG channels closed. The pulse pattern is applied repeatedly every 10 seconds (0.1 Hz). The instantaneous current at -50mV before stepping to +20mV is designated as the leak current and is subtracted from the corresponding peak tail current for data processing. Each concentration-response experiment starts with a buffer control to determine the maximal hERG tail current (0% inhibition) in the absence of drugs. Each drug concentration is applied three times (50 µl each at 25µl per second) with 11 seconds between additions, and each addition is preceded by aspiration of the 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 Database script is activated between eachconcentration. When the drug effect reaches a steady state (less than 0.1% difference from last measurement) or after a maximum of 5 minutes, the next concentration is queued for delivery. Immediately 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 the wash-out, a positive control (usually Cisapride at 3 µM) is applied to inhibit any recovered or remaining hERG channel activity.

2.9.5. 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 leakcurrent that is as much as 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 56. Voltage protocol (upper) and corresponding hERG tail current recording (lower) for the automated planar patch clamp assays.



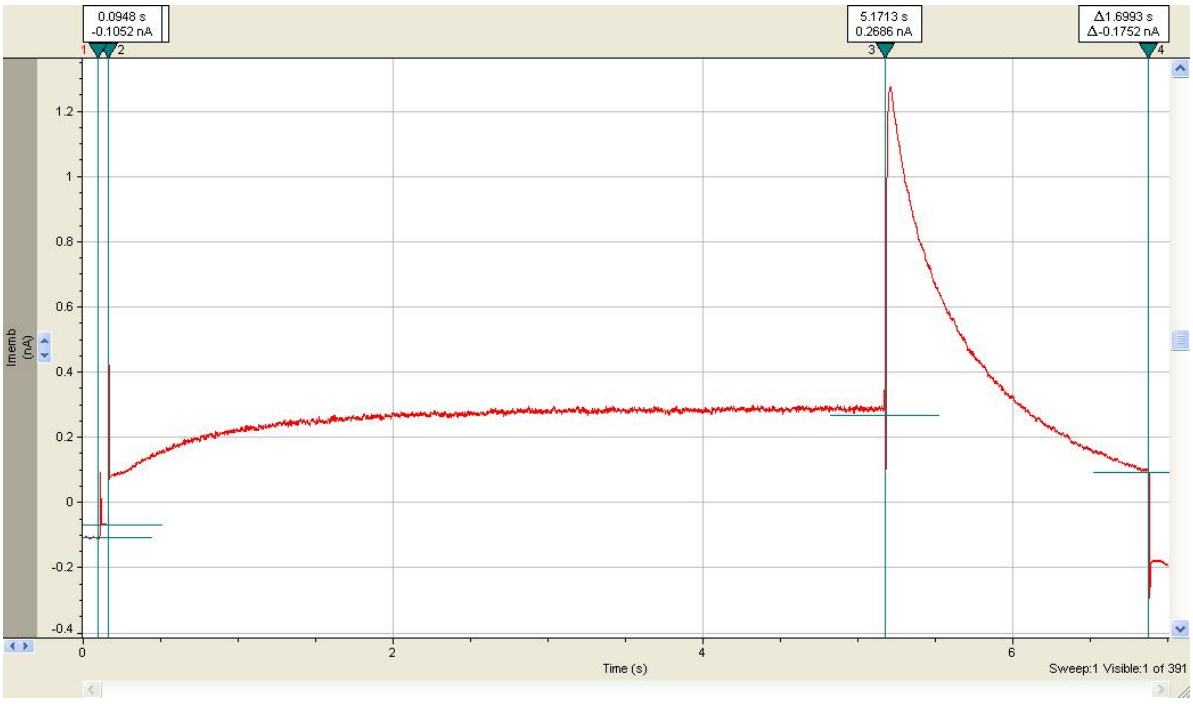


Figure 57. A representative whole-cell patch clamp recording showing that the hERG channel current (nA) was severely inhibited by a PDSP compound in a concentration-dependent manner, which was partially recovered during a wash-out cycle, and then completely inhibited again by a positive control (3 µM 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, the green vertical lines indicate triple additions of drugs with concentrations (µM) above the green lines, the dark blue vertical line indicates wash-out with buffer, and the pink vertical lines indicate the positive control, Cisapride. The red crosses indicate the time when the hERG tail current was measured by the system.

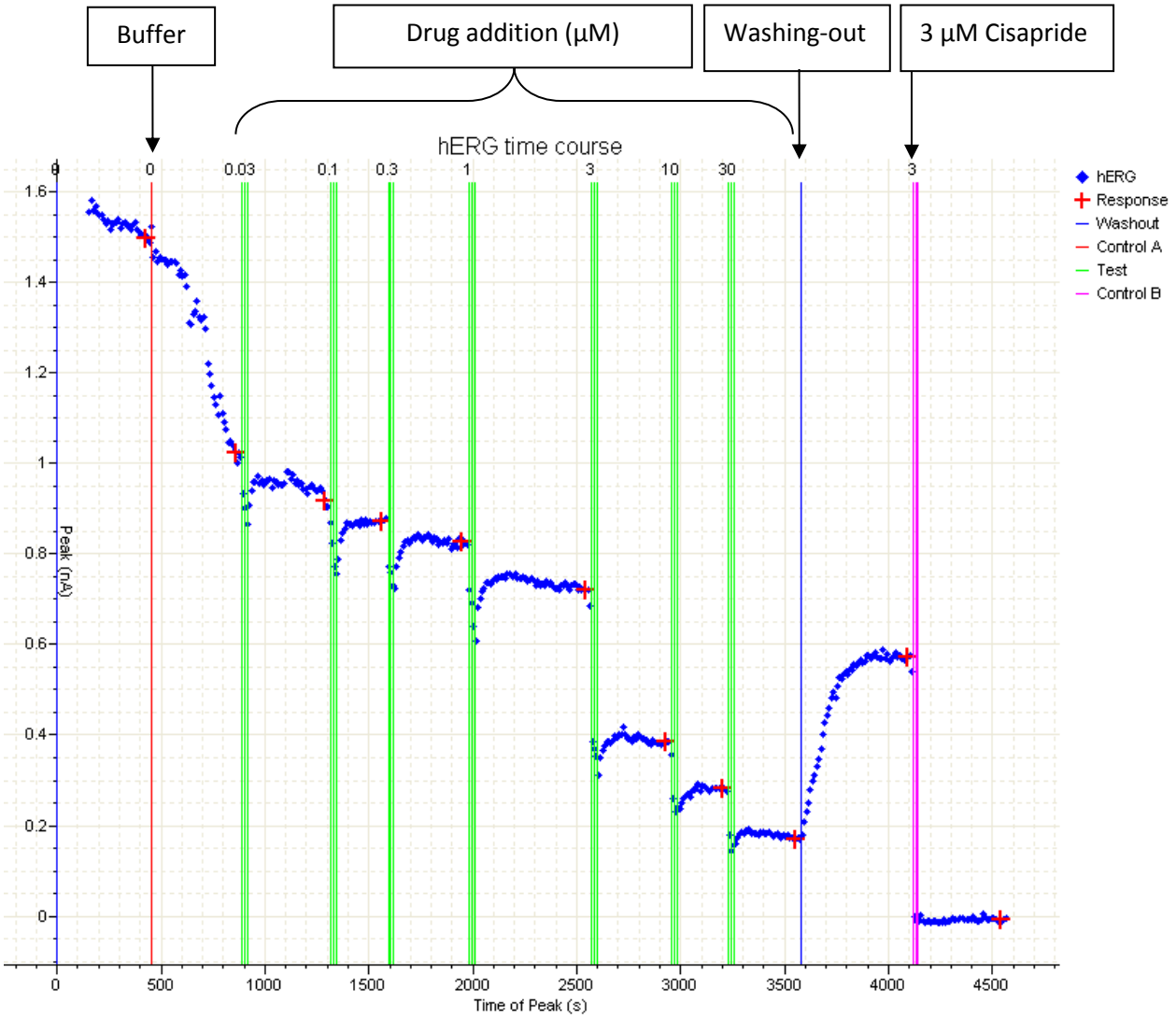


Figure 58. A captured screen in the DataXpress program showing a representative whole-cell patch clamp recording of hERG tail currents in the absence and presence of cisapride. The red vertical line indicates addition of buffer control and the green vertical triple lines indicate triple additions of cisapride with concentrations (μM) listed above the green lines. The red crosses indicate the times when the hERG tail current was measured by the system.

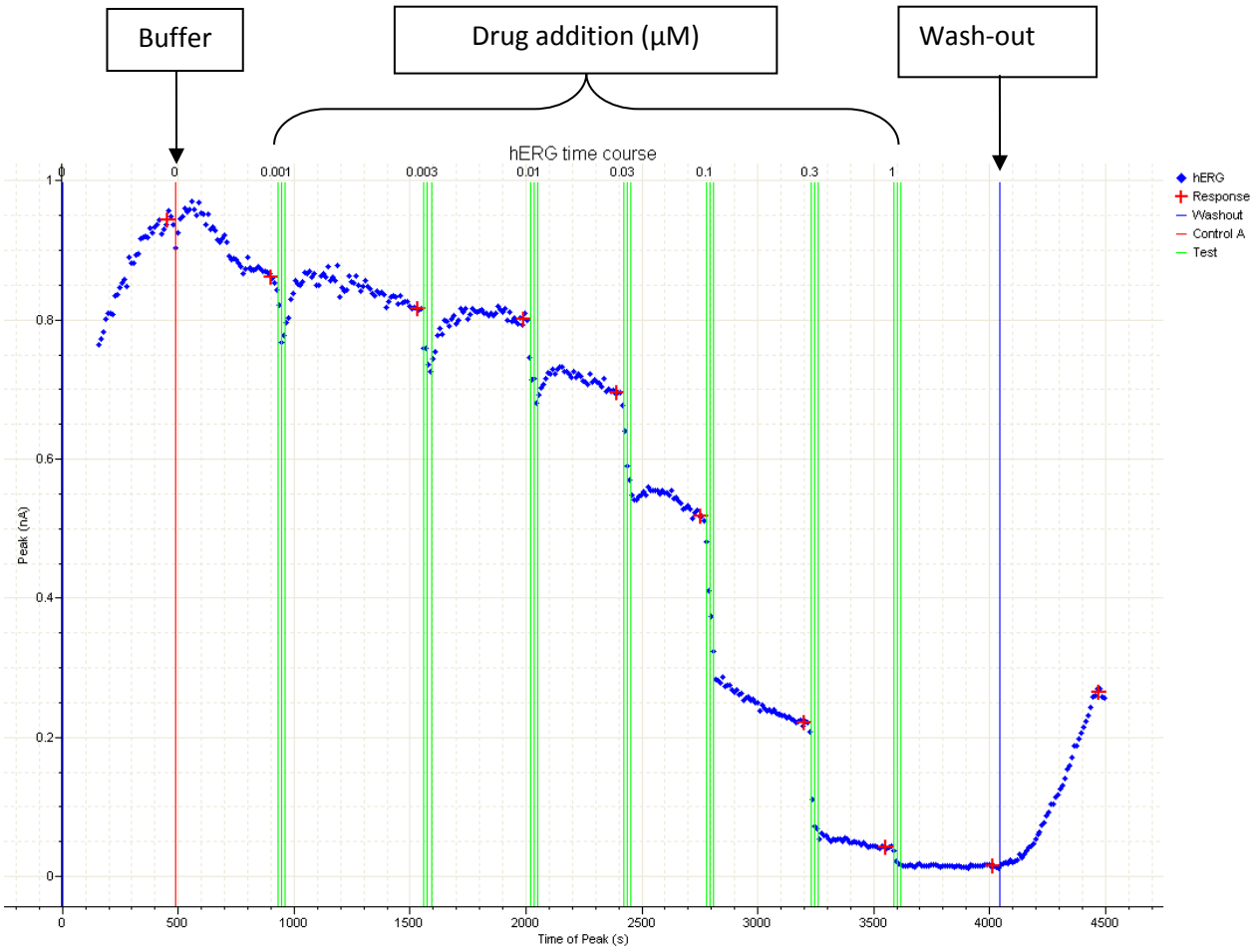


Figure 59. The hERG tail currents in the **Figure 58** were extracted from the program DataXpress, plotted again cisapride concentrations, and fitted to a four-parameter logistic function using GraphPad Prism 5.0.

