University of North Carolina

At Chapel Hill

National Institute of Mental Health

Psychoactive Drug Screening Program

Assay Protocol Book

What follows is a detailed description of the experimental protocols followed for measurements of compound affinity (K_i), agonist/antagonist potency (EC_{50}/IC_{50}), and agonist relative efficacy at recombinant, heterologously expressed or endogenous targets.

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I. Primary Radioligand Binding Assays

Compounds submitted to the PDSP are typically subjected to a 'primary assay' designed to identify a subset of potential receptors, transporters, ion channels, etc. for which the compounds display affinity.

Experimental Procedure and Data Analysis:

Test and reference compounds (see Table 1) are diluted to 5X final assay concentration (50 µM for a final assay concentration of 10 µM) in the appropriate radioligand binding buffer (see Table 1). Then, 50-µl aliquots of buffer (negative control), test compound, and reference compound are added in quadruplicate to the wells of a 96-well plate, each of which contains 50 µl of 5X radioligand (see Table 1 for final assay concentration for each radioligand) and 100 µl of buffer (see Table 1). Finally, receptor-containing, crude membrane fractions (prepared as detailed in IV) are resuspended in an appropriate volume of buffer (see Table 1) and dispensed (50 µl per well) into 96-well plate. Radioligand binding is allowed to equilibrate (typically for 1.5 hours at room temperature, see Table 1), and then bound radioactivity is isolated by filtration onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harverster. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter.

Raw dpm data from the Microbeta counter are analyzed on the PDSP DB. Total bound radioactivity is estimated from quadruplicate wells containing no test or reference compound and adjusted to 100%; non-specifically bound radioactivity is assessed from quadruplicate wells containing 10 µM of a suitable reference compound (see Table 1) and adjusted to 0%. The average bound radioactivity in the presence of the test compound (10 µM final assay concentration, quadruplicate determinations) is then expressed on the percent scale. The percent inhibition of radioligand binding is calculated as follows:

$$\% \text{ inhibition} = 100\% - \% \text{ radioactivity bound}$$

The PDSP on-line data entry and analysis system calculates the variance of the quadruplicate determinations (for the total, non-specific, and test compound binding values) and variances greater than 20% are flagged for further inspection and assays are repeated if necessary. Additionally, % inhibition values that are greater than the total binding (i.e., 100%) by at least 20% are also flagged for inspection; such results could indicate allosteric modulation of radioligand binding.

Receptor	Radioligand (Assay Conc.)	Reference	Assay Buffer
5-HT1A	[^{3}H]8-OH-DPAT (0.5 nM)	Methysergide	Standard Binding Buffer
5-HT1B	[^{3}H]GR127543 (0.3 nM)	Ergotamine	Standard Binding Buffer
5-HT1D	[^{3}H]GR127543 (0.3 nM)	Ergotamine	Standard Binding Buffer
5-HT1E	[^{3}H]5-HT (3 nM)	5-HT	Standard Binding Buffer
5-HT2A	[^{3}H]Ketanserin (0.5 nM)	Chlorpromazine	Standard Binding Buffer
5-HT2B	[^{3}H]LSD (1 nM)	Methysergide	Standard Binding Buffer
5-HT2C	[^{3}H]Mesulergine (0.5 nM)	Chlorpromazine	Standard Binding Buffer
5-HT3	[^{3}H]LY278584 (0.3 nM)	LY278584	Standard Binding Buffer
5-HT5a	[^{3}H]LSD (1 nM)	Ergotamine	Standard Binding Buffer
5-HT6	[^{3}H]LSD (1 nM)	Chlorpromazine	Standard Binding Buffer
5-HT7	[^{3}H]LSD (1 nM)	Chlorpromazine	Standard Binding Buffer
D1	[^{3}H]SCH233930 (0.2 nM)	SKF38393	Dopamine Binding Buffer
D2	[^{3}H]N-methylspiperone (0.2 nM)	Haloperidol	Dopamine Binding Buffer
D3	[^{3}H]N-methylspiperone (0.2 nM)	Chlorpromazine	Dopamine Binding Buffer
D4	[^{3}H]N-methylspiperone (0.3 nM)	Chlorpromazine	Dopamine Binding Buffer
D5	[^{3}H]SCH233930 (0.2 nM)	SKF38393	Dopamine Binding Buffer
Delta OR	[^{3}H]DADLE (0.3 nM)	Naltrindole	Standard Binding Buffer
Kappa OR	[^{3}H]U69593 (0.3 nM)	Salvinorin A	Standard Binding Buffer
Mu OR	[^{3}H]DAMGO (0.3 nM)	DAMGO	Standard Binding Buffer
H1	[^{3}H]Pyrilamine (0.9 nM)	Chlorpheniramine	Histamine Binding Buffer
H2	[^{3}H]Tiotidine (3 nM)	Cimetidine	Histamine Binding Buffer

H3	[^{3}H]alpha-methylhistamine (0.4 nM)	Histamine	Histamine Binding Buffer
H4	[^{3}H]Histamine (5 nM)	Clozapine	Histamine Binding Buffer
SERT	[^{3}H]Citalopram (0.5 nM)	Amitriptyline	Transporter Binding Buffer
NET	[^{3}H]Nisoxetine (0.5 nM)	Desipramine	Transporter Binding Buffer
DAT	[^{3}H]WIN35428 (0.5 nM)	GBR12909	Transporter Binding Buffer
V1	[^{3}H]Vasopressin (1 nM)	Vasopressin	Vasopressin Binding Buffer
V2	[^{3}H]Vasopressin (1 nM)	Vasopressin	Vasopressin Binding Buffer
V3	[^{3}H]Vasopressin (1 nM)	Vasopressin	Vasopressin Binding Buffer
EP3	[^{3}H]PGE2 (10 nM)	EP2	Prostaglandin Binding Buffer
EP4	[^{3}H]PGE2 (10 nM)	EP2	Prostaglandin Binding Buffer
PKCalpha	[^{3}H]PDBU (3 nM)	PDBU	PKC Binding Buffer
PKCbeta	[^{3}H]PDBU (3 nM)	PDBU	PKC Binding Buffer
PKCgamma	[^{3}H]PDBU (3 nM)	PDBU	PKC Binding Buffer
PKCdelta	[^{3}H]PDBU (3 nM)	PDBU	PKC Binding Buffer
PKCepsilon	[^{3}H]PDBU (3 nM)	PDBU	PKC Binding Buffer
A1	[^{3}H]NECA (5 nM)	NECA	Adenosine Binding Buffer
A2	[^{3}H]NECA (10 nM)	NECA	Adenosine Binding Buffer
VMAT2	[^{3}H]Tetrabenezine (1.5 nM)	Reserpine	VMAT Binding Buffer
GABAA	[^{3}H]Muscimol (1 nM)	GABA	50 mM Tris Acetate, pH 7.4
GABAB	[^{3}H]Baclofen (20 nM)	GABA	50 mM Tris Acetate, pH 7.4
PBR	[^{3}H]PK11195 (1 nM)	PK11195	50 mM Tris HCl, pH 7.4
AMPA	[^{3}H]AMPA (1 nM)	Glutamic Acid	50 mM Tris HCl, 2.5 mM $CaCl_2$, pH 7.4

BZP	[^{3}H]Flunitrazepam (0.5 nM)	Diazepam	50 mM Tris HCl, 2.5 mM $CaCl_2$, pH 7.4
Kainate	[^{3}H]Kainic Acid	Glutamic Acid	50 mM Tris HCl, 2.5 mM $CaCl_2$, pH 7.4
Na Channel	[^{3}H]Batrachotoxin	Veratridine	Na Channel Buffer
NMDA	[^{3}H]MK801 (1 nM)	MK801	5 mM Tris, pH 7.4
Oxytocin	[^{3}H]Oxytocin	Oxytocin	Oxytocin Binding Buffer
Alpha1A	[^{3}H]Prazosin (0.7 nM)	Urapidil	Alpha1 Binding Buffer
Alpha1B	[^{3}H]Prazosin (0.7 nM)	Corynanthine	Alpha1 Binding Buffer
Alpha2A	[^{3}H]Clonidine (1 nM)	Oxymetazoline	Alpha2 Binding Buffer
Alpha2B	[^{3}H]Clonidine (1 nM)	Prazosin	Alpha2 Binding Buffer
Alpha2C	[^{3}H]Clonidine (1 nM)	Prazosin	Alpha2 Binding Buffer
Beta1	[^{125}I]Iodopindolol (0.1 nM)	Atenolol	Beta Binding Buffer
Beta2	[^{125}I]Iodopindolol (0.1 nM)	ICI118551	Beta Binding Buffer
Beta3	[^{125}I]Iodopindolol (0.1 nM)	ICI118551	Beta Binding Buffer
M1	[^{3}H]QNB (0.5 nM)	Atropine	Muscarinic Binding Buffer
M2	[^{3}H]QNB (0.5 nM)	Atropine	Muscarinic Binding Buffer
M3	[^{3}H]QNB (0.5 nM)	Atropine	Muscarinic Binding Buffer
M4	[^{3}H]QNB (0.5 nM)	Atropine	Muscarinic Binding Buffer
M5	[^{3}H]QNB (0.5 nM)	Atropine	Muscarinic Binding Buffer
Alpha2Beta 2	[^{3}H]Epibatidine (0.5 nM)	(-)-Nicotine	50 mM Tris HCl, pH 7.4
Alpha2Beta 4	[^{3}H]Epibatidine (0.5 nM)	(-)-Nicotine	50 mM Tris HCl, pH 7.4
Alpha3Beta 2	[^{3}H]Epibatidine (0.5 nM)	(-)-Nicotine	50 mM Tris HCl, pH 7.4
Alpha3Beta	[^{3}H]Epibatidine (0.5 nM)	(-)-Nicotine	50 mM Tris HCl,

4			pH 7.4
Alpha4Beta 2	[^{3}H]Epibatidine (0.5 nM)	(-)-Nicotine	50 mM Tris HCl, pH 7.4
Alpha4Beta 4	[^{3}H]Epibatidine (0.5 nM)	(-)-Nicotine	50 mM Tris HCl, pH 7.4
Alpha4Beta 2 (endog.)	[^{3}H]Epibatidine (0.5 nM)	(-)-Nicotine	50 mM Tris HCl, pH 7.4
CB1	[^{3}H]CP55940	CP55940	Cannabinoid Binding Buffer
CB2	[^{3}H]CP55940	CP55940	Cannabinoid Binding Buffer
Sigma1	[^{3}H]Pentazocine (3 nM)	Haloperidol	Sigma Binding Buffer
Sigma2	[^{3}H]DTG (3 nM)	Haloperidol	Sigma Binding Buffer
AT1	[^{125}I]ATII (0.1 nM)	Candesartan	Angiotensin Binding Buffer
AT2	[^{125}I]ATII (0.1 nM)	PD123319	Angiotensin Binding Buffer
Ca++ Channel	[^{3}H]Nitrendipine (0.1 nM)	Nifendipine	Calcium Channel Buffer
Imidazoline 1	[^{125}I]Clonidine (0.1 nM)	Naphazoline	Imidazoline Binding Buffer
NT1	[^{3}H]Neurotensin (2 nM)	Neurotensin	50 mM Tris HCl, 0.2% BSA, pH 7.4
NT2	[^{3}H]Neurotensin (2 nM)	Neurotensin	50 mM Tris HCl, 0.2% BSA, pH 7.4

Table 1. Assay conditions for primary radioligand binding assays.

Buffer Compositions:

Standard Binding Buffer: 50 mM Tris HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4
Dopamine Binding Buffer: 50 mM HEPES, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4
Histamine Binding Buffer: 50 mM Tris HCl, 0.5 mM EDTA, pH 7.4
Transporter Binding Buffer: 50 mM Tris HCl, 150 mM NaCl, 5 mM KCl, 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
Prostaglandin Binding Buffer: 25 mM Tris HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4
PKC Binding Buffer: 50 mM Tris HCl, 1 mM $CaCl_2$, 4 mg/ml BSA, 100 µg/ml phosphatidylserine, pH 7.4
Adensoine Binding Buffer: 50 mM Tris HCl, 1 U/ml adenosine deaminase, pH 7.4

VMAT Binding Buffer: 50 mM HEPES, 300 mM sucrose, pH 8.0
Na Channel Buffer: 130 mM choline chloride, 5.4 mM KCl, 0.8 $MgSO_4$, 5.5 mM glucose, 50 mM HEPES, 1 µM tetrodotoxin, 1 mg/ml BSA, 30 µg/well scorpion venom, pH 7.4 at 37 degrees centigrade
Oxytocin Binding Buffer: 50 mM HEPES, 10 mM $MnCl_2$, pH 7.4
Alpha1 Binding Buffer: 20 mM Tris HCl, 145 mM NaCl, pH 7.4
Alpha2 Binding Buffer: 50 mM Tris HCl, 5 mM $MgCl_2$, pH 7.7
Beta Binding Buffer: 50 mM Tris HCl, 3 mM $MnCl_2$, pH 7.7
Muscarinic Binding Buffer: 50 mM Tris HCl, pH 7.7
Cannabinoid Binding Buffer: 50 mM Tris HCl, 1 mM EDTA, 3 mM $MgCl_2$, 5 mg/ml fatty acid-free BSA, pH 7.4
Sigma Binding Buffer: 50 mM Tris HCl, pH 8.0
Angiotensin Binding Buffer: 50 mM Tris HCl, 5 mM $MgCl_2$, 150 mM NaCl, 0.5 mg/ml BSA, 100 mM bacitracin, protease inhibitor, pH 7.4
Calcium Channel Buffer: 50 mM Tris HCl, 50 mM NaCl, 1 mM $CaCl_2$, pH 7.4
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

II. Secondary Radioligand Binding Assays

Serotonin Receptors: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_{5A}, 5-HT_6 and 5-HT_7

Note: 5-HT_{1F} and 5-HT_4 radioligand competition binding assays are currently under development.

Assay Buffer: Standard Binding Buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4)

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Roth *et al*. *J Pharmacol Exp Ther* 238(2):480-485 (1986), Roth *et al*. *J Pharmacol Exp Ther* 268(3):1403-1410 (1994).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in Standard Binding Buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference (see Table) compounds are prepared in Standard Binding Buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand (see Table) is diluted to five times the assay concentration (see Table 1) in Standard Binding Buffer. Typically, the assay concentration of radioligand is a value between one half the K_D and the K_D of a particular radioligand at its target.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of Standard Binding Buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled Standard Binding Buffer and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 (GraphPad Software) using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x\text{-log}IC50})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

Receptor	Radioligand (Assay Conc.)	Reference Compound
5-HT$_{1A}$	[^{3}H]8-OH-DPAT (0.5 nM)	Methysergide
5-HT$_{1B}$	[^{3}H]GR125743 (0.3 nM)	Ergotamine
5-HT$_{1D}$	[^{3}H]GR125743 (0.3 nM)	Ergotamine
5-HT$_{1E}$	[^{3}H]5-HT (3 nM)	5-HT
5-HT$_{2A}$	[^{3}H]Ketanserin (0.5 nM)	Chlorpromazine
5-HT$_{2B}$	[^{3}H]LSD (1 nM)	5-HT
5-HT$_{2C}$	[^{3}H]Mesulergine (0.5 nM)	Chlorpromazine
5-HT$_3$	[^{3}H]LY278584 (0.3 nM)	LY278584
5-HT$_{5A}$	[^{3}H]LSD (1 nM)	Ergotamine
5-HT$_6$	[^{3}H]LSD (1 nM)	Chlorpromazine
5-HT$_7$	[^{3}H]LSD (1 nM)	Chlorpromazine

Table 2. Serotonin (5-HT) receptor radioligands, radioligand assay concentrations, and reference compounds.

Figure 1. Schematic of binding assay plate. Increasing concentrations (from left to right) of reference or test compound (diluted in buffer) are added (50 µl aliquots, in duplicate) from 5x stocks to wells containing 50 µl of 5x radioligand (fixed concentration, prepared in buffer) and 100 µl of buffer. Finally, 50 µl of receptor-containing membrane homogenate (5x suspension in buffer) are added to achieve a final assay volume of 250 µl. Final concentrations of reference or test compound are listed above the columns.

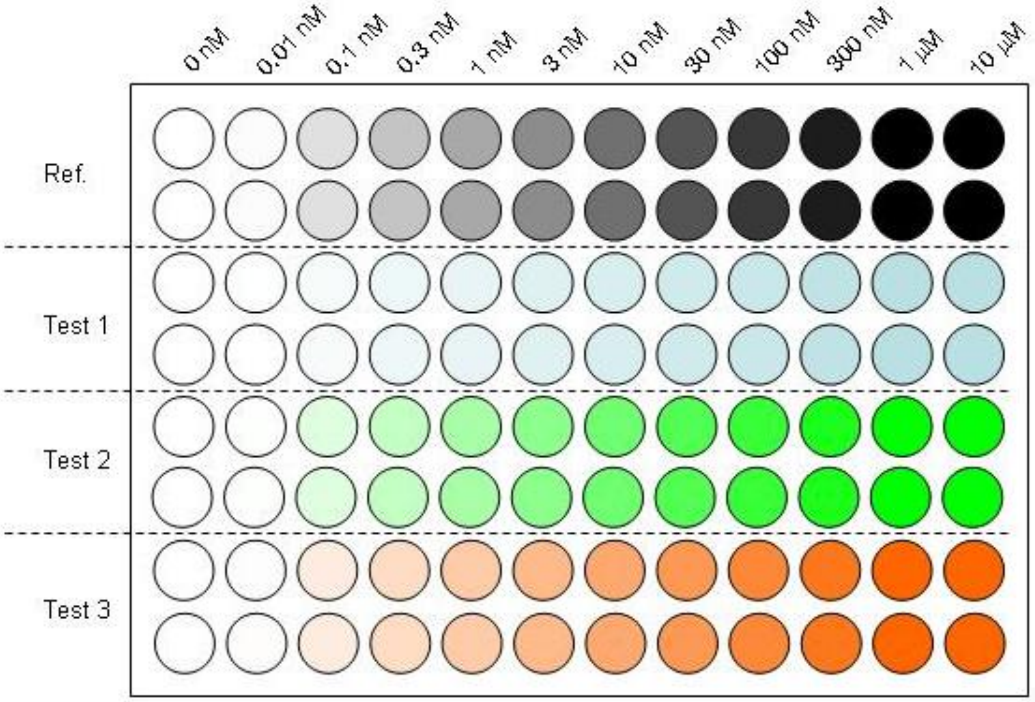
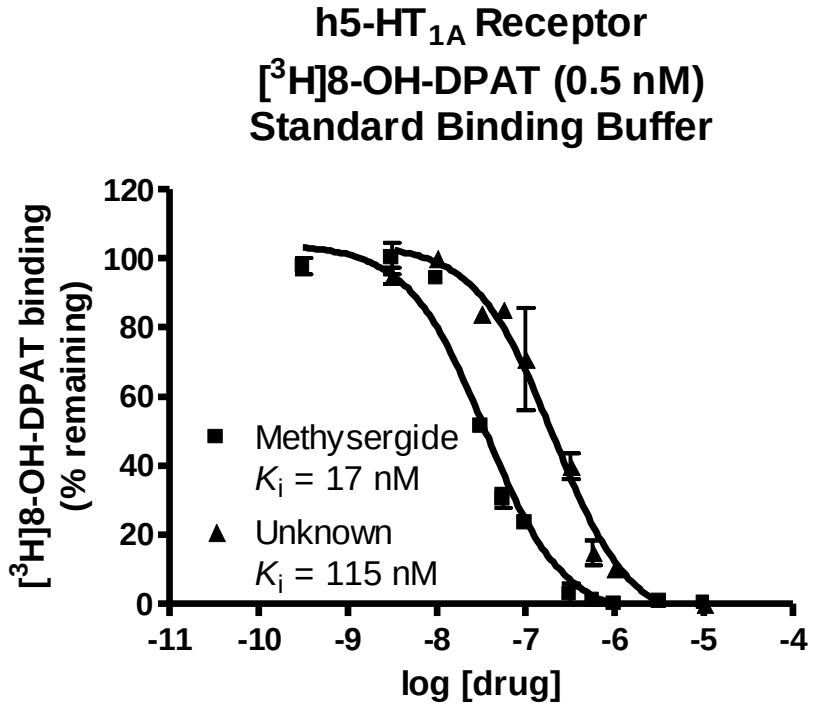
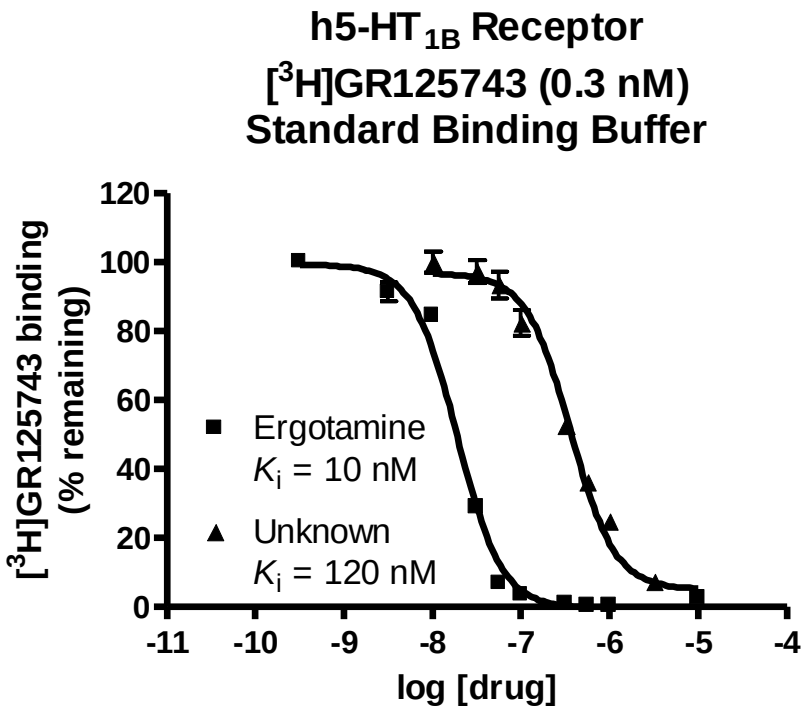
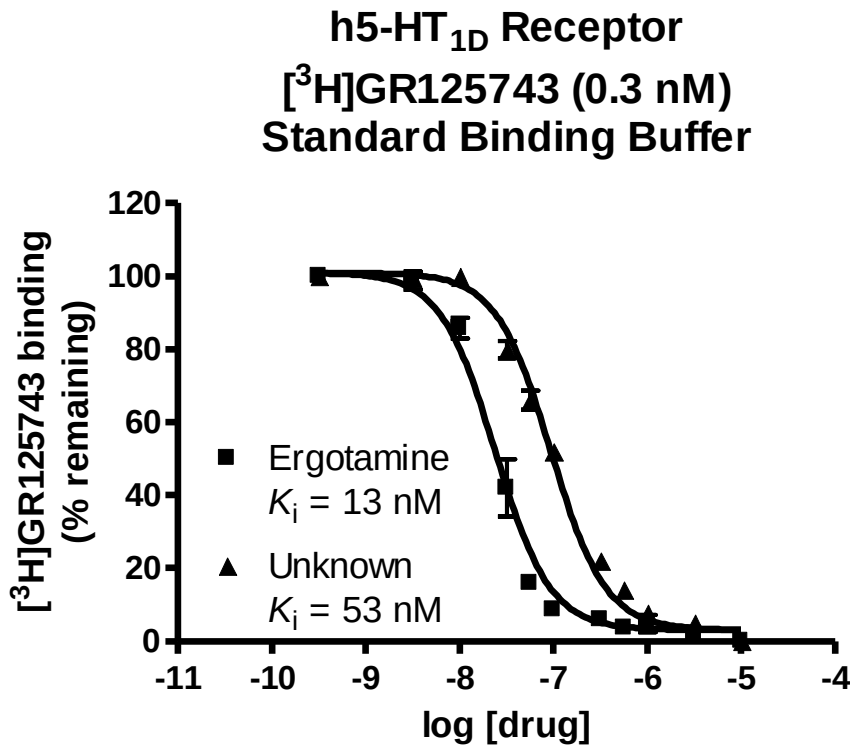
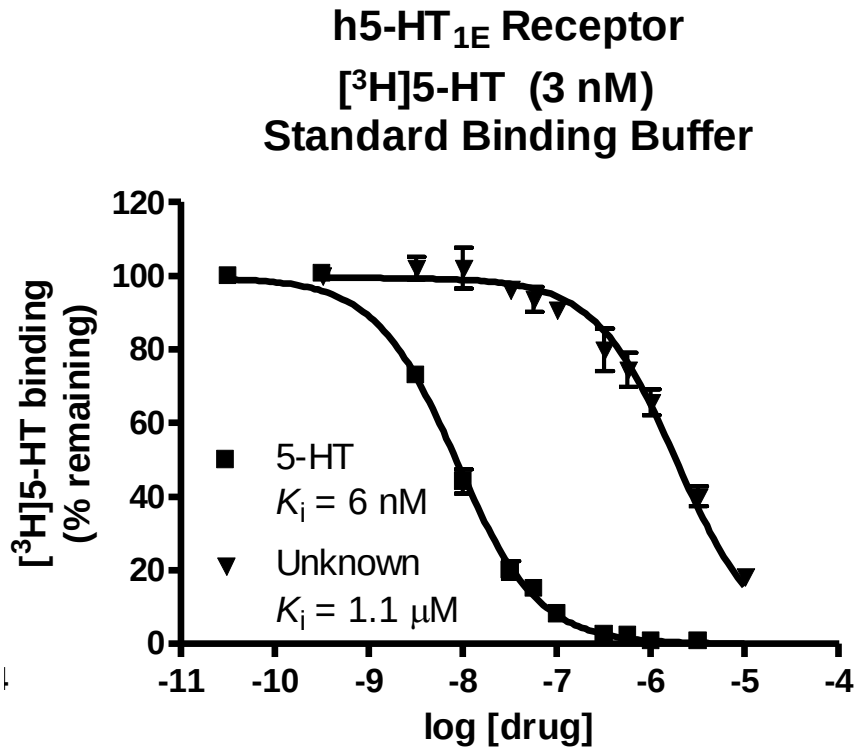


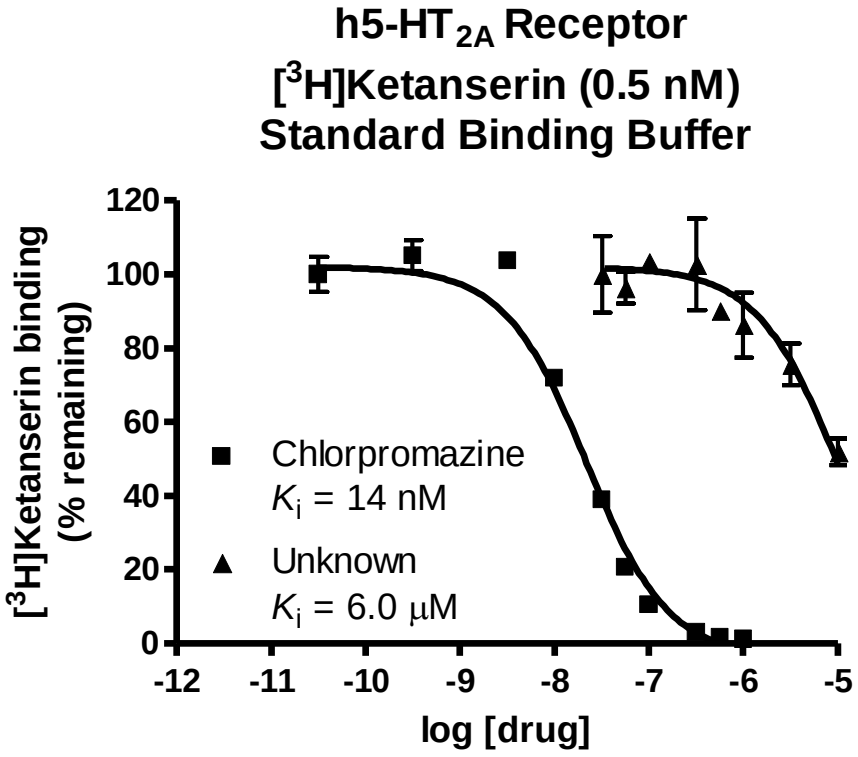
Figure 2. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the serotonin receptors available for screening.

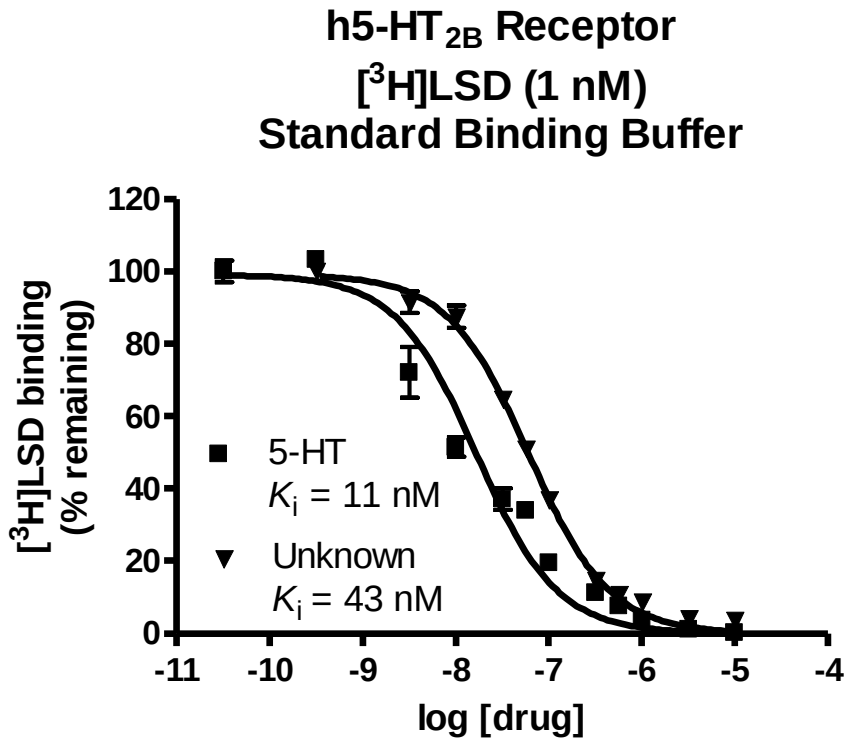


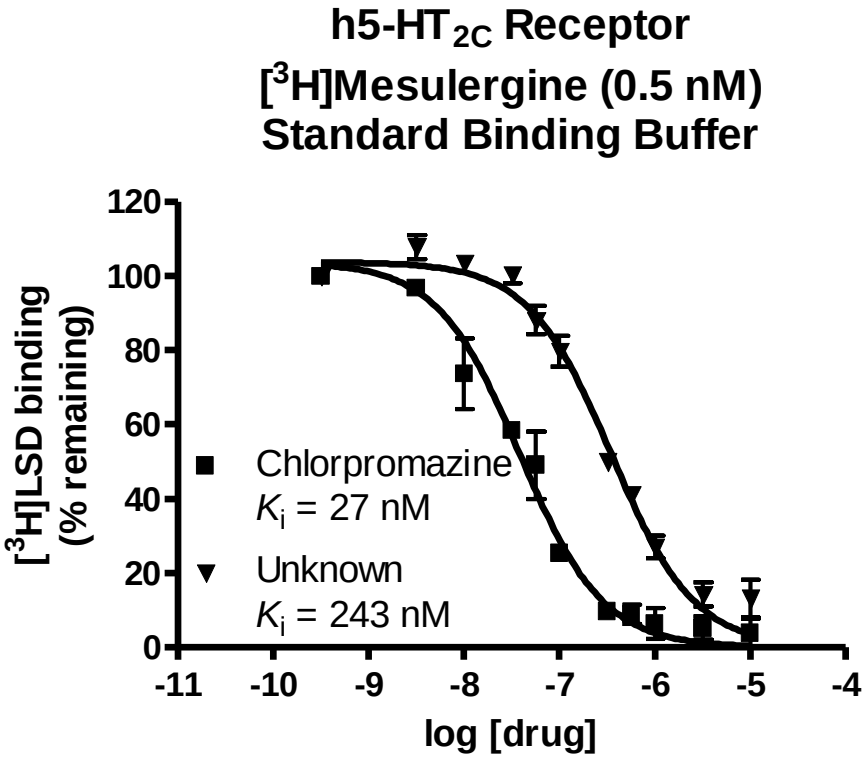


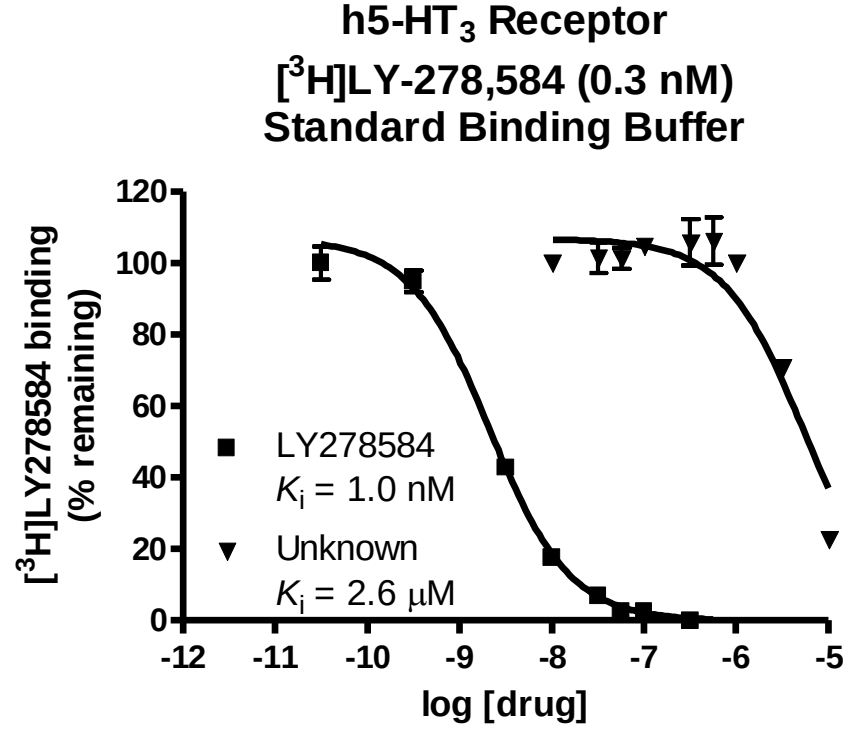

h5-HT1D Receptor
[3H]GR125743 (0.3 nM)
Standard Binding Buffer
[3H]GR125743 binding
(% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
Ergotamine
Ki = 13 nM
Unknown
Ki = 53 nM

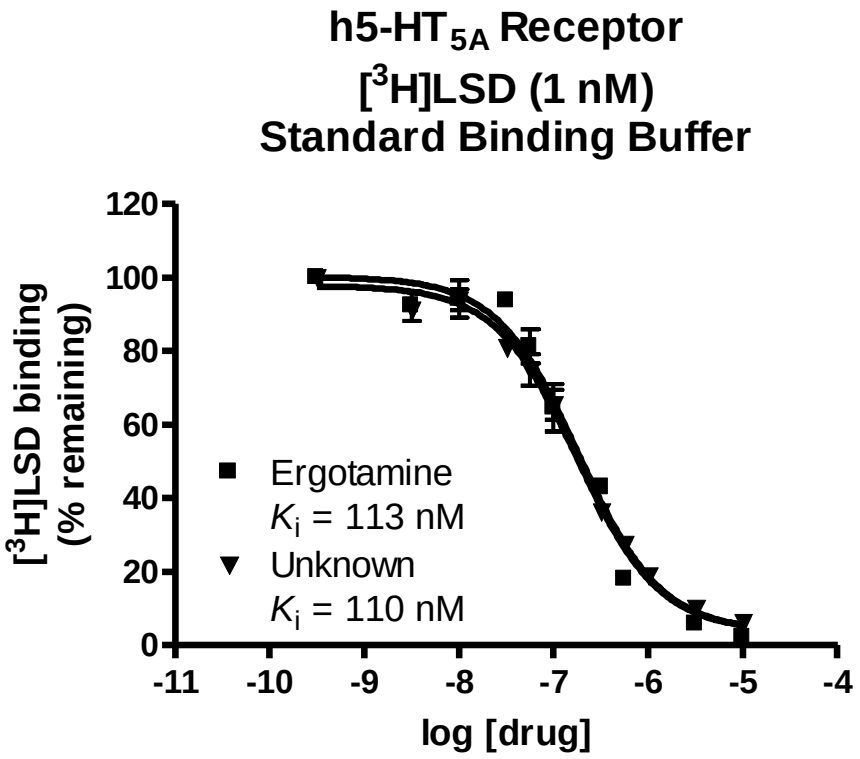

h5-HT1E Receptor
[3H]5-HT (3 nM)
Standard Binding Buffer
[3H]5-HT binding
(% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
5-HT
Ki = 6 nM
Unknown
Ki = 1.1 μM

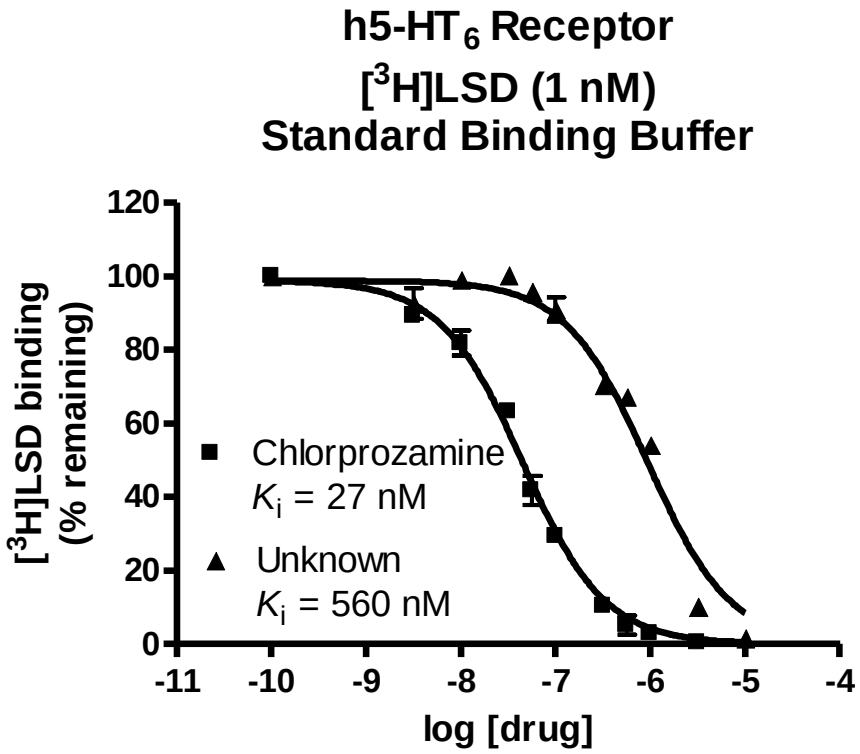

h5-HT2A Receptor
[3H]Ketanserin (0.5 nM)
Standard Binding Buffer
[3H]Ketanserin binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Chlorpromazine
Ki = 14 nM
Unknown
Ki = 6.0 μM

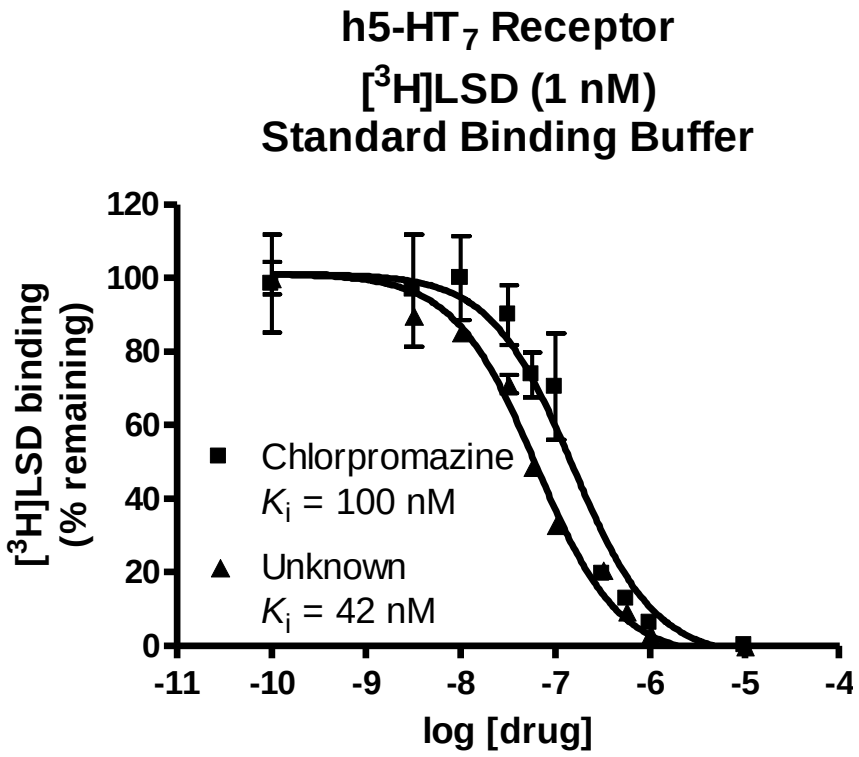

h5-HT2B Receptor
[3H]LSD (1 nM)
Standard Binding Buffer
[3H]LSD binding
(% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
5-HT
Ki = 11 nM
Unknown
Ki = 43 nM


h5-HT2C Receptor
[3H]Mesulergine (0.5 nM)
Standard Binding Buffer
[3H]LSD binding
(% remaining)
120
100
80
60
40
20
0
Chlorpromazine
Ki = 27 nM
Unknown
Ki = 243 nM
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]


h5-HT3 Receptor
[3H]LY-278,584 (0.3 nM)
Standard Binding Buffer
[3H]LY278584 binding
(% remaining)
120
100
80
60
40
20
0
LY278584
Ki = 1.0 nM
Unknown
Ki = 2.6 µM
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]


h5-HT5A Receptor
[3H]LSD (1 nM)
Standard Binding Buffer
[3H]LSD binding (% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
Ergotamine
Ki = 113 nM
Unknown
Ki = 110 nM


h5-HT6 Receptor
[3H]LSD (1 nM)
Standard Binding Buffer
[3H]LSD binding (% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
Chlorprozamine
Ki = 27 nM
Unknown
Ki = 560 nM


h5-HT7 Receptor
[3H]LSD (1 nM)
Standard Binding Buffer
[3H]LSD binding
(% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
Chlorpromazine
Ki = 100 nM
Unknown
Ki = 42 nM

Dopamine Receptors: D_1, D_2, D_3, D_4, D_5

Assay Buffers: Dopamine Binding Buffer (50 mM NaCl, 50 mM HEPES-HCl, 5 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4)

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Roth *et al*. *Psychopharmacology* 120(3):365-368 (1995).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in Dopamine Binding Buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference (see Table) compounds are prepared in Dopamine Binding Buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand (see Table 2) is diluted to five times the assay concentration (see Table 2) in Dopamine Binding Buffer. Typically, the assay concentration of radioligand is a value between one half the K_D and the K_D of a particular radioligand at its target.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of Dopamine Binding Buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled Dopamine Binding Buffer and homogenized by several passages through a 26 gauge needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight.