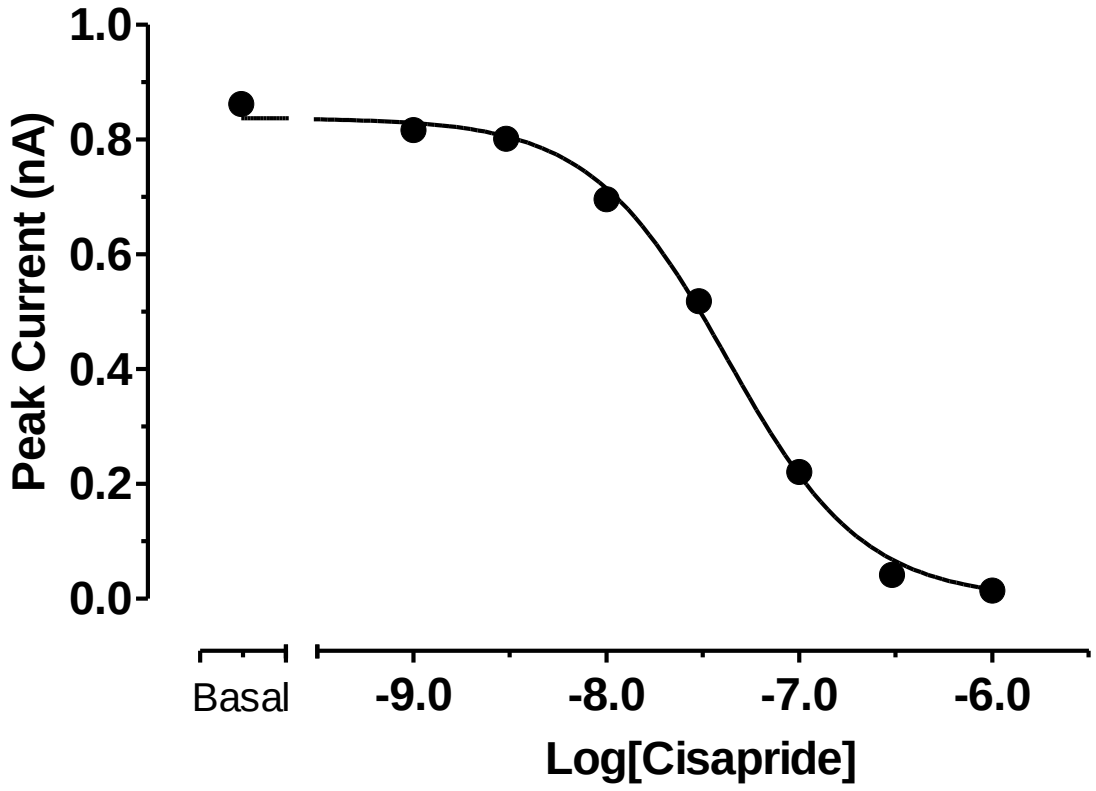
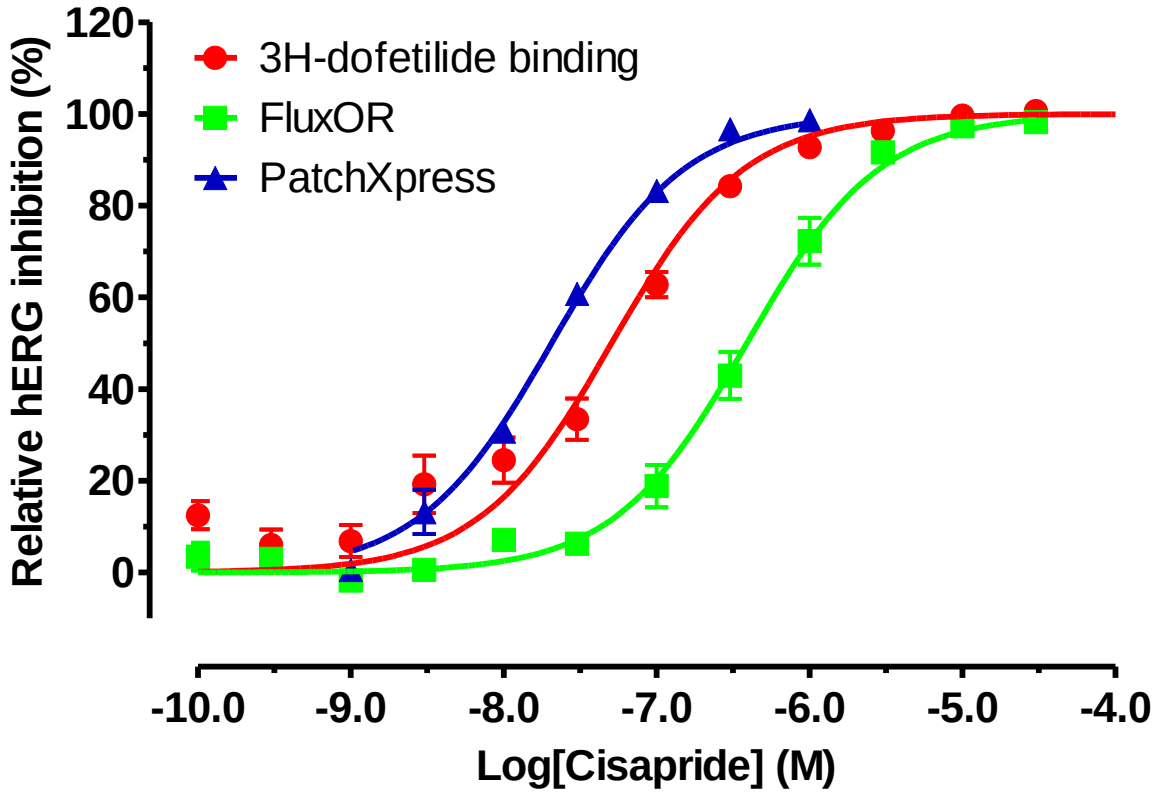


Figure 60. Comparison of concentration-dependent activity of cisapride in ^{3}H-dofetilide competition binding assay; Tl^{+} flux assay, and APPC assay. Results from multiple assays ($n \geq 2$) are normalized to percentage inhibition and pooled for curve-fitting in GraphPad Prism 5.0. The Results indicate that the PatchXpress assay is the most sensitive and the Tl^{+} flux assay was the least sensitive.



2.10. 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) (Molecular Devices)

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

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

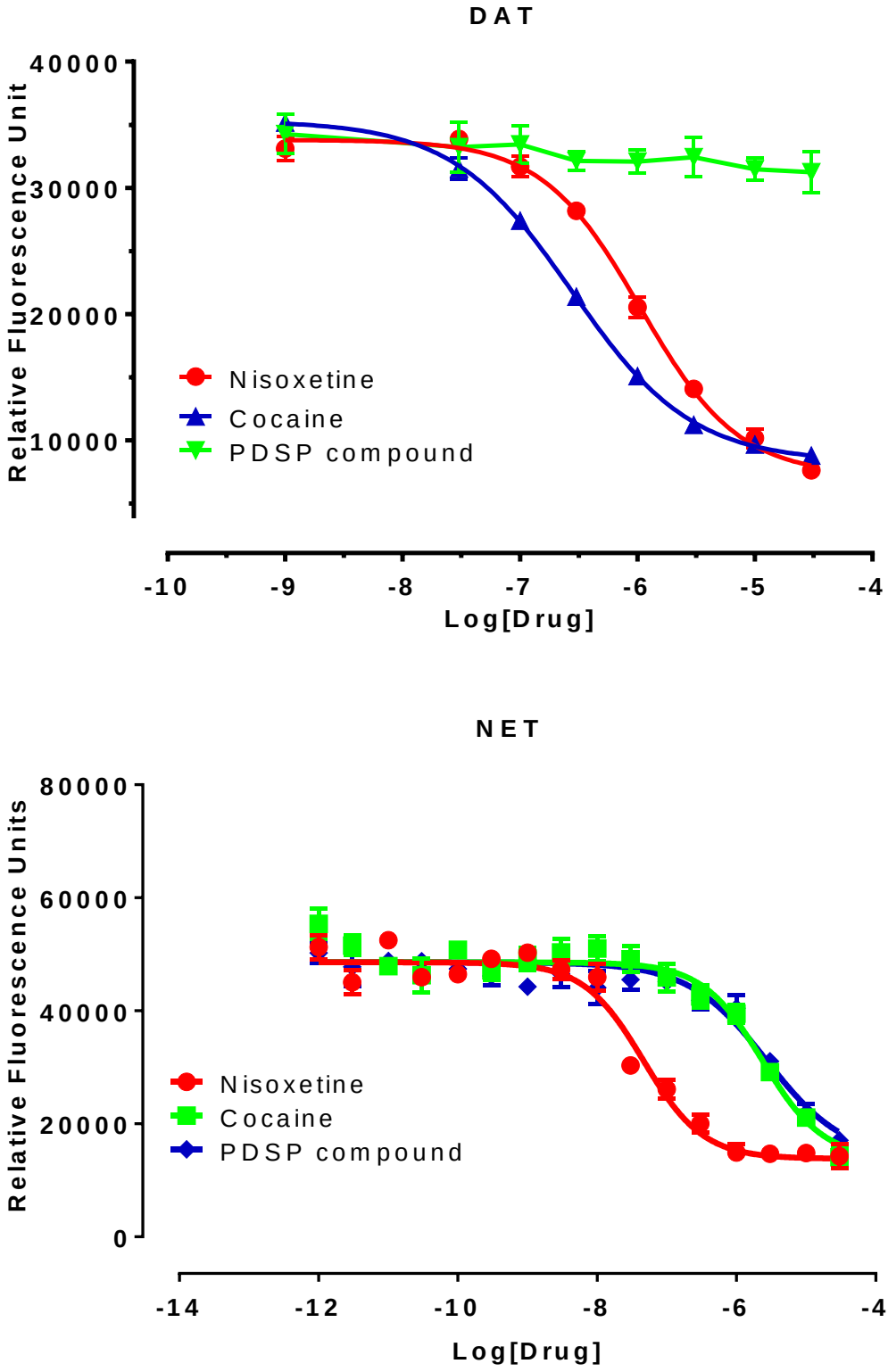
2.10.2. Assay procedure: Neurotransmitter transporter assays are conducted using Molecular Devices' Neurotransmitter Transporter Uptake Assay Kit (R8174) with HEK293 cells stably expressing human DAT, NET, or SERT. In brief, cells are plated in Poly-L-Lys (PLL) coated 384-well black clear bottom cell culture plates in DMEM + 1% dialyzed FBS, at a density of 15,000 cells per well in a total volume of 40 µl. The cells are incubated for a minimum of 6 hours before being used for assays. Medium is removed, and 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**). The plate is incubated at 37°C for 30 min. After incubation, 25 µl of dye solution is added and the fluorescence intensity is measured after 30 min at 37°C, using the FlexStation II (bottom read mode, Excitaion at 440 nm, Emission at 520 nm with 510 nm cut-off). Results (in Relative Fluorescence Units, RLU) are exported and plotted against drug concentrations in Prism 5.0 for nonlinear regression