The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 (GraphPad Software) using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x\text{-log}IC50})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

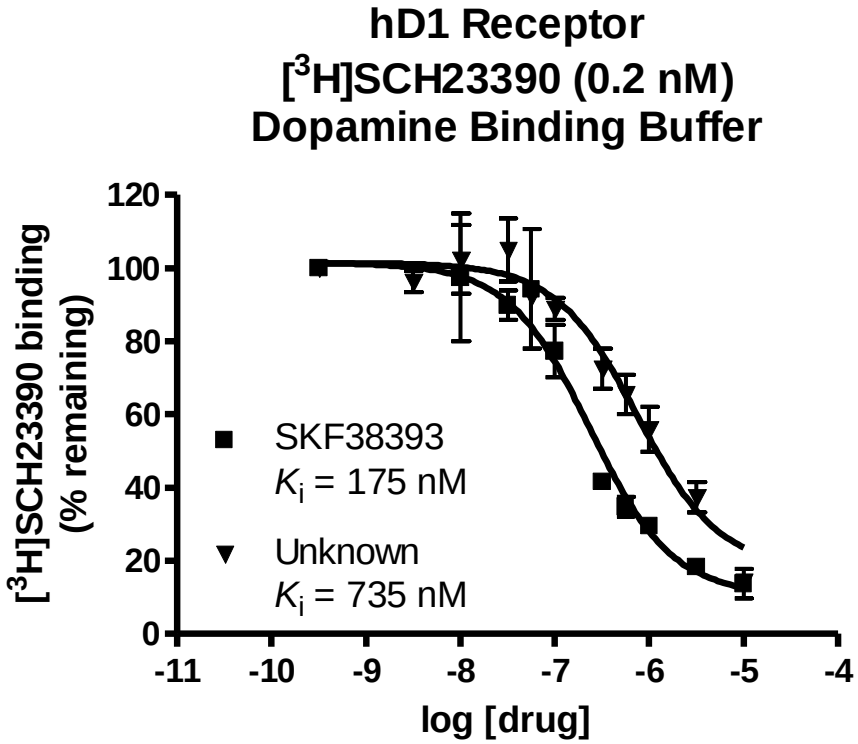
$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

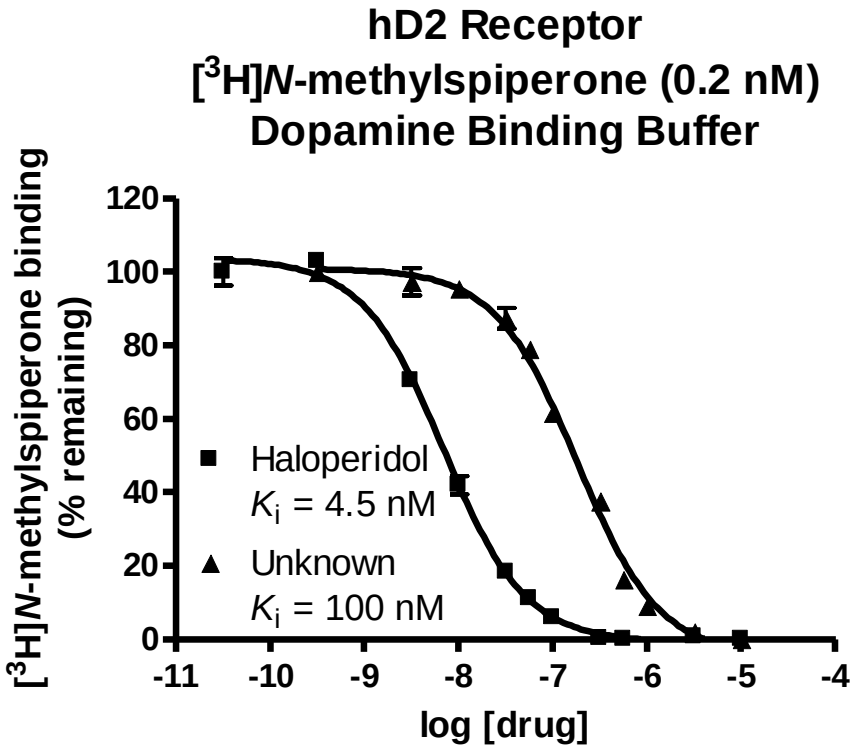
where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

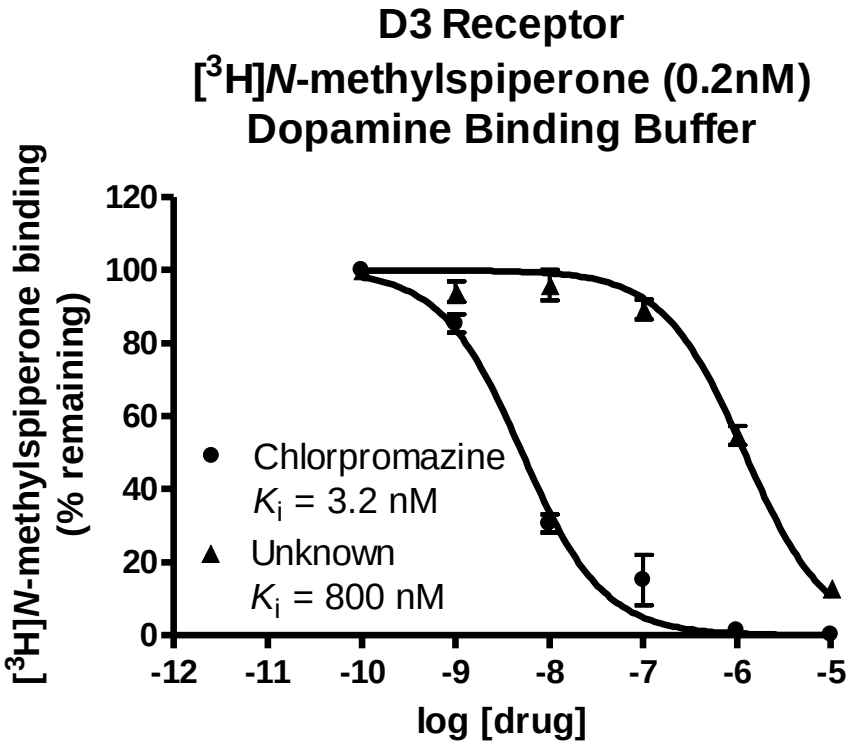
Receptor	Radioligand (Assay Conc.)	Reference Compound
D_1	[^{3}H]SCH23390 (0.2 nM)	SKF38393
D_2	[^{3}H]*N*-methylspiperone (0.2 nM)	Haloperidol
D_3	[^{3}H] *N*-methylspiperone (0.2 nM)	Chlorpromazine
D_4	[^{3}H] *N*-methylspiperone (0.3 nM)	Chlorpromazine
D_5	[^{3}H]SCH23390 (0.2 nM)	SKF38393

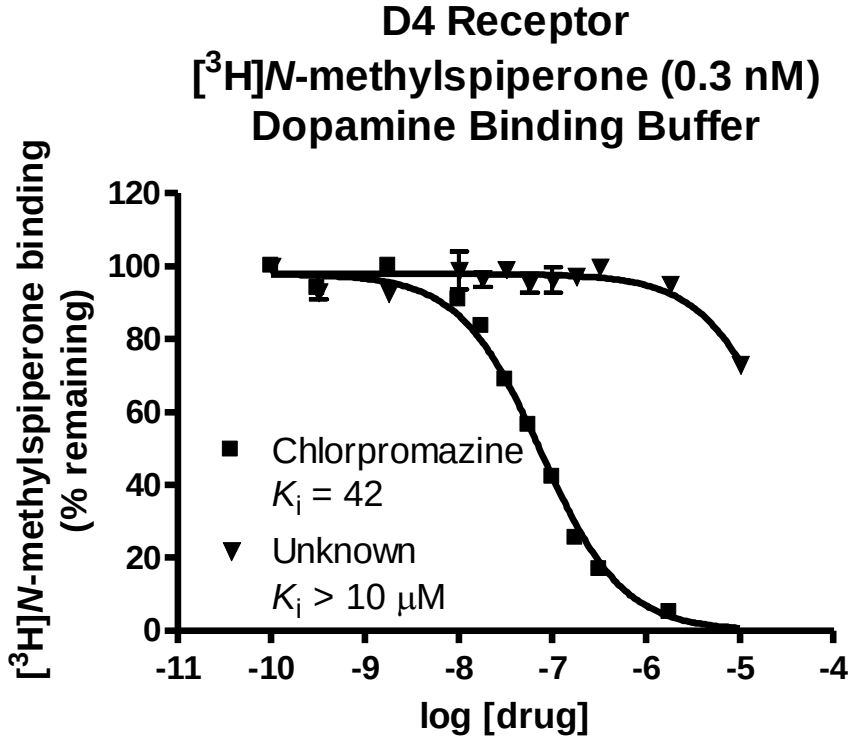
Table 3. Dopamine receptor radioligands, radioligand assay concentrations, and reference compounds.

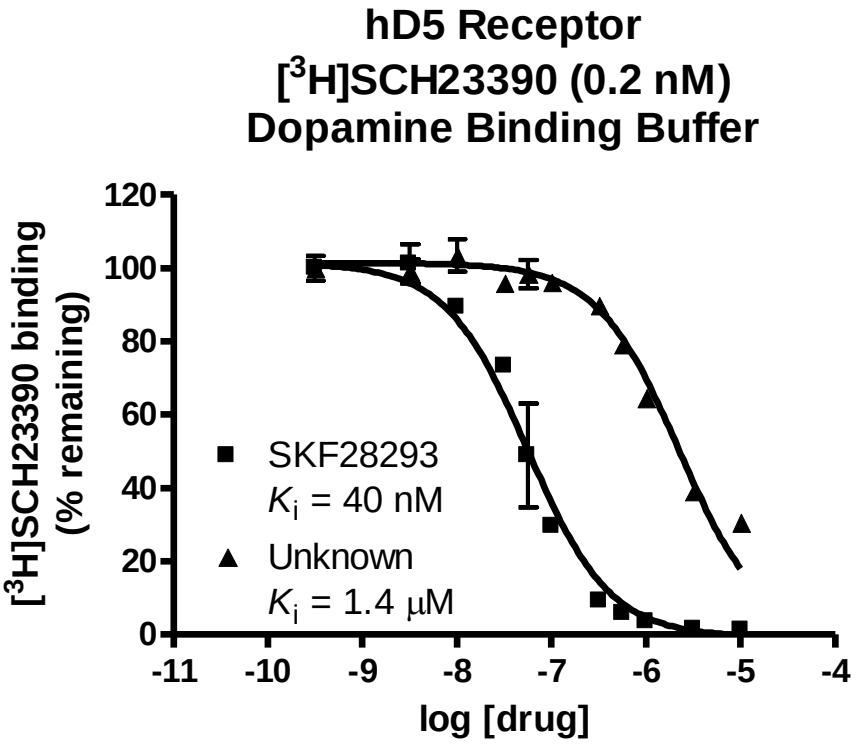
Figure 3. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the dopamine receptors.




hD2 Receptor
[3H]N-methylspiperone (0.2 nM)
Dopamine Binding Buffer
[3H]N-methylspiperone binding
(% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
Haloperidol
Ki = 4.5 nM
Unknown
Ki = 100 nM


D3 Receptor
[3H]N-methylspiperone (0.2nM)
Dopamine Binding Buffer
[3H]N-methylspiperone binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Chlorpromazine
Ki = 3.2 nM
Unknown
Ki = 800 nM


D4 Receptor
[3H]N-methylspiperone (0.3 nM)
Dopamine Binding Buffer
[3H]N-methylspiperone binding
(% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
Chlorpromazine
Ki = 42
Unknown
Ki > 10 μM


hD5 Receptor
[3H]SCH23390 (0.2 nM)
Dopamine Binding Buffer
[3H]SCH23390 binding
(% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
SKF28293
Ki = 40 nM
Unknown
Ki = 1.4 μM

Glutamate Receptors (Ionotropic): NMDA Receptor (MK801 site)

Note: NMDA receptor NMDA site and glycine site, and Kainate and AMPA receptor radioligand competition binding assays are currently under development.

Assay Buffer: 50 mM Tris-HCl, **pH 8.0**

Membrane Fraction Source: Rat brain homogenate

Protocol adapted from Kozikowski *et al*. *J Med Chem* 33(6):1561-1571 (1990).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in 50 mM Tris-HCl, pH 8.0 or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference (unlabeled MK801) compounds are prepared in 50 mM Tris-HCl by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand ([^{3}H]MK801) is diluted to 5 nM (five times the assay concentration) in 50 mM Tris-HCl, pH 8.0.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of 50 mM Tris-HCl, pH 8.0. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions (prepared from rat brain homogenate; see III for details) are resuspended in 3 ml of chilled 50 mM Tris-HCl, pH 8.0 and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-

well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed In Prism 4.0 (GraphPad Software) using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

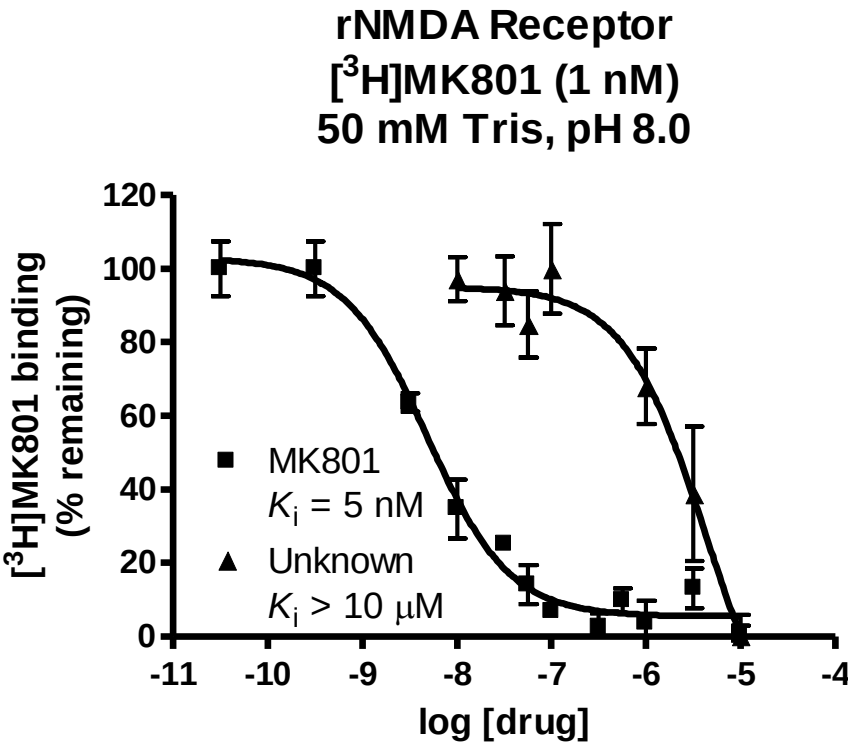
$$y = bottom + [(top-bottom)/(1 + 10^{x-logIC50})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

$$K_i = IC_{50}/(1 + [ligand]/K_D)$$

where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

Figure 4. Radioligand binding isotherms for competition binding assays wherein a reference compound and a PDSP investigator-submitted compound were assayed in parallel at the NMDA MK801 site.



Glutamate Receptors (metabotropic): mGluR5

Assay Buffer: Standard Binding Buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4)

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Kozikowski *et al*. *J Med Chem* 41(10):1641-1650 (1998).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in the Standard Binding Buffer or DMSO according to its solubility. A similar stock of reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference (MTEP) compounds are prepared in Standard Binding Buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand ([^{3}H]MPEP) is diluted to 4.5 nM (five times the assay concentration) in Standard Binding Buffer.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of Standard Binding Buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled Standard Binding Buffer and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1 hour, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid

scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 (GraphPad Software) using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

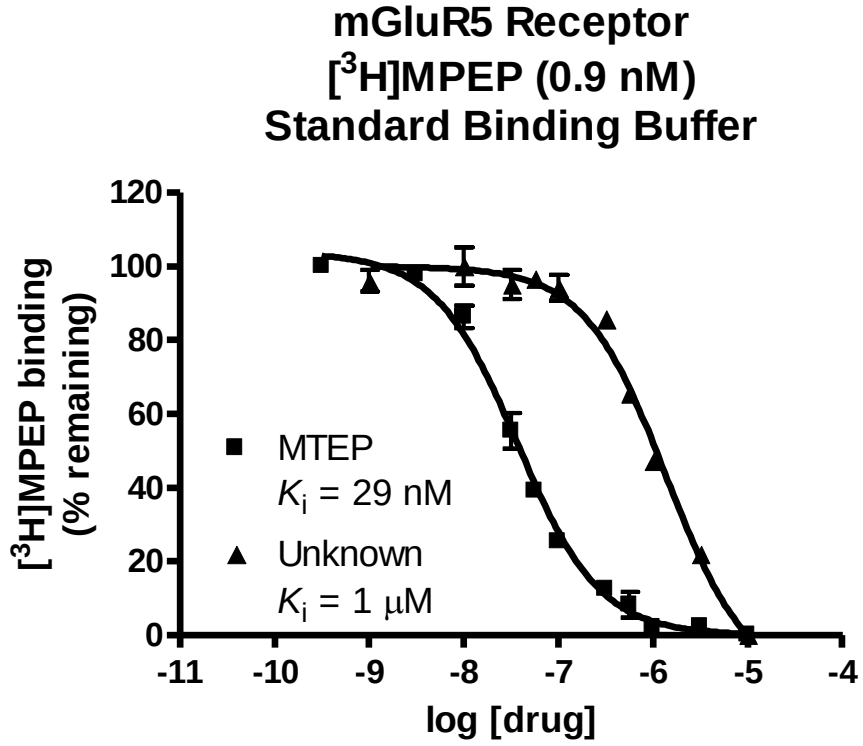
$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x\text{-log}IC50})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

Figure 5. Radioligand binding isotherms for competition binding assays wherein a reference compound and a PDSP investigator-submitted compound were assayed in parallel at mGluR5.



GABA Receptors: $GABA_A$ (rat brain muscimol site), PBR (rat kidney PK11195 site), recombinant α1β2γ2, α2β2γ2, α3β2γ2, α5β2γ2

Note: $GABA_B$ receptor radioligand competition binding assays are under development.

Assay Buffer: 50 mM Tris-**acetate**, pH 7.4 for $GABA_A$ and recombinant $GABA_A$; 50 mM Tris-HCl, pH 7.4 for PBR

Membrane Fraction Source: Rat brain homogenate ($GABA_A$ muscimol site and PBR PK11195 site); stably or transiently transfected cell lines (HEK293, COS, CHO, NIH3T3) for recombinant α1β2γ2, α2β2γ2, α3β2γ2, and α5β2γ2 receptors

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

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in the appropriate 50 mM Tris buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference (see Table 3) compounds are prepared in the appropriate 50 mM Tris buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand (see Table 3) is diluted to five times the assay concentration (see Table 3) in 50 mM Tris-acetate, pH 7.4. Typically, the assay concentration of radioligand is a value between one half the K_D and the K_D of a particular radioligand at its target.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of 50 mM Tris-acetate, pH 7.4. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions prepared from rat brain homogenate for GABAA muscimol site and PBR PK11195 site (see III for details) or, for recombinant α1β2γ3, α2β2γ2, α3β2γ2, and α5β2γ2 receptors, from transfected cells in 10-cm plates (by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-acetate, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled 50 mM Tris-acetate, pH 7.4 and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled 50 mM Tris-acetate to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 (GraphPad Software) using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x\text{-log}IC50})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

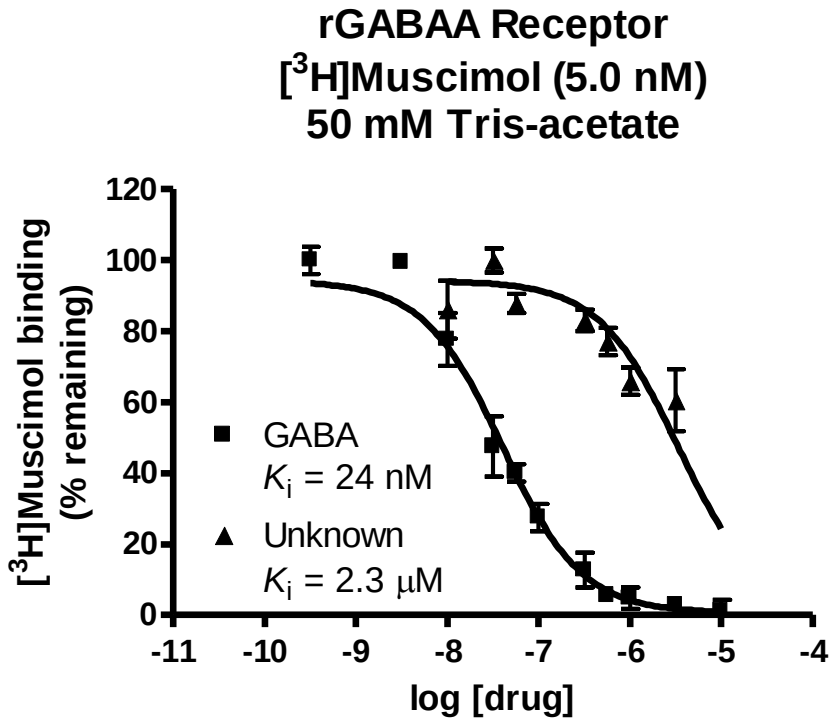
$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

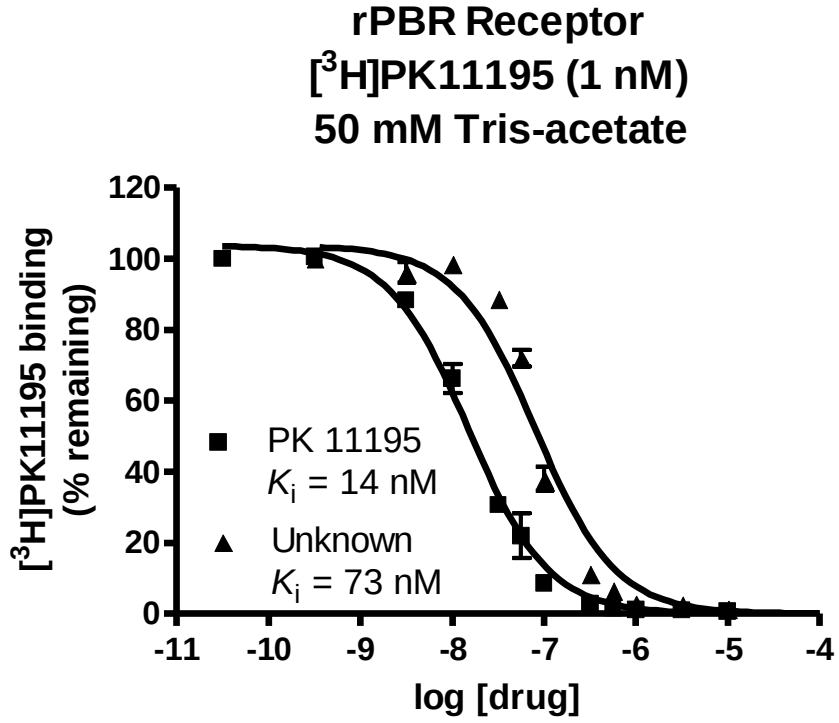
where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

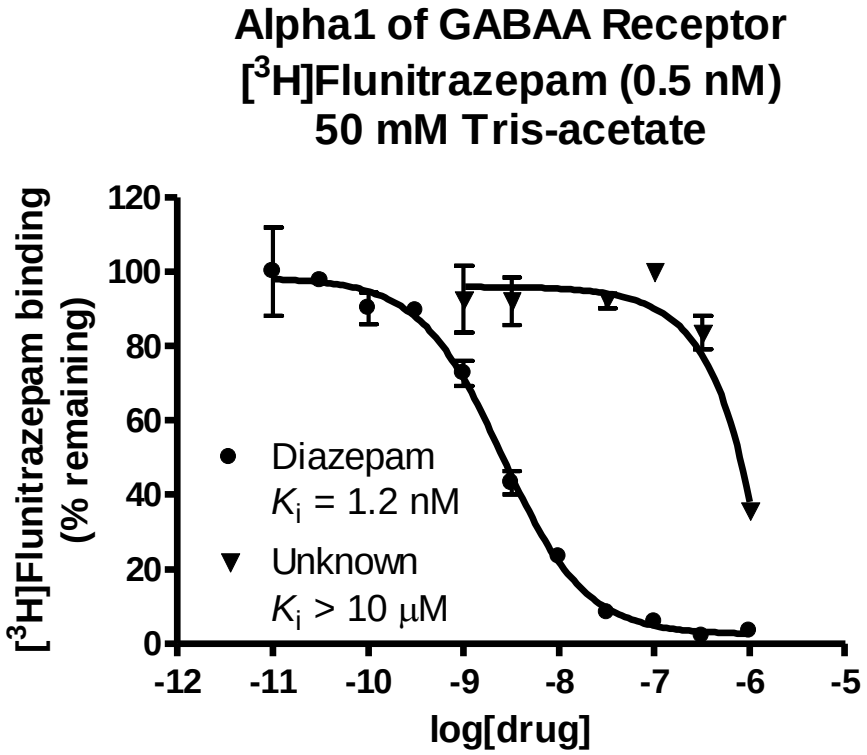
Receptor	Radioligand (Assay Conc.)	Reference Compound
$GABA_A$	[^{3}H]Muscimol (5 nM)	GABA
PBR	[^{3}H]PK11195 (1 nM)	PK11195
α1β2γ2	[^{3}H]Flunitrazepam (0.5 nM)	Diazepam
α2β2γ2	[^{3}H]Flunitrazepam (0.5 nM)	Diazepam
α3β2γ2	[^{3}H]Flunitrazepam (0.5 nM)	Diazepam
α5β2γ2	[^{3}H]Flunitrazepam (0.5 nM)	Diazepam

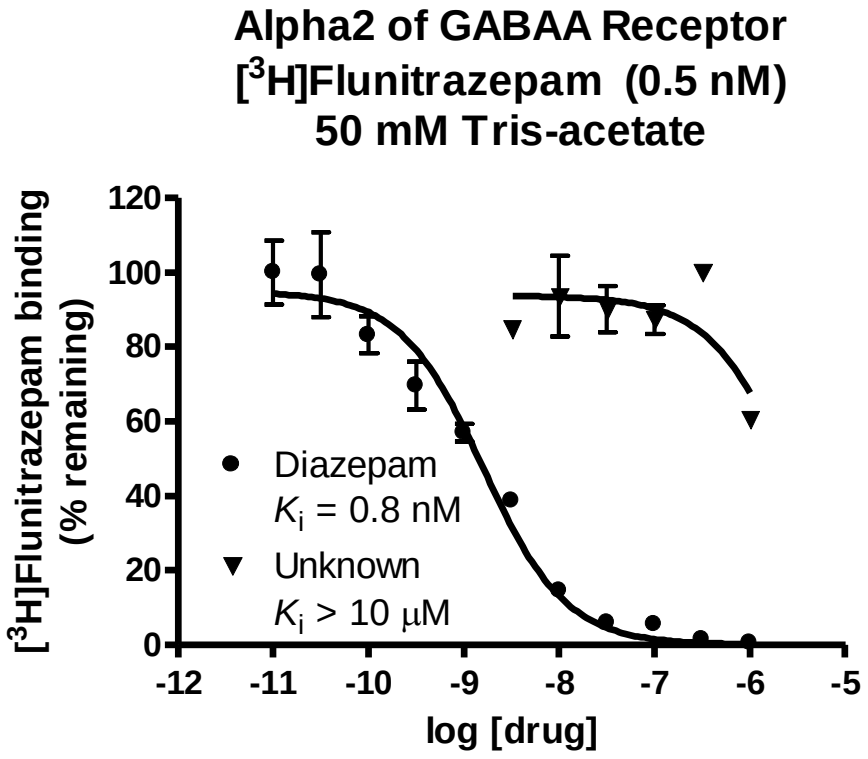
Table 4**. GABA receptor radioligands, radioligand assay concentrations, and reference compounds**

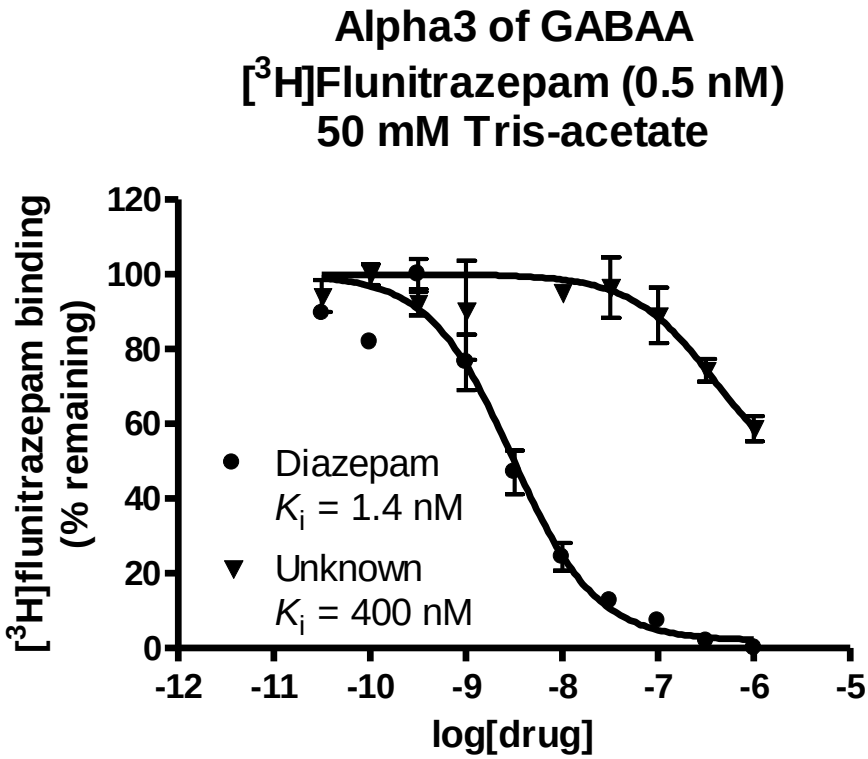
Figure 6. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the GABAA receptors available for screening.

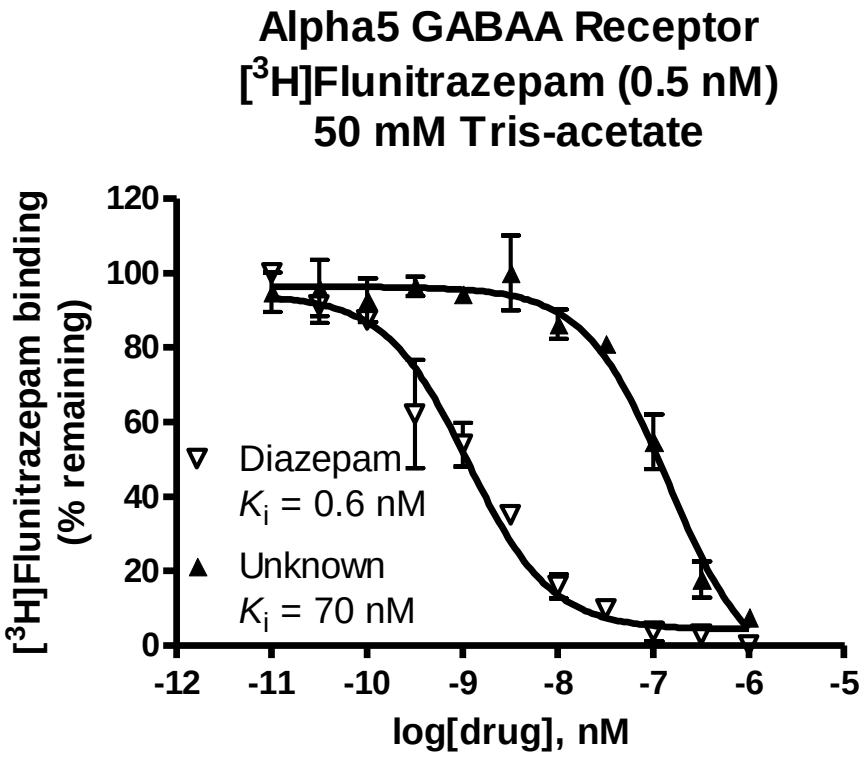



rPBR Receptor
[3H]PK11195 (1 nM)
50 mM Tris-acetate
[3H]PK11195 binding
(% remaining)
120
100
80
60
40
20
0
PK 11195
Ki = 14 nM
Unknown
Ki = 73 nM
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]


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

Biogenic Amine Transporters: SERT, NET, DAT

Note: Glutamate EAAC1, glycine GLYT2, GABA GAT1 and GAT3, VMAT1 and VMAT2 radioligand competition binding assays are under development.

Assay Buffer: Transporter Binding Buffer (150 mM NaCl, 5 mM KCl, 50 mM Tris-HCl, pH 7.4)

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Nakaki *et al*. *J Neurochem* 45(3):920-925 (1985).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in Transporter Binding Buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference (see Table 4) compounds are prepared in Transporter Binding Buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand (see Table 4) is diluted to five times the assay concentration (see Table 4) in Transporter Binding Buffer. Typically, the assay concentration of radioligand is a value between one half the K_D and the K_D of a particular radioligand at its target.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of Transporter Binding Buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled Transporter Binding Buffer and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Transporter Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x\text{-log}IC50})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

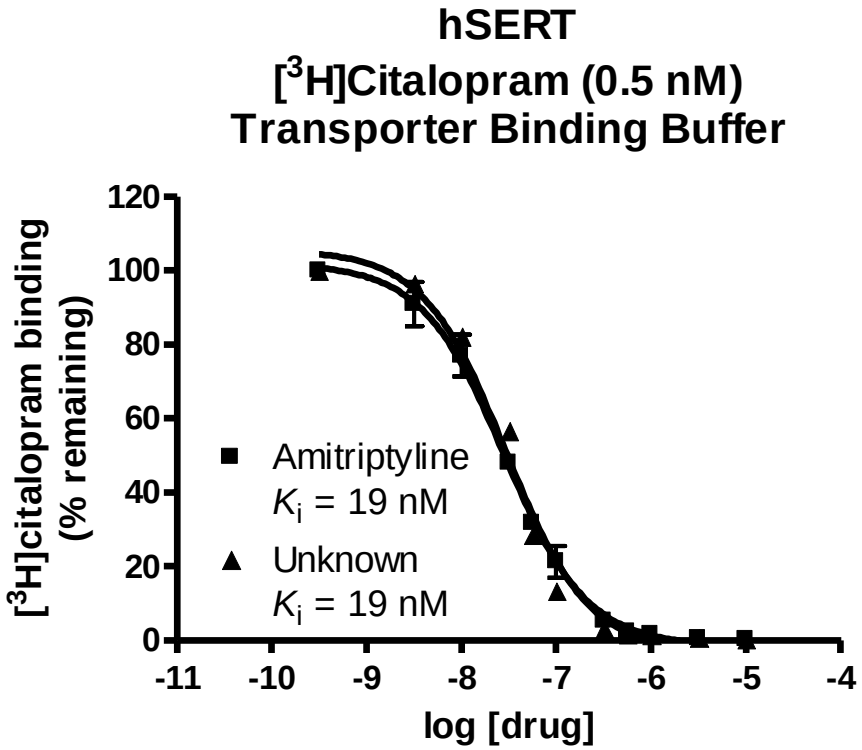
$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

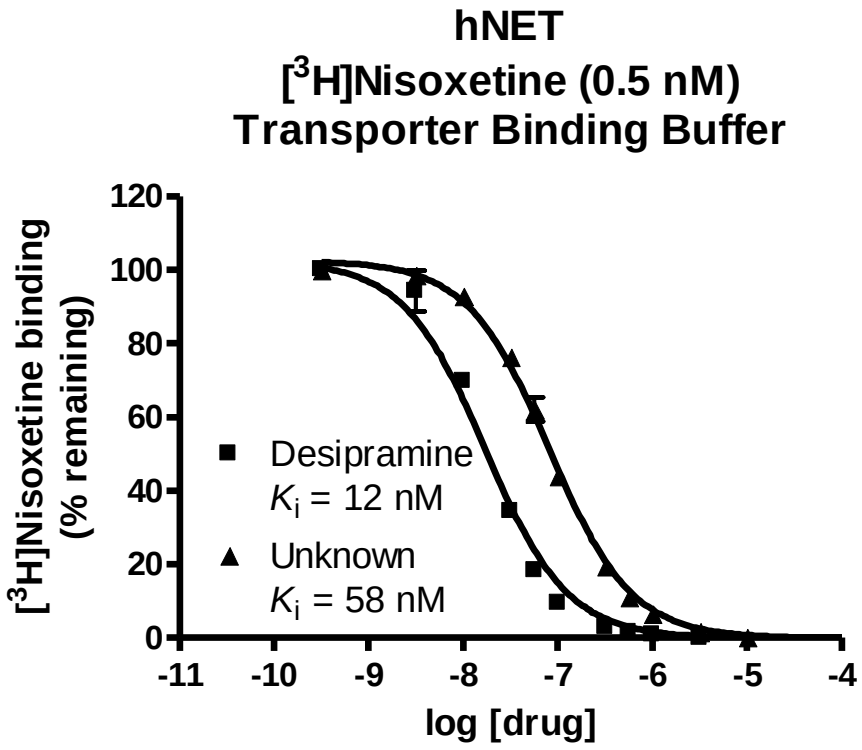
where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

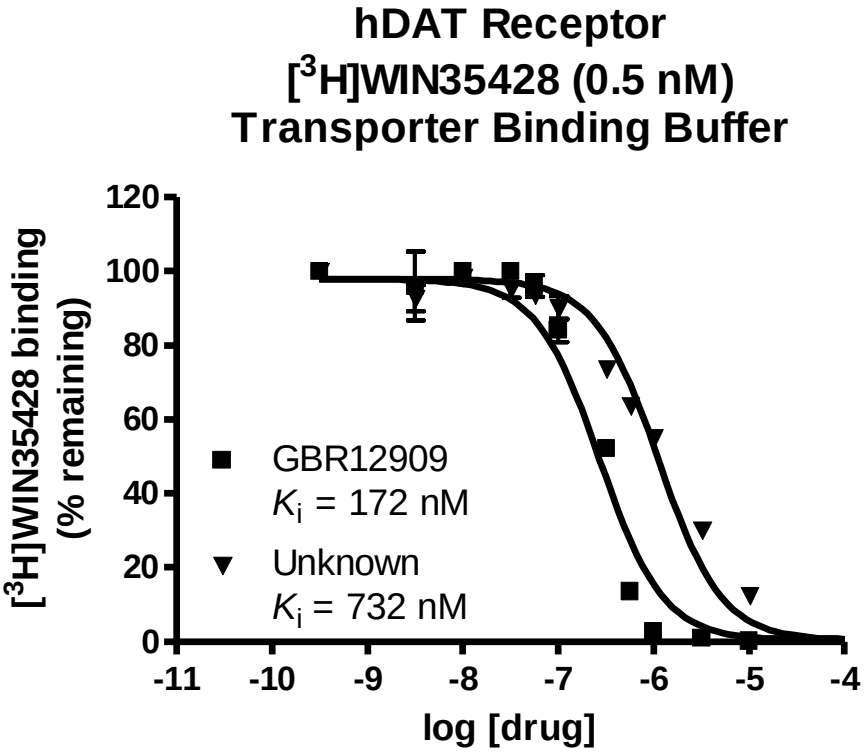
Transporter	Radioligand (Assay Conc.)	Reference Compound
SERT	[^{3}H]Citalopram (0.5 nM)	Amitriptyline
NET	[^{3}H]Nisoxetine (0.5 nM)	Desipramine
DAT	[^{3}H]WIN35428 (0.5 nM)	GBR12909

Table 5. Monoamine transporter radioligands, radioligand assay concentrations, and reference compounds.