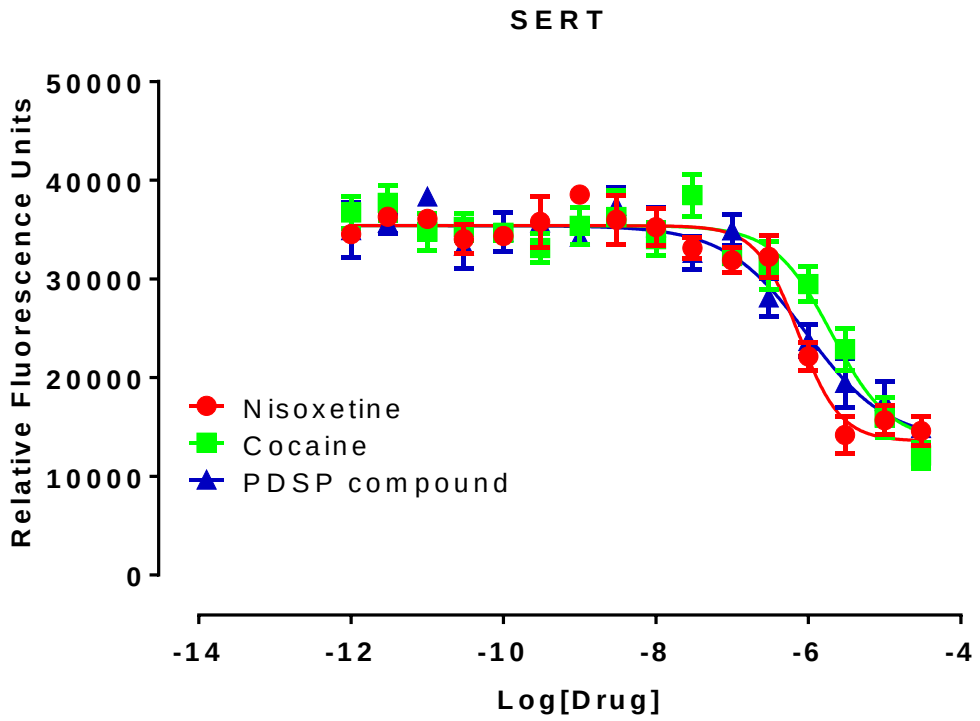
to obtain inhibitory potency. Nisoxetine and Cocaine serve as positive controls for DAT, NET, and SERT.

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

2.10.4. Representative figures

Figure 61. Representative curves of neurotransmitter reuptake inhibition at DAT, NET, and SERT.




SERT
Relative Fluorescence Units
50000
40000
30000
20000
10000
0
Nisoxetine
Cocaine
PDSP compound
-14
-12
-10
-8
-6
-4
Log[Drug]

2.11. 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)

2.11.1. MDR assay Background: Assays for modulation of MDR activity are performed using Caco-2 cells, a cultured line derived from human colonic epithelium, or HEK human kidney cells that express MDR. The assay relies on calcein acetoxymethyl ester (calcein-AM), a lipophilic compound that enters cells by passive diffusion across the plasma membrane. Once inside cells, esterases can hydrolyze calcein-AM to calcein, which is trapped in the cytoplasm because it is negatively charged. Importantly, calcein is highly fluorescent, but calcein-AM is not.

The assay is based upon the principle that calcein-AM, but not free calcein, is transported out of cells by MDR. In cells that express MDR, the lipophilic calcein-AM that crosses the plasma membrane will be pumped out of the cells before it can be converted to calcein, and thus the accumulation of fluorescence will be low. Compounds that compete with calcein-AM for MDR will prevent calcein-AM transport, resulting in increased conversion to calcein and increased fluorescence. Therefore, the effect of a compound on fluorescence accumulation can be used to measure its interaction with the MDR protein.

2.11.2. 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 96-well clear bottom cell culture 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 increase in fluorescence. All compounds are assayed in quadruplicate and each assay contains wells with no test compound (negative control) and wells with 25 µM cyclosporin A (positive control), an efficient MDR inhibitor. Results for test compounds are calculated from the slope of the fluorescence increase and are normalized so that the value for untreated cells is 0% and the value for cyclosporin A is 100%.

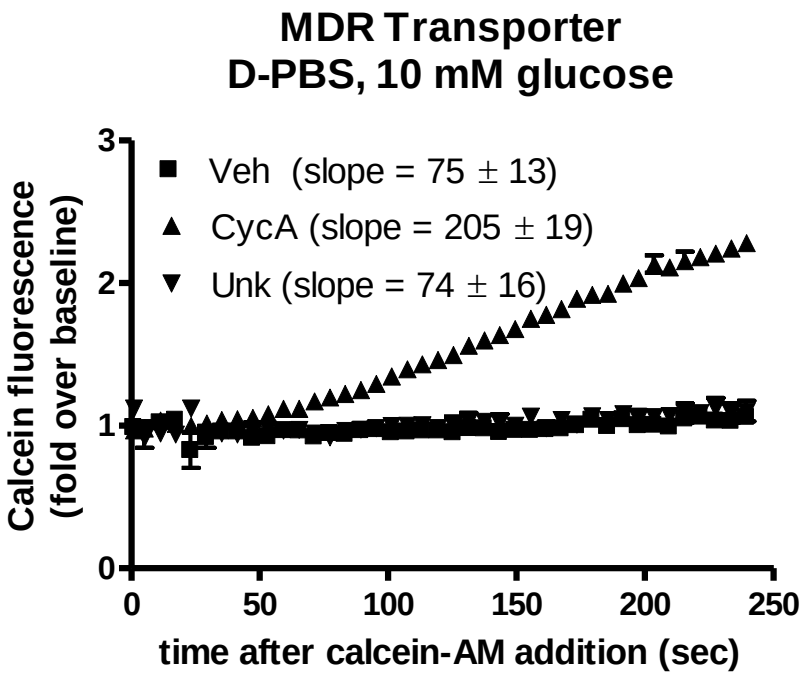
This assay has several features that make it ideal for initial screening of compounds for interaction with MDR. Because the assay is carried out in live cells, compounds must diffuse across lipid bilayers to interact with MDR sites on the cytoplasmic face of the protein. This is similar to the situation 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.

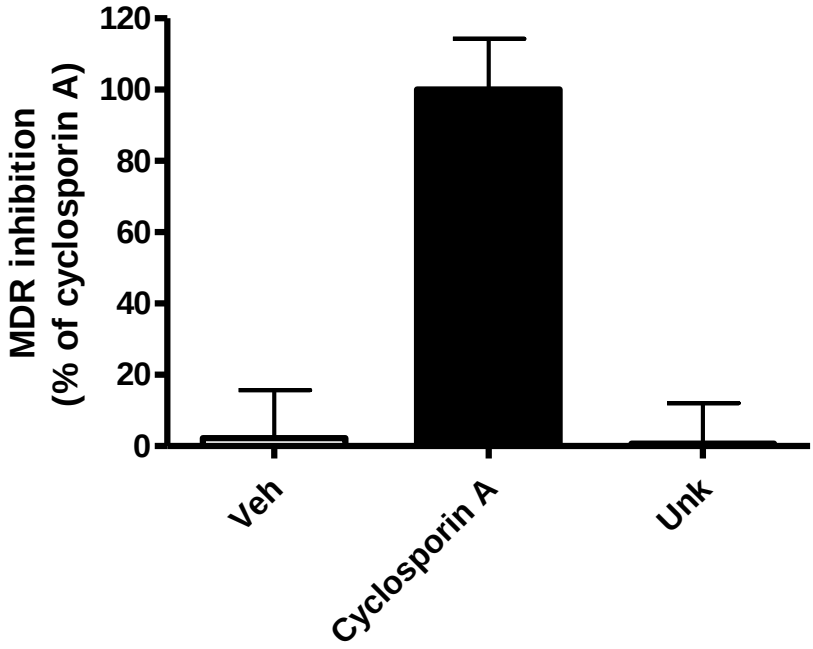
Although this assay is excellent for initial screening, users should be aware that the assay has several drawbacks. i) The assay does not distinguish between MDR substrates and inhibitors. Both will give similar signals in the assay because they prevent the transport of calcein-AM. ii) Some compounds 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 from 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.

2.11.3. Data process. Fluorescence instensity is exported and analyzed in Prism 5.0 using non-linear least-squares curve fitting as above.

2.11.4. Representative figures

Figure 62. Representative data from an MDR inhibition assay.





2.12. 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

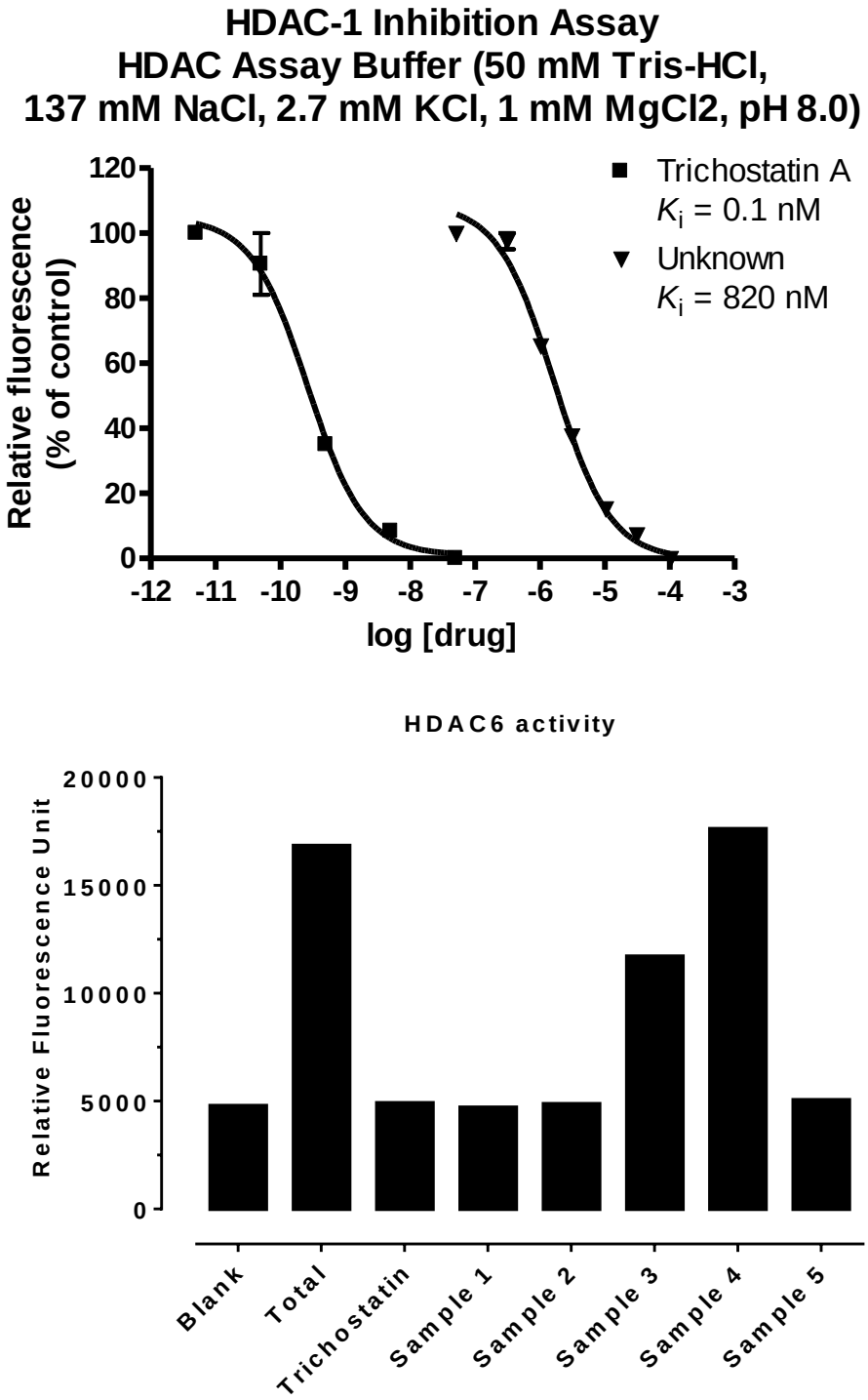
Main reagent: The HDAC assay protocol was adapted from BioMol Fluor de Lys assay system (Plymouth Meeting, PA)

2.12.1. General Assay Procedure. To identify potential inhibitors of HDAC, we utilize the fluorimetric Fluor de Lys HDAC Assay Kit (Biomol) as instructed by the manufacturer. 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 appropriate wells of a proprietary 96-well plate (Biomol). Nuclear extracts containing HDAC activity (Biomol) are diluted to 4X and 12.5 µl are added to the wells containing test or reference compound (each concentration is 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 wavelength 350-380 nm, emission wavelength 440-460 nm).