Figure 7. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the biogenic amine transporters available for screening.




hNET
[3H]Nisoxetine (0.5 nM)
Transporter Binding Buffer
[3H]Nisoxetine binding
(% remaining)
120
100
80
60
40
20
0
Desipramine
Ki = 12 nM
Unknown
Ki = 58 nM
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]


hDAT Receptor
[3H]WIN35428 (0.5 nM)
Transporter Binding Buffer
[3H]WIN35428 binding
(% remaining)
120
100
80
60
40
20
0
GBR12909
Ki = 172 nM
Unknown
Ki = 732 nM
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]

Adrenergic Receptors: α_{1A}, α_{1B}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, β_3

Assay Buffers: For α_1 receptors, α_1 Binding Buffer (20 mM Tris-HCl, 145 mM NaCl, pH 7.4); for α_2 receptors, α_2 Binding Buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, **pH 7.7**); for β receptors, β Binding Buffer (50 mM Tris-HCl, 3 mM **$MnCl_2$**, **pH 7.7**)

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Arango *et al*. *Brain Res* 630(1-2):271-282 (1993), Arango *et al*. *Gen Psychiatry* 47 :1038-1047 (1990)

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in the appropriate buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference (see Table 5) compounds are prepared in the appropriate buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand (see Table 5) is diluted to five times the assay concentration (see Table 5) in the appropriate buffer. Typically, the assay concentration of radioligand is a value between one half the K_D and the K_D of a particular radioligand at its target.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of the appropriate buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled binding buffer and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1 hour (for α_1 receptors, 40 min for α_2 receptors, and 1.5 hours for β receptors), then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes

are performed with chilled distilled water (for α_1 and β receptors) or 0.1% polyethyleneimine (for α_2 receptors) to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x\text{-logIC50}})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

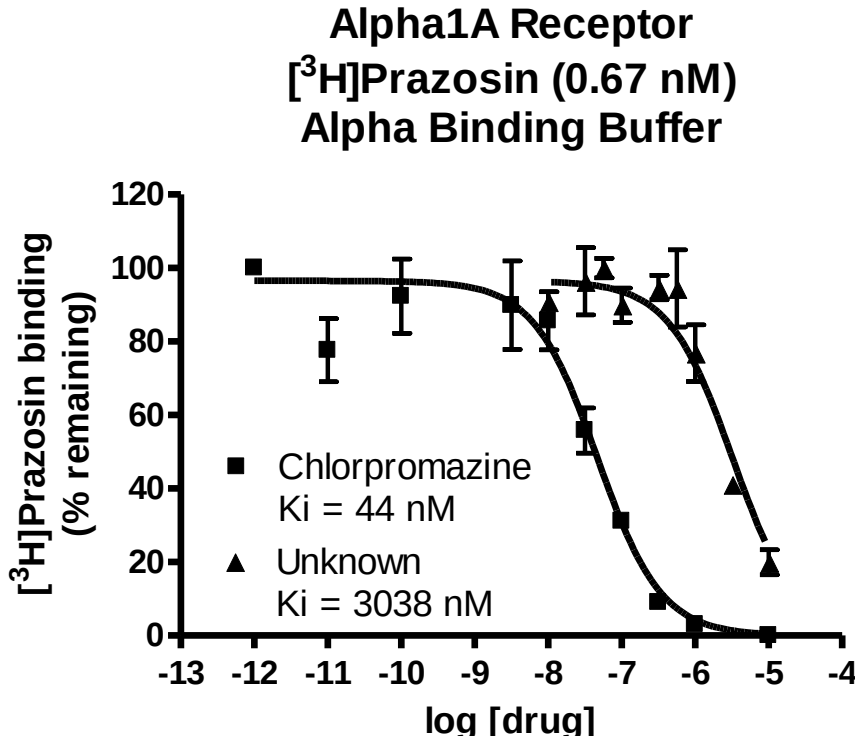
$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

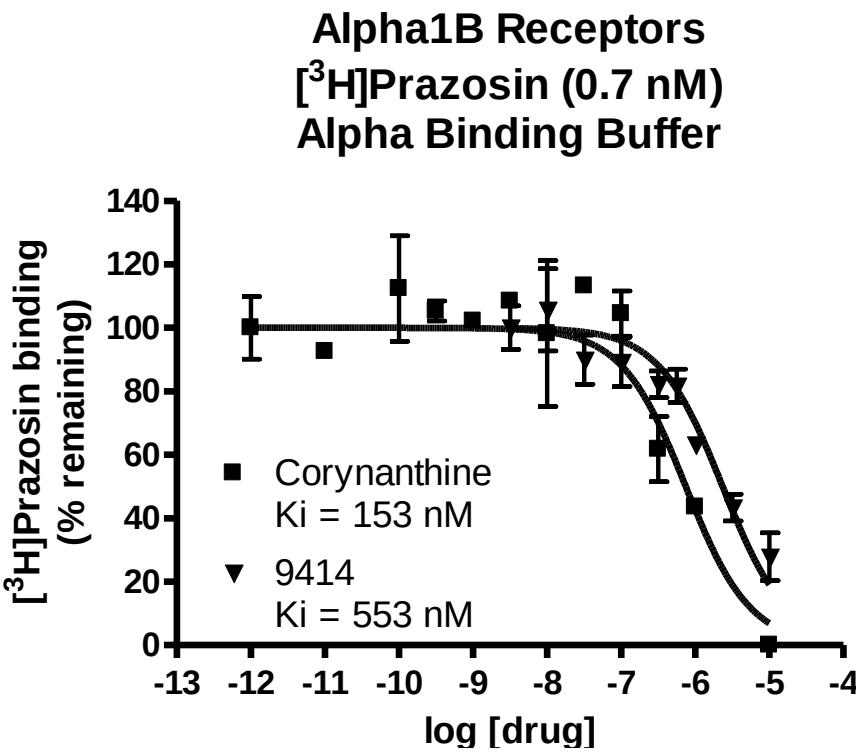
where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

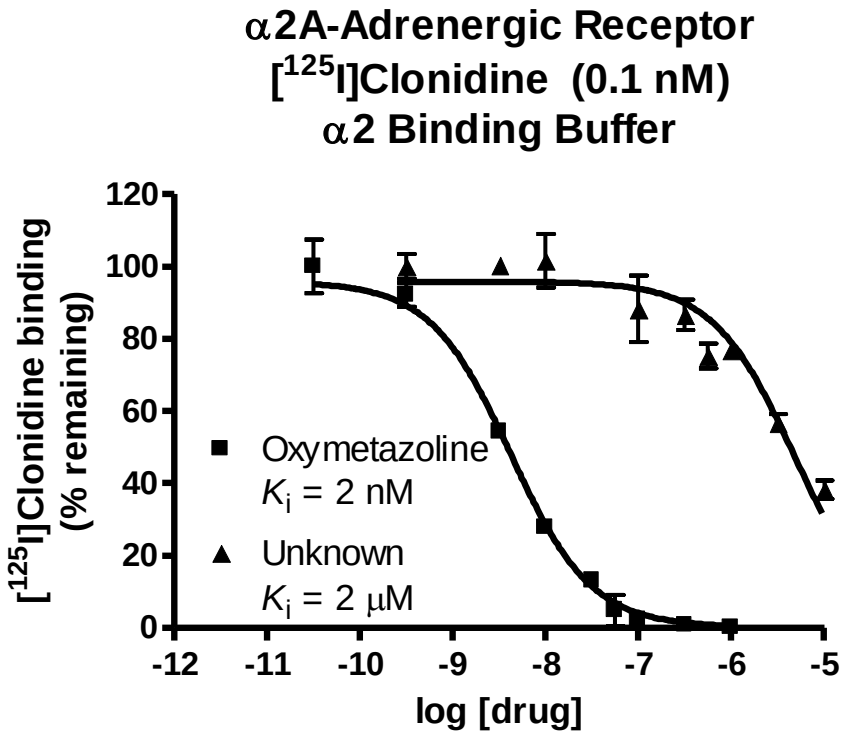
Receptor	**Radioligand (Assay Conc.)**	Reference Compound
α_{1A}	[^{125}I]HEAT (0.1 nM)	Urapidil
α_{1B}	[^{125}I]HEAT (0.1 nM)	Corynanthine
α_{2A}	[^{125}I]Iodoclonidine (0.1 nM)	Oxymetazoline
α_{2B}	[^{125}I]Iodoclonidine (1 nM)	Prazosin
α_{2C}	[^{125}I]Iodoclonidine (1 nM)	Prazosin
β_1	[^{125}I]Iodopindolol (0.1 nM)	Alprenolol
β_2	[^{125}I]Iodopindolol (0.1 nM)	Alprenolol
B_3	[^{125}I]Iodopindolol (0.1 nM)	Alprenolol

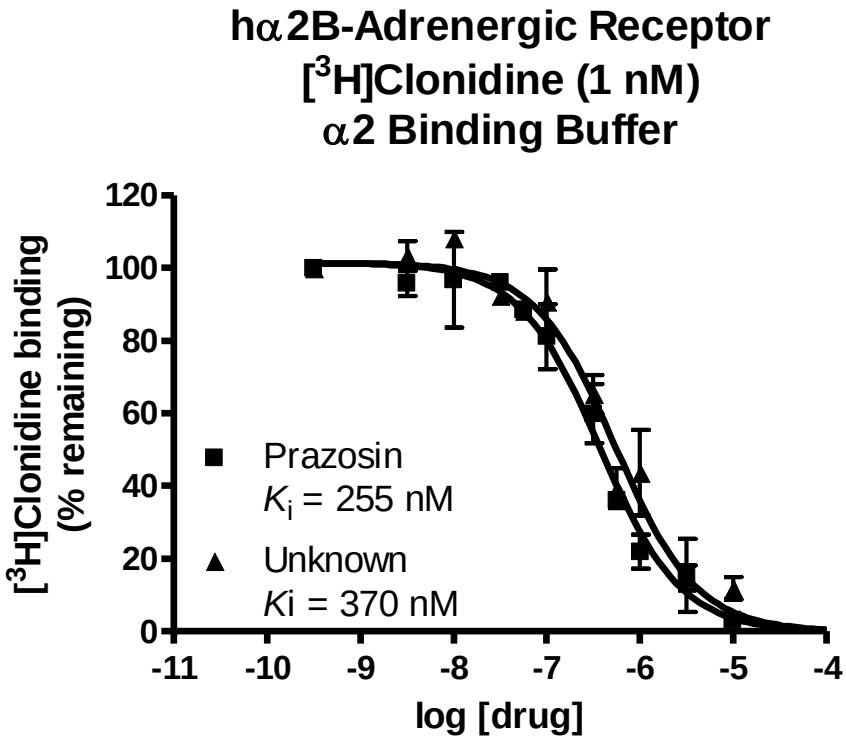
Table 6. **Adrenergic receptor radioligands, radioligand assay concentrations, and reference compounds.**

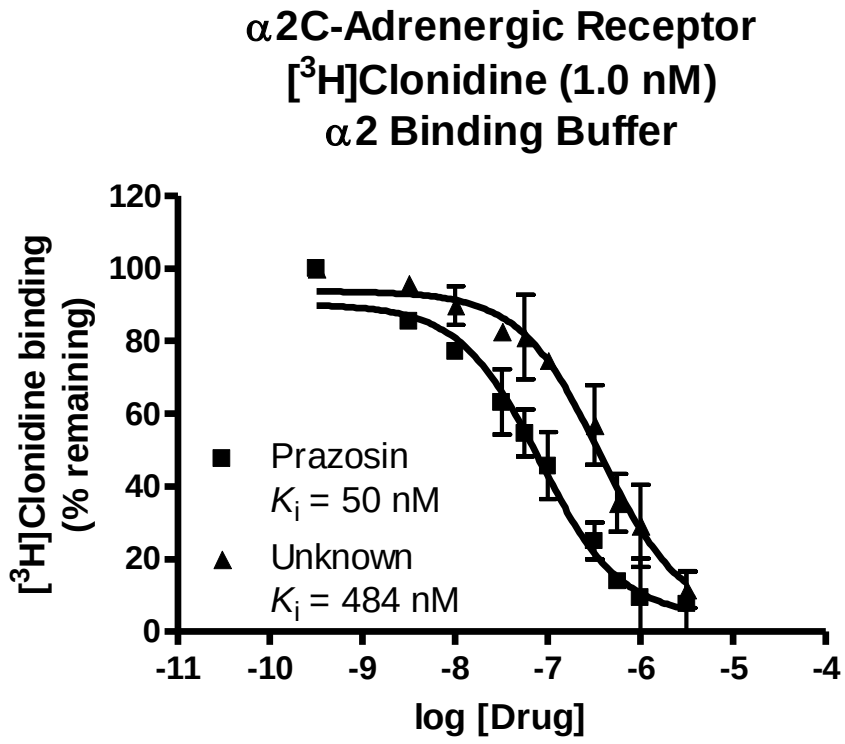
Figure 8. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the adrenergic receptors available for screening.

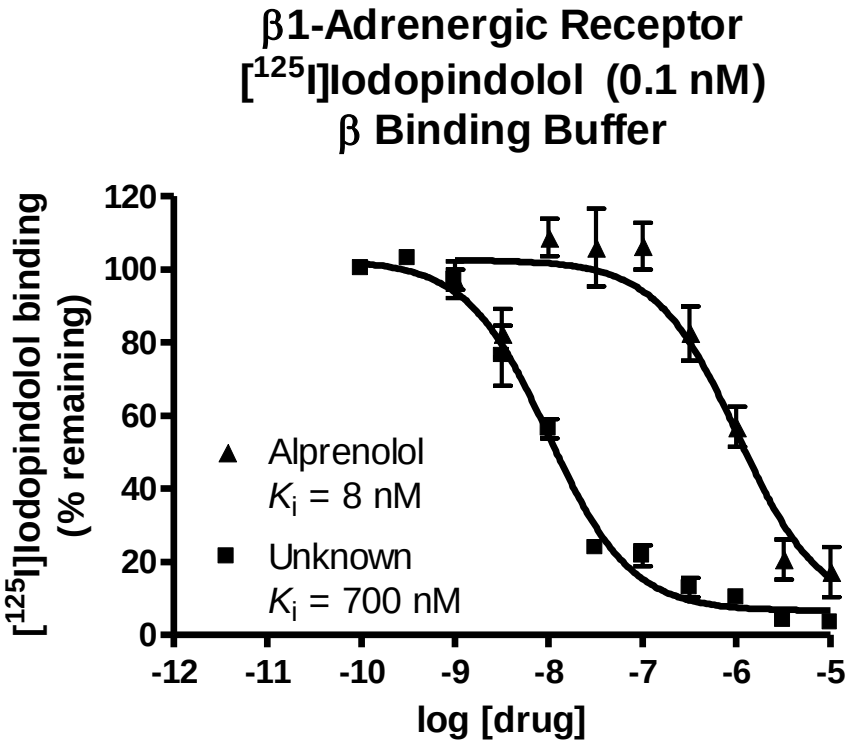


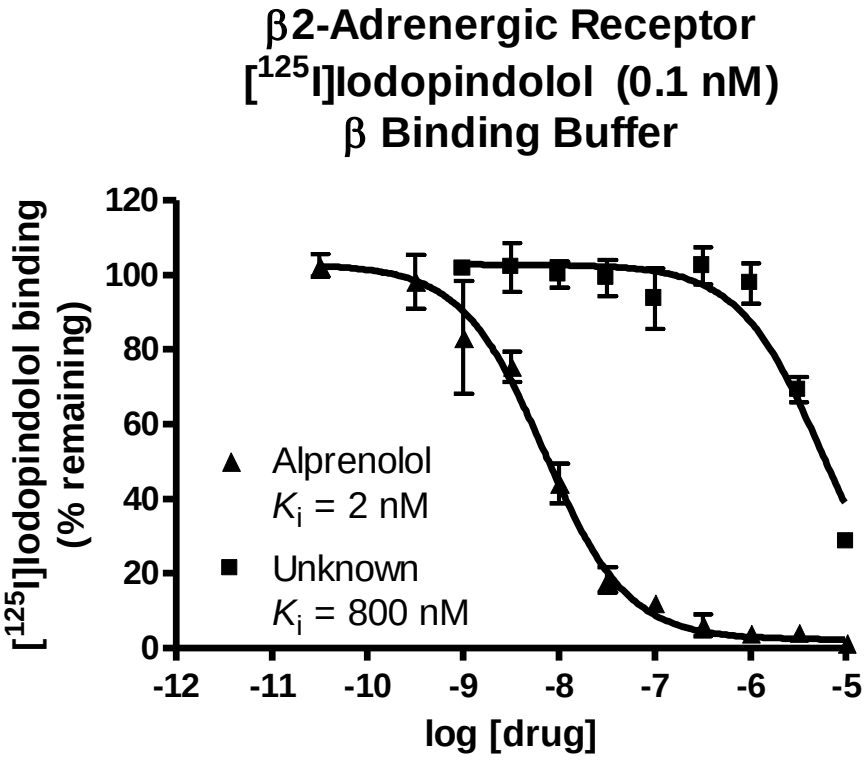


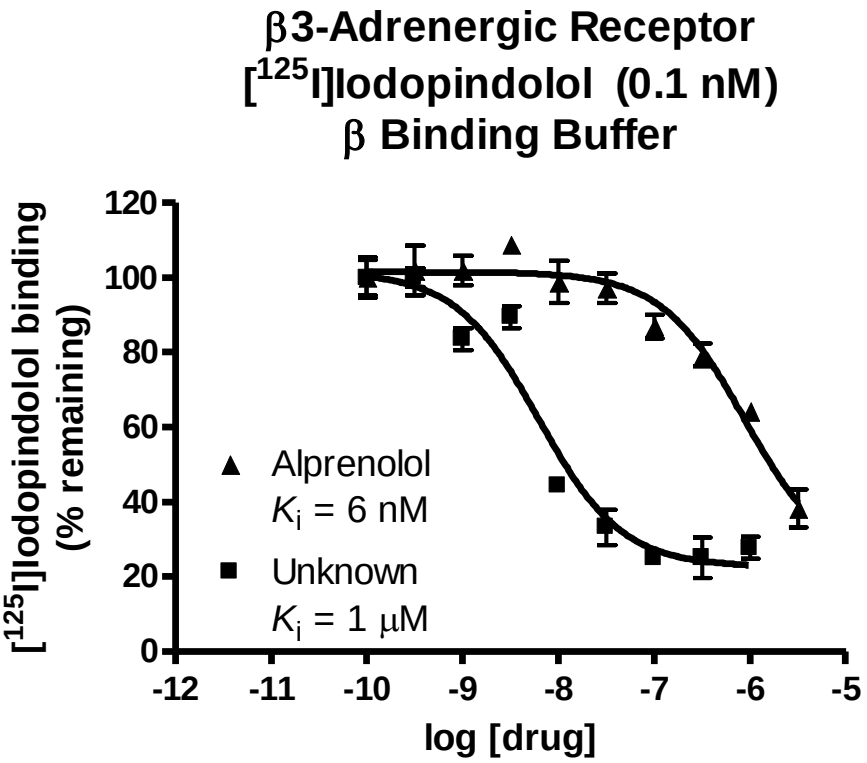

α2A-Adrenergic Receptor
[125I]Clonidine (0.1 nM)
α2 Binding Buffer
[125I]Clonidine binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Oxymetazoline
Ki = 2 nM
Unknown
Ki = 2 μM


hα2B-Adrenergic Receptor
[3H]Clonidine (1 nM)
α2 Binding Buffer
[3H]Clonidine binding
(% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
Prazosin
Ki = 255 nM
Unknown
Ki = 370 nM


α2C-Adrenergic Receptor
[3H]Clonidine (1.0 nM)
α2 Binding Buffer
[3H]Clonidine binding
(% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [Drug]
Prazosin
Ki = 50 nM
Unknown
Ki = 484 nM


β1-Adrenergic Receptor
[125I]Iodopindolol (0.1 nM)
β Binding Buffer
[125I]Iodopindolol binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Alprenolol
Ki = 8 nM
Unknown
Ki = 700 nM


β2-Adrenergic Receptor
[125I]Iodopindolol (0.1 nM)
β Binding Buffer
[125I]Iodopindolol binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Alprenolol
Ki = 2 nM
Unknown
Ki = 800 nM


β3-Adrenergic Receptor
[125I]Iodopindolol (0.1 nM)
β Binding Buffer
[125I]Iodopindolol binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Alprenolol
Ki = 6 nM
Unknown
Ki = 1 μM

Muscarinic Receptors: M_1, M_2, M_3, M_4, M_5

Assay Buffer: Muscarinic Binding Buffer (50 mM Tris-HCl, **pH 7.7**)

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Ernsberger *et al*. *Brain Res* 452(1-2):336-344.

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in Muscarinic Binding Buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference compound (atropine) are prepared in Muscarinic Binding Buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand ([^{3}H]QNB) is diluted to 2.5 nM (five times the assay concentration) in Muscarinic Binding Buffer. Typically, the assay concentration of radioligand is a value between one half the K_D and the K_D of a particular radioligand at its target.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of Muscarinic Binding Buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled Muscarinic Binding Buffer and homogenized by several passages through a 26 gauge needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid

scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 (GraphPad) using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

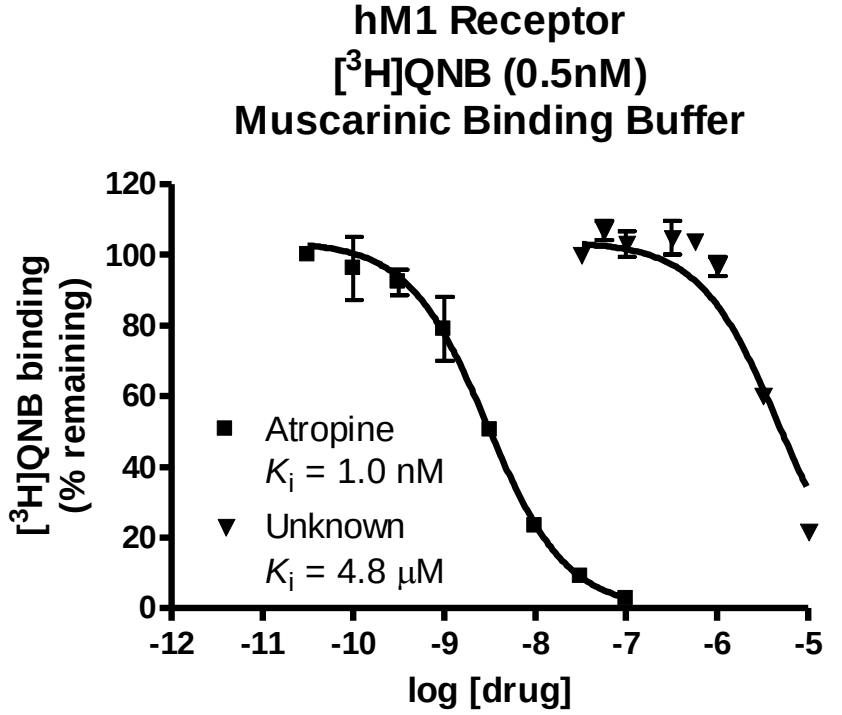
$$y = bottom + [(top-bottom)/(1 + 10^{x-logIC50})]$$

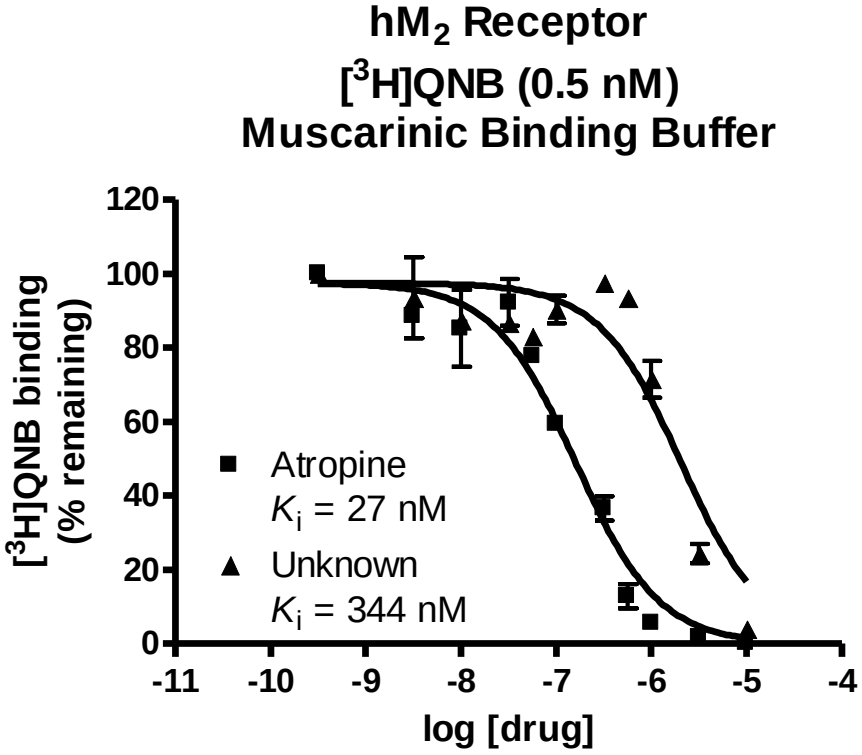
where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

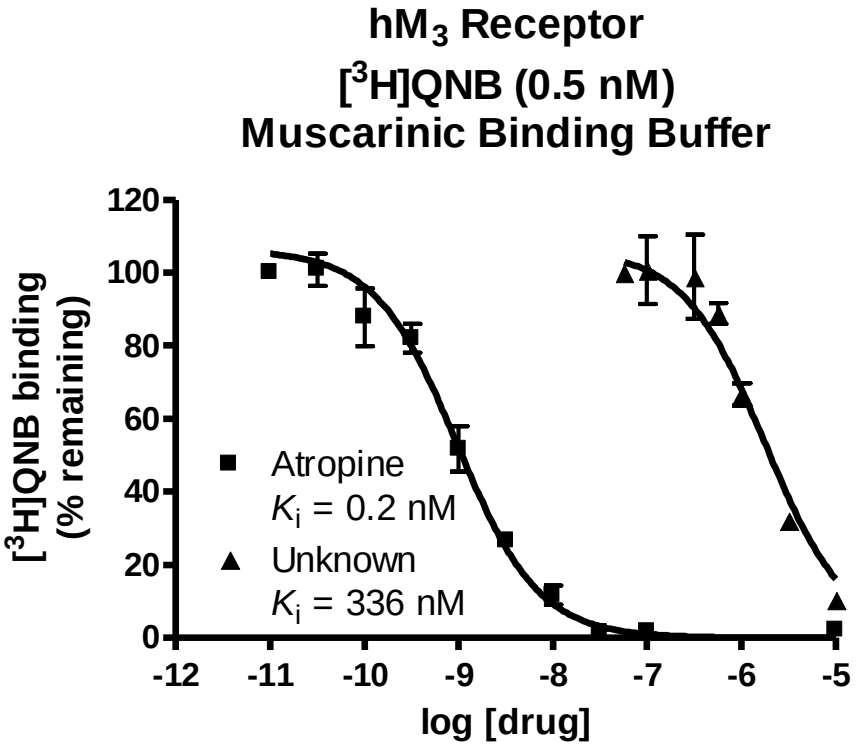
$$K_i = IC_{50}/(1 + [ligand]/K_D)$$

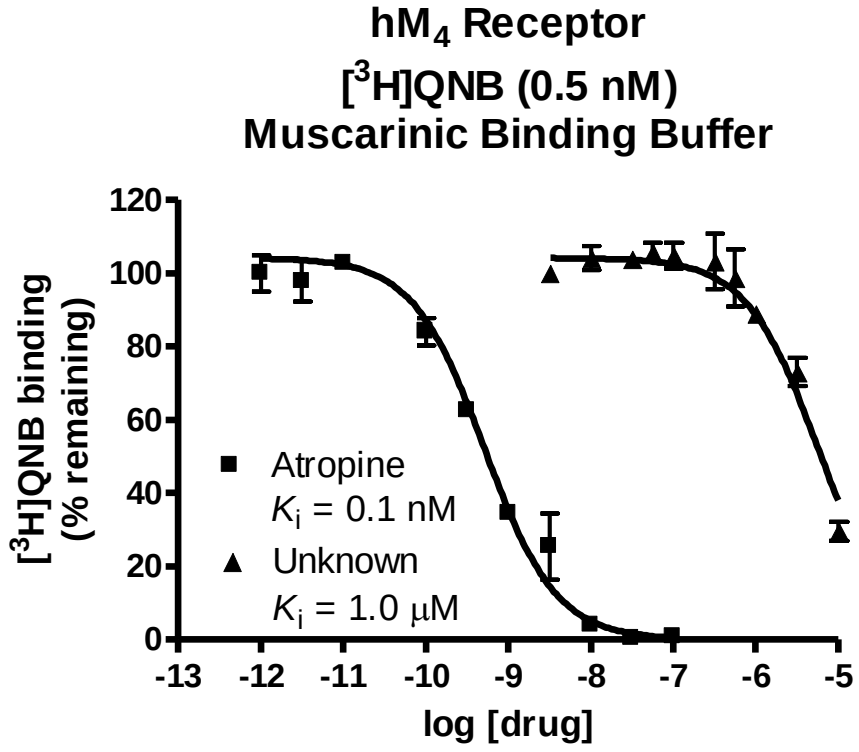
where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

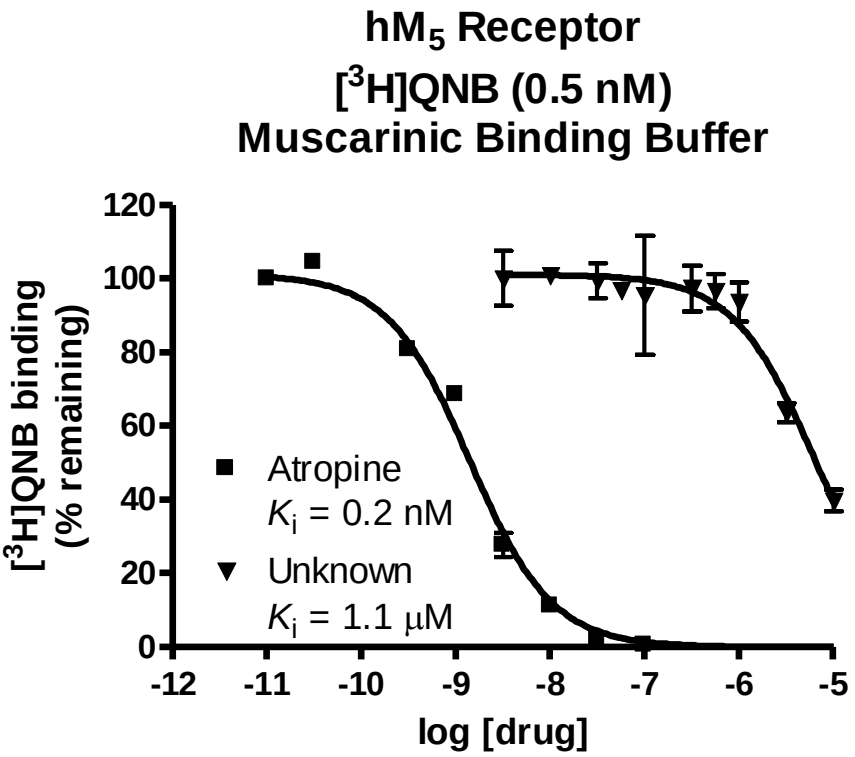
Figure 9. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the muscarinic receptors.






hM3 Receptor
[3H]QNB (0.5 nM)
Muscarinic Binding Buffer
[3H]QNB binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Atropine
Ki = 0.2 nM
Unknown
Ki = 336 nM


hM4 Receptor
[3H]QNB (0.5 nM)
Muscarinic Binding Buffer
[3H]QNB binding
(% remaining)
120
100
80
60
40
20
0
-13
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Atropine
Ki = 0.1 nM
Unknown
Ki = 1.0 μM


hM5 Receptor
[3H]QNB (0.5 nM)
Muscarinic Binding Buffer
[3H]QNB binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Atropine
Ki = 0.2 nM
Unknown
Ki = 1.1 μM

Nicotinic Receptors: α2β2, α2β4, α3β2, α3β4, α4β2, α4β4, enodegnous α4β2

Assay Buffer: 50 mM Tris-HCl, pH 7.4

Membrane Fraction Source: Stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3) (α2β2, α2β4, α3β2, α3β4, α4β2, α4β4) or rat forebrain (α4β2)

Protocol adapted from Xiao *et al*. *Mol Pharm* 54(2):322-333 (1998).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in assay buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference compound [(-)-nicotine] are prepared in assay buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand ([^{3}H]epibatidine) is diluted to 2.5 nM (five times the assay concentration) in assay buffer.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of assay buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled assay buffer and homogenized by several passages through a 26 gauge needle, then 50 µl are dispensed into each well. (See III for details of crude membrane fraction preparation from rat forebrain for endogenous α4β2 assays.)

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 4 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.5% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 (GraphPad) using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

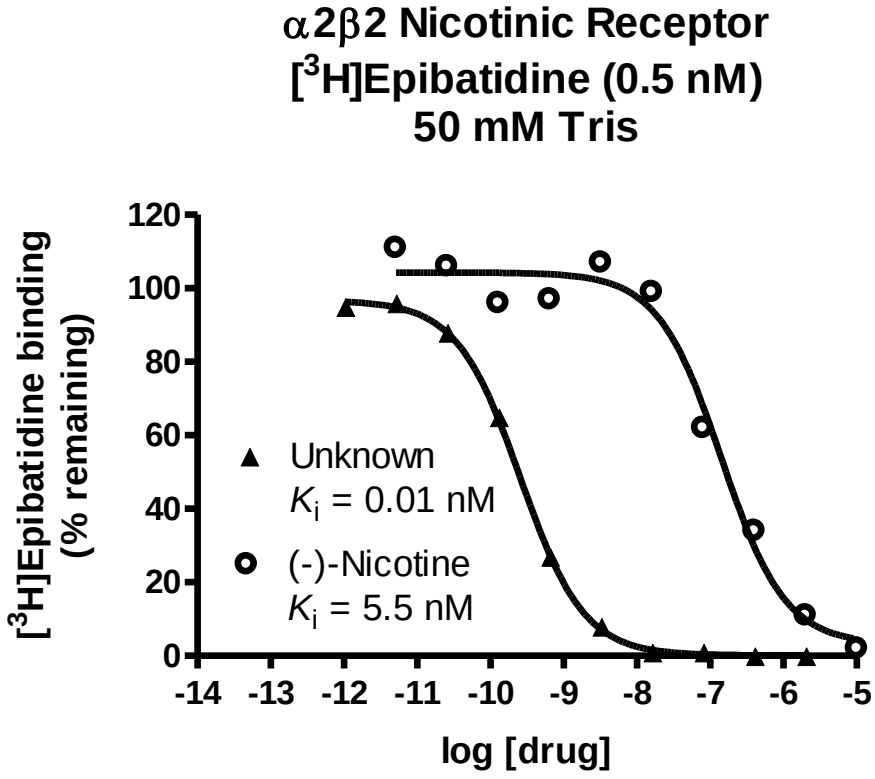
$$y = bottom + [(top-bottom)/(1 + 10^{x-logIC50})]$$

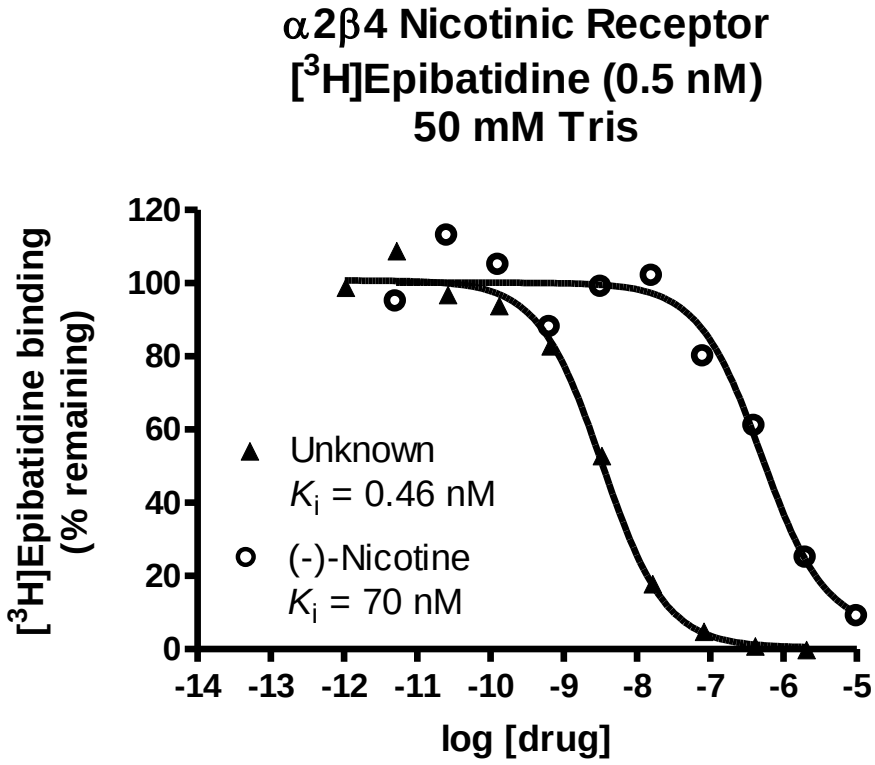
where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

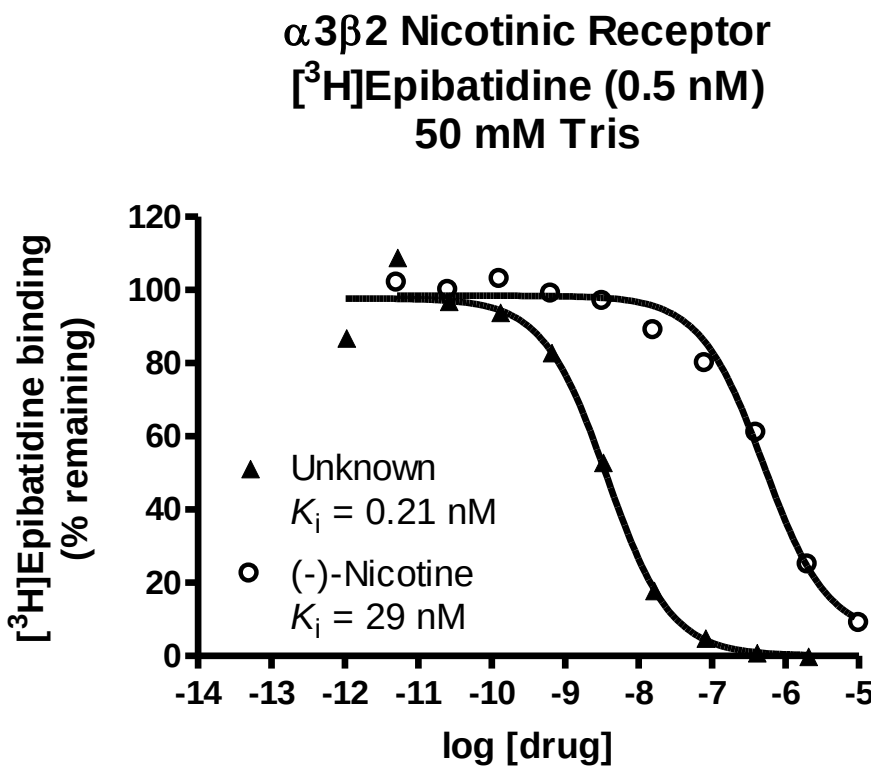
$$K_i = IC_{50}/(1 + [ligand]/K_D)$$

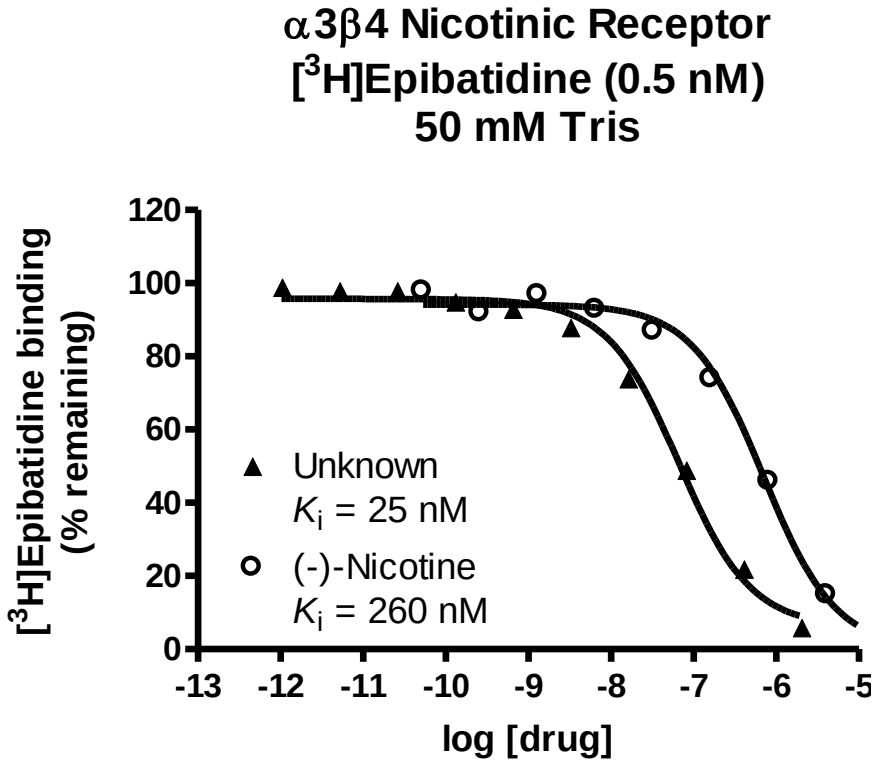
where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

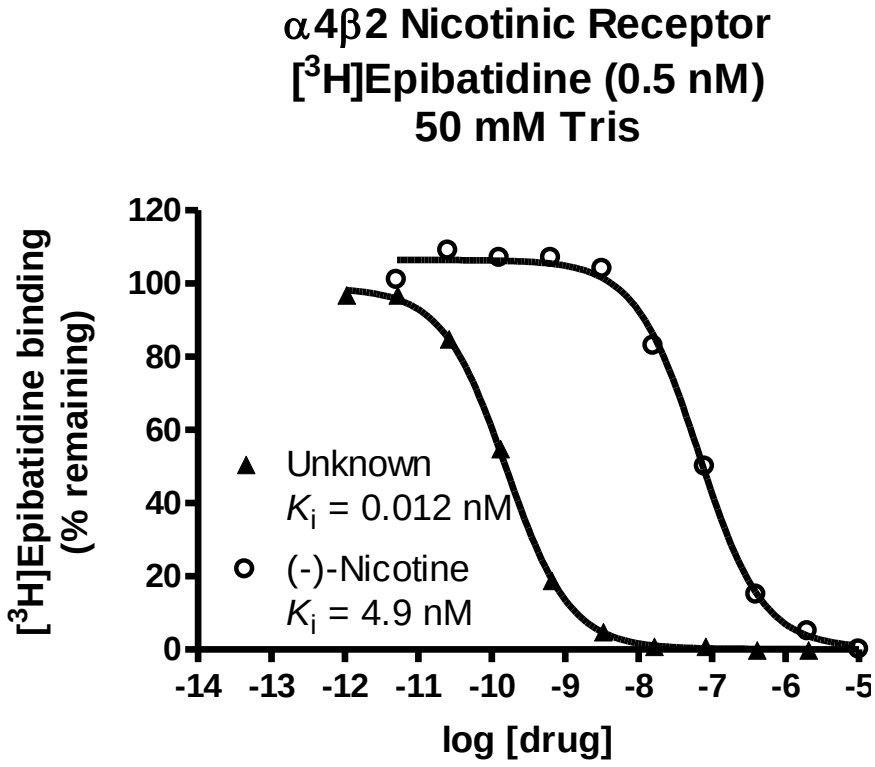
Figure 10. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the nicotinic receptors.

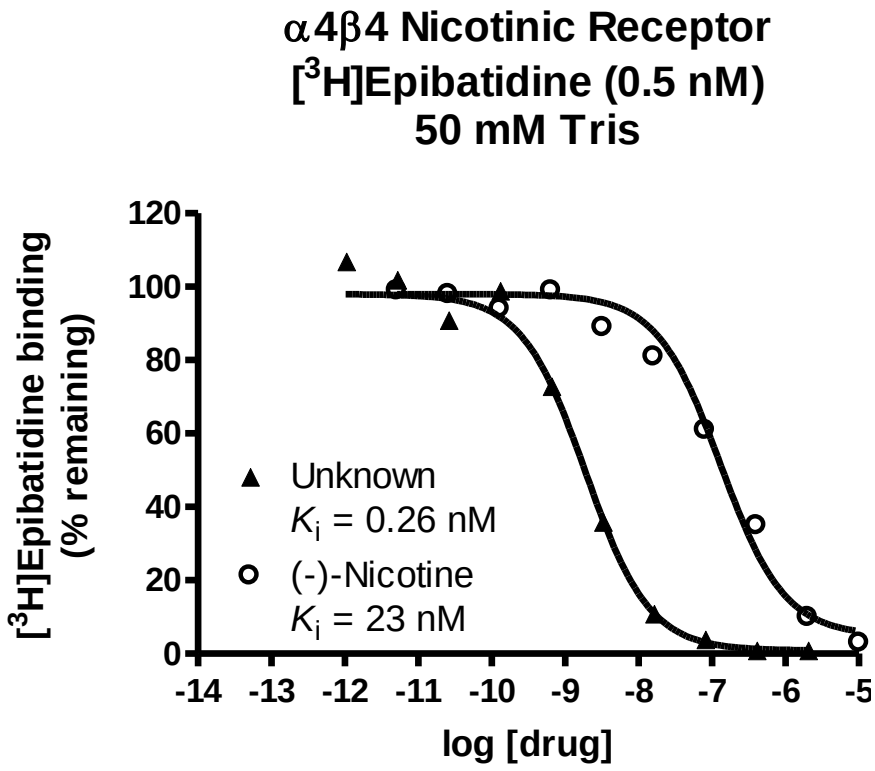


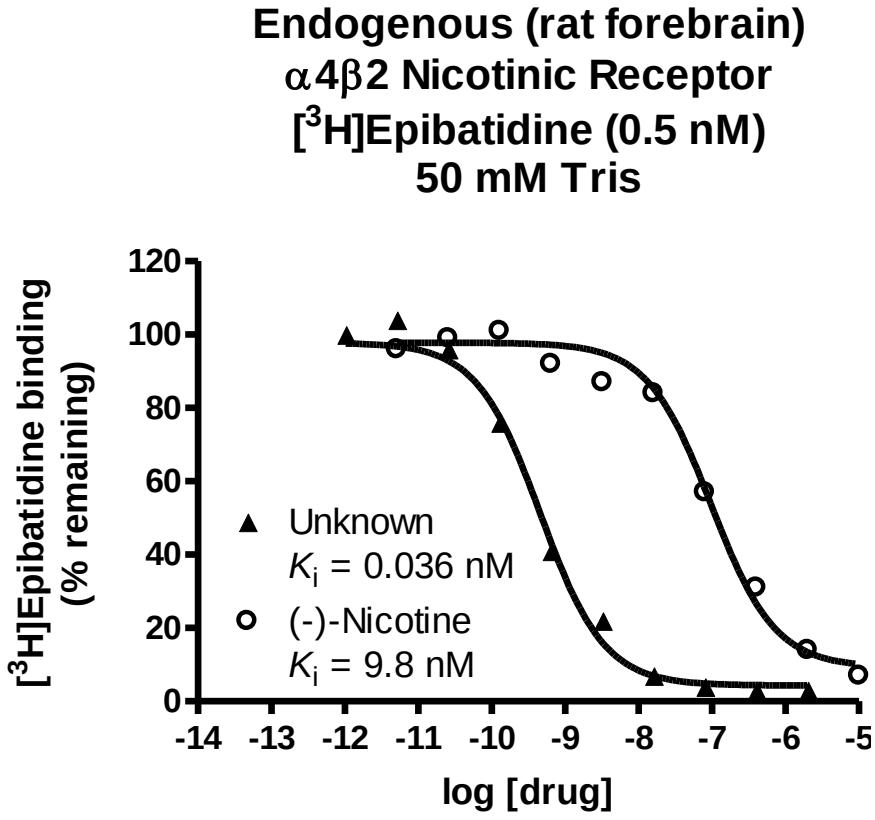

α2β4 Nicotinic Receptor
[3H]Epibatidine (0.5 nM)
50 mM Tris
[3H]Epibatidine binding
(% remaining)
120
100
80
60
40
20
0
Unknown
Ki = 0.46 nM
(-)-Nicotine
Ki = 70 nM
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5
log [drug]


α3β2 Nicotinic Receptor
[3H]Epibatidine (0.5 nM)
50 mM Tris
[3H]Epibatidine binding
(% remaining)
120
100
80
60
40
20
0
Unknown
Ki = 0.21 nM
(-)-Nicotine
Ki = 29 nM
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5
log [drug]


α3β4 Nicotinic Receptor
[3H]Epibatidine (0.5 nM)
50 mM Tris
[3H]Epibatidine binding
(% remaining)
120
100
80
60
40
20
0
-13
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Unknown
Ki = 25 nM
(-)-Nicotine
Ki = 260 nM


α4β2 Nicotinic Receptor
[3H]Epibatidine (0.5 nM)
50 mM Tris
[3H]Epibatidine binding
(% remaining)
120
100
80
60
40
20
0
-14
-13
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Unknown
Ki = 0.012 nM
(-)-Nicotine
Ki = 4.9 nM


α4β4 Nicotinic Receptor
[3H]Epibatidine (0.5 nM)
50 mM Tris
[3H]Epibatidine binding
(% remaining)
120
100
80
60
40
20
0
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5
log [drug]
Unknown
Ki = 0.26 nM
(-)-Nicotine
Ki = 23 nM


Endogenous (rat forebrain)
α4β2 Nicotinic Receptor
[3H]Epibatidine (0.5 nM)
50 mM Tris
[3H]Epibatidine binding
(% remaining)
120
100
80
60
40
20
0
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5
log [drug]
Unknown
Ki = 0.036 nM
(-)-Nicotine
Ki = 9.8 nM

Opioid Receptors: MOR, KOR, DOR

Assay Buffer: Standard Binding Buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4)

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Roth *et al*. *J Biol Chem* 256(19):10017-10023 (1981).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in Standard Binding Buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference (see Table 6) compounds are prepared in Standard Binding Buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand (see Table 6) is diluted to five times the assay concentration (see Table 6) in Standard Binding Buffer. Typically, the assay concentration of radioligand is a value between one half the K_D and the K_D of a particular radioligand at its target.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of Standard Binding Buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled Standard Binding Buffer and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight.

The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

$$y = bottom + [(top-bottom)/(1 + 10^{x-logIC50})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

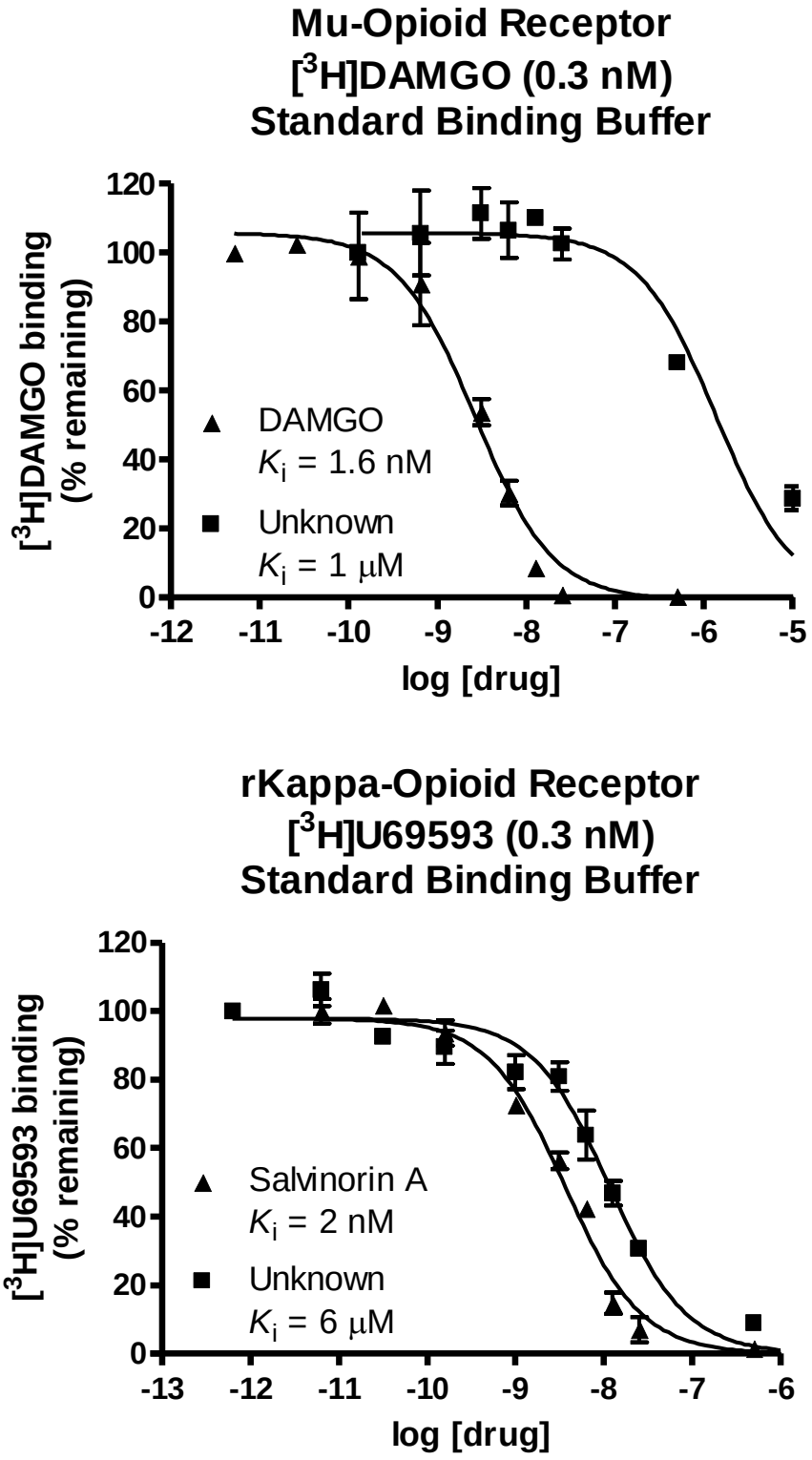
$$K_i = IC_{50}/(1 + [ligand]/K_D)$$

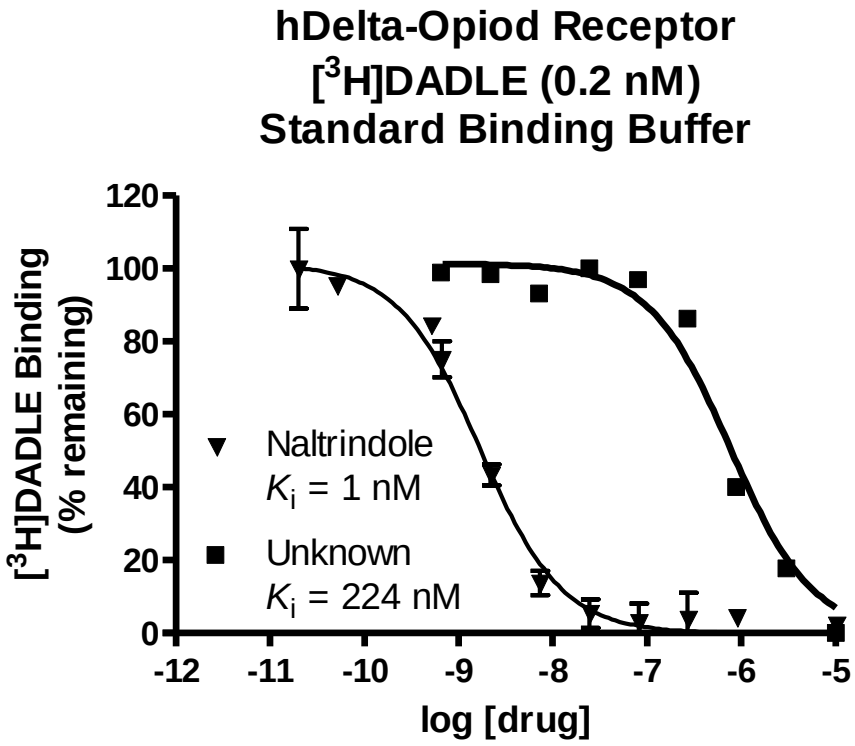
where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

Receptor	**Radioligand (Assay Conc.)**	Reference Compound
MOR	[^{3}H]DAMGO (0.3 nM)	DAMGO
KOR	[^{3}H]U69593 (0.3 nM)	Salvinorin A
DOR	[^{3}H]DADLE (0.2 nM)	Naltrindole

Table 7. **Opioid receptor radioligands, radioligand assay concentrations, and reference compounds.**

Figure 11. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the opioid receptors.




hDelta-Opiod Receptor
[3H]DADLE (0.2 nM)
Standard Binding Buffer
[3H]DADLE Binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
log [drug]
Naltrindole
Ki = 1 nM
Unknown
Ki = 224 nM

Sigma Receptors: Sigma1, Sigma2

Assay Buffer: Sigma Binding Buffer (50 mM Tris-HCl, **pH 8.0**)

Membrane Fraction Source: Rat brain homogenate (Sigma1), PC12 cells (Sigma2)

Protocol adapted from Kovacs and Larson. *Eur J Pharmacol* 350(1):47-52 (1998).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in Sigma Binding Buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference (see Table 7) compounds are prepared in Sigma Binding Buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand (see Table 7) is diluted to five times the assay concentration (see Table 7) in Sigma Binding Buffer. Typically, the assay concentration of radioligand is a value between one half the K_D and the K_D of a particular radioligand at its target.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of Sigma Binding Buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions prepared from rat brain homogenate for Sigma1 receptors (see III for details) or, for Sigma2 receptors, from PC12 cells in 10-cm plates (by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled 50 mM Tris-HCl, pH 8.0 and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at 37 degrees (for Sigma1 receptors) or room temperature (for Sigma2 receptors) and shielded from light (to prevent photolysis of light-sensitive ligands) for 2.5 hours (for Sigma 1 receptors) or 2 hours (for Sigma2 receptors), then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel

harverster. Four rapid 500-µl washes are performed with chilled 10 mM Tris-HCl, pH 8.0 to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x\text{-log}IC50})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

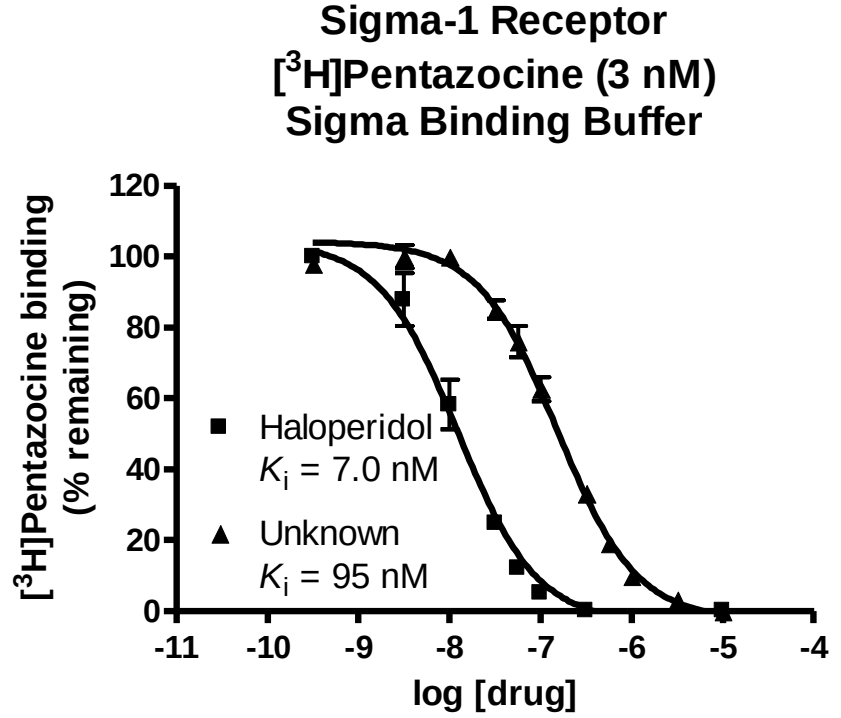
$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

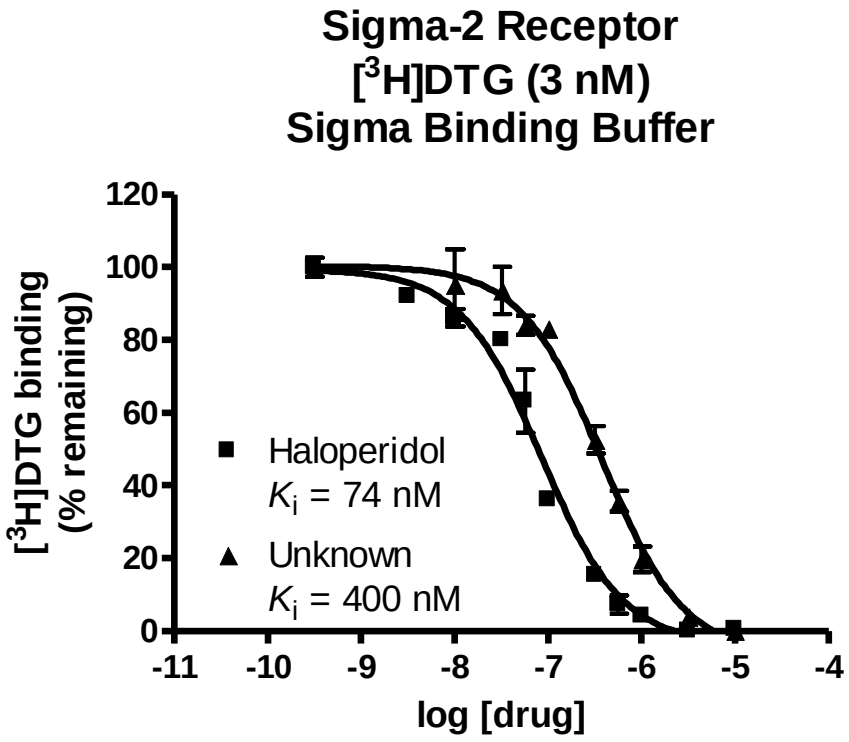
where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

Receptor	**Radioligand (Assay Conc.)**	Reference Compound
Sigma1	[^{3}H]Pentazocine (3 nM)	Haloperidol
Sigma2	[^{3}H]DTG (3 nM)	Haloperidol

Table 8. **Sigma receptor radioligands, radioligand assay concentrations, and reference compounds.**

Figure 12. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the Sigma receptors.





Histamine Receptors: H_1, H_2, H_3, H_4

Assay Buffer: Histamine Binding Buffer (50 mM Tris-HCl, 0.5 mM EDTA, pH 7.4)

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Moguilevsky *et al*. *Eur J Pharmacol* 224:489-495 (1994).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in Histamine Binding Buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference (see Table 8) compounds are prepared in Histamine Binding Buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand (see Table 8) is diluted to five times the assay concentration (see Table 8) in Histamine Binding Buffer. Typically, the assay concentration of radioligand is a value between one half the K_D and the K_D of a particular radioligand at its target.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of Histamine Binding Buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled Histamine Binding Buffer and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are

added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x\text{-log}IC50})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

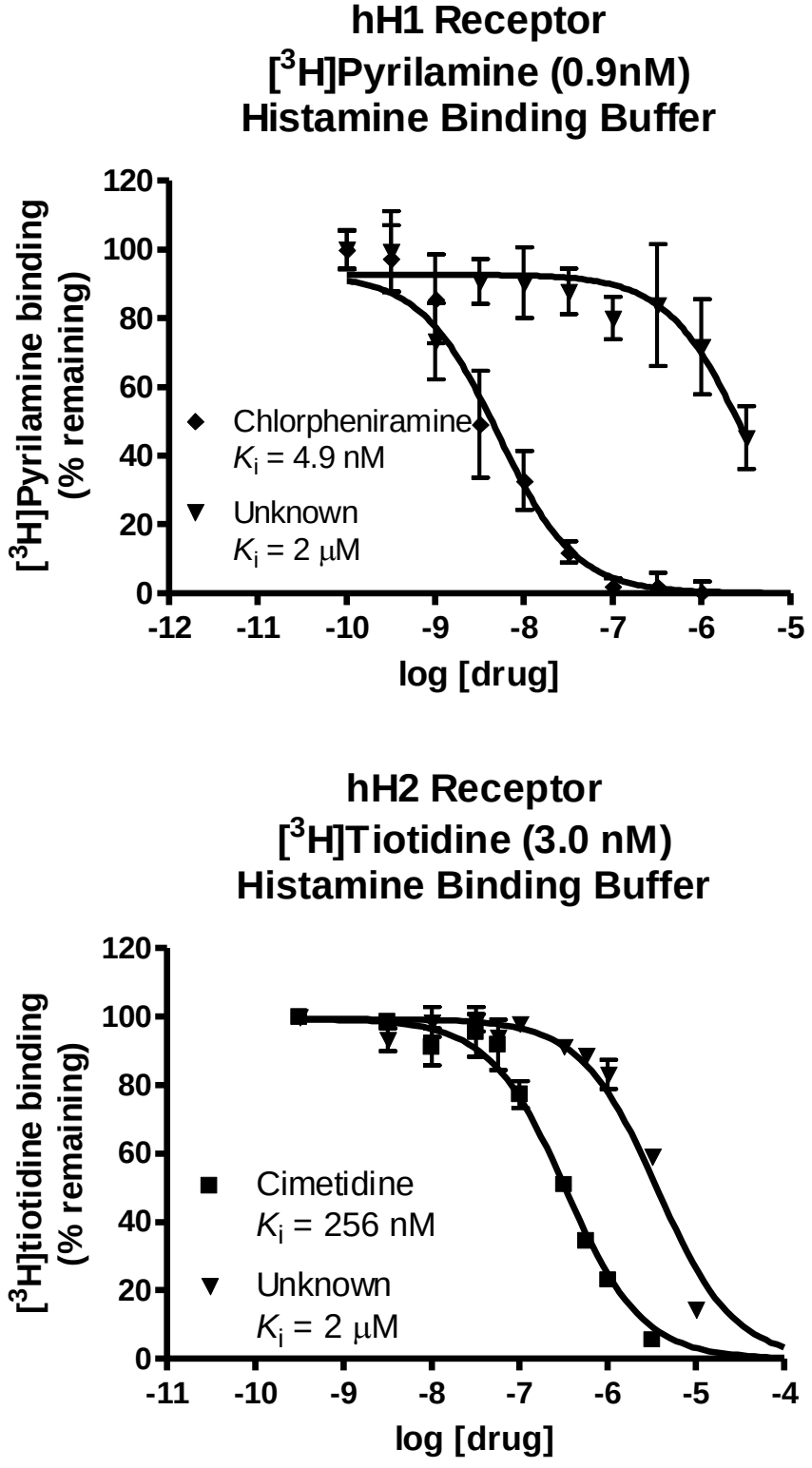
$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

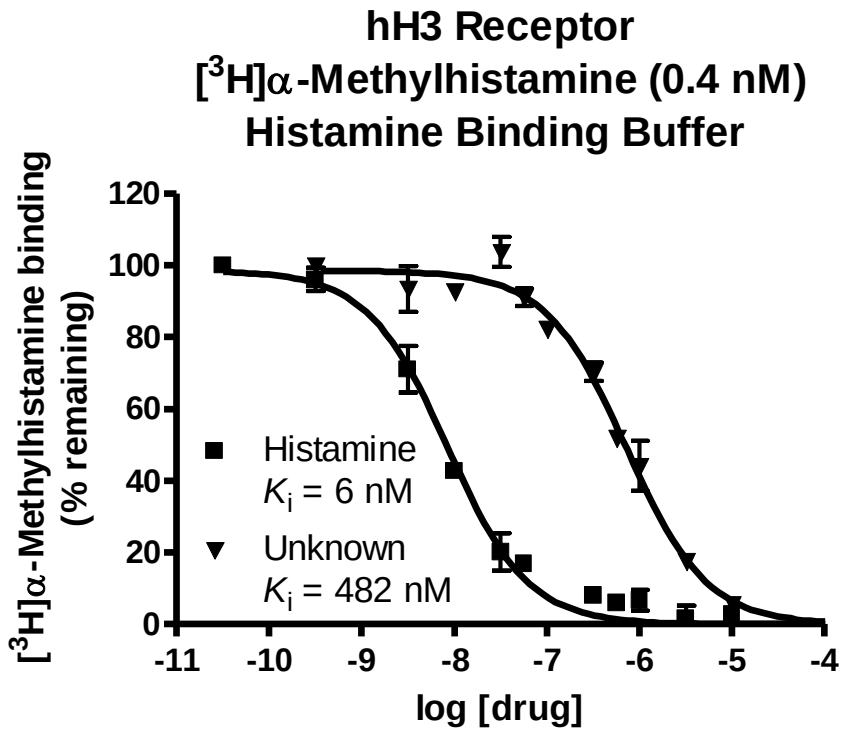
where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

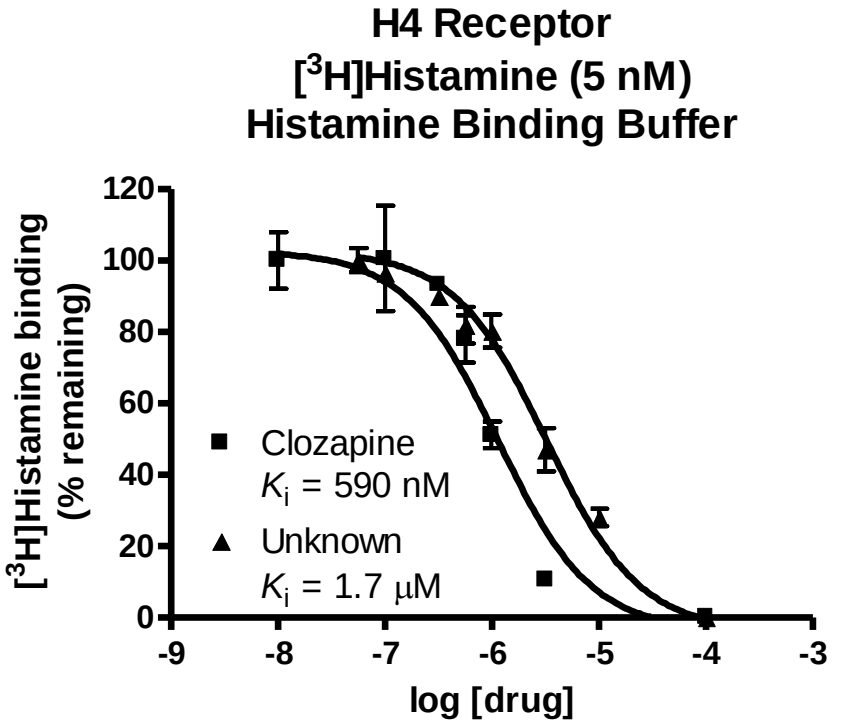
Receptor	**Radioligand (Assay Conc.)**	Reference Compound
H_1	[^{3}H]Pyrilamine (0.9 nM)	Chlorpheniramine
H_2	[^{3}H]Tiotidine (3 nM)	Cimetidine
H_3	[^{3}H]α-Methylhistamine (0.4 nM)	Histamine
H_4	[^{3}H]Histamine (5 nM)	Clozapine

Table 9. **Histamine receptor radioligands, radioligand assay concentrations, and reference compounds.**

Figure 13. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the histamine receptors.




hH3 Receptor
[3H]α-Methylhistamine (0.4 nM)
Histamine Binding Buffer
[3H]α-Methylhistamine binding
(% remaining)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
Histamine
Ki = 6 nM
Unknown
Ki = 482 nM


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

Vasopressin Receptors: V1, V2, V3

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

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Roth and Spitzer. *Am J Physiol* 252(5 Pt 1):E699-702 (1987).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in Vasopressin Binding Buffer or DMSO according to its solubility. A similar stock of a reference compound (positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference compound (vasopressin) are prepared in Vasopressin Binding Buffer by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand ([^{3}H]vasopressin) is diluted to 5 nM (five times the assay concentration) in Vasopressin Binding Buffer.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of Vasopressin Binding Buffer. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled Vasopressin Binding Buffer and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are

added to each tube. The tubes are capped, labeled, and counted by liquid scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

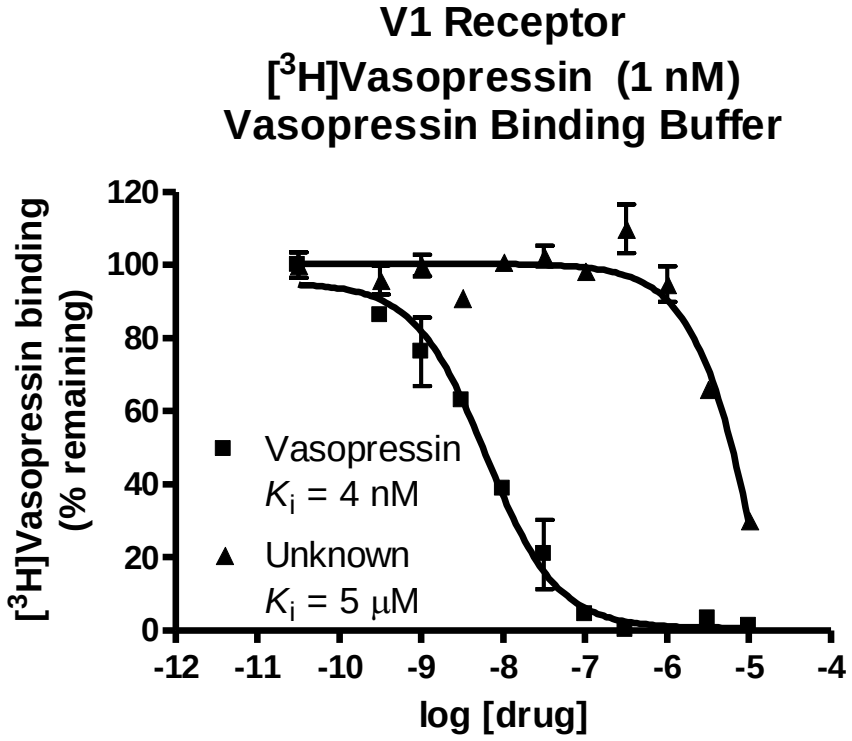
$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x\text{-log}IC50})]$$

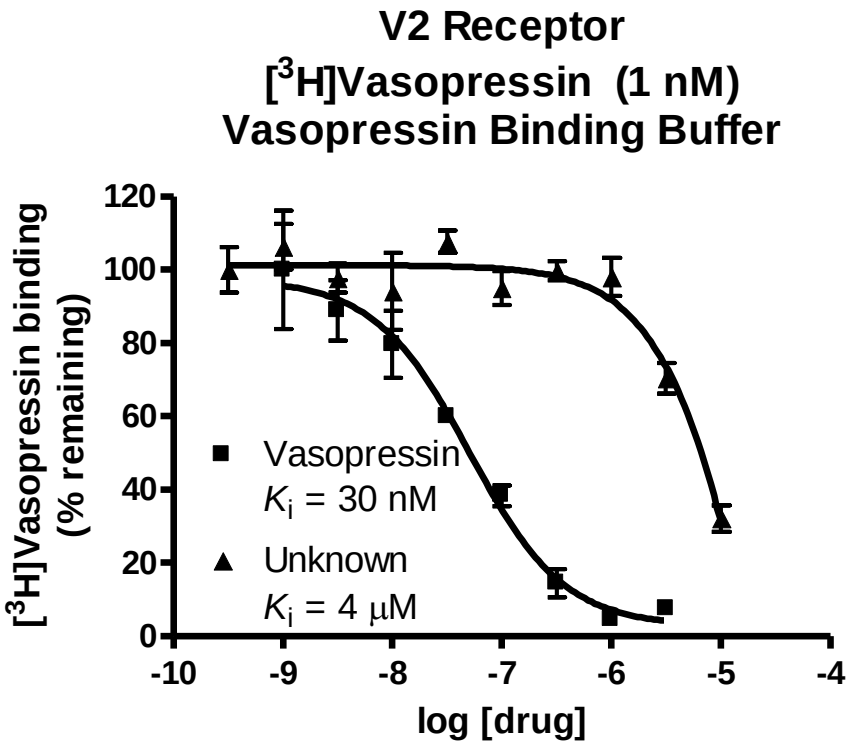
where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

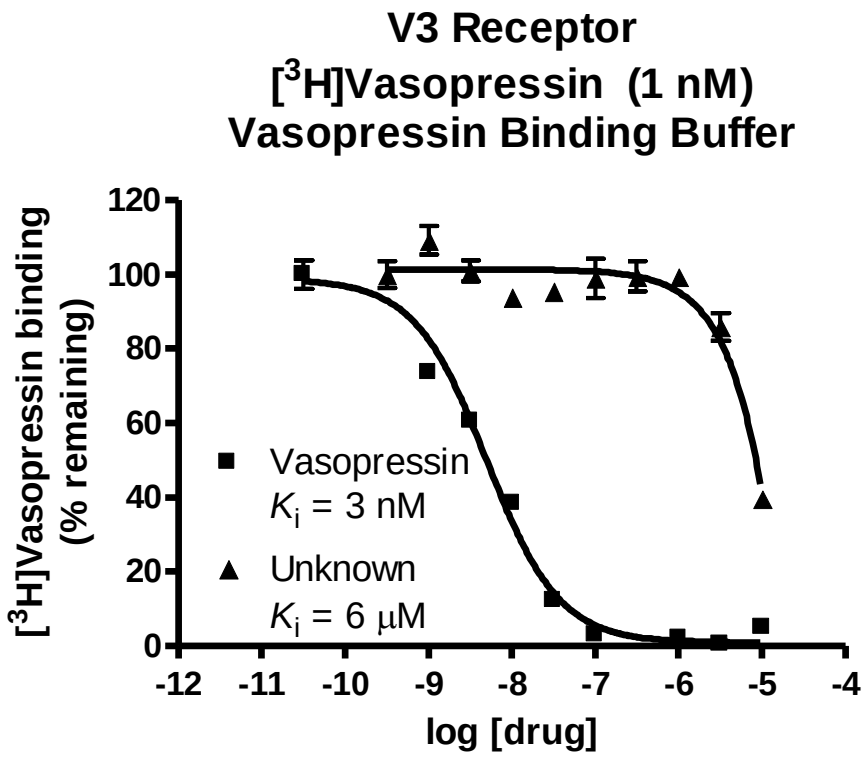
$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

Figure 14. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the vasopressin receptors.