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

2.12.3. Representative figures.

Figure 63. Representative HDAC 1 inhibition curves (upper panel) and HDAC 6 inhibition in the absence and presence of compounds at 10 μM (lower panel).



2.13. 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)

2.13.1. Brief background. Monoamine oxidases (MAO) are flavin-containing enzymes that catalyse 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 selectivity. 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.

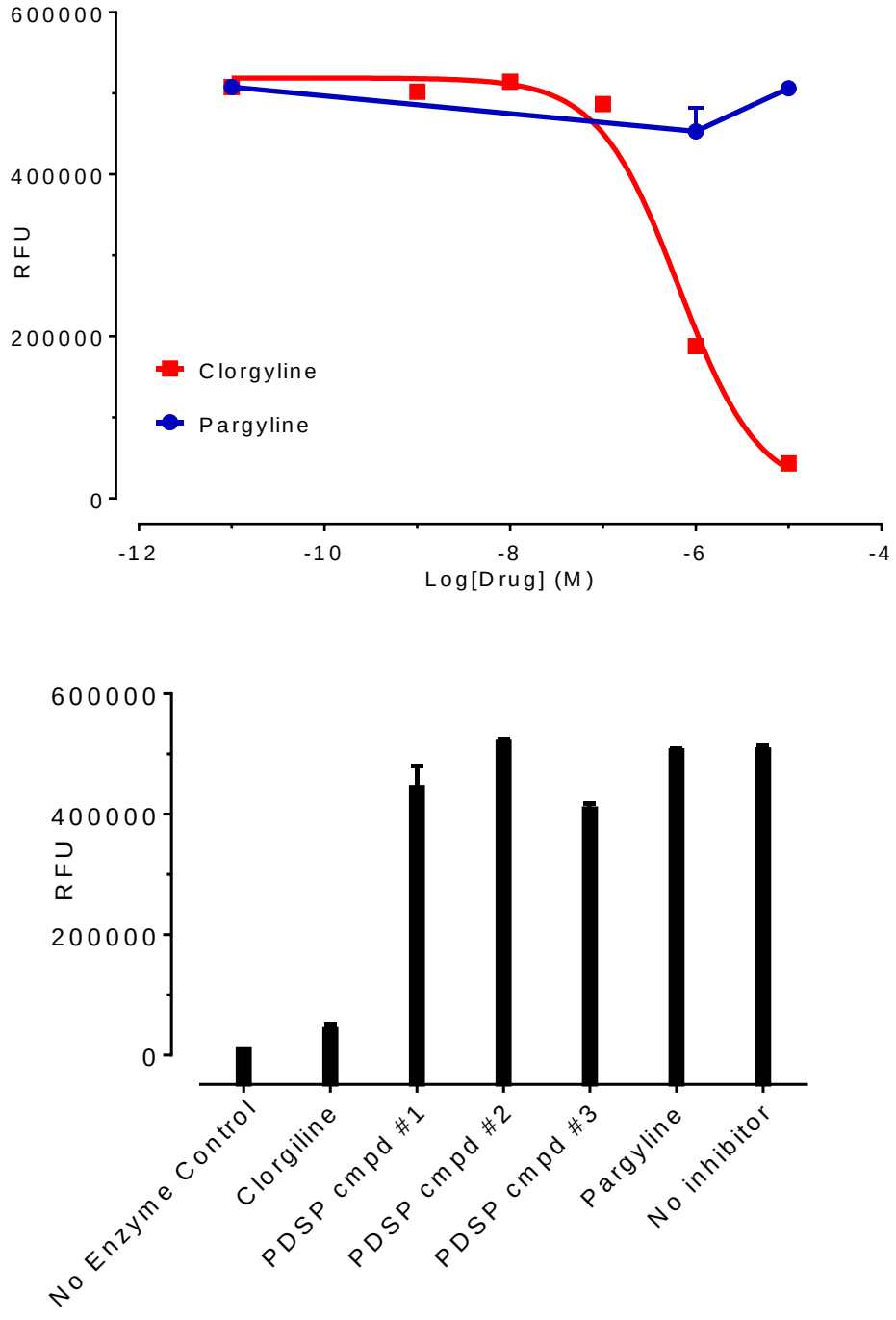
2.13.2. Assay principle. We use the MAO-A and -B Detection Kit (Cell Technology) for Monoamine oxidase-A and -B enzyme assays. The assay system utilizes a non-fluorescent proprietary substrate to detect H_2O_2 released by the conversion of a MAO substrate (Benzylamine for MAO A and Tyramine for MAO B)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

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

2.13.4. Data processing. Fluorescence intensity values are exported and analyzed in Prism v 5.0 using non-linear least-squares curve fitting as above.

2.13.5. Representative figures

Figure 64. Representative curves for MAO-A activity in the presence of Clorgyline and Pargyline (upper panel) and bar graph showing MAO-A activity in the presence of various test compounds at 10 μM (lower panel).



2.14. Portein Kinase C 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)

2.14.1. Brief background. Protein Kinase C (PKC) has 12 isoforms and is classified into three groups (conventional PKCs, Novel PKCs, and Atypical PKCs) based on their requirement for activators (Calcium ions and/or 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.

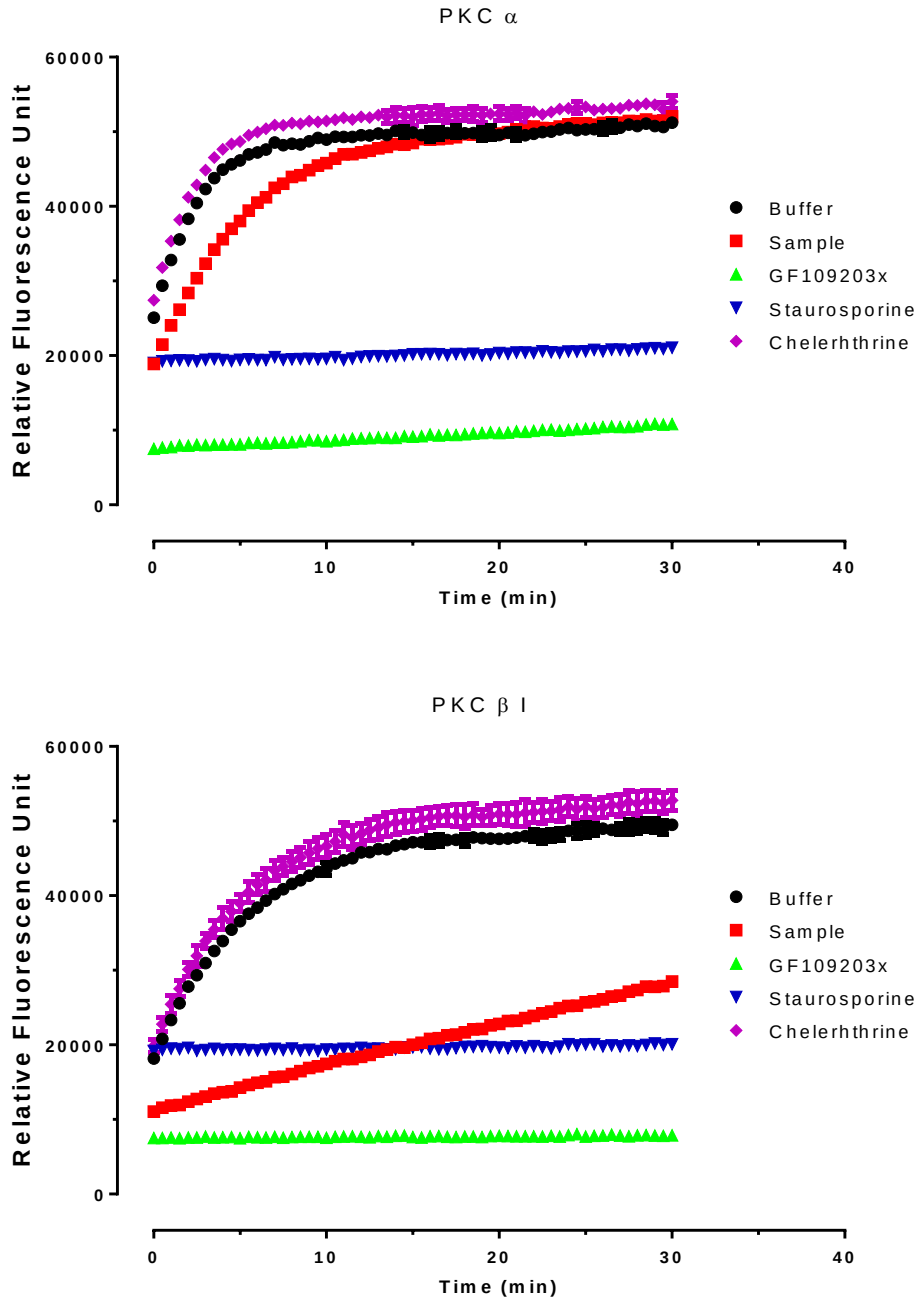
2.14.2. Principle of the PKC assay. The assay uses a Ser/Thr containing peptide substrate conjugated with the chelation-enhanced fluorophore (CHEF) 8-hydro-5-(N,N-dimethylsulfonamido)-2-methylquinoline (Sox). Phosphorylation of the peptide sbustrate results in Mg^{2+} chelation and formation of an ion-ion interaction bridge between the Sox moiety and the phosphate group, leading to an increase in fluorescence at 485 nm when being excited at 360 nm.

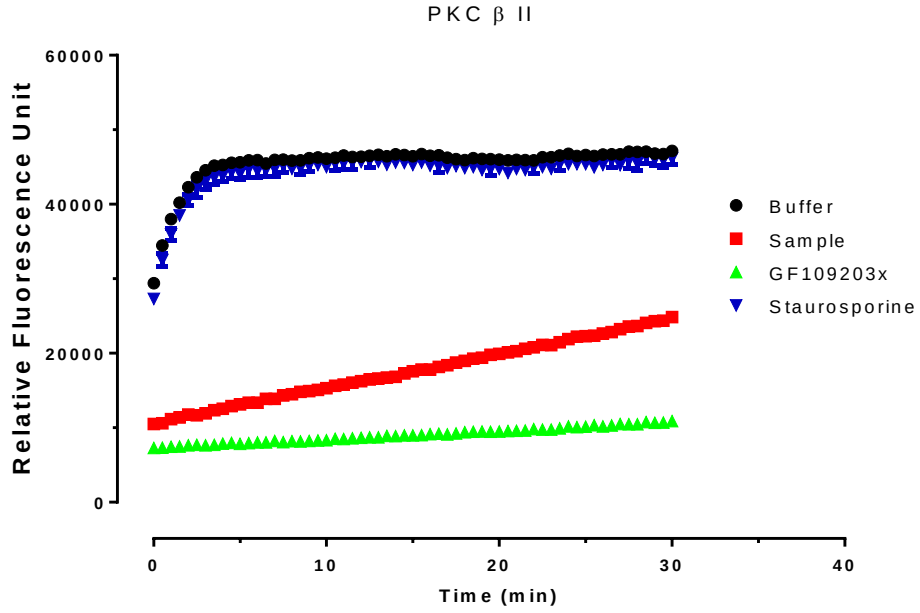
2.14.3. Assay procedure. The PKC kinase activity assays are performed in 96-well plates (1/2 area and low protein binding surface) according to the manufacturer's suggested procedures. In brief, a master reaction mix is made containing the following components: assay buffer, peptide substrate, ATP, DTT, Calcium and/or lipid activator (use buffer for atypical PKC isoforms). This mixture is aliquoted to appropriate wells, drug working solutions (samples) or buffer (as negative control) or known inhibitors (as positive controls) are added, and the mixture is warmed in the FlexStation to 30°C for about 10 min. The reactions start when the PKC isoform is added, and plates are read every minute for60 min, with an excitation wavelength of 360 nm and an emission wavelength of 485 nM.