V3 Receptor
[3H]Vasopressin (1 nM)
Vasopressin Binding Buffer
[3H]Vasopressin binding
(% remaining)
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
Vasopressin
Ki = 3 nM
Unknown
Ki = 6 μM

Neurotensin Receptors: NT1, NT2

Assay Buffer: 50 mM Tris-HCl, 0.2% BSA

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Pettibone *et al*. *J Pharmacol Exp Ther.* 300(1):305-313 (2002).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in 50 mM Tris-HCl, 0.2% BSA or DMSO according to its solubility. A similar stock of a reference compound (neurotensin, positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference compound (neurotensin) are prepared in 50 mM Tris-HCl, 0.2% BSA by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand ([^{3}H]neurotensin) is diluted to 10 nM (five times the assay concentration) in 50 mM Tris-HCl, 0.2% BSA.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of 50 mM Tris-HCl, 0.2% BSA. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled 50 mM Tris-HCl, 0.2% BSA and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid

scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

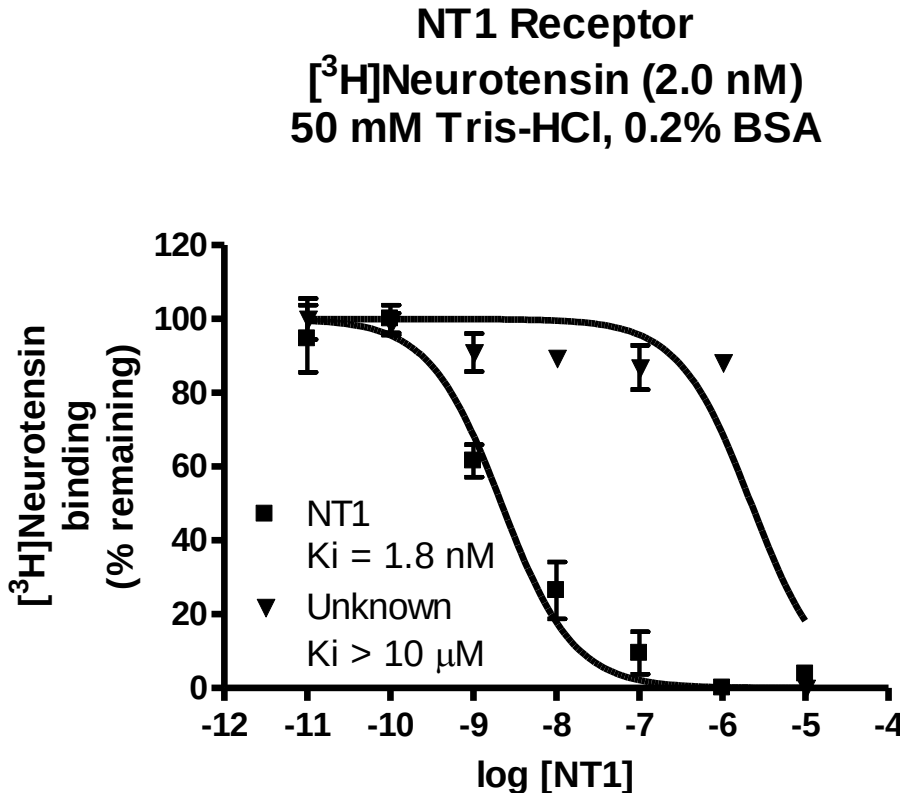
$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x\text{-log}IC50})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

Figure 15. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the neurotensin NT1 receptor.



VMAT2

Assay Buffer: 50 mM HEPES, 300 mM sucrose, **pH 8.0**

Membrane Fraction Source: Transiently or stably transfected cell lines (*e.g.*, HEK293, COS, CHO, NIH3T3)

Protocol adapted from Zucker *et al*. 69(2001):2311-2317 (2001).

Experimental Procedure and Data Analysis:

A solution of the compound to be tested is prepared as a 1-mg/ml stock in 50 mM HEPES, 300 mM sucrose, **pH 8.0** or DMSO according to its solubility. A similar stock of a reference compound (reserpine, positive control) is also prepared. Eleven dilutions (5 x assay concentration) of the test and reference compound (neurotensin) are prepared in 50 mM HEPES, 300 mM sucrose, **pH 8.0** by serial dilution: 0.05 nM, 0.5 nM, 1.5 nM, 5 nM, 15 nM, 50 nM, 150 nM, 500 nM, 1.5 µM, 5 µM, 50 µM (thus, the corresponding assay concentrations span from 10 pM to 10 µM and include semilog points in the range where high-to-moderate affinity ligands compete with radioligand for binding sites).

Radioligand ([^{3}H]tetrabenezine) is diluted to 7.5 nM (five times the assay concentration) in 50 mM HEPES, 300 mM sucrose, **pH 8.0**.

Aliquots (50 µl) of radioligand are dispensed into the wells of a 96-well plate (see Figure 1) containing 100 µl of 50 mM HEPES, 300 mM sucrose, **pH 8.0**. Then, duplicate 50-µl aliquots of the test and reference compound dilutions are added (see Figure 1).

Finally, crude membrane fractions of cells expressing recombinant target (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 3 ml of chilled 50 mM Tris-HCl, 0.2% BSA and homogenized by several passages through a 26 guage needle, then 50 µl are dispensed into each well.

The 250-µl reactions are incubated at room temperature and shielded from light (to prevent photolysis of light-sensitive ligands) for 1.5 hours, then harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harverster. Four rapid 500-µl washes are performed with chilled Standard Binding Buffer to reduce non-specific binding. Filters are placed in 6-ml scintillation tubes and allowed to dry overnight. The next day, 4 ml of EcoScint scintillation cocktail (National Diagnostics) are added to each tube. The tubes are capped, labeled, and counted by liquid

scintillation counting. For higher throughput assays, bound radioactivity is harvested onto 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester. The filter mats are dried, then scintillant is melted onto the filters and the radioactivity retained on the filters is counted in a Microbeta scintillation counter. Currently, nearly all of our radioligand binding assays have been adapted to this higher throughput approach.

Raw data (dpm) representing total radioligand binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent radioligand binding compared to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 using the built-in three parameter logistic model describing ligand competition binding to radioligand-labeled sites:

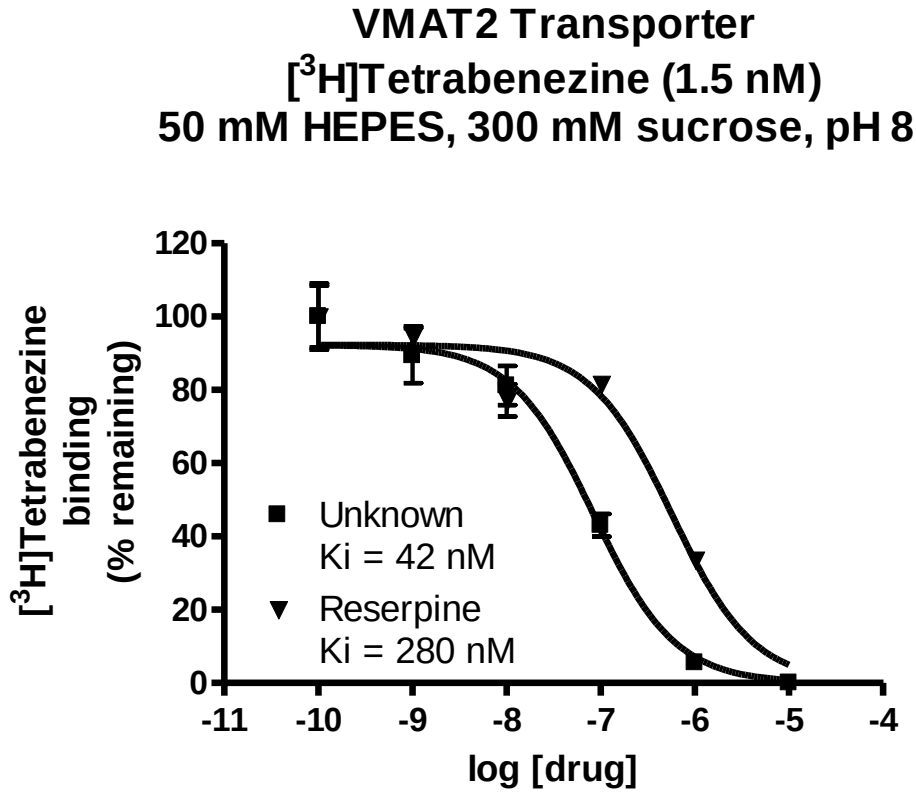
$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{\text{x-logIC50}})]$$

where bottom equals the residual radioligand binding measured in the presence of 10 µM reference compound (*i.e.*, non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The log IC_{50} (*i.e.*, the log of the ligand concentration that reduces radioligand binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

$$K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$$

where [ligand] equals the assay radioligand concentration and K_D equals the affinity constant of the radioligand for the target receptor.

Figure 16. Radioligand binding isotherms for competition binding assays wherein reference compounds and PDSP investigator-submitted compounds were assayed in parallel at the VMAT2 transporter.



III. Functional Assays

All functional assays are performed on transiently or stably transfected cell lines (e.g., HEK293, COS, CHO, NIH3T3).

Gi/o-Coupled Targets: [^{35}S]GTPγS binding to crude membrane fractions

Receptors: Serotonin 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{5A}; Dopamine D_2, D_4; Norepinephrine α_{2A}, α_{2B}, α_{2C}; Muscarinic M_2, M_4; Opioid MOR, KOR, DOR; Histamine H_3, H_4

Protocol adapted from Jensen *et al*. *Neuropsychopharmacology* 5 Dec (2007).

Experimental Procedure and Data Analysis:

Assay buffer: 50 mM HEPES, 5 mM $MgCl_2$, 150 mM NaCl, 0.2 mM EDTA, 100 mg/l ascorbic acid, pH 7.4

Reference (endogenous) agonist and compounds to be tested are dissolved in assay buffer or DMSO according to solubility. Serial dilutions of the test and reference compounds are made in binding buffer at 2x assay concentration (final assay concentrations ranging from 0.1 nM to 10 µM). Crude membrane fractions (1.8 to 3.5 cm^2/well) (prepared from 10-cm plates by harvesting PBS-rinsed monolayers, resuspending and lysing in chilled, hypotonic 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000 x *g*, decanting the supernatant and storing at -80 degrees centigrade; typically, one 10-cm plate provides sufficient material for 24 wells) are resuspended in 1.2 ml of assay buffer containing 20 µM GDP, wheat germ agglutinin (WGA)-coated scintillation proximity beads (2.4 mg), and [^{35}S]GTPγS (300 pM final). The suspension is then added (50 µl/well) to 50 µl of the 2x test or reference compounds (each concentration assayed in triplicate) in flexible transparent PET 96-well plates. The reaction plate is sealed and incubated for 90 min at room temperature, then centrifuged for 5 min at 216 × *g*, and finally loaded into a Wallac MicroBeta TriLux counter. Non-specific [^{35}S]GTPγS binding is assessed at the maximum concentration of reference agonist in the presence of 10 µM antagonist (see Table 9). The background signal is measured at 10 µM reference agonist in the presence of 10 µM unlabeled GTPγS.

Raw data (dpm) representing total [^{35}S]GTPγS binding (*i.e.*, specific + non-specific binding) are plotted as a function of the logarithm of the molar concentration of the drug (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, fold increase in [^{35}S]GTPγS binding over that observed in the absence of test or reference compound) data is performed in Prism 4.0 (GraphPad Software) using the built-in three parameter logistic model (*i.e.*, sigmoidal concentration-response) describing agonist-stimulated activation of one receptor population:

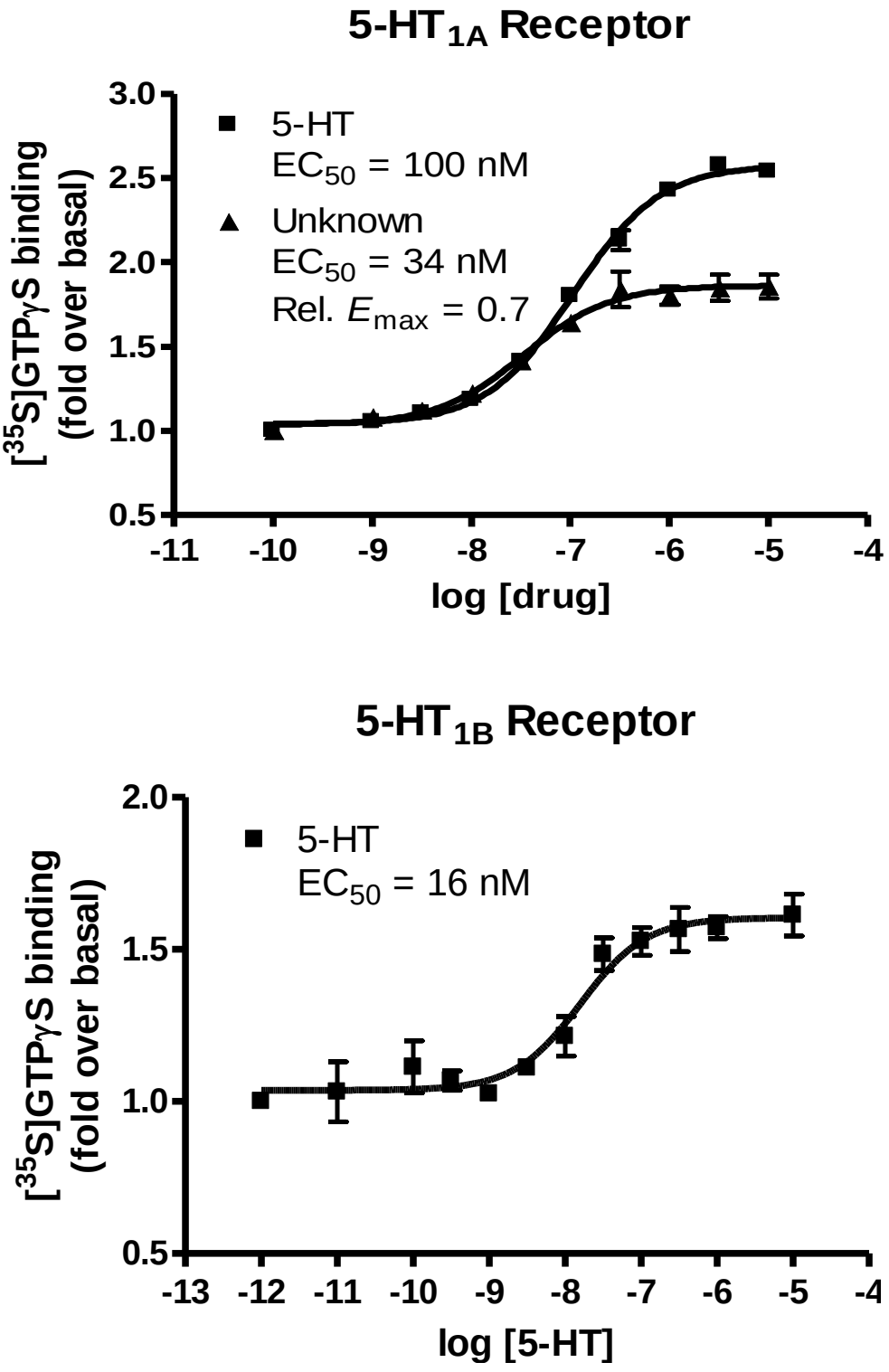
$$y = bottom + [(top-bottom)/(1 + 10^{x-logEC50})]$$

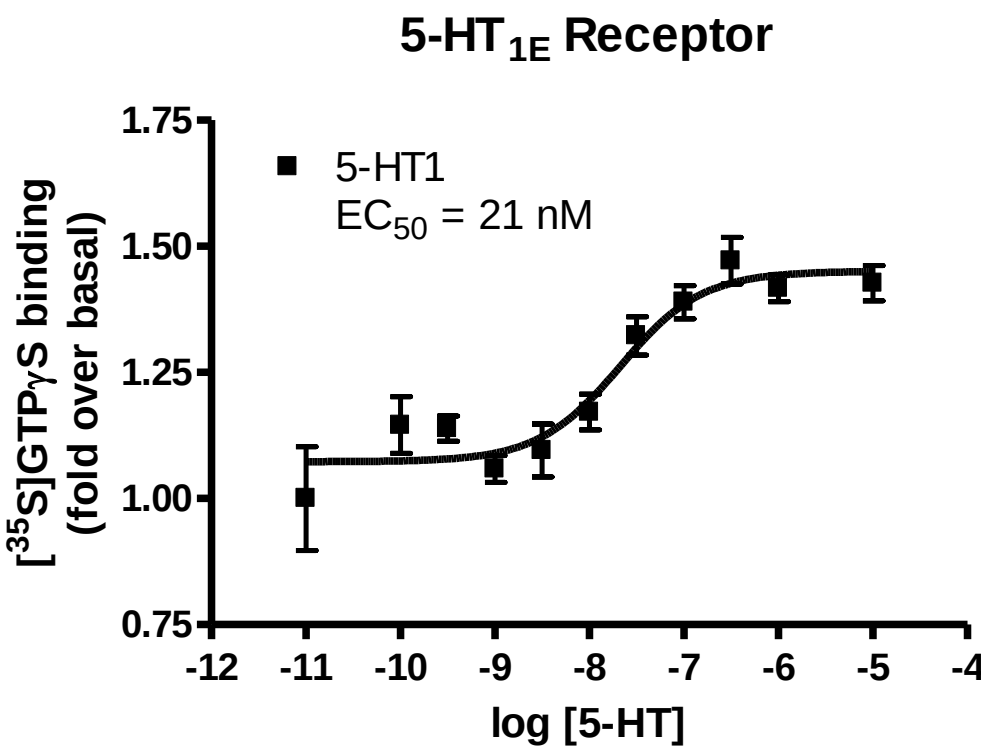
where bottom equals the best-fit basal [^{35}S]GTPγS binding and top equals the best-fit maximum [^{35}S]GTPγS binding. The log EC_{50} (*i.e.*, the log of the drug concentration that increases [^{35}S]GTPγS binding by 50% of the top) is thus estimated from the data, and the EC_{50} (agonist potency) is obtained. To obtain an estimate of the relative efficacy of the test compound (Rel. E_{max}), its data-fit top is compared to and expressed as a ratio of that for the reference agonist (Rel. E_{max} of 1.00).

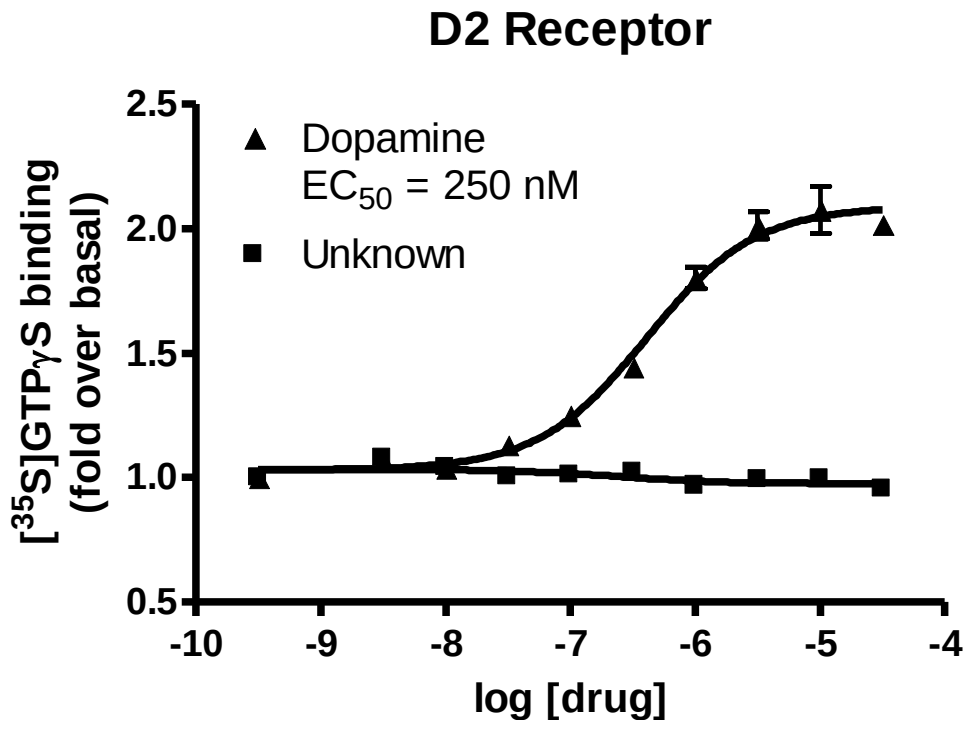
Receptor	Antagonist
5-HT_{1A}	**WAY100635**
5-HT_{1B}	**SB216641**
5-HT_{1D}	**BRL15572**
5-HT_{1E}	**Methylergonovine**
5-HT_{5A}	**Methiothepin**
D_2	**L741626**
D_4	**L745870**
α_{2A}	**Yohimbine**
α_{2B}	**Yohimbine**
α_{2C}	**Yohimbine**
M_2	**Methoctramine**
M_4	**Tropicamide**
MOR	**Cyprodime**
KOR	**Nor-BNI**
DOR	**Naltrindole**
H_3	**Ciproxifan**
H_4	**JNJ7777120**

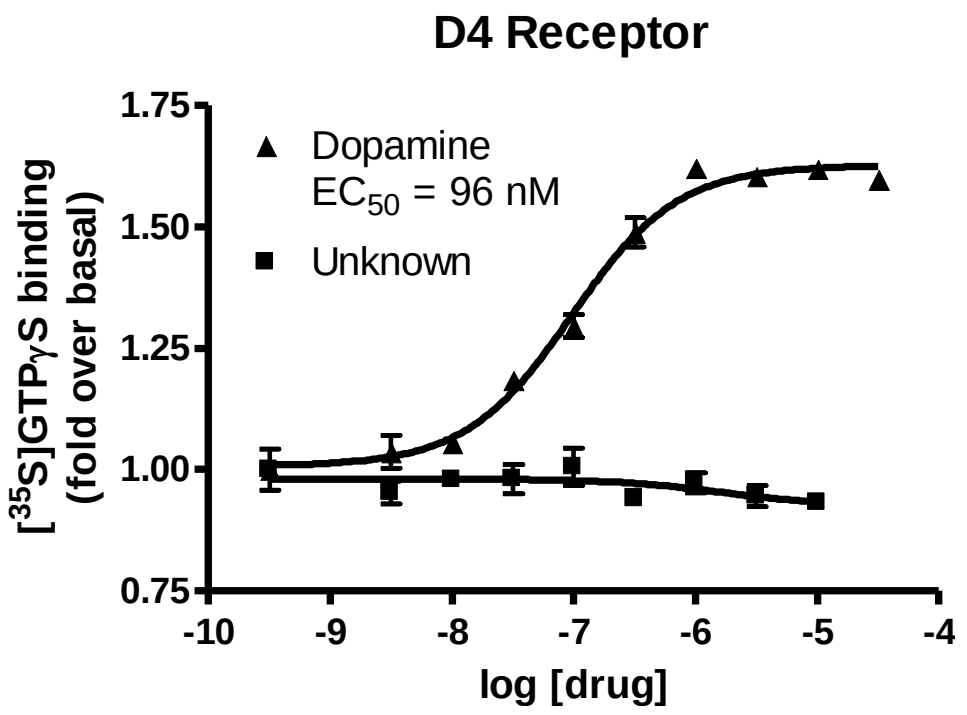
Table 10. Potency (EC_{50}) of 5-HT at Gi/o-coupled 5-HT receptors and selective antagonists used to assess non-specific [^{35}S]GTPγS binding. NS, no selective antagonist available.

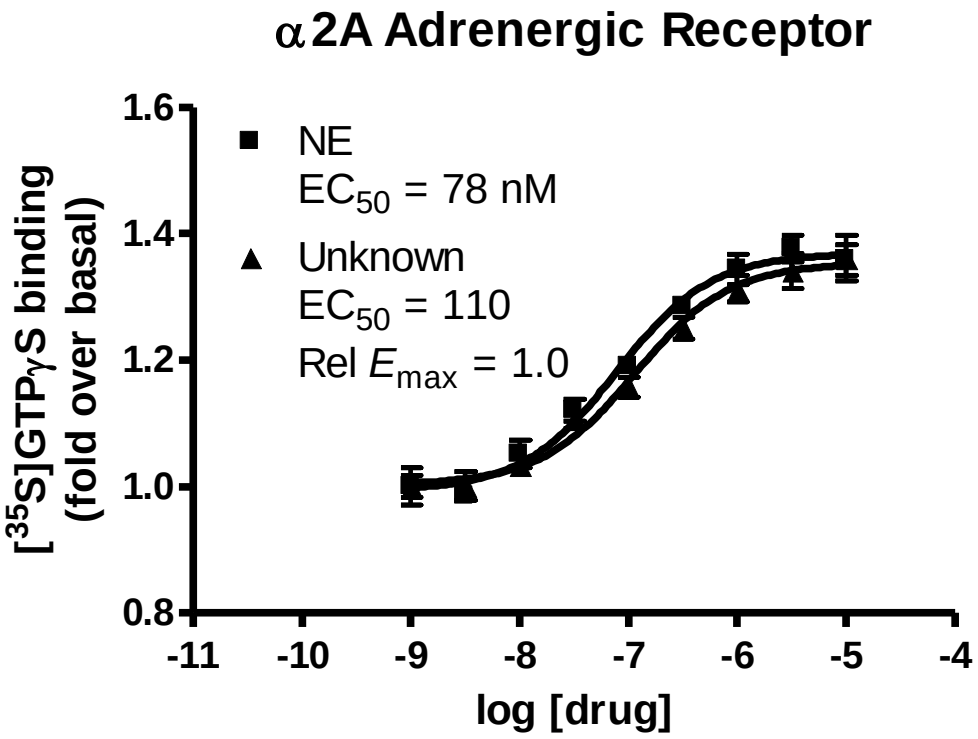
Figure 17. Representative drug-stimulated [^{35}S]GTPγS binding isotherms, wherein a reference full agonist and a PDSP investigator-submitted unknown were assayed in parallel.

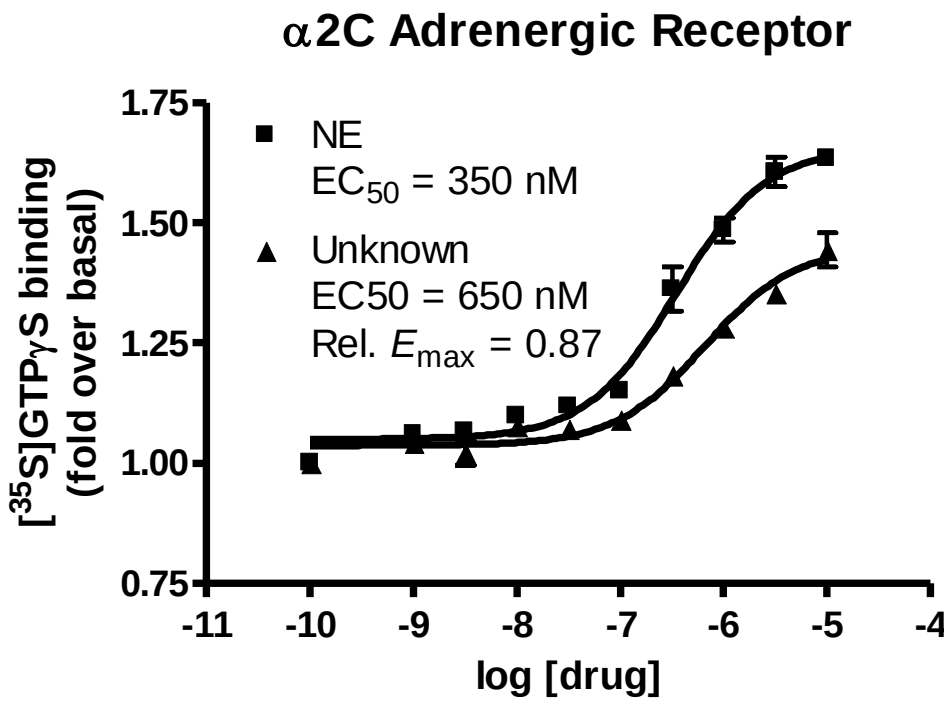


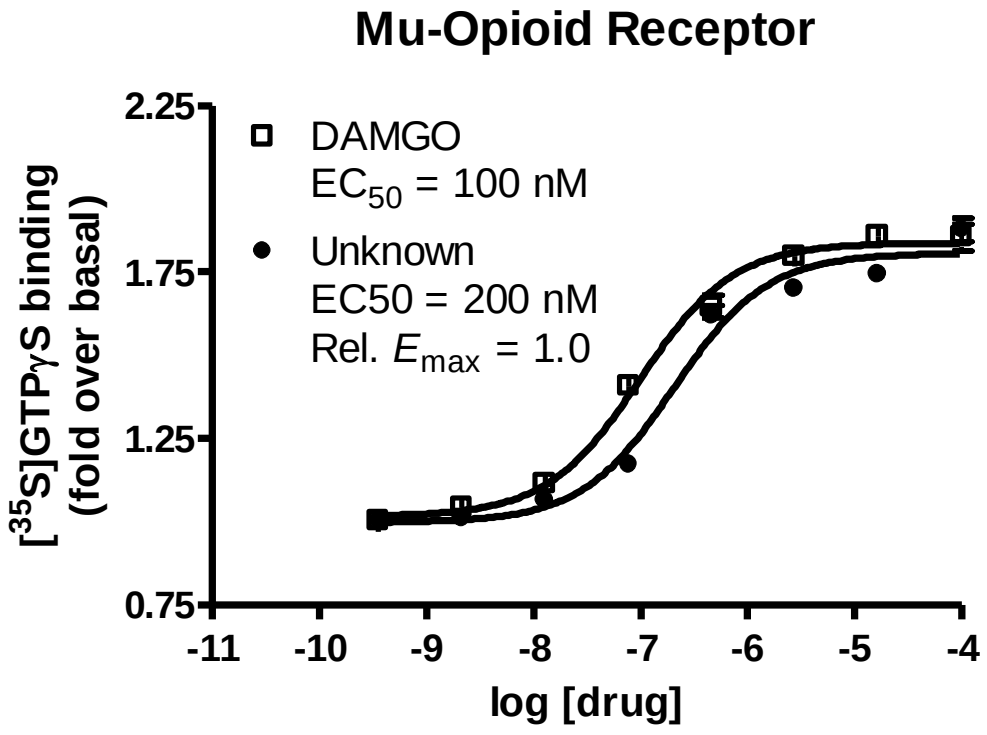

5-HT1E Receptor
5-HT1
EC50 = 21 nM
[35S]GTPγS binding
(fold over basal)
1.75
1.50
1.25
1.00
0.75
-12
-11
-10
-9
-8
-7
-6
-5
-4
log [5-HT]


D2 Receptor
Dopamine
EC50 = 250 nM
Unknown
[35S]GTPγS binding
(fold over basal)
2.5
2.0
1.5
1.0
0.5
-10
-9
-8
-7
-6
-5
-4
log [drug]

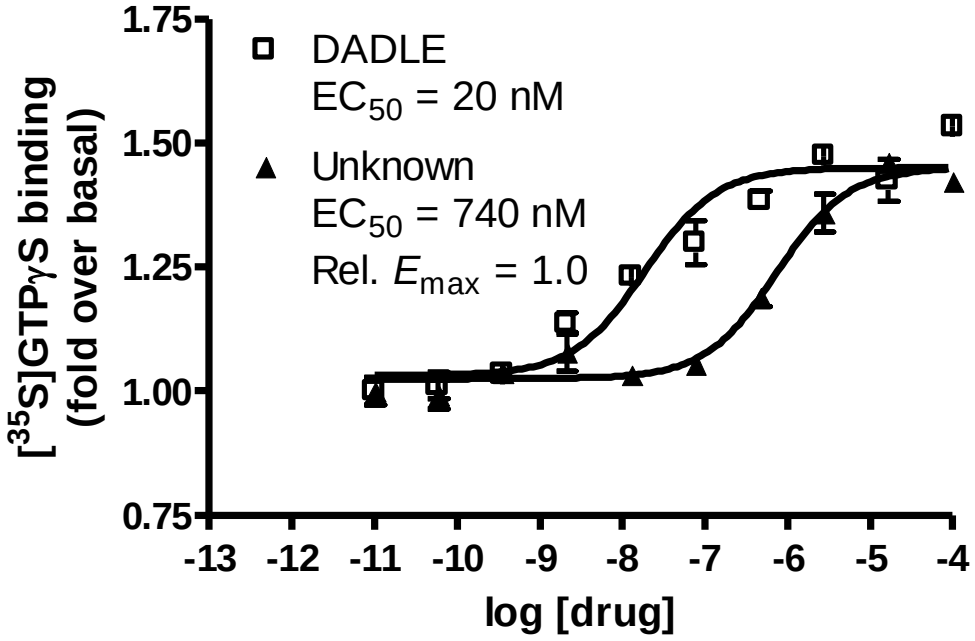

D4 Receptor
[35S]GTPγS binding
(fold over basal)
1.75
1.50
1.25
1.00
0.75
Dopamine
EC50 = 96 nM
Unknown
-10
-9
-8
-7
-6
-5
-4
log [drug]


α2A Adrenergic Receptor
[35S]GTPγS binding
(fold over basal)
1.6
1.4
1.2
1.0
0.8
NE
EC50 = 78 nM
Unknown
EC50 = 110
Rel Emax = 1.0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]

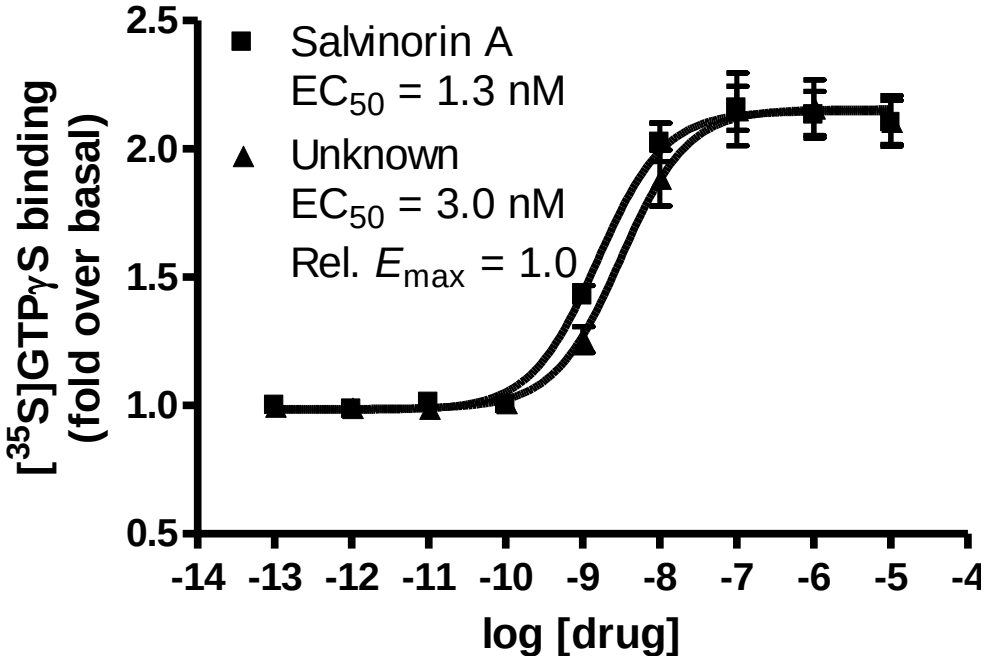

α2C Adrenergic Receptor
[35S]GTPγS binding (fold over basal)
1.75
1.50
1.25
1.00
0.75
NE
EC50 = 350 nM
Unknown
EC50 = 650 nM
Rel. Emax = 0.87
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]


Mu-Opioid Receptor
[35S]GTPγS binding (fold over basal)
2.25
1.75
1.25
0.75
DAMGO
EC50 = 100 nM
Unknown
EC50 = 200 nM
Rel. Emax = 1.0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]

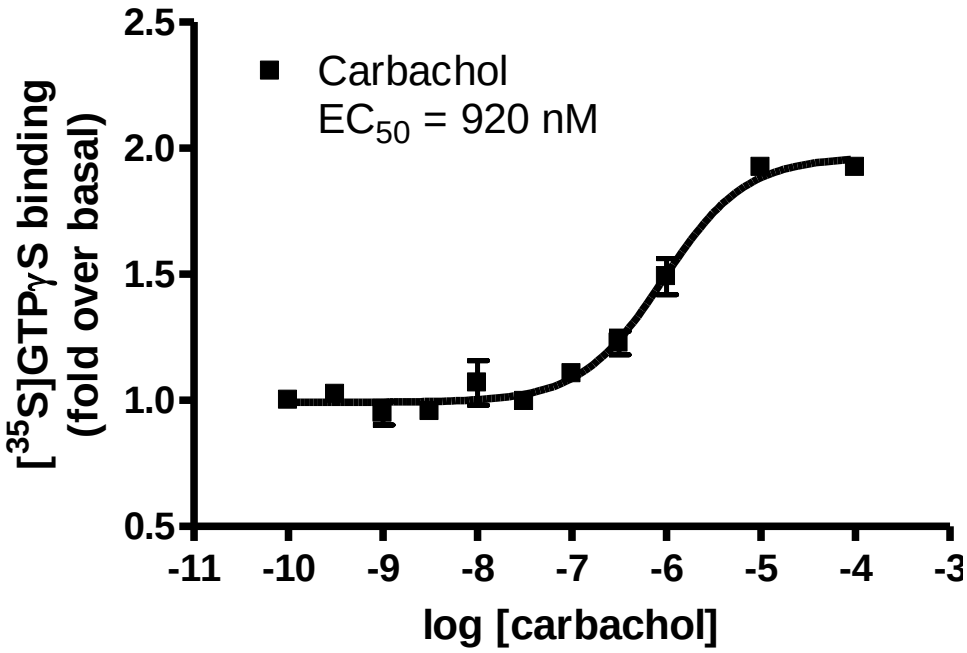
Delta-Opioid Receptor



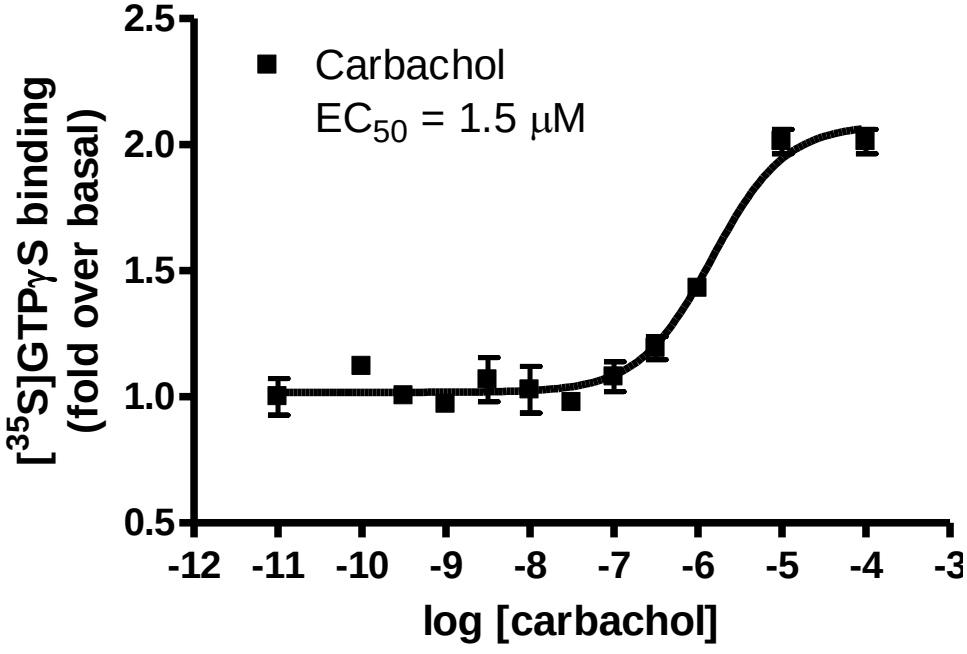
Kappa-Opioid Receptor



M2 Receptor



M4 Receptor



Gq/11-Coupled Targets: intracellular calcium mobilization

Receptors: Serotonin 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}; Glutamate $mGluR_{1-8}$; Norepinephrine α_{1A}, $\alpha_{1B,}$ α_{1D}; Muscarinic M_1, M_3, M_5; Histamine H_1, H_2; Purinergic P2Y1, P2Y2, P2Y4, P2Y6; Oxytocin; Vasopressin V_1, V_2, V_3; GPR40, GPR41, GPR43; MC3, MC4

Assay Buffer: 1X Hanks Balanced Salt Solution, 20 mM HEPES, 2.5 mM probenecid, pH 7.4 is used to reconstitute lyophilized Calcium Plus Assay Kit dye (Molecular Devices)

Protocol adapted from Davies *et al*. *Psychopharmacology* 178(4) :451-460 (2005).