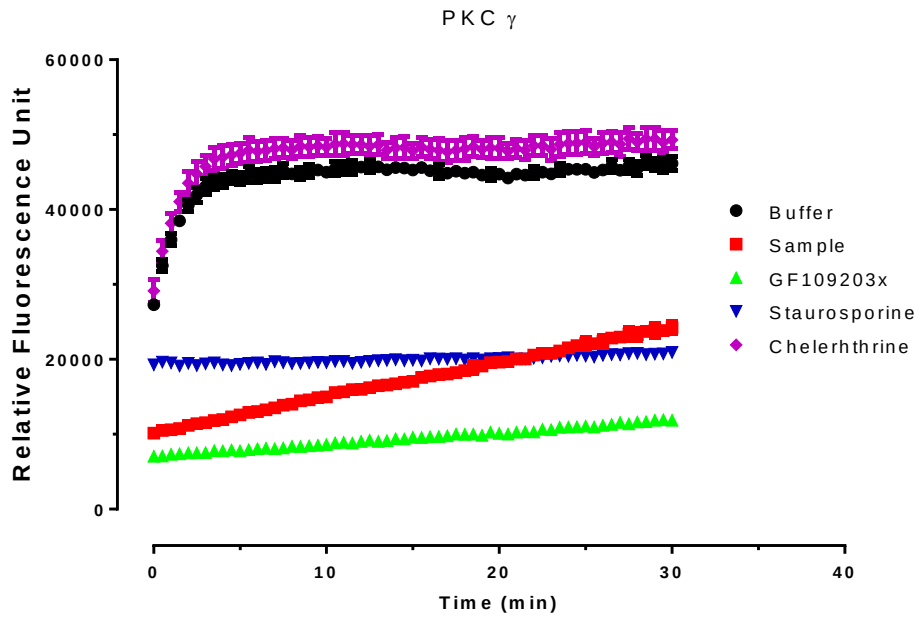
2.14.4. Data analysis. Fluorescence intensity increases over time. The intensity values are exported when the total activity reaches a plateau (or at 30 min) and are analyzed in Prism v 5.0 using non-linear least-squares curve fitting as above.

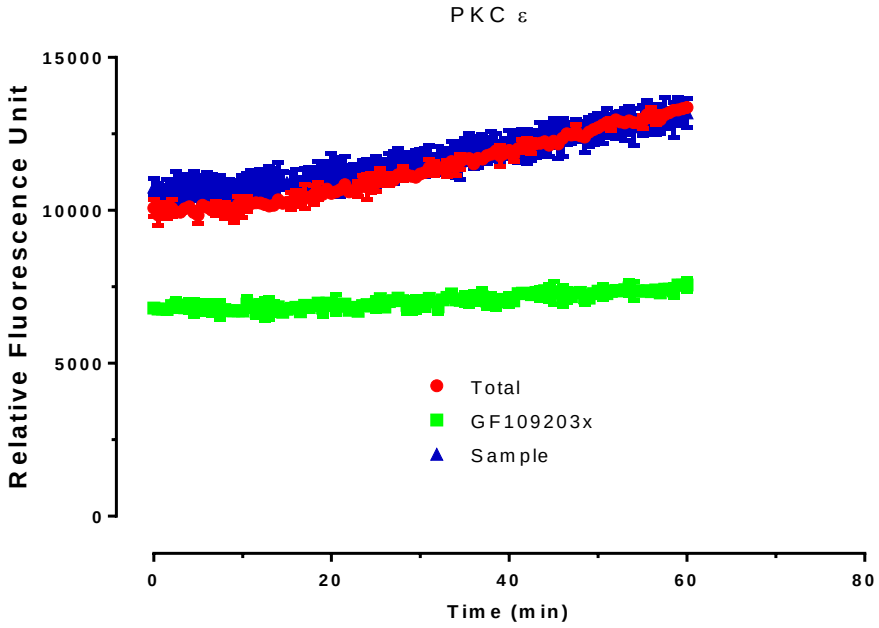
2.14.5. Representative figures.

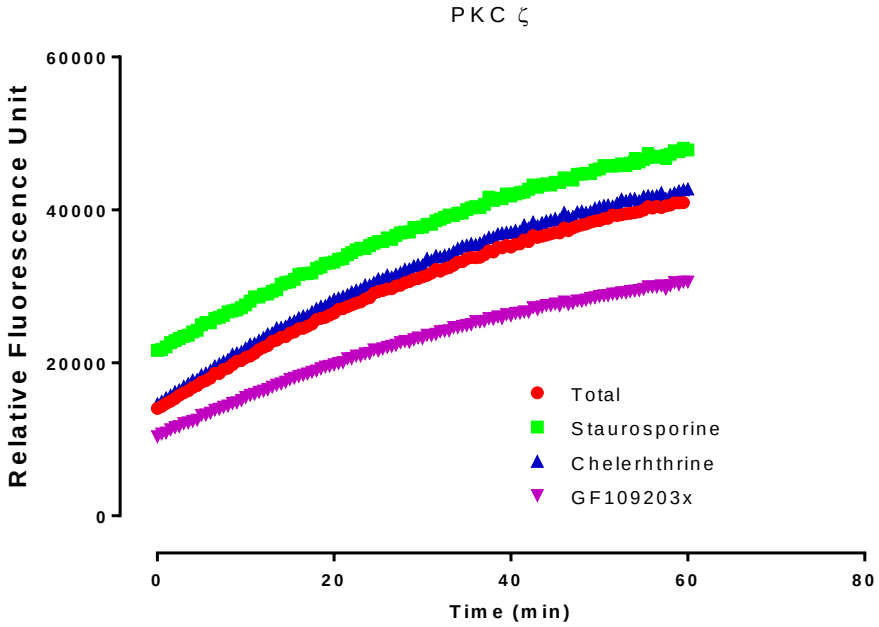
Figure 65. Time course of PKC activity in the absence and presence of various compounds (10 μM).

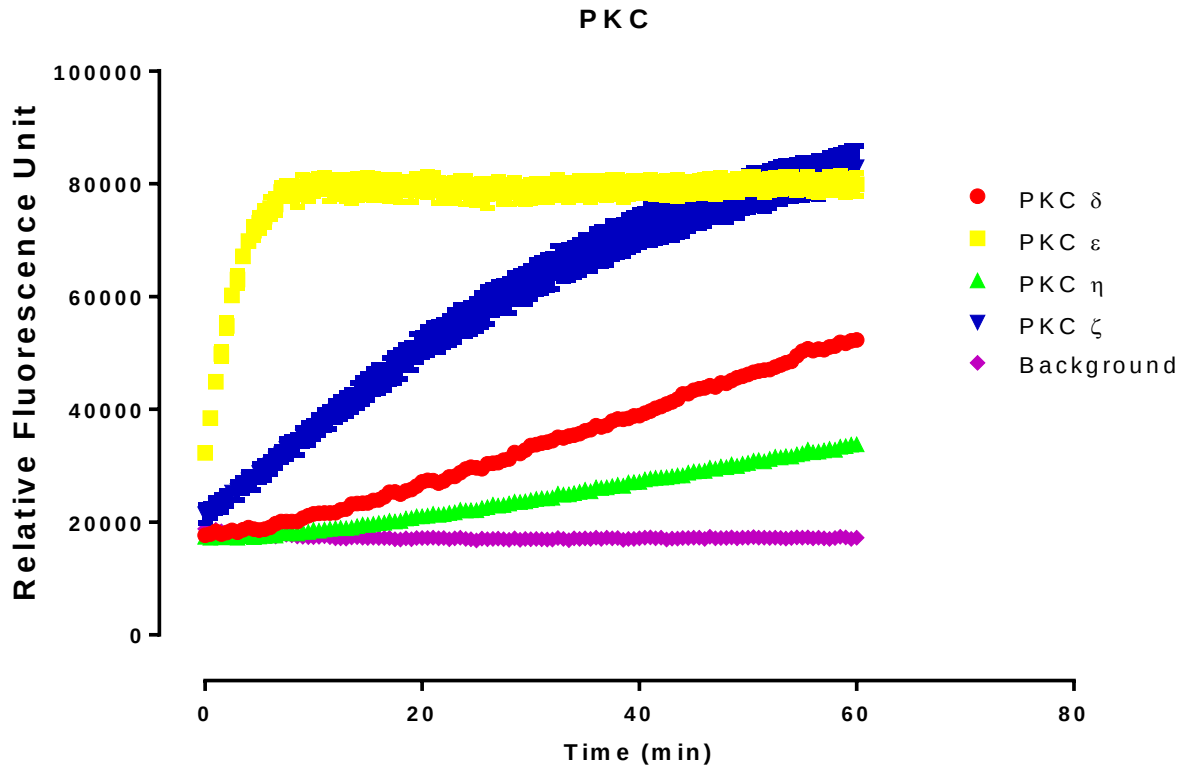


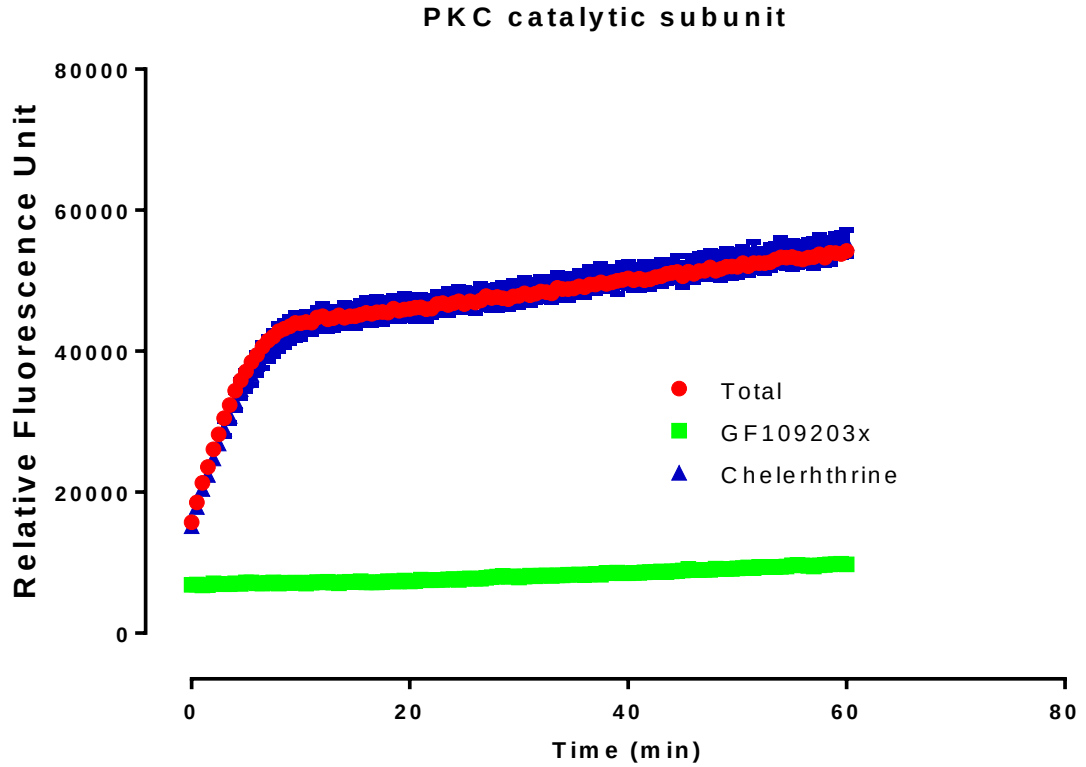

PKC β II
Relative Fluorescence Unit
60000
40000
20000
0
0
10
20
30
40
Time (min)
Buffer
Sample
GF109203x
Staurosporine


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


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


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





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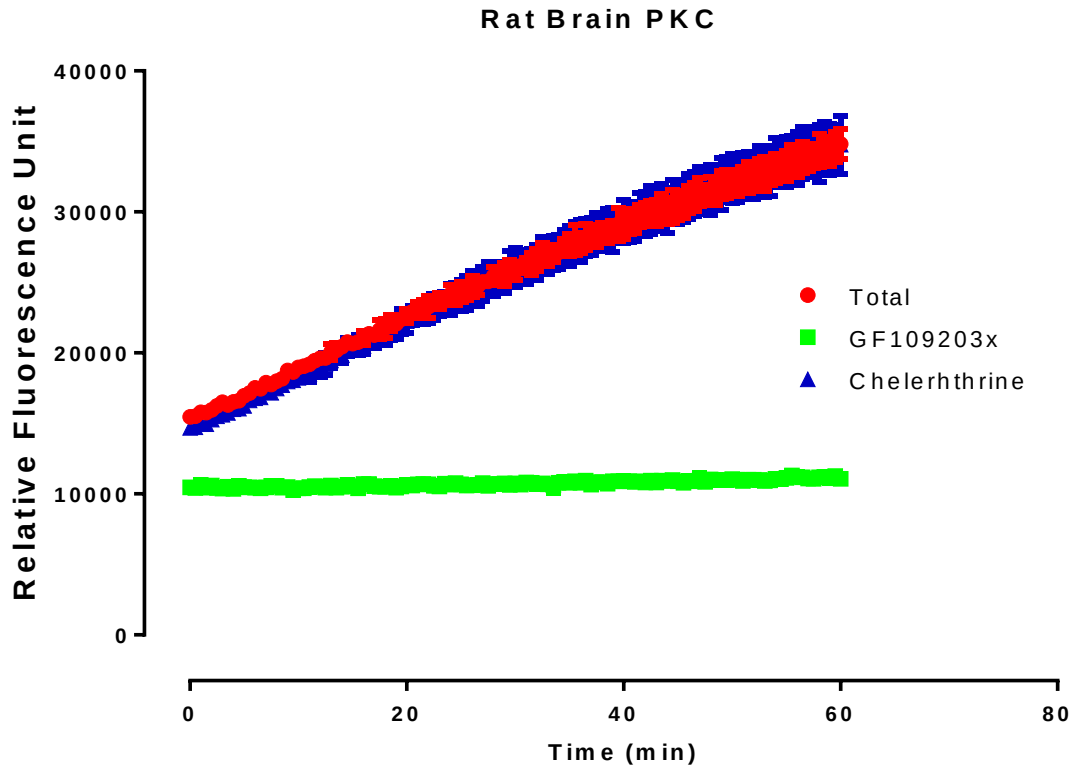


Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

2.15. 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) is also purchased from Invitrogen.

2.15.1. Brief 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, these kinases play a critical role in determining cellular responses to DNA damage. Inhibitors for kinases like CHK2 may represent novel anticancer therapies.

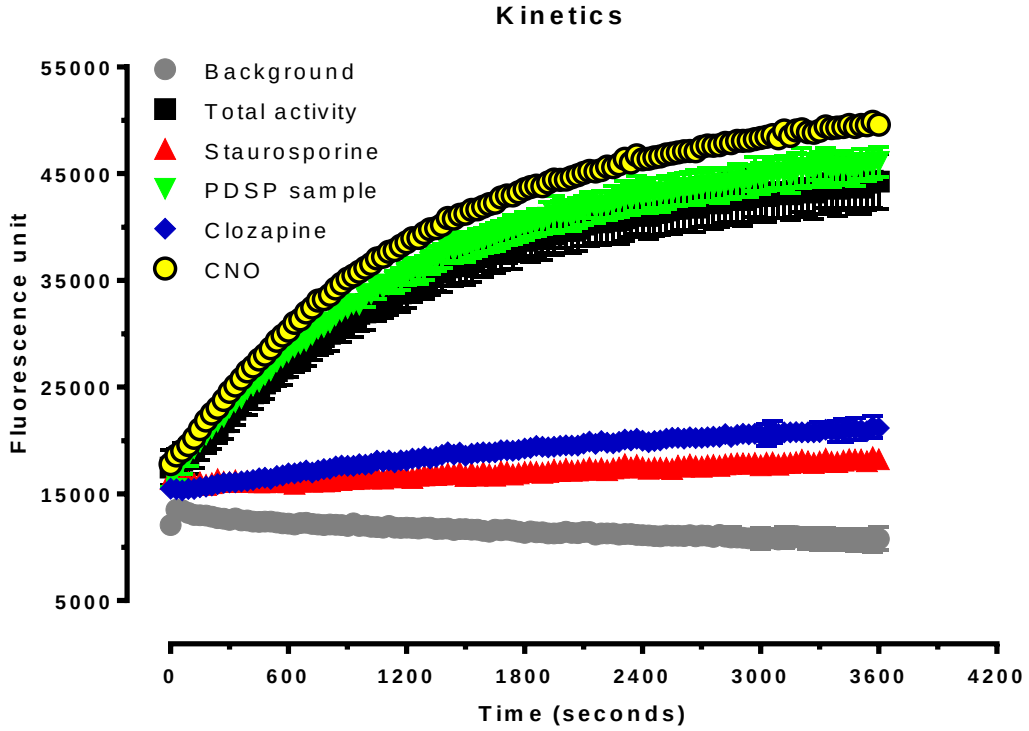
2.15.2. Principle of the CHK2 kinase assay. We use Invitrogen's Kinase Activity Assay Kit (#KNZ1031, Omnia Ser/Thr Recombinant Kit 3) to measure CHK2 activity. The Omnia® Kinase assay uses a Ser/Thr-containing peptide substrate conjugated with the chelation-enhanced fluorophore (CHEF) 8-hydro-5-(N,N-dimethylsulfonamido)-2-methylquinoline (Sox). Phosphorylation of the peptide 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.

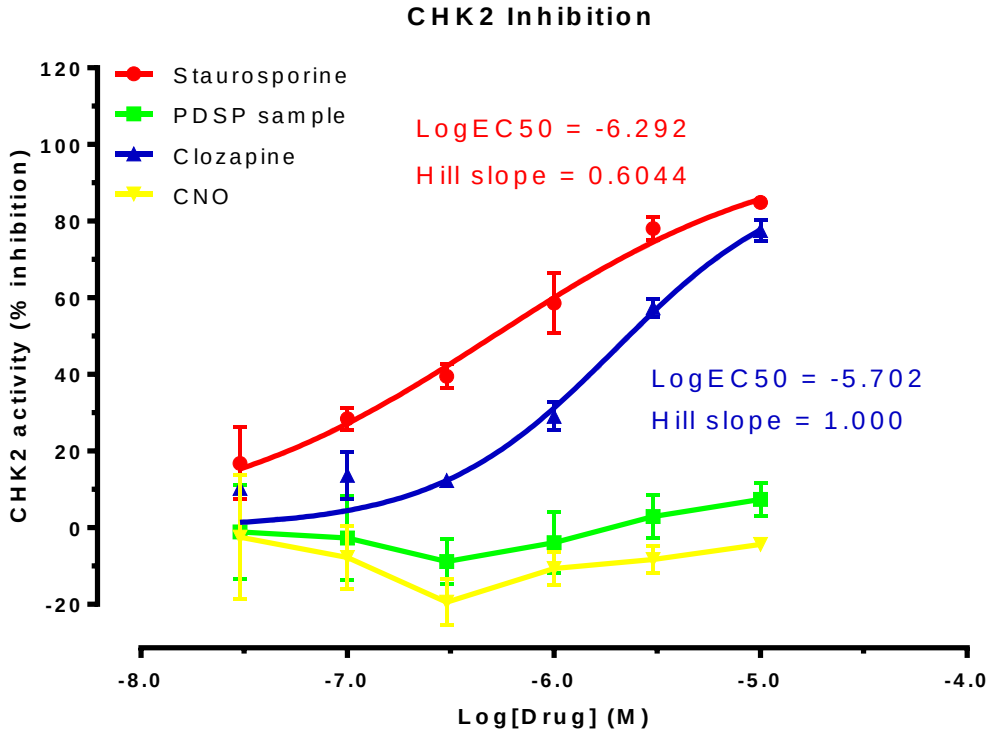
2.15.3. Assay procedure. CHK2 kinase activity assays are performed in 96-well plates according to the manufacturer's suggested procedure. In brief, a master reaction mix is made with following componets: kinase assay buffer, peptide substrate, ATP, DTT, and samples or controls; the mixture and the empty assay plate are warmed for 5 min at 30°C. To start the reaction, kinase solution is added first to the warmed plate, followed by the master mix to appropriate wells. The plate is incubated at 30°C. During the incubation, the plate is read every minute for 60 min, with excitation wavelength of 360 nm and emission wavelength of 485 nM.

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

2.15.5. Representative figures

Figure 66. Representative figures for CHK2 kinase activity kinetics (upper panel) and percentage inhibition (lower panel).





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

2.16.1. Brief background. Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels, that differ 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-termini.

2.16.2. Cell culture. Cells for nAChR functional assays are maintained as in Section 1.5.1.

2.16.3. General 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 (Xiao et al. 1998; Xiao et al., 2006). In brief, aliquots of cells in 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. The loading mixture is 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 the $^{86}Rb^{+}$ 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.

2.16.3.1. Primary functional assays – $^{86}Rb^{+}$ efflux experiments. For assessing agonist activity, 4 concentrations of a PDSP compound to be tested, 0.1, 1, 10 and 100 μM, are applied. Agonist activity is scaled as a percentage of the stimulation by 100 μM nicotine (100%). If a PDSP compound shows a concentration-dependent activation, and shows 25% stimulation at any concentration, it is subject to a secondary functional assay for agonist activity. For assessing

antagonist activity, 4 concentrations of a PDSP compound to be tested, 0.1 1, 10 and 100 μM, are applied in the presence of 100 μM nicotine. Antagonist activity is scaled as a percentage of the inhibition of $^{86}Rb^{+}$ efflux stimulated by 100 μM nicotine. If a PDSP compound shows concentration-dependent inhibition, and shows more than 50% inhibition at 100 μM, it is subject to a secondary functional assay for antagonist activity. All efflux assays are performedin quadruplicate. Nicotine is included in assays to define 100% agonist activity, as well as to function as a control.