Experimental Procedure and Data Analysis:

Receptor-expressing cell lines are seeded in glass-bottom 96- or 384-well, poly-L-lysine-coated plates 48 hours prior to the assay (40,000 cells per well or 6,700 cells, respectively) in DMEM containing 5% dialyzed serum. Twenty hours prior to the assay, the medium is changed to serum-free DMEM. Then, the cells are preincubated in 30 µl (96-well plates) or 20 µl (384-well plates) of calcium dye-containing assay buffer (the lyophilized dye is reconstituted with 15 ml of assay buffer) at 37 degrees centigrade for 75 min in a humidified incubator. During that time, serial dilutions of the reference and test compounds are made at 2x assay concentration (final assay concentrations ranging from 0.1 nM to 10 µM). Just prior to the assay, the plates are allowed to cool to room temperature for 10 min and then are transferred to a FLIPR Tetra fluorescence image plate reader (Molecular Devices). Basal fluorescence (excitation 488 nm, emission 510-570 nm) is measured for 20 sec, then test compound or reference agonist dilutions (2x assay concentration) are added (30 µl for 96-well plates, 20 µl for 384-well plates, each concentration assayed in triplicate) and fluorescence is measured for 60 sec. The maximum fluorescence values during the baseline and test compound or reference agonist addition phases (for agonist assays) are exported for analysis.

For agonist tests, raw data (maximum fluorescence, fluorescence units) for each concentration of test compound or reference are normalized to the baseline fluorescence (reported as fold increase over basal) and plotted as a function of the logarithm of the molar concentration of the drug (*i.e.*, test or reference compound). Non-linear regression of the normalized data is performed in Prism 4.0 (GraphPad Software) using the built-in three parameter logistic model (*i.e.*, sigmoidal concentration-response) describing agonist-stimulated activation of one receptor population:

$$y = bottom + [(top-bottom)/(1 + 10^{x-logEC50})]$$

where bottom equals the best-fit basal fluorescence and top equals the best-fit maximal fluorescence stimulated by the test compound or reference agonist. The log EC_{50} (*i.e.*, the log of the drug concentration that increases fluorescence by 50% of the maximum fluorescence observed for the test compound or reference agonist) is thus estimated from the data, and the EC_{50} (agonist potency) is obtained. To obtain an estimate of the relative efficacy of the test compound (Rel. E_{max}), its best-fit top is compared to and expressed as a ratio of that for the reference agonist (Rel. E_{max} of the reference agonist is 1.00).

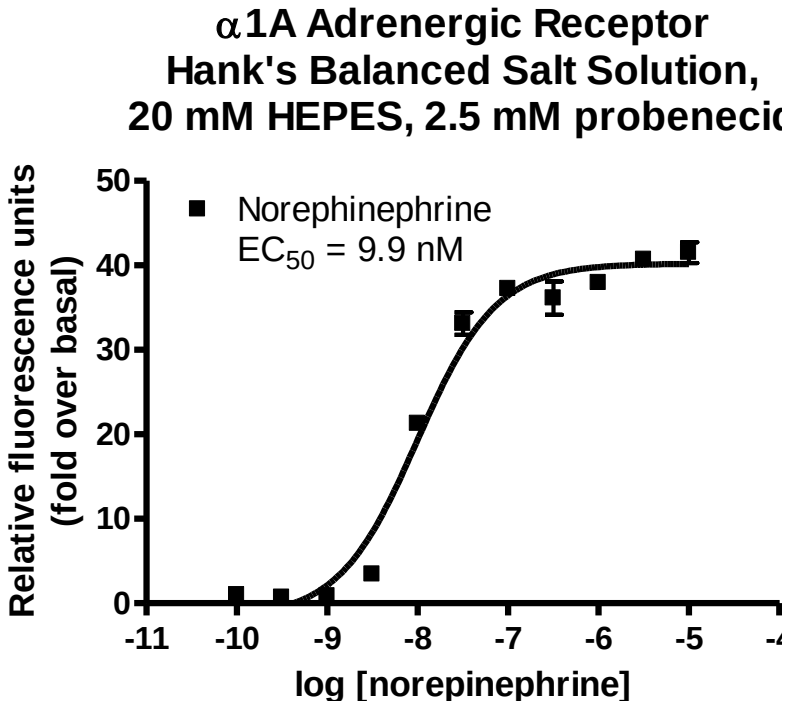
To ascertain whether test compounds are antagonists, a double-addition paradigm is employed. After measuring baseline fluorescence for 20 seconds, 30 µl of test compound (20 µM) is added (10 µM final concentration, assayed in triplicate) and fluorescence is measured for an additional 15 min. Then, 30 µl of reference agonist (3X; EC_{90}) is added (final concentration of agonist is EC_{30}) and fluorescence is measured for 60 sec. Maximum baseline-normalized fluorescence evoked by the reference agonist in the presence of test compound is compared to the maximum baseline-normalized fluorescence elicited by the reference agonist following addition of vehicle instead of test compound and expressed as a ratio. 'Hits' (compounds that antagonize reference agonist-stimulated increases in baseline-normalized fluorescence by at least 50%) are then characterized by a modified Schild analysis.

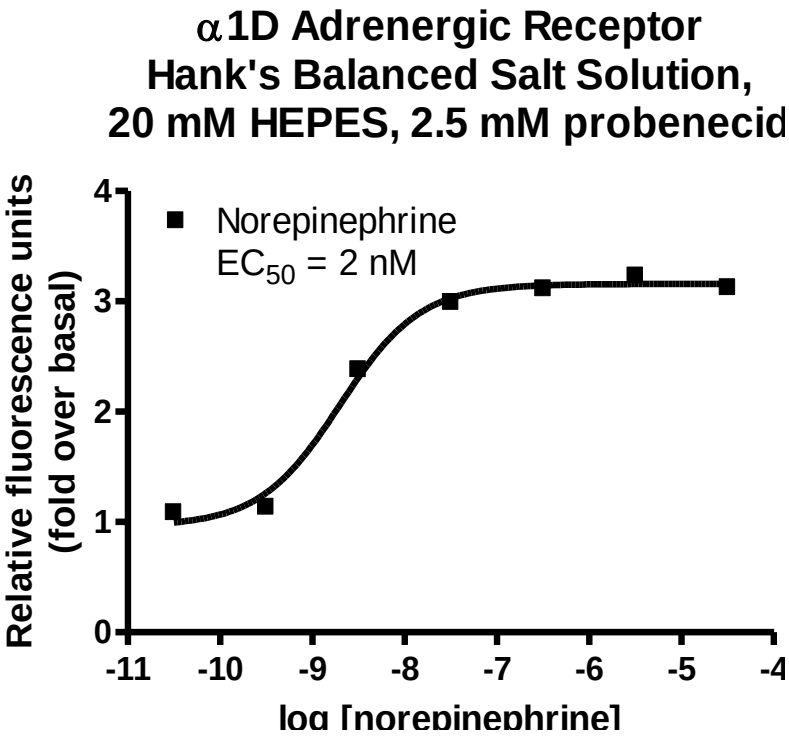
For modified Schild analysis, a family of reference agonist concentration-response isotherms is generated in the absence and presence of graded concentrations of test compound (added 15 min prior to reference agonist). Theoretically, compounds that are competitive antagonists cause a dextral shift of agonist concentration-response isotherms without reducing the maximum response to agonist (i.e., surmountable antagonism). However, on occasion, factors such as non-competitive antagonism, hemiequilibria, and/or receptor reserve cause apparent insurmountable antagonism. To account for such deviations, we apply the modified Lew-Angus method to ascertain antagonist potency (Christopoulos et al., 1999). Briefly, equieffective concentrations of agonist (concentrations of agonist that elicit a response equal to the $EC_{25\%}$ of the agonist control curve) are plotted as a function of the test compound concentration present in the wells in which they were measured. Non-linear regression of the baseline-normalized data is performed in Prism 4.0 using the following equation:

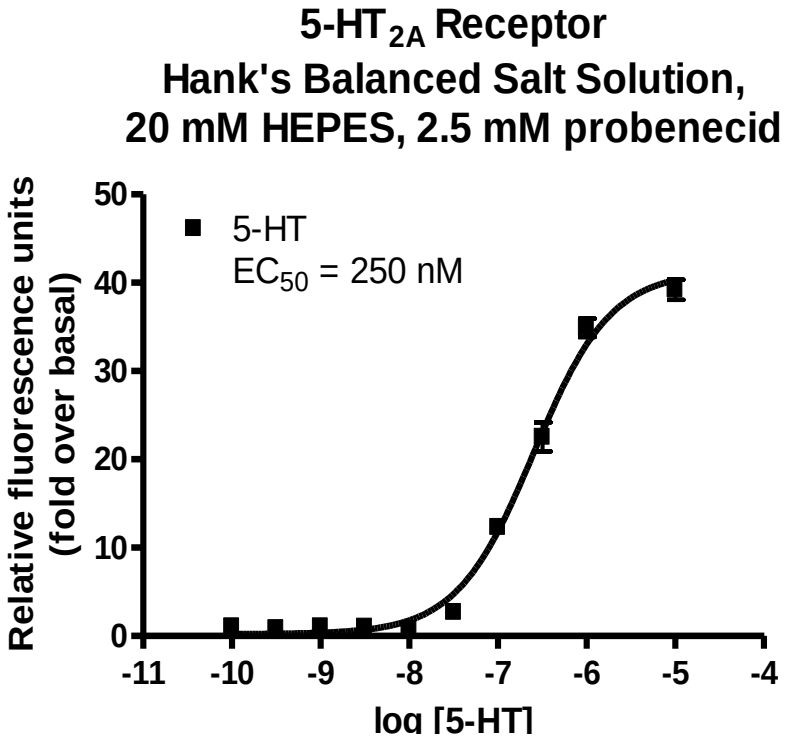
$$pEC_{25\%} = -\log ([B] + 10^{-pK}) - \log c$$

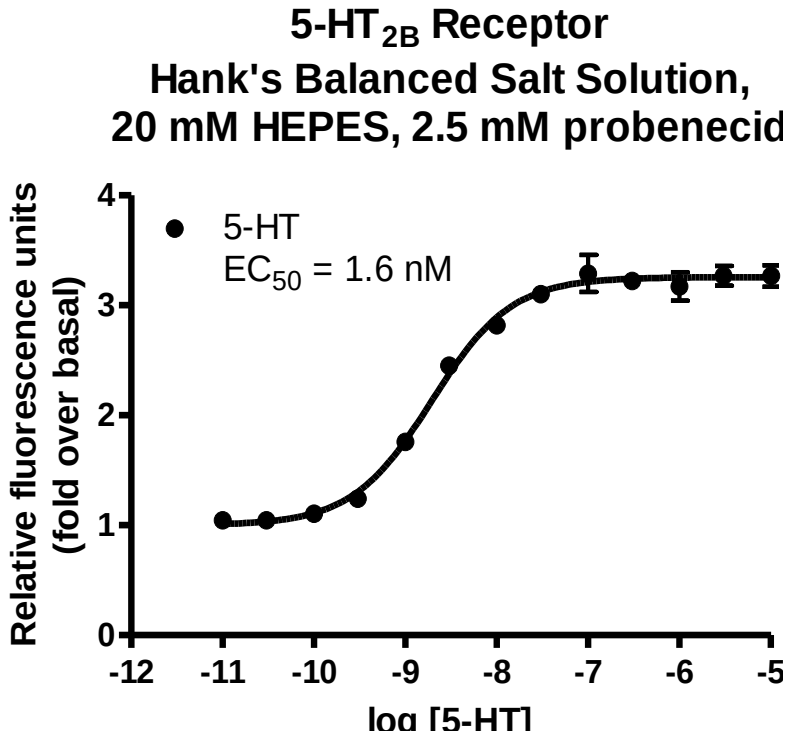
where $EC_{25\%}$ equals the concentration of agonist that elicits a response equal to 25% of the maximum agonist control curve response and [B] equals the antagonist concentration; K, c, and s are fit parameters. The parameter s is equal to the Schild slope factor. If s is not significantly different from unity, pK equals pK_B; otherwise, pA_2 is calculated (pA_2 = pK/s). The parameter c equals the ratio $EC_{25\%}$/[B].

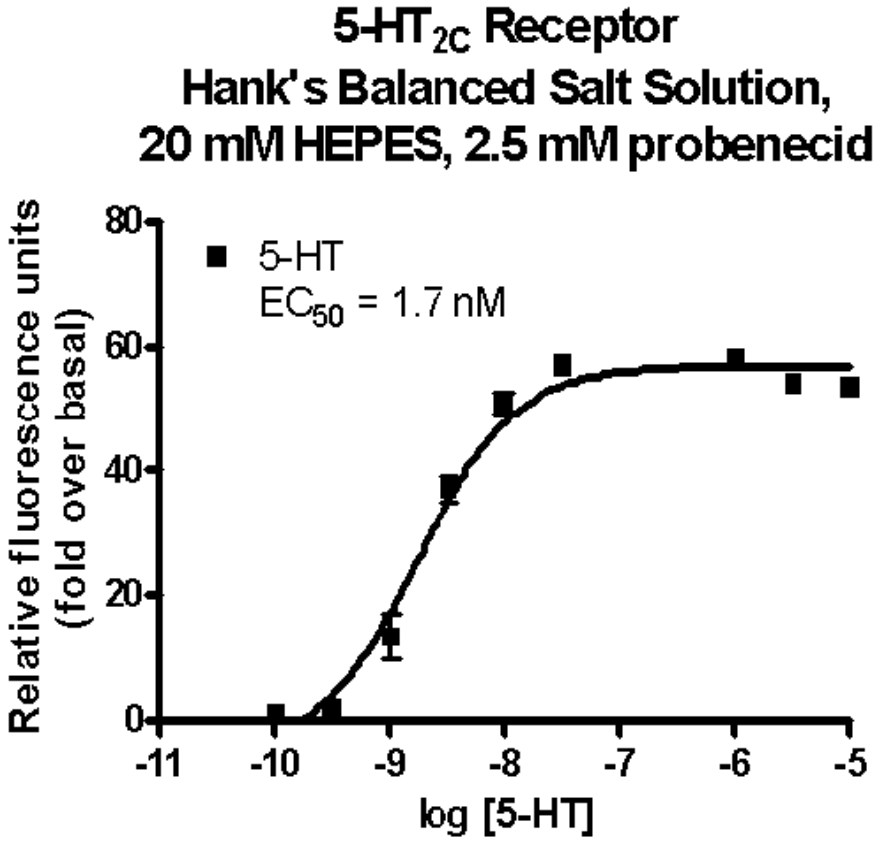
Figure 18. Representative reference agonist-stimulated intracellular calcium mobilization isotherms for selected Gq/11-coupled receptors available for screening.

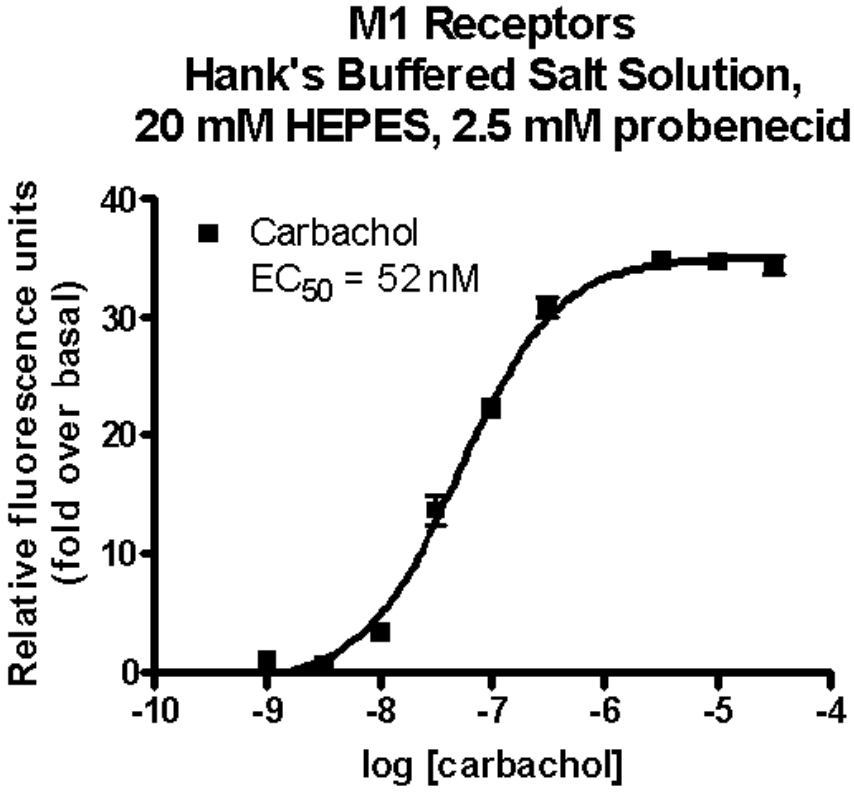


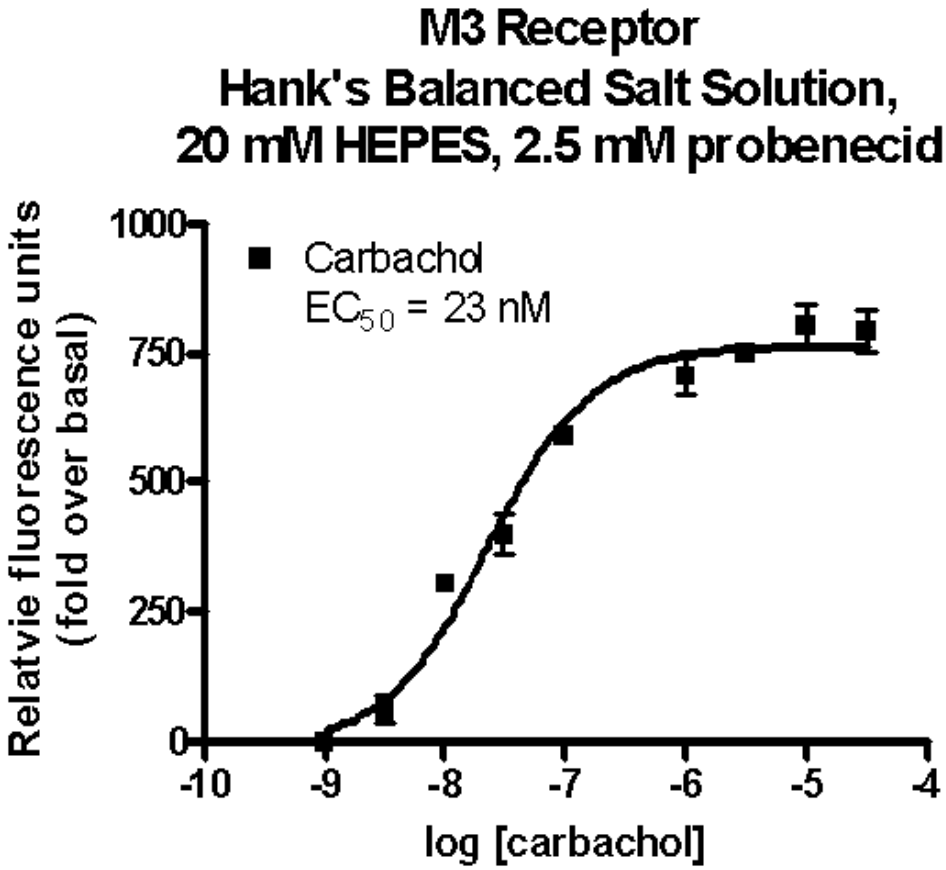


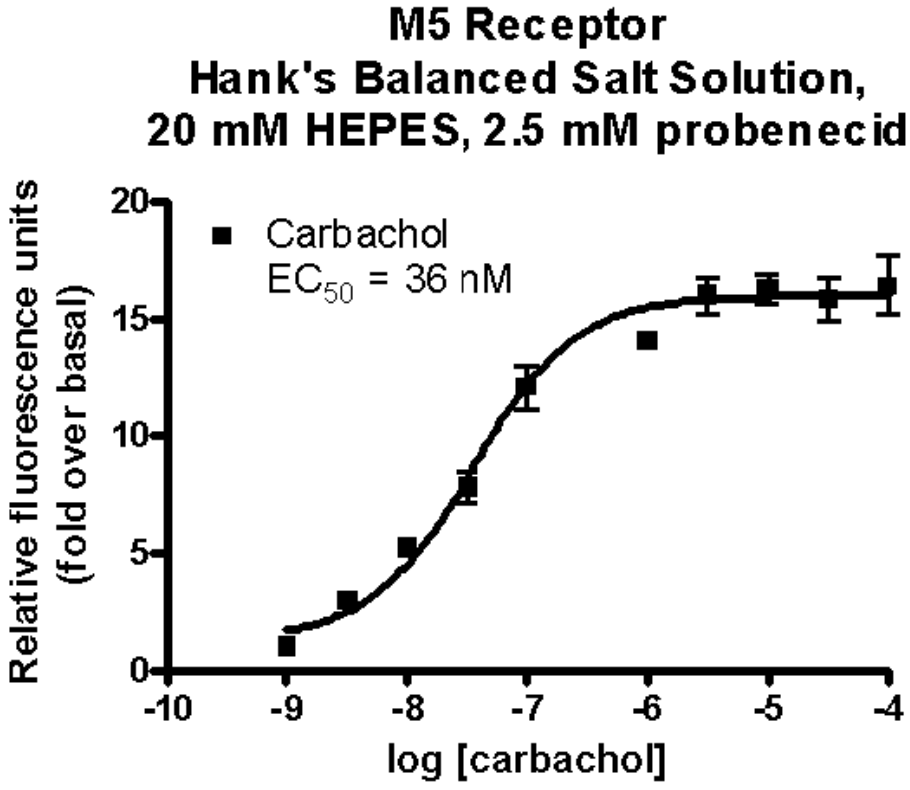

5-HT2A Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence units
(fold over basal)
5-HT
EC50 = 250 nM
50
40
30
20
10
0
-11
-10
-9
-8
-7
-6
-5
-4
log [5-HT]

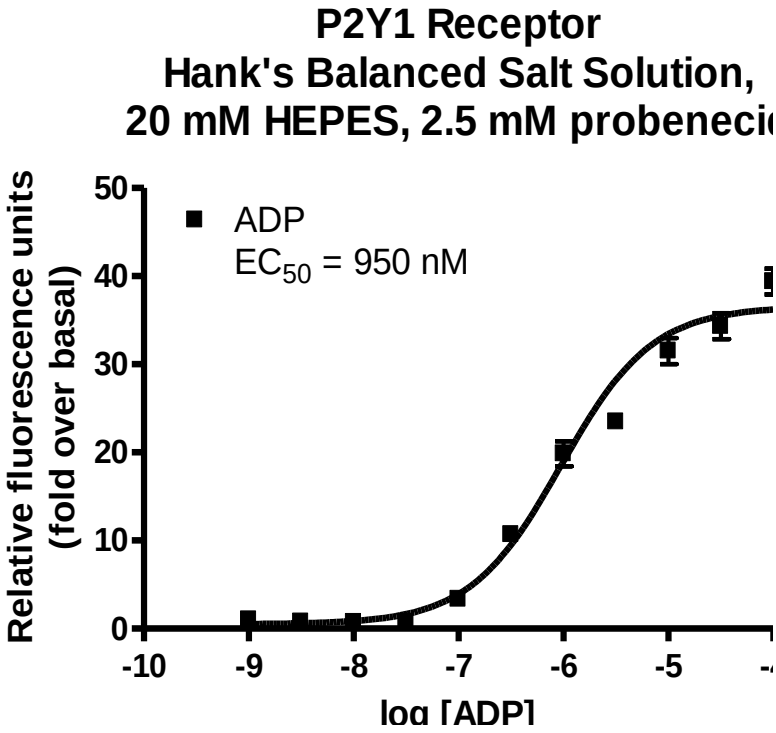

5-HT2B Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence units
(fold over basal)
5-HT
EC50 = 1.6 nM
4
3
2
1
0
-12
-11
-10
-9
-8
-7
-6
-5
log [5-HT]

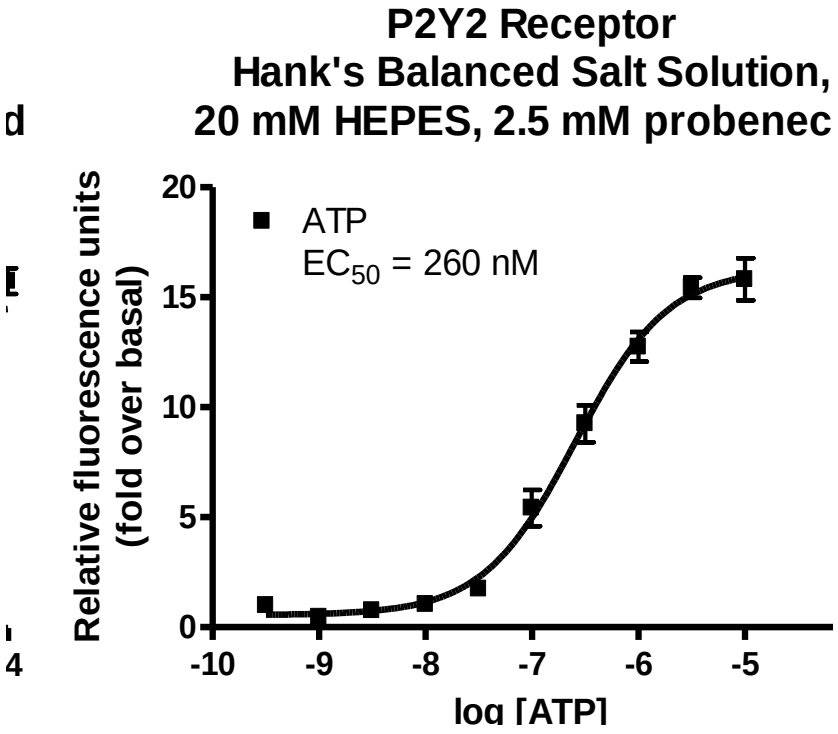

5-HT2C Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence units
(fold over basal)
5-HT
EC50 = 1.7 nM
log [5-HT]

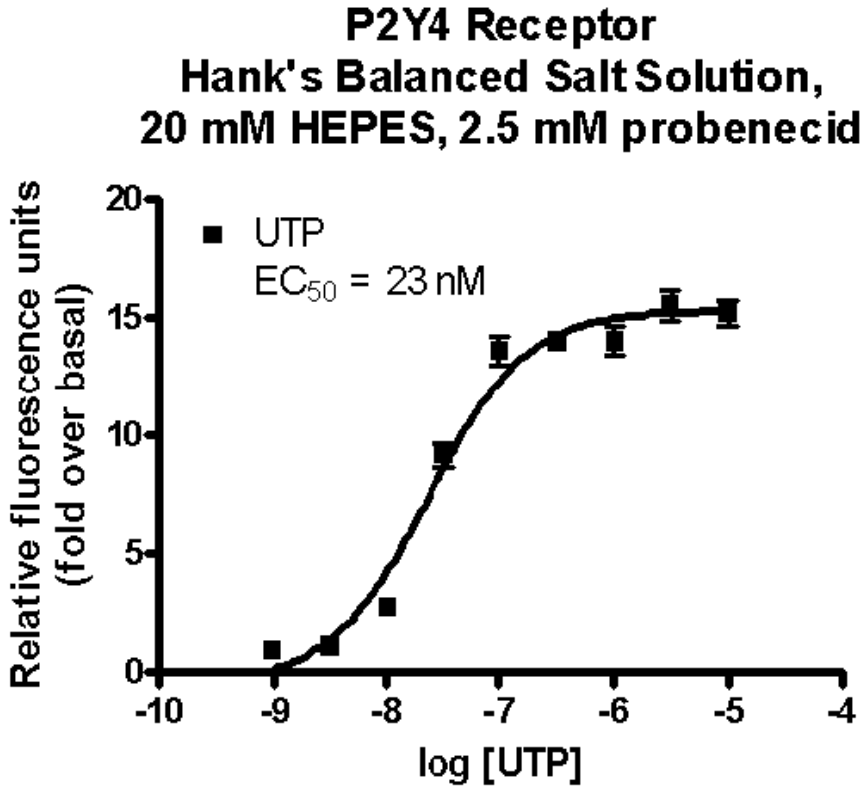

M1 Receptors
Hank's Buffered Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence units
(fold over basal)
Carbachol
EC50 = 52 nM
log [carbachol]

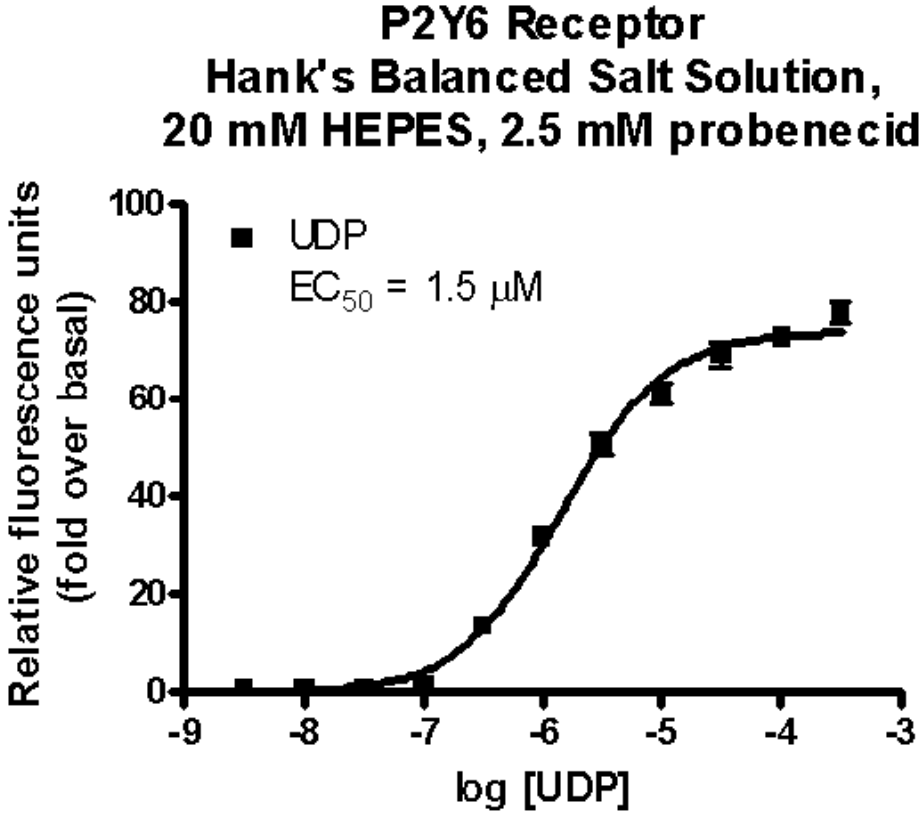

M3 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relatvie fluorescence units
(fold over basal)
1000
750
500
250
0
Carbachol
EC50 = 23 nM
-10
-9
-8
-7
-6
-5
-4
log [carbachol]

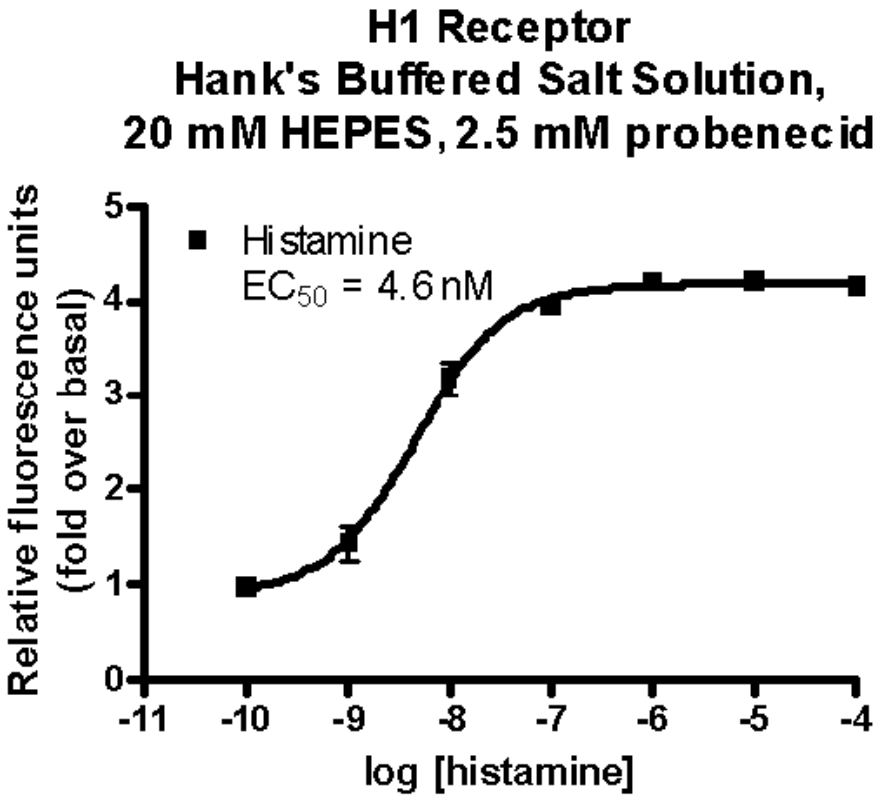

M5 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence units
(fold over basal)
20
15
10
5
0
Carbachol
EC50 = 36 nM
-10
-9
-8
-7
-6
-5
-4
log [carbachol]

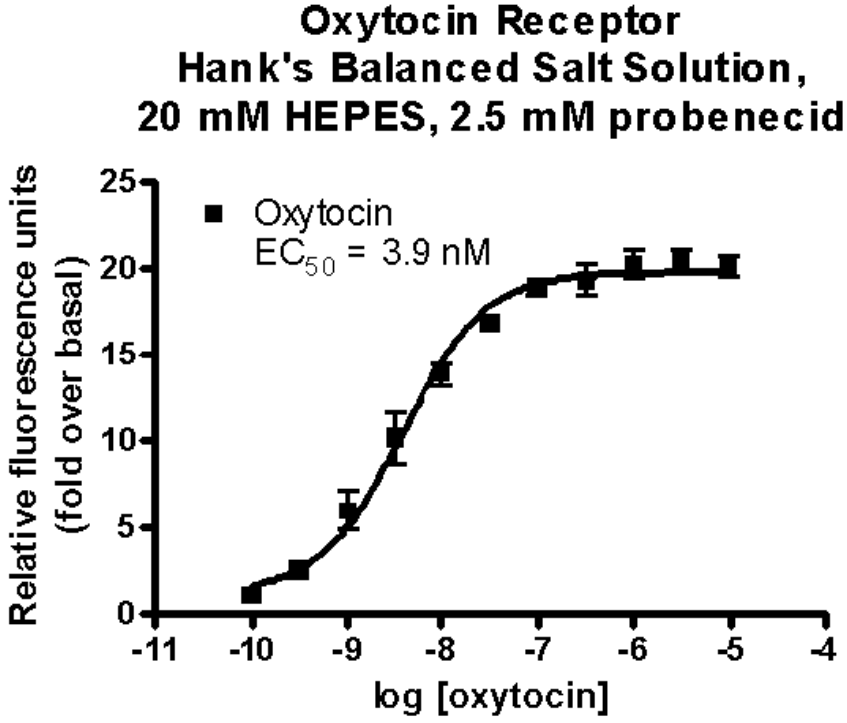

P2Y1 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence units
(fold over basal)
ADP
EC50 = 950 nM
log [ADP]


P2Y2 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenec
Relative fluorescence units
(fold over basal)
ATP
EC50 = 260 nM
log [ATP]

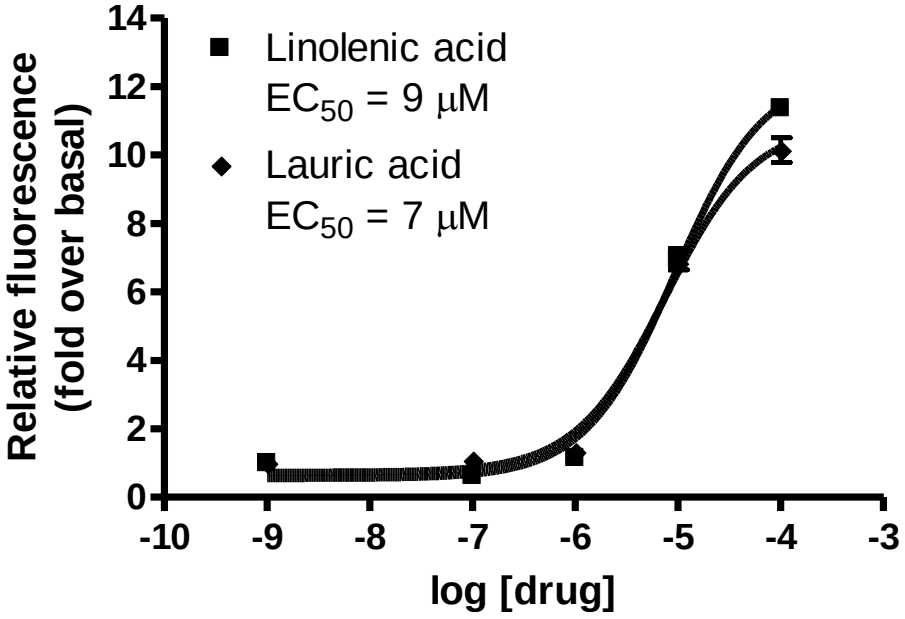

P2Y4 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence units
(fold over basal)
UTP
EC50 = 23 nM
log [UTP]


P2Y6 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence units
(fold over basal)
UDP
EC50 = 1.5 µM
log [UDP]

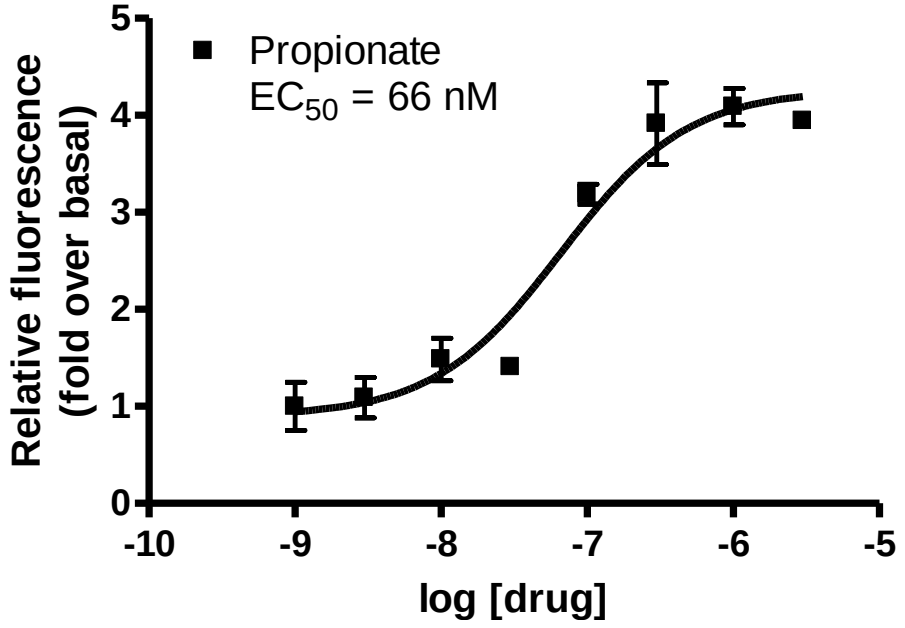

H1 Receptor
Hank's Buffered Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence units
(fold over basal)
Histamine
EC50 = 4.6 nM
log [histamine]


Oxytocin Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence units
(fold over basal)
Oxytocin
EC50 = 3.9 nM
log [oxytocin]

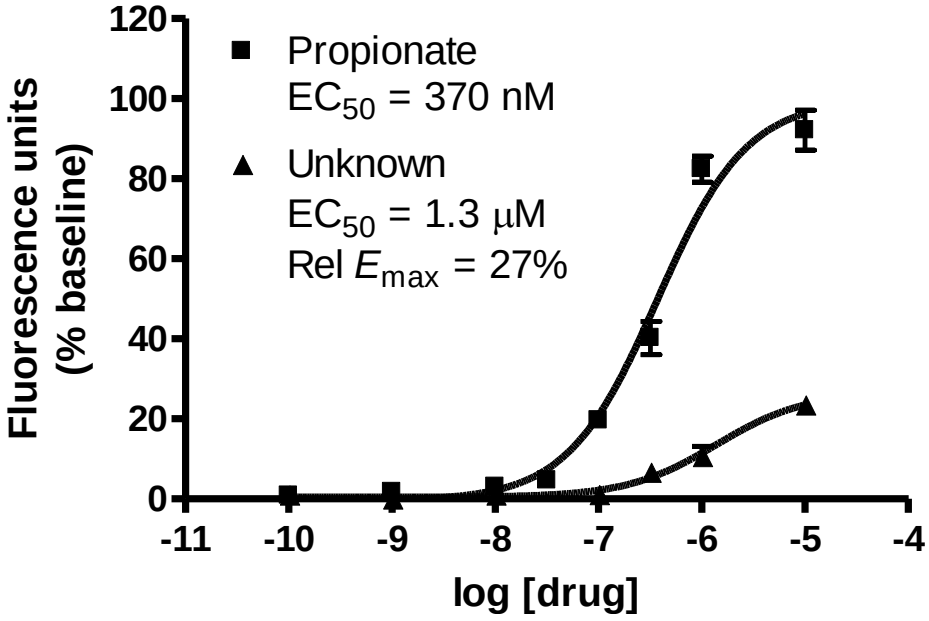
GPR40 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid



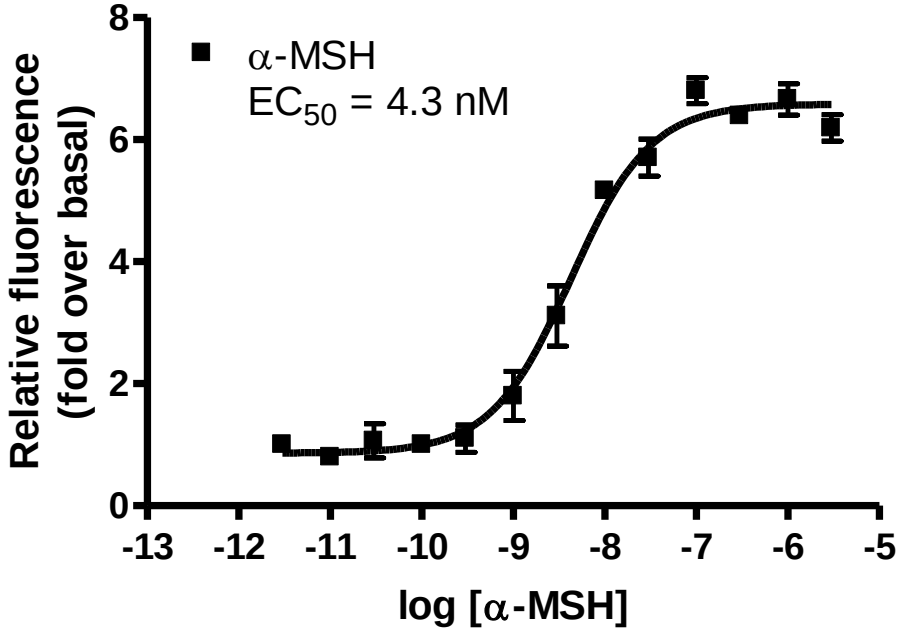
GPR41 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid



GPR43 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid



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



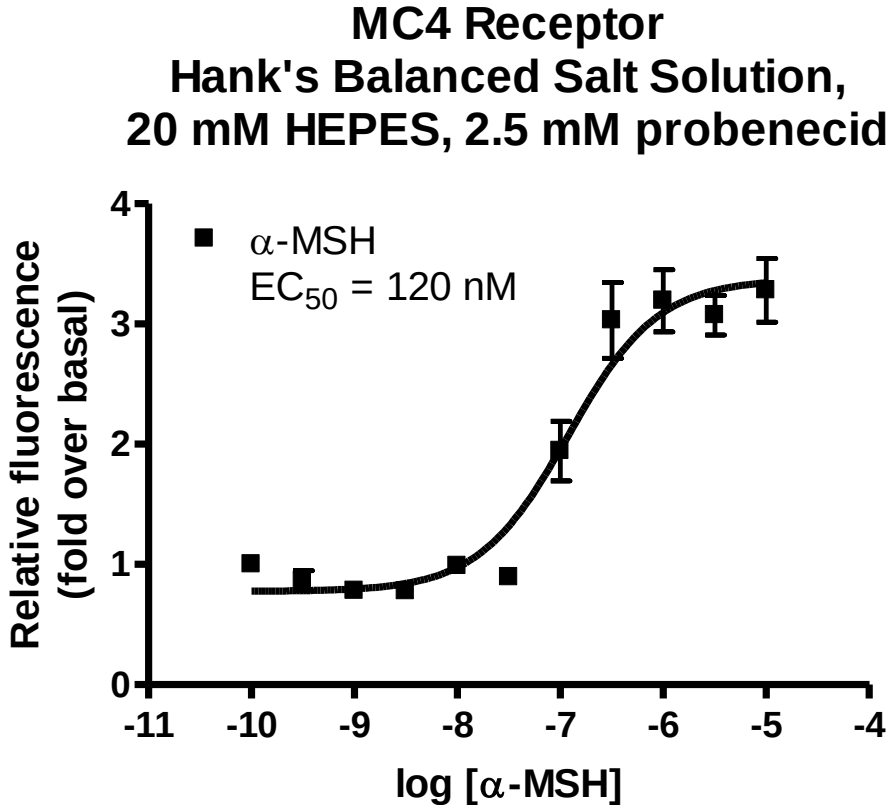
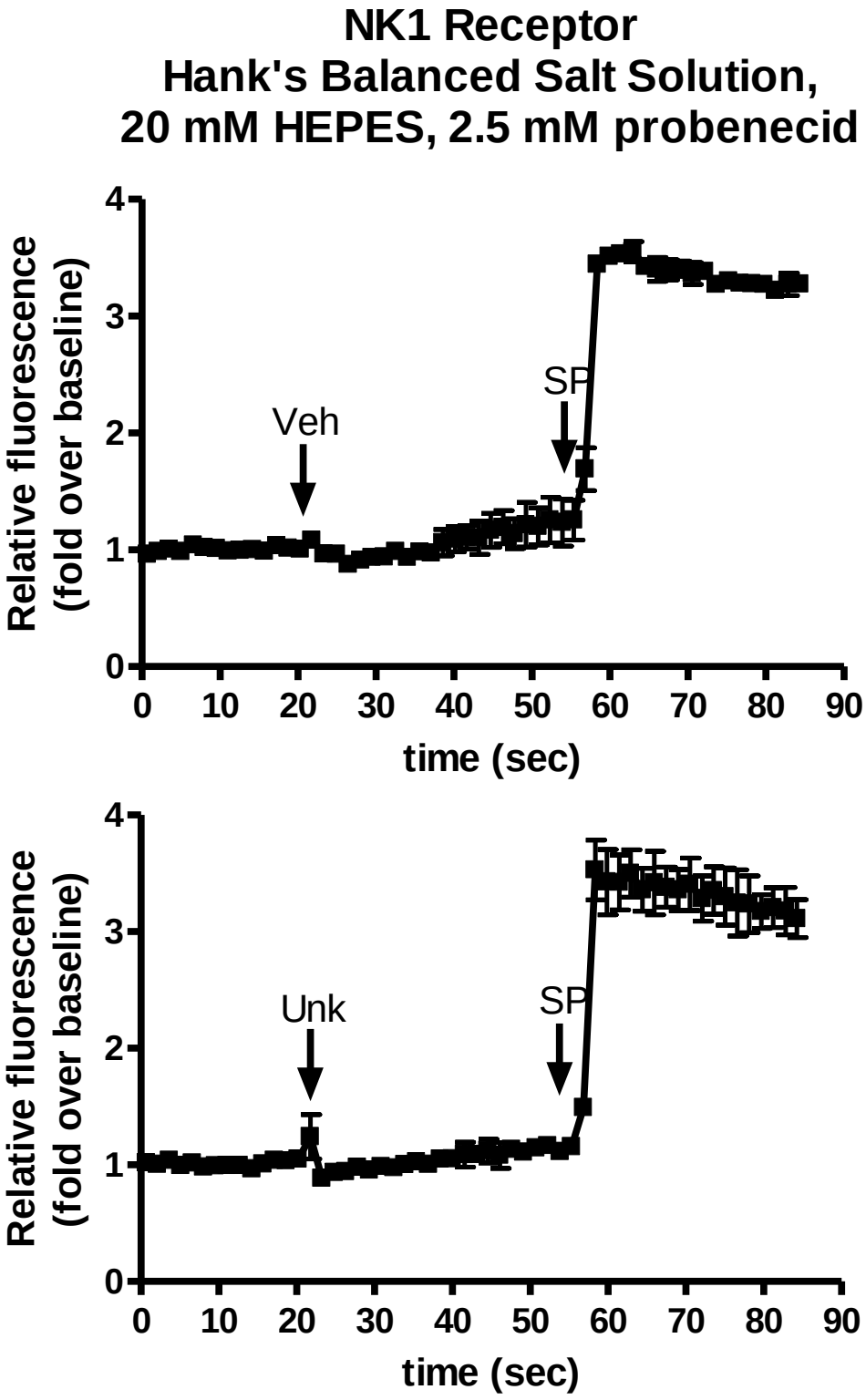
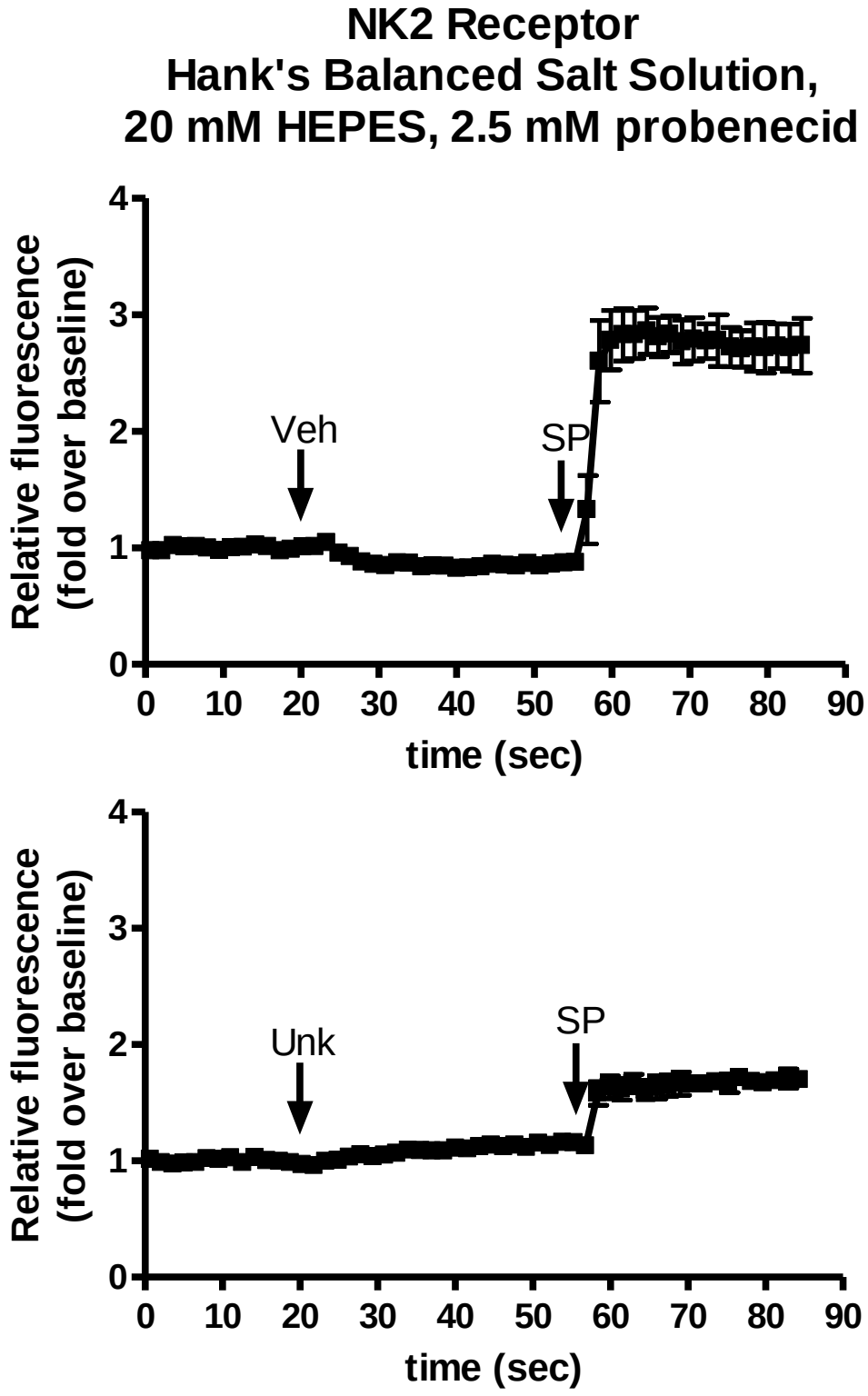
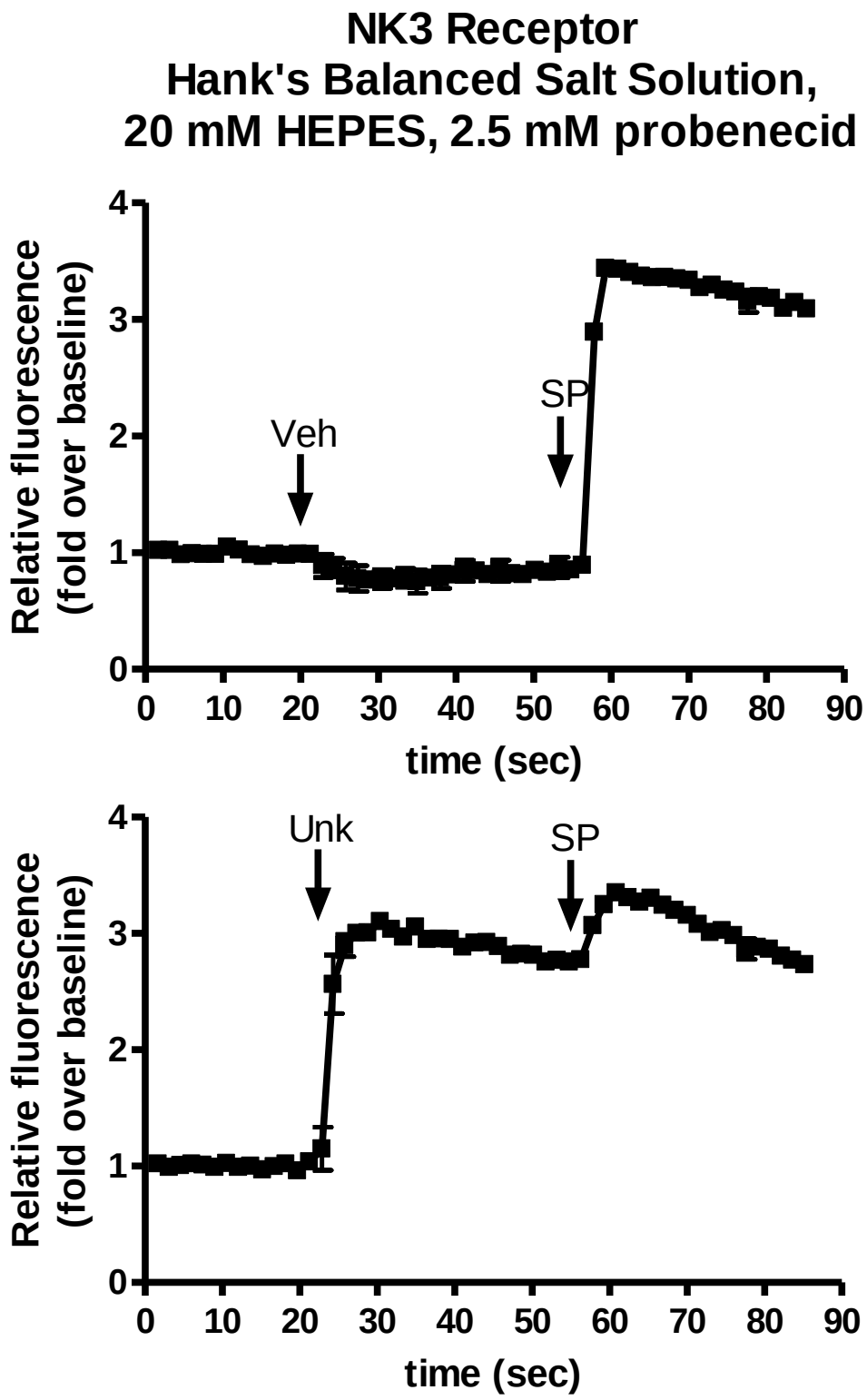
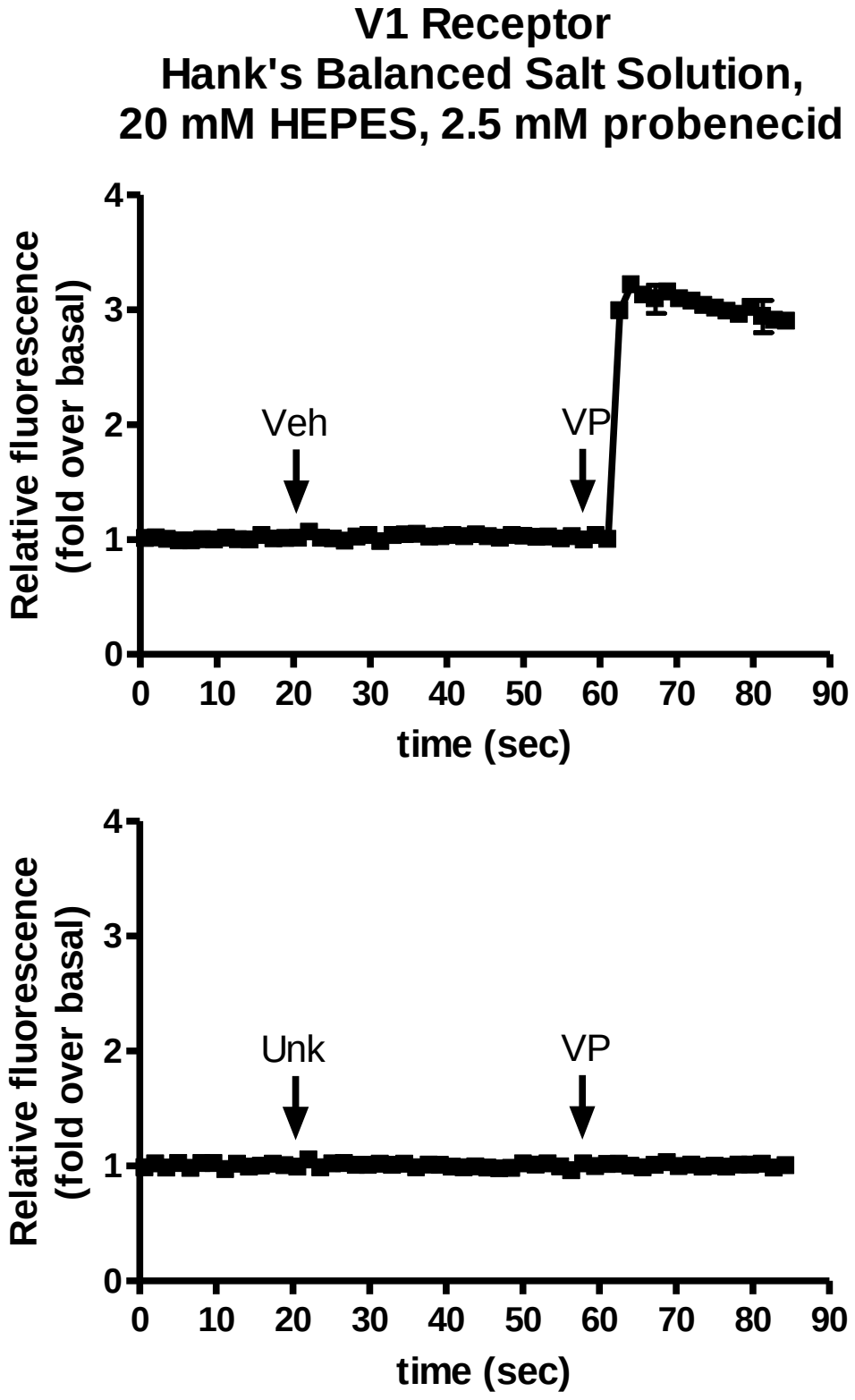

MC4 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
α-MSH
EC50 = 120 nM
Relative fluorescence
(fold over basal)
0
1
2
3
4
-11
-10
-9
-8
-7
-6
-5
-4
log [α-MSH]

Figure 19. Representative primary functional screen results of NIMH PDSP Investigator-submitted unkowns (Unk) assayed against Neurokinin NK1, NK2, and NK3 receptors, and Vasopressin V1 and V2 receptors.




NK2 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence
(fold over baseline)
Veh
SP
time (sec)
Unk
SP
time (sec)


NK3 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence
(fold over baseline)
Veh
SP
time (sec)
Unk
SP
time (sec)


V1 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence
(fold over basal)
Veh
VP
time (sec)
Relative fluorescence
(fold over basal)
Unk
VP
time (sec)

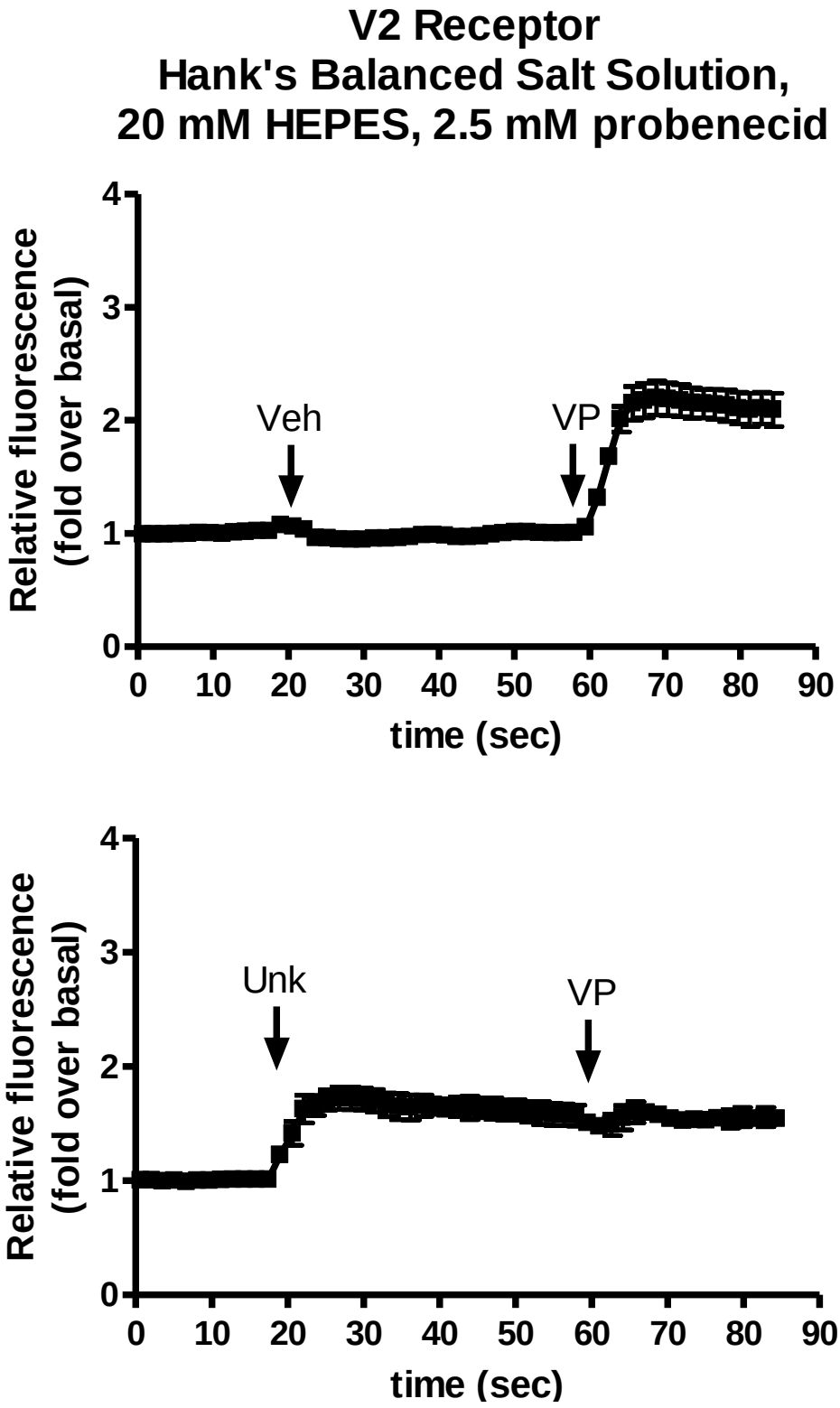
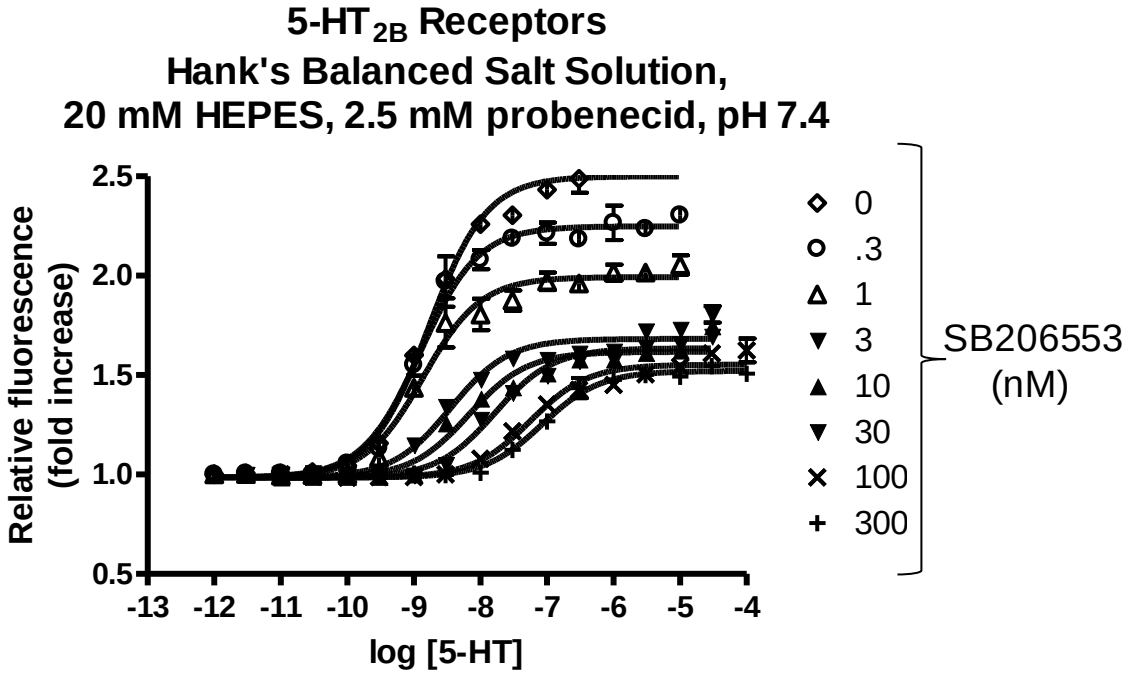
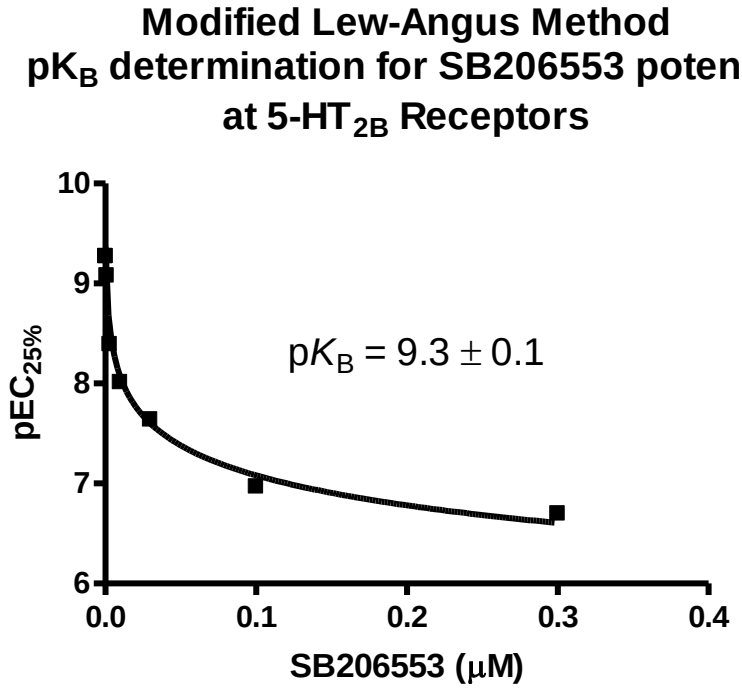

V2 Receptor
Hank's Balanced Salt Solution,
20 mM HEPES, 2.5 mM probenecid
Relative fluorescence
(fold over basal)
Veh
VP
time (sec)
Unk
VP
time (sec)

Figure 20. Representative antagonist potency determination using the modified Lew-Angus method (Christopoulos et al., 1999).





Gq/11/i9-Coupled Targets: intracellular inositol phosphate accumulation

Receptors: Glutamate $mGluR_1$, $mGluR_5$, $mGluR_6$

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)

Protocol adapted from Shi *et al*. *J Pharmacol Exp Ther* 305(1):131-142 (2003).

Experimental Procedure and Data Analysis:

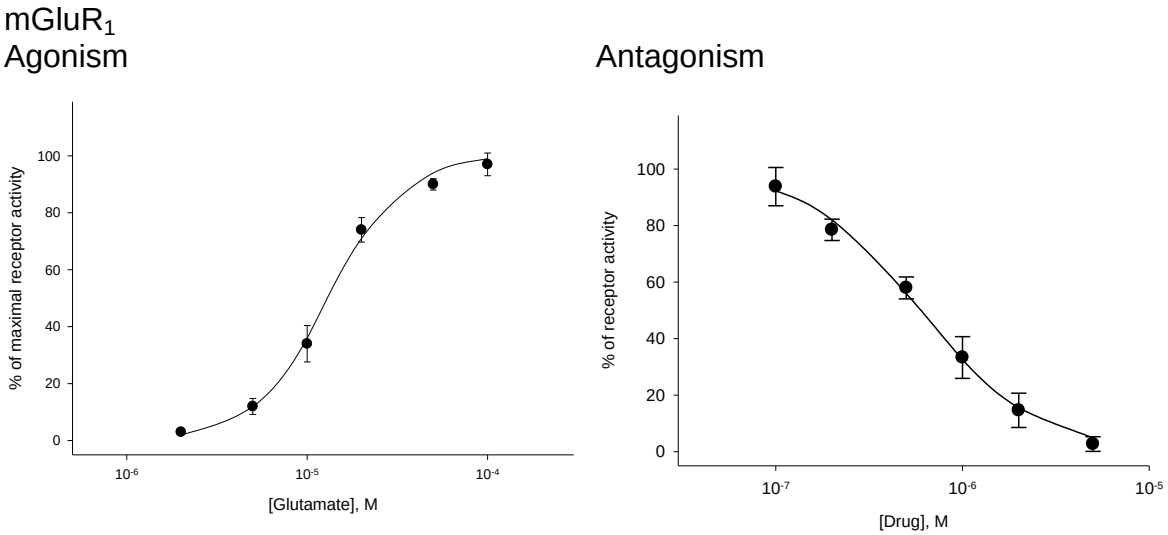
Compounds are tested using cell lines with stable expression of mGluR receptors in functional assays. The signal transduction assays used in these studies fall in two categories. The first is the assay of PI hydrolysis, used for phospholipase C-coupled receptors. Cells, cultured in 96-well plates to confluency (3 to 4 days), are incubated overnight in glutamine-free culture medium supplemented with 0.5 μCi *myo*-[^{3}H]inositol (NEN) to label the cell membrane phosphoinositides. After washing the cultures, incubations with agonist (10 μM glutamate) are carried out for 45 min at 37 degrees centigrade in 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, pH 7.4) containing 20 mM LiCl to block the degradation of inositol phosphates (IPs). The reaction is terminated by aspiration, and IPs are extracted with 0.1 M HCl. The separation of [^{3}H]IPs is performed by anion exchange chromatography. This assay is used with CHO cells expressing $mGluR_1$ and $mGluR_5$ receptors, but also with CHO cell lines expressing $mGluR_8$ receptors. This cell line was created by co-transfection of a chimeric G protein (G_{qi9}) that allows the coupling of these receptors to phospholipase C and, hence, measurements of PI hydrolysis. 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, 15 μM and 5 μM glutamate is used for $mGluR_1$ and $mGluR_5$, respectively, while 0.5 μM 4-aminophosphonobutyrate (AP4) is used for $mGluR_6$.

Primary assays - Single concentration assays. Each new compound is tested on all receptors at a single concentration (to be agreed upon, usually 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 per cent of maximal activity (as obtained with maximal agonist concentrations) and for antagonist as the percent of inhibition of receptor activity

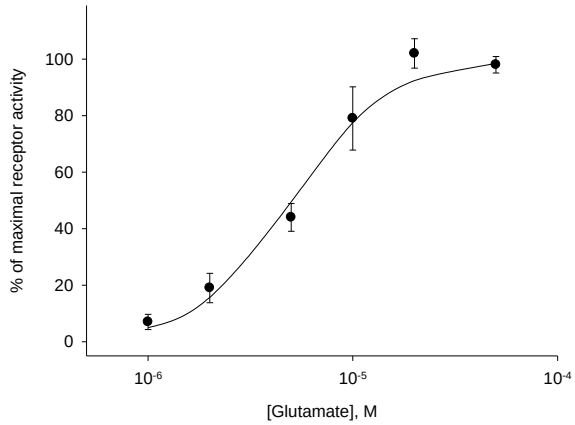
(in presence of an EC_{50} concentration of the agonist). Results are expressed as means ± SEM from four replications.

Secondary assays - Concentration-response assays. Compounds determined to be active as agonists or antagonists may be tested for their potency in concentration-response experiments. Six-point concentration-response curves are 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 replications are averaged and then either EC_{50} or IC_{50} values are calculated by non-linear regression using the 4-parameter logistic equation. Results are reported as EC_{50} or IC_{50} values for each tested compound (and receptor) and also include the EC_{50} or IC_{50} values of a known agonist or antagonist for comparison purposes.

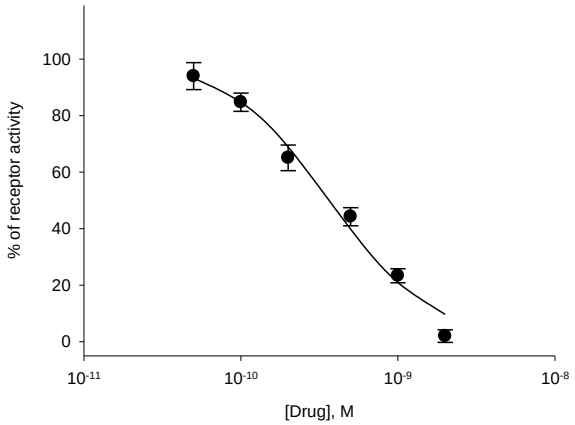
Figure 21. Representative intracellular inositol phosphate accumulation concentration-response isotherms for Gq/11/i9-coupled metabotropic gluatamate receptors.



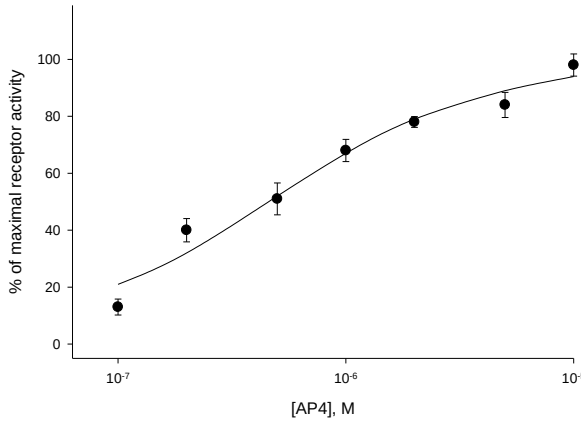
$mGluR_5$
Agonism



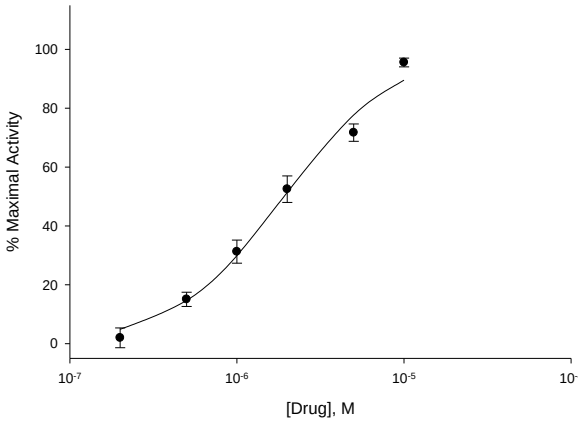
Antagonism



$mGluR_6$
Agonism



Antagonism



Gi-Coupled Targets: inhibition of forskolin-stimulated intracellular cAMP accumulation

Receptors: Glutamate $mGluR_2$, $mGluR_4$, $mGluR_8$

Assay Buffer: IBMX-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, 300 μM IBMX, pH 7.4)

Protocol adapted from Shi *et al*. *J Pharmacol Exp Ther* 305(1):131-142 (2003).