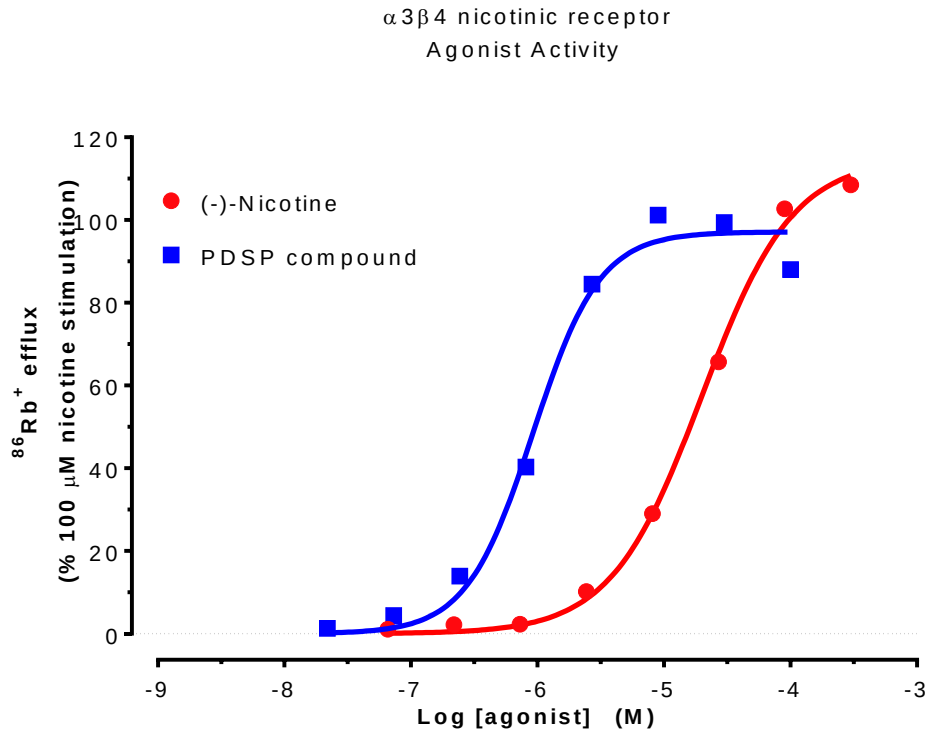
2.16.3.2. Secondary functional assays – $^{86}Rb^{+}$ efflux experiments. For assessing agonist activity, 8 concentrations of a PDSP compound to be tested are used. Agonist activity is scaled as % of the stimulation by 100 μM nicotine (100%). For assessing antagonist activity, 8 concentrations of a PDSP compound to be tested are used in the presence of 100 μM nicotine. Antagonist activity is scaled as a percentage of the inhibition of $^{86}Rb^{+}$ efflux stimulated by 100 μM nicotine. All efflux assays are performedin quadruplicate. Nicotine is included in the assays to define 100% agonist activity, as well as to function as a control.

2.16.4. Data analysis and representative figures. Raw counts of radioactivity (in cpm/well) are exported and analyzed in Prism 5.0 by nonlinear least-squares regression to estimate EC_{50} or IC_{50} values.

References

- Xiao Y, Meyer EL, Thompson JM, Surin A, Wroblewski J, Kellar KJ (1998). Rat alpha3/beta4 subtype of neuronal nicotinic acetylcholine receptor stably expressed in a transfected cell line: pharmacology of ligand binding and function. Mol Pharmacol 54(2): 322-333.
- Xiao Y, Fang H, Musachio JL, Wei ZL, Chellappan SK, Kozikowski AP, Kellar KJ (2006). Sazetidine-A, a novel ligand that desensitizes alpha4beta2 nicotinic acetylcholine receptors without activating them. Mol Pharmacol 70(4): 1454-1460.

Figure 67. Representative figures of agonist activity at nAChRs



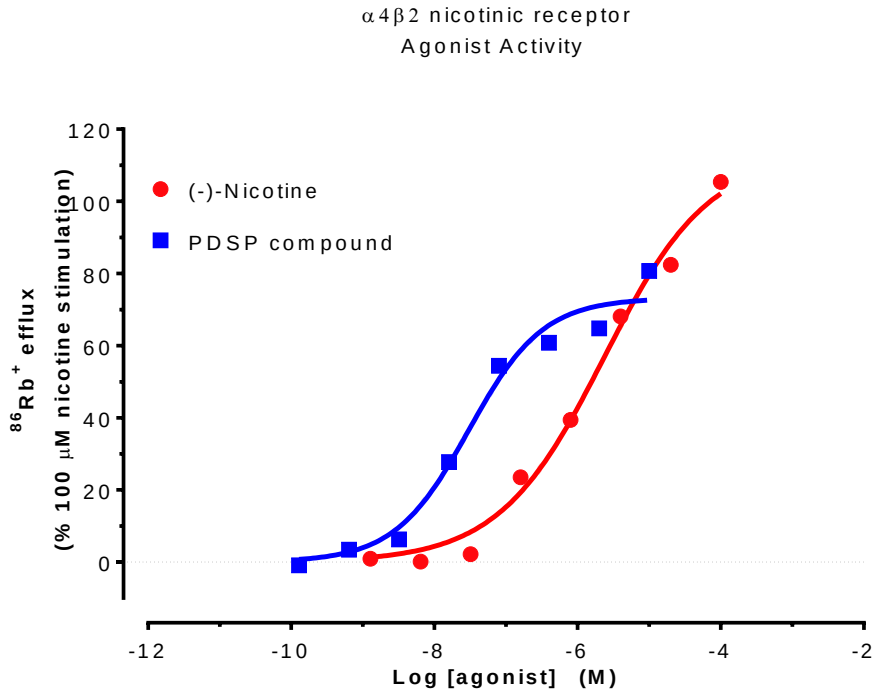
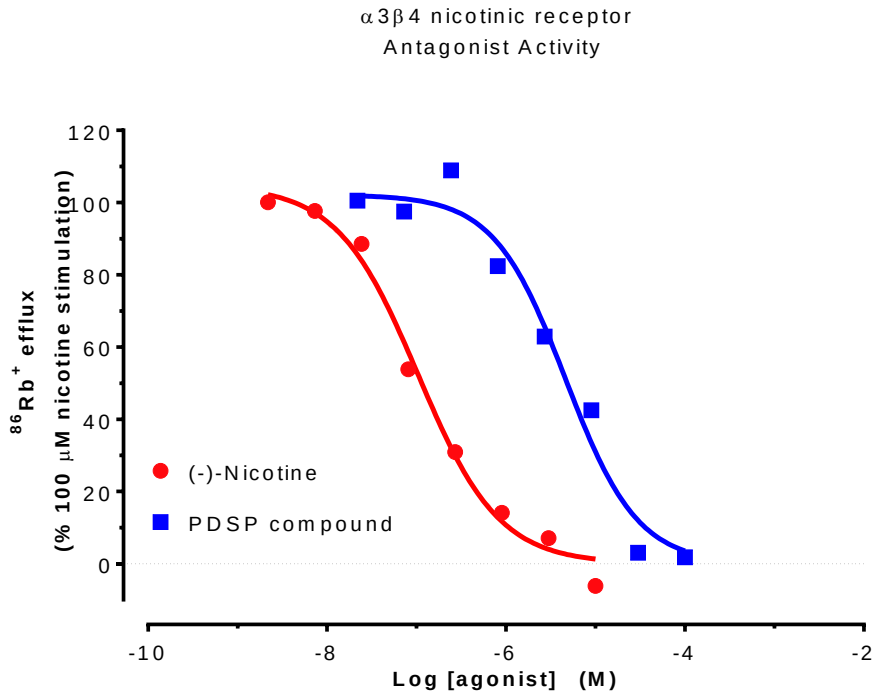
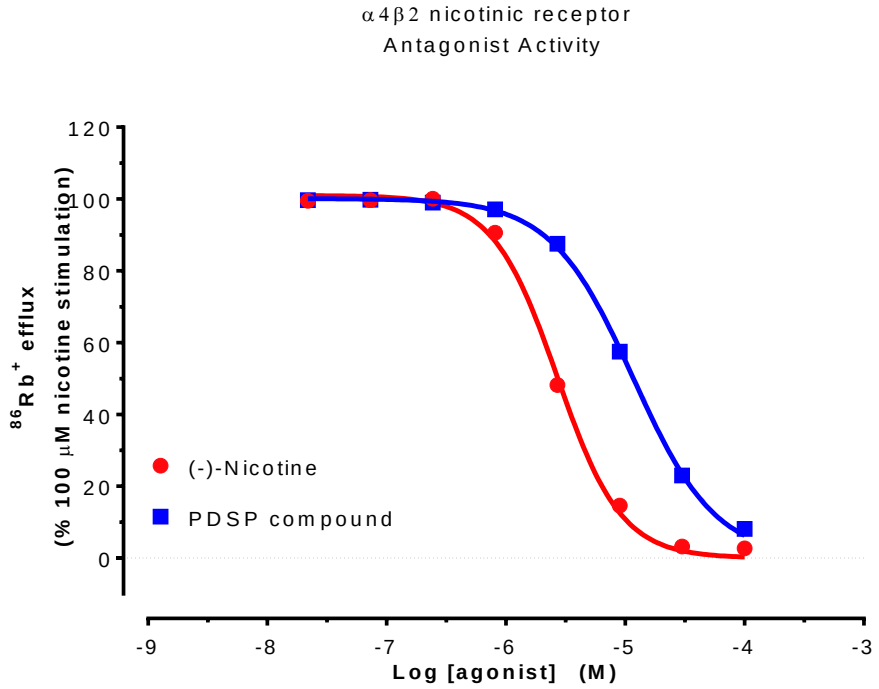


Figure 68. Representative figures of antagonist activity at nAChRs.





Section 3. Master table of PDSP targets.

Table 30. Complete list of targets and corresponding functional and radioligand binding assays available at the PDSP. ^{3}H or ^{125}I radioligands are available for radioligand binding assays. A checkmark (√) indicates that the functional assay is available: Gq - Calcium mobilization or Inositol phosphate accumulation assay; Gi or Gs - split luciferase cAMP biosensor assay; β-arrestin for GPCR mediated arrestin translocation assay; "Other Assay" indicates target-specific assays as detailed in the main text and indicated in the specific section; "?" indicates assays being developed, verified or optimized. The rightmost column (PDSP V1) indicates assays available under the previous PDSPcontract, in which 'B' represents radioligand binding assays and 'F' represents functional assays. See individual sections for detailed information regarding binding and functional assays.

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
5-HT receptors							
5-HT1A	3H		√	√			B, F
5-HT1B	3H		√	√			B, F
5-HT1D	3H		√	√			B, F
5-HT1E	3H		√	√			B, F
5-HT1F			√	√			B, F
5-HT2A	3H	√		√			B, F
5-HT2B	3H	√		√			B, F
5-HT2C (INI)	3H	√		√			B, F
5-HT2C (VGI)	3H	√					B, F
5-HT2C (VGV)	3H	√					
5-HT2C (VNV)	3H	√		√			
5-HT2C (VSV)	3H	√		√			
5-HT3	3H						B
5-HT4	3H		√	√			B, F
5-HT5A	3H		?	√			B, F
5-HT6	3H		√	√			B, F
5-HT7A	3H		√	√			B, F
5-HT7B							B, F
5-HT7D							
Muscarinic receptors							
M1	3H	√		√			B, F
M2	3H		√	√			B, F
M3	3H	√		√			B, F
M4	3H		√	√			B, F

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
M5	3H	√		√			B, F
Muscarinic DREADDs							
M1D	3H	√					
M2D	3H		√				
M3D	3H	√		√			
M4D	3H		√				
M5D	3H	√					
Nicotinic receptors							
α2ß2	3H				√	Section 2.16	B
α2ß4	3H						B
α3ß2	3H						B
α3ß4	3H				√	Section 2.16	B
α4ß2	3H				√	Section 2.16	B
α4ß4	3H						B
α4ß2 (Rat Brain)	3H						B
α2ß2	3H						B
α7	125I						B
Adrenergic receptors							
α1A	3H	√					B, F
α1B	3H	√		√			B, F
α1D	3H	√		√			B, F
α2A	3H		√				B, F
α2B	3H		√	√			B, F
α2C	3H		√	√			B, F
ß1	125I		√	√			B, F
ß2	3H		√	√			B, F
ß3	3H		√				B, F
Adenosine receptors							
A1	3H		√	√			B, F
A2A	3H		√				B, F
A2B	3H						B, F
A3	3H						B, F
Angiotensin II receptors							
AT1	3H	√		√			B
AT2	3H						B
Apelin receptor							
Apelin				√			
Bile Acid receptors							
GPBA				√			
Bombesin receptors							
BB1		√		√			
BB2		√		√			
BB2, mouse		√					

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
BB3		√		√			
Bradykinin receptors							
B1				√			F
B2		√		√			F
Calcitonin receptors							
CT							
CT-like						CALCR	F
Calcium sensing receptors							
CaS				√			F
GPRC6							
Cannabinoid receptors							
CB1 (rat brain)	3H			√			B, F
CB2	3H			√			B, F
Chemokine receptors							
CCR1							F
CCR2							F
CCR3							F
CCR4				√			F
CCR5							F
CCR6				√			F
CCR7							F
CCR8							F
CCR9							F
CCR10							F
CXCR1				√			F
CXCR2				√			F
CXCR3							F
CXCR4				√			F
CXCR5							F
CXCR6				√			F
CXCR7							F
CX3CR1				√			F
XCR1							F
CCRL1						CCBP2	F
CCRL2							F
Cholecystokinin receptors							
CCK1				√			
CCK2		√					
Complement peptide receptors							
C3a							F
C5a1							F
C5a2						C5L2	F
Corticotropin-releasing factor receptors							
CRF1	3H		√				B, F

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
CRF2	3H		√				B, F
Dopamine receptors							
D1	3H		√	√			
D2	3H		√	√			
D3	3H			√			
D4	3H		√				
D5	3H		√	√			
Endothelin receptors							
ETA				√			F
ETB							F
Estrogen receptor							
GPER							
Formylpeptide receptors							
FPR1				√			
FPR2/ALX				√		FPRL1	F
FPR3				√		FPRL2	F
Free fatty acid receptors							
FFA1		√				GPR40	
FFA2							
FFA3							
Frizzled receptors							
FZD1							
FZD2							
FZD3							
FZD4							
FZD5							
FZD6							
FZD7							
FZD8							
FZD9							
FZD10							
SMO	3H		√?	?	Gli-Luc (?)		
GABAA receptors (BZP = benzodiazepine)							
GABAA a1	3H, BZP						B
GABAA a2	3H, BZP						B

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
GABAA a3	3H, BZP						B
GABAA a5	3H, BZP						B
GABAA a6	3H, BZP						B
GABAA (rat brain)	3H, BZP						B
GABAB (rat brain)	3H						B
GABAB receptors							
GABAB1							B
GABAB2							
Galanin receptors							
GAL1				√			
GAL2				√			
GAL3				√			
Ghrelin receptor							
Ghrelin	3H	√		√			F
Glucagon receptors							
GHRH							F
GIP							F
GLP-1				√			F
GLP-2							F
Glucagon						GCGR	F
Secretin				√		SCTR	F
Glycoprotein receptors							
FSH							
LH							
TSH							
Gonadotrophin-releasing hormone receptors							
GnRH				√			
GnRH2							
Histamine receptors							
H1	3H	√		√			B, F
H2	3H	√	√	√			B, F
H3	3H		√	√			B, F
H4				√			B, F
Hydroxycarboxylic acid receptors							
HCA1							
HCA2							
HCA3							
Imidazoline receptors							
I1 (rat brain)	3H						B
I2 (rat brain)	3H						B
Kisspeptin receptor							
KISS (GPR54)							
Leukotriene receptors							

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
BLT1				√			F
BLT2							F
CysLT1				√			F
CysLT2							F
OXE							
FRP2/ALX				√			
Lysophospholid (LPA) receptors							
LPA1				√		EDG2	F
LPA2				√		EDG4	F
LPA3						EDG7	F
LPA4							F
LPA5				√			F
Lysophospholipid (S1P) receptors							
S1P1				√			F
S1P2				√		EDG5	F
S1P3				√		EDG3	F
S1P4						EDG6	F
S1P5						EDG8	F
Melanin-concentrating hormone receptors							
MCH1				√			F
MCH2				√			F
Melanocortin receptors							
MC1		√		√			F
MC2							F
MC3		√					F
MC4				√			F
MC5				√			F
Melantonin receptors							
MT1				√			
MT2				√			
Glutamate receptors							
NMDA (rat brain)	3H						
AMPA (rat brain)	3H						
PCP (rat brain)	3H						
Kainate (rat brain)	3H						
NR1 (rat brain)	3H						
NR2B (rat brain)	3H						
Metabotropic Glutamate receptors							
mGluR1	3H?						F
mGluR2	3H?						F
mGluR3	3H?						F
mGluR4	3H?						F
mGluR5	3H?						F

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
mGluR5 (rat brain)	3H						
mGluR6	3H?						
mGluR7	3H?						F
mGluR8	3H?						F
Motolin receptor							
Motilin				√			F
Neuromedin U receptors							
NMU1				√			F
NMU2				√			F
Neuropeptide FF receptors							
NPFF1							
NPFF2							
Neuropeptide S receptor							
NPS				√			
Neuropeptide W/Neuropeptide B receptor							
NPBW1							
NPBW2							
Neuropeptide Y receptors							
Y1				√			B, F
Y2				√			B, F
Y4				√			B, F
Y5							B, F
Y6							B, F
Neurotensin receptors							
NTS1	3H	√					F
NTS2	3H			√			F
Opioid receptors							
δ (DOR)	3H		√	√			B,F
κ (KOR)	3H		√	√			B,F
μ (MOR)	3H		√	√?			B,F
NOP	3H		√	√			B,F
Orexin receptors							
OX1				√			
OX2				√			F
P2Y receptors							
P2Y1		√		√			F
P2Y2		√		√			F
P2Y4		√		√			F
P2Y6		√		√			F
P2Y11		√		√			F
P2Y12				√			
P2Y13				√			
P2Y14				√			

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
Parathyroid hormone receptors							
PTH1				√			F
PTH2							F
Peptide P518 receptor							
QRFP							
Platelet-activating factor receptor							
PAF	3H	√		√			
Prokineticin receptors							
PKR1							
PKR2							
Prolactin-releasing peptide receptor							
PRRP							
Prostanoid receptors							
DP1				√			
DP2							
EP1	3H			√			B, F
EP2	3H			√			B, F
EP3	3H			√			B, F
EP4	3H			√			B, F
FP				√			
IP1				√			B, F
TP							
Protease-activated receptors							
PAR1		√					
PAR2							
PAR3							
PAR4							
Relaxin family peptide receptors							
RXFP1							
RXFP2							
RXFP3						SALPR	F
RXFP4							
Sigma receptors							
Sigma 1 (Guinea Pig)	3H						B
Sigma 2 (PC12)	3H						B
Somatostatin receptors							
sst1				√			F
sst2				√			F
sst3				√			F
sst4				√			F
sst5			√	√			F
Tachykinin receptors (Neurokinin receptors)							
NK1		√		√			F

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
NK2		√		√			F
NK3		√		√			F
Thyrotropin-releasing hormone receptors							
TRH1							
TRH2							
Trace amine receptor							
TA1				√			
Urotensin receptor							
UT				√			F
Vasopressin and Oxytocin receptors							
V1a	3H	√		√			B, F
V1b	3H	√		√			B, F
V2	3H	√		√			B, F
OT	3H	√		√			B, F
VIP and PACAP receptors							
PAC1						ADCYAP1R	F
VPAC1				√			
VPAC2				√			F
Neurotransmitter transporters							
DAT	3H				√	Section 2.10	B,F
NET	3H				√	Section 2.10	B,F
SERT	3H				√	Section 2.10	B,F
VMAT1							
VMAT2	3H						
Ion channels							
hERG K+	3H				√ (Tl^+ Flux) √ (Patch)	Section 2.8 Section 2.9	B,F
Ca2+ (rat brain)	3H						B
Na+ site II	3H						B
Other targets							
MDR1					√	Section 2.11	
PKCα	3H				√	Section 2.14	B, F
PKCβ I	3H				√	Section 2.14	B, F
PKCβ II	3H				√	Section 2.14	B, F
PKCγ	3H				√	Section 2.14	B, F
PKCδ	3H				√	Section 2.14	B, F
PKCε	3H				√	Section 2.14	B, F
PKCη					√	Section 2.14	
PKCζ					√	Section 2.14	
PKCτ					√	Section 2.14	
PBR (rat brain)	3H						
PNR							F
Vanilloid receptor						TRPs	F

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
CHK2							F
HDAC							F
MAO A							F
MAO B							F
	Class A orphan GPCRs						
CMKLR1				√		CHEMERINR	F
GPR1							
GPR3							
GPR4							
GPR6							
GPR12							
GPR15							
GPR17							
GPR18							
GPR19							
GPR20							
GPR21							F
GPR22							
GPR25							
GPR26							
GPR27							F
GPR31							
GPR32							
GPR33							
GPR34							
GPR35							
GPR37						EDNRBL	F
GPR37L1							
GPR39							
GPR45							F
GPR50							
GPR52							
GPR55							
GPR61							F
GPR62							
GPR63							
GPR65							
GPR68		?	√				
GPR75							
GPR78							
GPR79							
GPR82							
GPR83							

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
GPR84							
GPR85							
GPR87							
GPR88			√	?			
GPR101							
GPR119							
GPR120							
GPR132							
GPR135							
GPR139							
GPR141							
GPR142							
GPR146							
GPR148							
GPR150							
GPR151							
GPR152							
GPR153							
GPR160							
GPR161							
GPR162							
GPR171							
GPR173							
GPR174							
GPR176							
GPR182							
GPR183							
LGR4							
LGR5							
LGR6							
LPAR6							
MAS1							
MAS1L							
MRGPRD							
MRGPRE							
MRGPRF							
MRGPRG							
MRGPRX1							
MRGPRX2							
MRGPRX3				√			
MRGPRX4				√			
OPN3							
OPN5							
OXGR1							

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
P2RY8							
P2RY10							
SUCNR1							
TAAR2							
TAAR3							F
TAAR4							F
TAAR5							F
TAAR6							
TAAR8							
TAAR9							
	Class B orphan GPCRs						
BAI1							
BAI2							
BAI3							
CD97							
CELSR1							
CELSR2							
CELSR3							
ELTD1							
EMR1							F
EMR2							F
EMR3							F
EMR4P							
GPR56							
GPR64							
GPR97							
GPR98						MASS1	F
GPR110							
GPR112							
GPR113							
GPR114							
GPR115							F
GPR116							
GPR123							F
GPR124							
GPR125							F
GRP126							
GPR127							
GPR128							F
GPR133							F
GPR144							
GPR157							F
LPHN1							
LPHN2							

Target (IUPHAR name)	Available assays at PDSP (new system)					Synonym Or Note	PDSP V1
	Binding	G_q	G_i or G_s	ß-arrestin	Other Assay		
LPHN3							
	Class C orphan GPCRs						
GPR156							
GPR158							
GPR179							
GPRC5A							
GPRC5B							
GPRC5C							
GPRC5D							
	Other 7TM receptors						
GPR107							
GPR137						TM7SF1	F
GPR143							

Section 4: List of publications supported by NIMH-PDSP

Complete list of research publications co-authored or otherwised supported by the NIMH PDSP. References are listed in the order to publication data, from the most recent to the oldest. Detailed validated protocols and modified protocols for all assays can also be found in these peer-reviewed publications.

1. Wang C, Jiang Y, Ma J, Wu H, Wacker D, Katritch V, Han GW, Liu W, Huang XP, Vardy E, McCorvy JD, Gao X, Zhou E, Melcher K, Zhang C, Bai F, Yang H, Yang L, Jiang H, **Roth BL**, Cherezov V, Stevens RC and Xu H Structural Basis for Molecular Recognition at Serotonin Receptors Science 2013. NIH Manuscript ID (NIHMSID) 350378.
2. Wacker D, Wang C, Katritch V, Han GW, Huang XP, Vardy E, McCorvy JD, Jiang Y, Chu M, Siu F, Liu W, Xu HE, Cherezov V, **Roth BL** and Stevens RC Structural Features for Functional Selectivity at Serotonin Receptors Science 2013. NIMH Manuscript ID (NIHMSID) 450382 (*BRL and RCS = Co-corresponding authors)
3. Vardy E, **Roth BL**. Conformational Ensembles in GPCR Activation. Cell 152(3): 385-6, 2013
4. Lin H, Sassano MF, **Roth BL**, Shoichet BK. A pharmacological organization of G protein-coupled receptors. Nat Methods 10(2): 140-6. (*BLR and BKS = Co-corresponding authors)
5. Iversen L, Gibbons S, Treble R, Setola V, Huang XP, **Roth BL**. Neurochemical profiles of some novel psychoactive substances. Eur J Pharmacol 700(1-3): 147-51, 2013
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