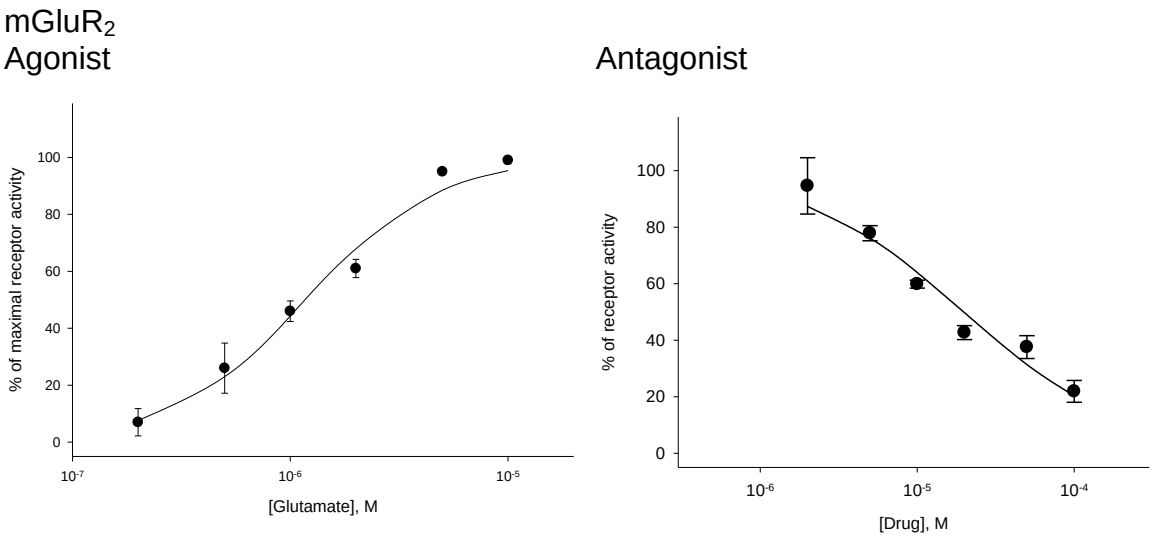
Experimental Procedure and Data Analysis:

The second type of glutamate receptor functional assays consists of measurements of cAMP formation and is used with receptors negatively coupled to adenylyl cyclase. These are $mGluR_2$ and $mGluR_6$ receptors expressed in CHO cells and $mGluR_4$ receptors expressed in BHK cells. The activity of agonists at these receptors is determined by measurements of their ability to decrease the forskolin-induced elevation of cyclic AMP formation. Cells cultured in 96-well culture plates are preincubated for 10 min at 37 degrees centigrade in Locke's medium containing 300 μM isobutylmethylxanthine to inhibit the activity of phosphodiesterases which degrade cAMP. Then 5 μM forskolin is added without or with mGluR agonists, and the incubation is continued for 10 min. After the incubation, the medium is rapidly aspirated and cAMP is extracted with 0.1 M HCl and measured by radioimmunoassay using a magnetic Amerlex RIA kit (Amersham). 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, 1 μM glutamate is used for $mGluR_2$, while 0.5 μM and 1 μM 4-aminophosphonobutyrate (AP4) are used for $mGluR_4$ and $mGluR_8$, respectively.

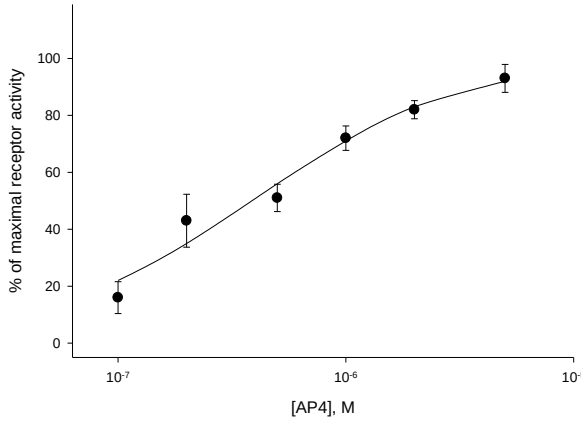
Primary assays - Single concentration assays. Each new compound is tested on all receptors at a single concentration (to be agreed upon, usually 10 μM) for activity as an agonist or an antagonist. Testing for antagonism is performed in presence of the EC_{50} concentration of a typical agonist (as described 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 per cent 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 replications.

Secondary assays - Dose-response assays. Compounds determined to be active as agonists or antagonists may be tested for their potency in dose-response experiments. Six-point dose-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 replications are averaged and then either EC_{50} or IC_{50} values are calculated by non-linear regression using the 4-parameter logistic equation. Results are reported as EC_{50} or IC_{50} values for each tested compound (and receptor) and also include the EC_{50} or IC_{50} values of a known agonist or antagonist for comparison purposes.

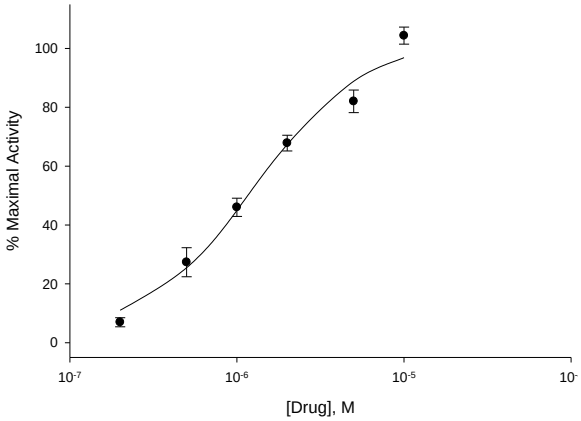
Figure 22. Representative inhibition of forskolin-stimulated intracellular cAMP accumulation concentration-response isotherms for Gi-coupled metabotropic gluatamate receptors.



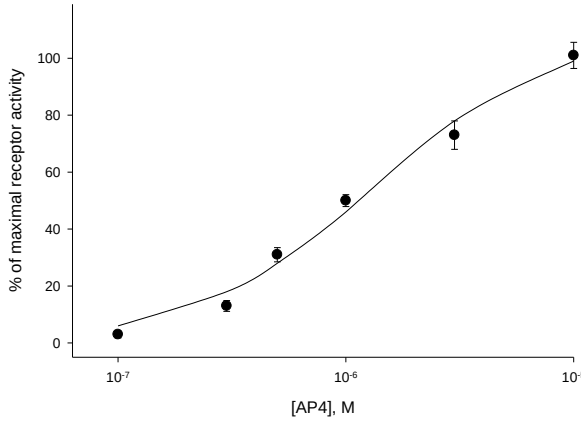
$mGluR_4$
Agonist



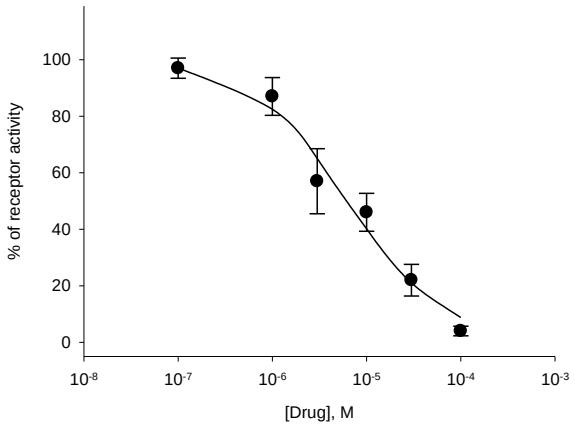
Antagonist



$mGluR_8$
Agonist



Antagonist



Gs-Coupled Targets: intracellular cAMP accumulation

Receptors: Serotonin 5-HT_6, 5-HT_7; Dopamine D_1, D_5; Norepinephrine β_1, β_2, β_3

Assay Buffer: 1X Krebs-Ringer bicarbonate glucose buffer, 0.75 mM IBMX, pH 7.4

Protocol adapted from Jensen *et al*. *Neuropsychopharmacology* 5 Dec (2007).

Experimental Procedure and Data Analysis:

Receptor-expressing cell lines are seeded in 96-well, poly-L-lysine-coated plates 48 hours prior to the assay (40,000 cells per well) in DMEM containing 5% dialyzed serum. Twenty hours prior to the assay, the medium is changed to serum-free DMEM. On the day of the assay, the DMEM is washed and replaced with 30 µl of assay buffer. A 10-min pre-incubation is performed in a 37-degree centigrade, humidified incubator. Then, the cells are stimulated by addition of 30 µl of 2X dilutions of test or reference compound (final concentrations ranging from 0.1 nM to 10 µM, each concentration assayed in triplicate). A positive control (100 µM forskolin) is also included. Accumulation of cAMP is allowed to continue for 15 min, after which the buffer is removed and the cells are lysed with Cell Lysis Buffer (CatchPoint cAMP Assay Kit, Molecular Devices). Next, the lysates are transferred to 96-well, glass-bottom plates coated with goat anti-rabbit IgG and adsorbed with rabbit anti-cAMP (Molecular Devices). Following a 5-min incubation, horseradish peroxidase-cAMP conjugate is added (Molecular Devices) and a 2-hour incubation is performed at room temperature. Then, after three washes with Wash Buffer (Molecular Devices), Stoplight Red substrate (Molecular Devices), reconstituted in Substrate Buffer (Molecular Devices) containing freshly-added 1 mM H_2O_2, is added and, after a 15-min incubation at room temperature, fluorescence is measured (excitation 510-545 nm, emission 565-625 nm). For each assay, a cAMP calibration curve is generated and controls without lysate and without antibody are included.

For agonist tests, raw data (maximum fluorescence, fluorescence units) for each concentration of test compound or reference agonist are normalized to the basal (vehicle-stimulated) fluorescence (reported as fold increase over basal) and plotted as a function of the logarithm of the molar concentration of the drug (*i.e.*, test or reference compound). Non-linear regression of the normalized data is performed in Prism 4.0 (GraphPad Software) using the built-in three parameter logistic model (*i.e.*, sigmoidal concentration-response) describing agonist-stimulated activation of one receptor population:

$$y = bottom + [(top-bottom)/(1 + 10^{x-logEC50})]$$

where bottom equals the best-fit basal fluorescence and top equals the best-fit maximal fluorescence stimulated by the test compound or reference agonist. The

log EC_{50} (*i.e.*, the log of the drug concentration that increases fluorescence by 50% of the maximum fluorescence observed for the test compound or reference agonist) is thus estimated from the data, and the EC_{50} (agonist potency) is obtained. To obtain an estimate of the relative efficacy of the test compound (Rel. E_{max}), its best-fit top is compared to and expressed as a ratio of that for the reference agonist (Rel. E_{max} of the reference agonist is 1.00).

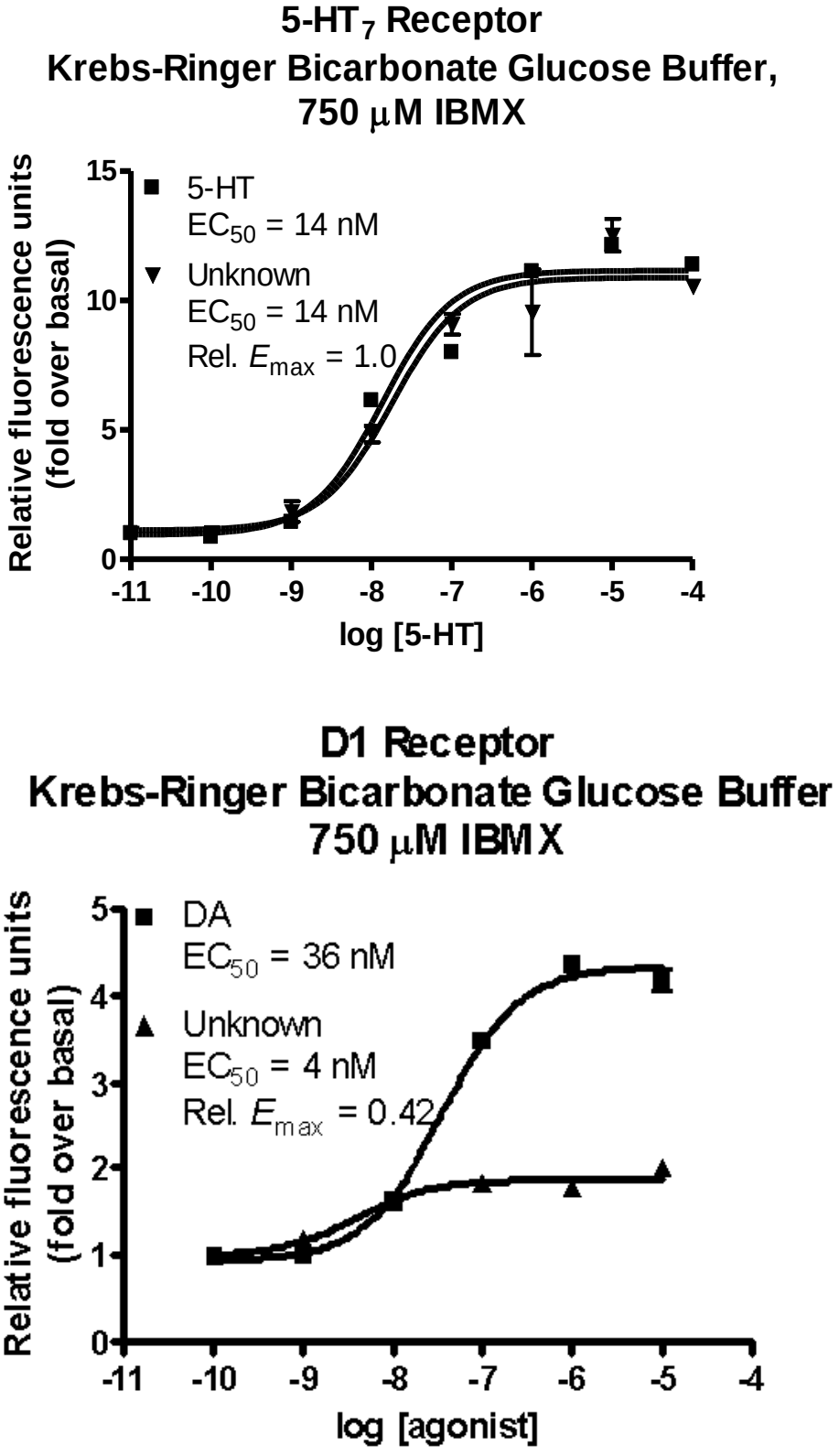
To ascertain whether test compounds are antagonists, a double-addition paradigm similar to that used in calcium mobilization assays is employed. First, 30 µl of test compound (20 µM) is added (10 µM final concentration) and a 15-min incubation is performed. Then, 30 µl of reference agonist (3X; EC_{90}) is added (final concentration of agonist is EC_{30}) and cAMP accumulation is allowed to proceed for 15 min. The samples are then processed for cAMP measurements as detailed above. Measurements of reference agonist-induced cAMP accumulation are compared to the signals elicited by the reference agonist following addition of vehicle instead of test compound and expressed as a ratio. 'Hits' (compounds that antagonize reference agonist-stimulated increases in baseline-normalized fluorescence by at least 50%) are then characterized by a modified Schild analysis.

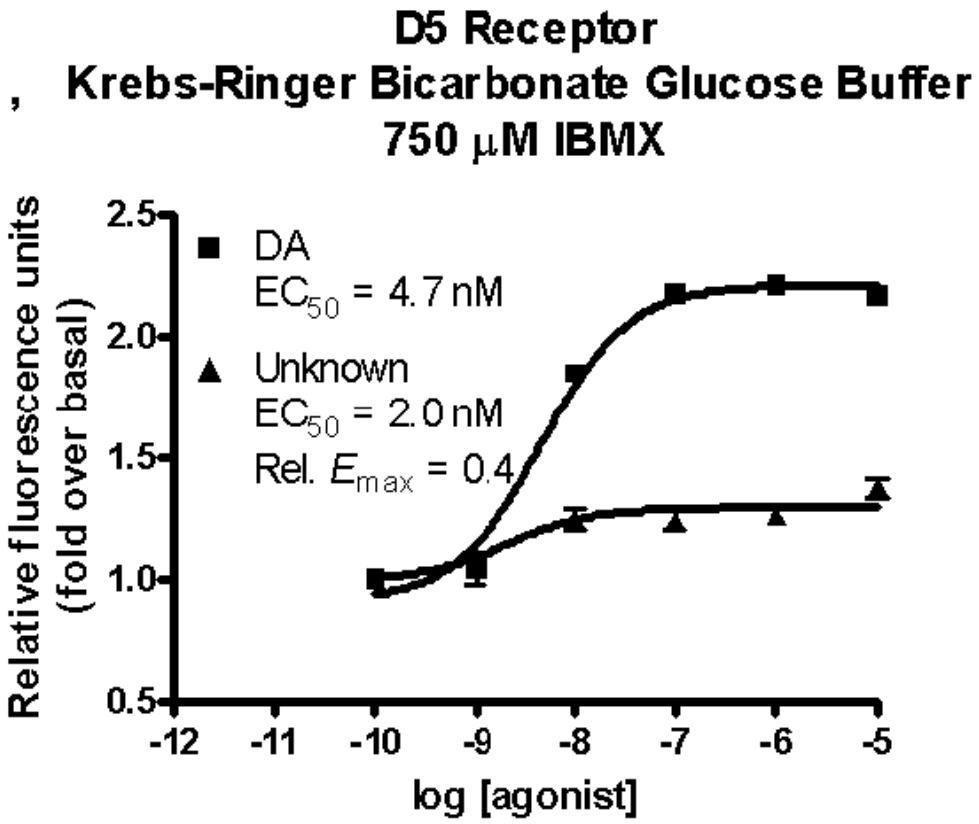
For modified Schild analysis, a family of reference agonist concentration-response isotherms is generated in the absence and presence of graded concentrations of test compound (added 15 min prior to reference agonist). Theoretically, compounds that are competitive antagonists cause a dextral shift of agonist concentration-response isotherms without reducing the maximum response to agonist (i.e., surmountable antagonism). However, on occasion, factors such as non-competitive antagonism, hemiequilibria, and/or receptor reserve cause apparent insurmountable antagonism. To account for such deviations, we apply the modified Lew-Angus method to ascertain antagonist potency (Christopoulos et al., 1999). Briefly, equieffective concentrations of agonist (concentrations of agonist that elicit a response equal to the $EC_{25\%}$ of the agonist control curve) are plotted as a function of the test compound concentration present in the wells in which they were measured. Non-linear regression of the baseline-normalized data is performed in Prism 4.0 using the following equation:

$$pEC_{25\%} = -\log([B] + 10^{-pK}) - \log c$$

where $EC_{25\%}$ equals the concentration of agonist that elicits a response equal to 25% of the maximum agonist control curve response and [B] equals the antagonist concentration; K, c, and s are fit parameters. The parameter s is equal to the Schild slope factor. If s is not significantly different from unity, pK equals pK_B; otherwise, pA_2 is calculated (pA_2 = pK/s). The parameter c equals the ratio $EC_{25\%}$/[B].

Figure 23. Representative cAMP accumulation isotherms for reference compounds and NIMH-PDSP investigator-submitted unknowns at selected Gs-coupled receptors available for screening.




D5 Receptor
, Krebs-Ringer Bicarbonate Glucose Buffer
750 μM IBMX
Relative fluorescence units
(fold over basal)
2.5
2.0
1.5
1.0
0.5
DA
EC50 = 4.7 nM
Unknown
EC50 = 2.0 nM
Rel. Emax = 0.4
-12
-11
-10
-9
-8
-7
-6
-5
log [agonist]

Monoamine transporters: inhibition of [^{3}H]monoamine uptake

Transporters: Serotonin 5-HT transporter (SERT); Dopamine transporter (DAT); Norepinephrine transporter (NET)

Assay Buffer: 1X Krebs-Ringer HEPES glucose buffer, 0.1 mM ascorbate, 0.1 mM pargyline, 0.1 mM tropolone (DAT and NET assays only), pH 7.4

Protocol adapted from Jensen *et al*. *Neuropsychopharmacology* 5 Dec (2007).

Experimental Procedure and Data Analysis:

Transporter-expressing cell lines are seeded in 96-well, poly-L-lysine-coated plates 48 hours prior to the assay (40,000 cells per well) in DMEM containing 5% dialyzed serum. Twenty hours prior to the assay, the medium is changed to serum-free DMEM. On the day of the assay, the DMEM is washed and replaced with 30 µl of assay buffer containing vehicle or dilutions of test or reference compound (final concentrations ranging from 0.1 nM to 10 µM, each concentration assayed in triplicate). A 10-min pre-incubation is performed in a 37-degree centigrade, humidified incubator. Then, monoamine transport is initiated by addition of 30 µl of 2X [^{3}H]monoamine, the specific activity of which is diluted 10- to 50-fold with non-labeled monoamine (final assay concentration of [^{3}H]monoamine is 1 µM). Uptake of [^{3}H]monoamine is allowed to proceed for 4 min, after which the buffer is aspirated and the cells are washed three times with ice-cold buffer containing 10 µM paroxetine (for SERT), GBR12909 (for DAT), or nisoxetine (for NET). Then, polystyrene-compatible scintillation cocktail (Microscint PS, PerkinElmer; 50 µl/well) is added and the plates are sealed and agitated on an orbital shaker at a high setting for 5 min. The plates are then counted in a Wallac MicroBeta TriLux scintillation counter.

Raw data (dpm) representing total [^{3}H]monoamine uptake (*i.e.*, specific + non-specific uptake) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.*, test or reference compound). Non-linear regression of the normalized (*i.e.*, percent of [^{3}H]monoamine uptake relative to that observed in the absence of test or reference compound) raw data is performed in Prism 4.0 using the built-in three parameter logistic model describing competitive inhibition (one-site):

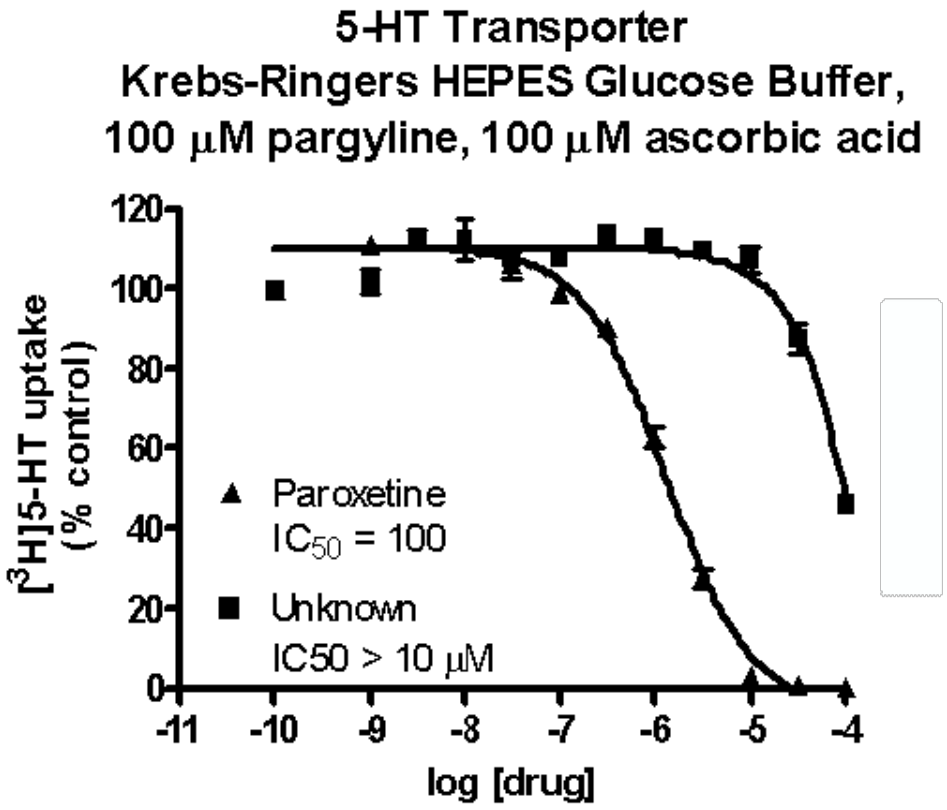
$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10^{x-\log IC50})]$$

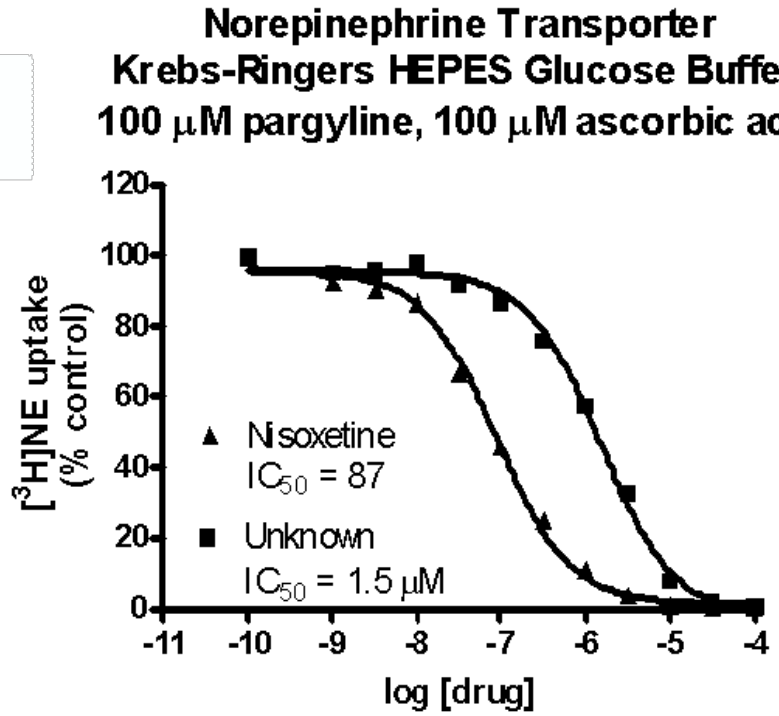
where bottom equals the best-fit non-specific [^{3}H]monoamine uptake (*i.e.*, non-specific uptake) and top equals the best-fit total [^{3}H]monoamine uptake (*i.e.*, uptake absent any competitor). The log IC_{50} (*i.e.*, the log of the test or reference compound concentration that reduces [^{3}H]monoamine uptake binding by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

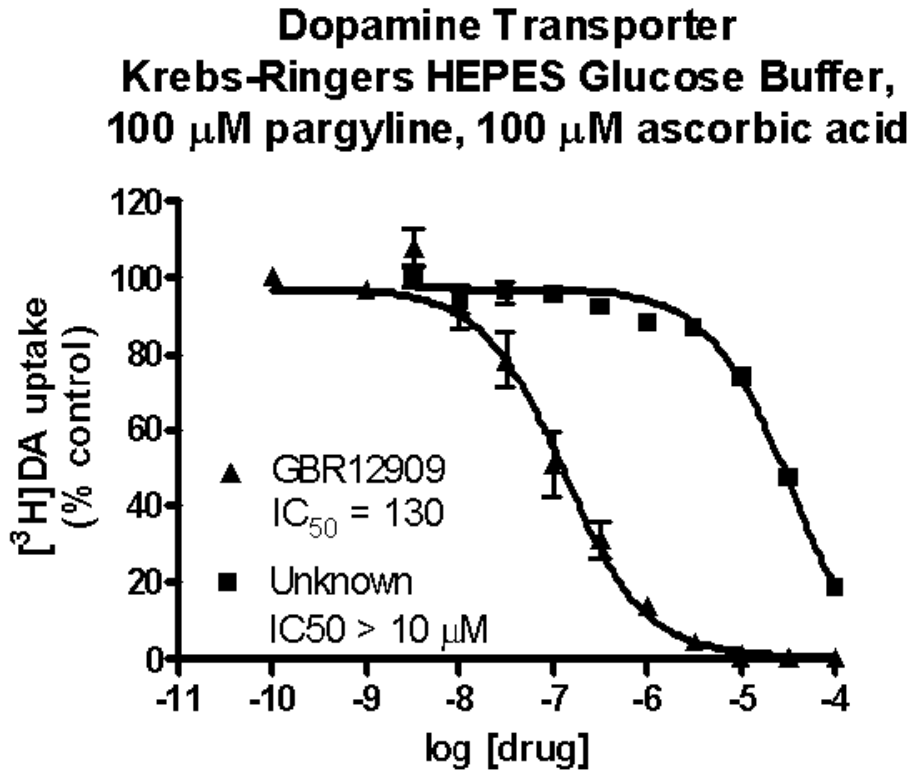
$$K_i = IC_{50}/(1 + [monoamine]/K_M)$$

where [monoamine] equals the assay [^{3}H]monoamine concentration and K_M equals the affinity constant of the monoamine for the transporter.

Figure 24. Representative [^{3}H]monoamine uptake inhibition isotherms for reference compounds and NIMH-PDSP investigator-submitted unknowns.




Norepinephrine Transporter
Krebs-Ringers HEPES Glucose Buffer,
100 μM pargyline, 100 μM ascorbic acid
[3H]NE uptake
(% control)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
Nisoxetine
IC50 = 87
Unknown
IC50 = 1.5 μM


Dopamine Transporter
Krebs-Ringers HEPES Glucose Buffer,
100 μM pargyline, 100 μM ascorbic acid
[3H]DA uptake
(% control)
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
log [drug]
GBR12909
IC50 = 130
Unknown
IC50 > 10 μM

Other functional assays

Histone Deacetylase (HDAC) Inhibition Assay

Assay Buffer: HDAC Assay Buffer (50 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, **pH 8.0**)

Protocol adapted from BioMol Fluor de Lys product insert.

Experimental Procedure and Data Analysis:

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

Raw data (RFUs) representing deacetylated substrate fluorescence are plotted as a function of the logarithm of the molar concentration of the test or reference compound. Non-linear regression of the normalized (to the fluorescence measured in the absence of HDAC inhibitor and test compound) raw data is performed in Prism 4.0 using the built-in three parameter logistic model describing competitive inhibition (one-site):

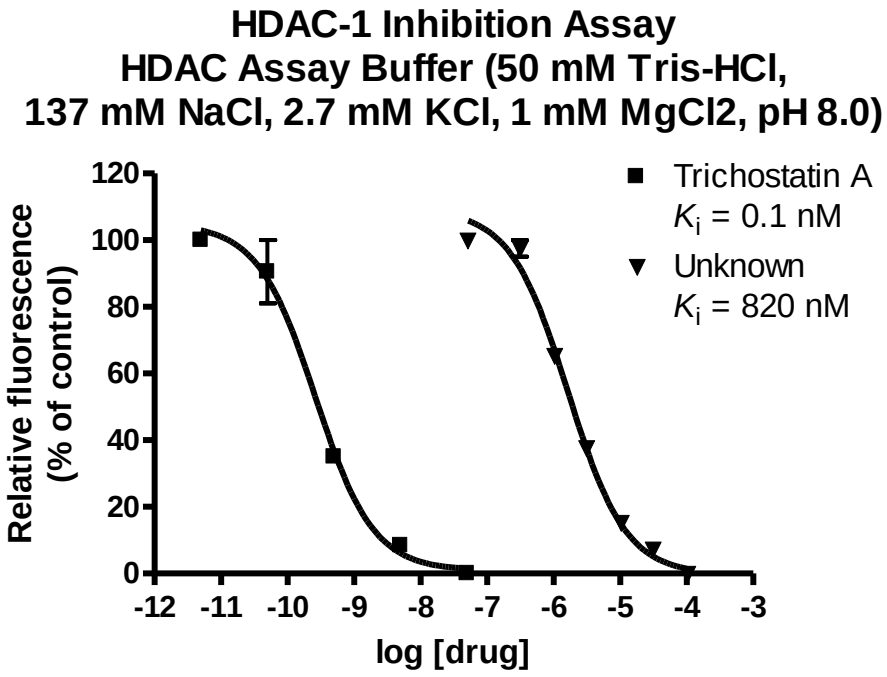
$$y = bottom + [(top-bottom)/(1 + 10^{x-logIC50})]$$

where bottom equals the best-fit baseline fluorescence (*i.e.*, maximal inhibition of HDAC) and top equals the best-fit maximum fluorescence (*i.e.*, maximum HDAC activity). The log IC_{50} (*i.e.*, the log of the test or reference compound concentration that reduces product fluorescence by 50%) is thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation:

$$K_i = IC_{50}/(1 + [substrate]/K_M)$$

where [substrate] equals the assay concentration of the substrate and K_M equals the affinity constant of the substrate for HDAC.

Figure 25. Representative HDAC inhibition isotherms for trichostatin A and an NIMH-PDSP investigator-submitted unknown.



HERG K^+ Channel Assay

Assay Buffer: Hank's Balanced Salt Solution, 20 mM HEPES, 2.5 mM probenecid, pH 7.4, membrane potential sensitive dye (Molecular Devices)

Protocol adapted from PubChem BioAssay ID 316 (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=376).

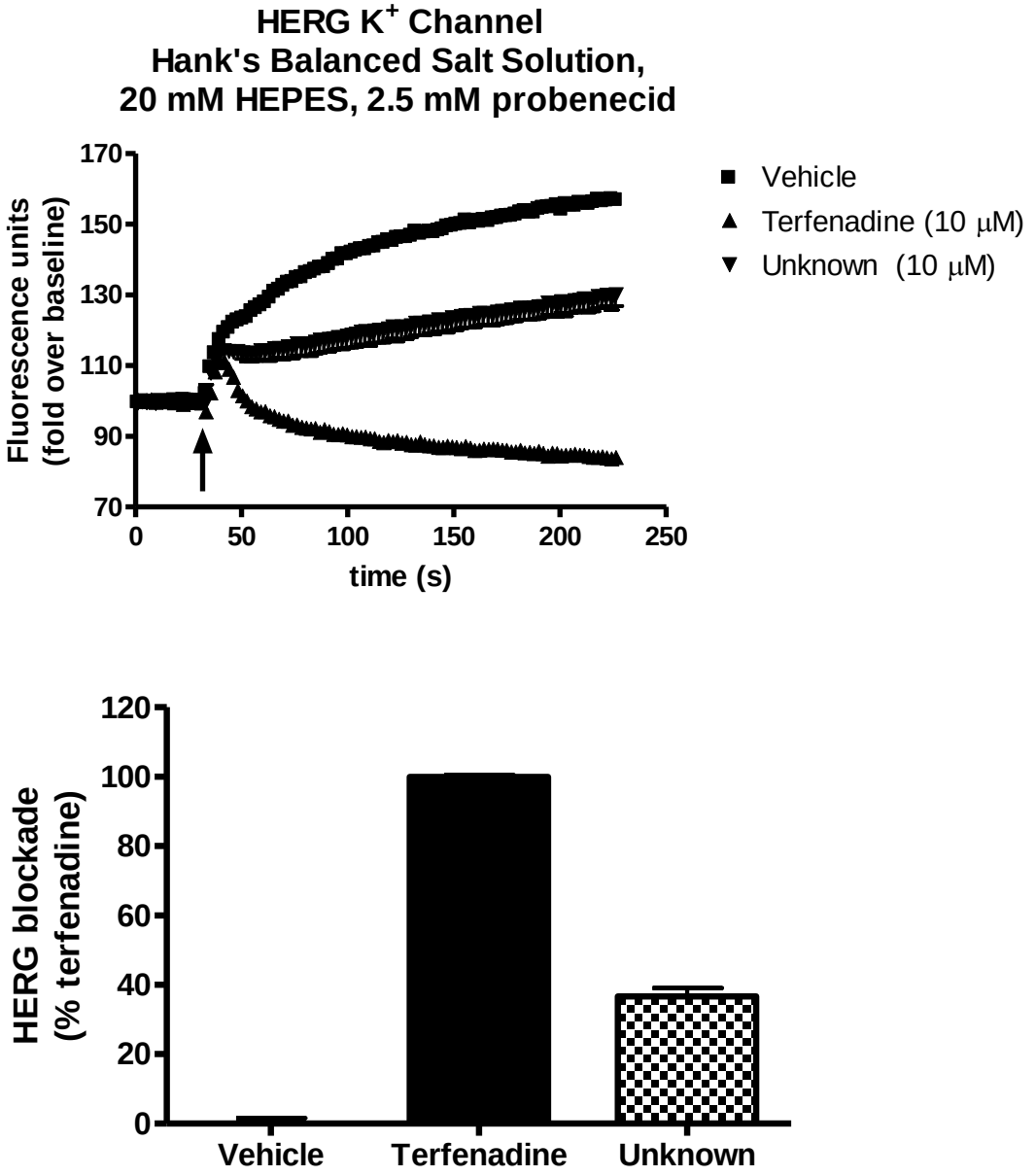
To screen for drugs that block or open HERG K^+ channels, we employ a fluorescence-based membrane potential assay (Molecular Devices). HEK293 cells stably expressing recombinant human HERG (provided by Dr. J. Overholt, Case Western Reserve Univeristy, Cleveland, OH; cells originally from Drs. A. Brown and E. Ficker, MetroHealth Medical Center, Cleveland, OH) are seeded in poly-L-lysine-coated 96-well plates (45,000 cells/100 µl DMEM supplemented with 10% fetal bovine serum/well) one day prior to assay. The next day, the medium is removed and replaced with 30 µl/well of assay buffer containing membrane potential dye (Molecular Devices) (the lyophilized dye is reconstituted with 15 ml of assay buffer). After a 15 min incubation at 37 degrees centigrade, 30 µl/well of 2X dilutions of terfenadine (a known HERG blocker used as a reference compound) or test compound (final assay concentration ranging from 0.1 nM to 10 µM) are added to the cells (each concentration assayed in triplicate). Baseline fluorescence (excitation 530 nM, emission 565 nM) is measured over 15 min, then 140 µl of depolarization solution (143 mM KCl in distilled water) containing test or reference compound (1x) are added to the cells and fluorescence is recorded for 3 min.

Raw fluorescence data are exported to GraphPad Prism 4.0 for further analysis. The last data value measured in each well (i.e., 3 minutes after addition of depolarization solution) is used for analysis, after subtraction of the mean background value obtained in the first 30 seconds before depolarization of the cells. For negative controls, the value obtained using assay buffer alone is defined as 0% HERG blockade, and for positive controls, the value obtained using 10 µM terfenadine is defined as 100% HERG blockade. Negative and positive terfenadine controls are done on each plate. Data from test compounds are scaled as follows:

% HERG blockade for test compound = 100 x (value for test compound - value for negative control)/(value for 10 µM terfenadine - value for negative control)

"Hits" (or "blockers") are defined as those compounds with 20% or greater HERG blockade. HERG "openers" are defined as those compounds with values for HERG blockade < -20%.

Figure 26. Representative raw data showing K^+-induced (50 mM K^+ final) depolarization in HERG-HEK293 cells pretreated with vehicle, terfenadine (10 µM), or an NIMH PDSP investigator-submitted unknown (10 µM) (top panel). Percent blockade (bottom panel) was calculated as described above.



Unknown is a HERG blocker exhibiting 37 ± 5 % blockade relative to terfenadine.

$^{86}Rb^{+}$ Efflux Assay (α3β4, α4β2 Nicotinic Receptors)

Assay 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

Protocol adapted from Xiao *et al*. *Mol Pharm* 54(2):322-333 (1998).

Agonist and antagonist activities of reference and test compounds are assayed using cells stably expressing on either the α3β4 or the α4β2 receptor subtype (Xiao et al., 1998; Xiao et al., 2006). Cells are grown as described previously (Xiao et al., 1998) in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.7 mg/ml of Geneticin (G418) at 37^{o}C with 5% CO_2 in a humidified incubator. In brief, receptor subtype-expressing cells are seeded into 24-well plates (approximately 100,000 cells per well) coated with poly-D-lysine. The plated cells are grown at 37 degrees centigrade for 18 to 24 so as to reach 70% to 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^{o}C. The loading mixture is then aspirated, and the cells are washed four times with assay buffer. Next, 1 ml of buffer, with or without dilutions (final assay concentrations typically rage from 1 nM to 300 µM) of test or reference [(-)-nicotine] compounds, is added to each well. For antagonist assays, 100 µM (-)-nicotine is added to the cells along with each dilution (final assay concentrations typically rage from 1 nM to 300 µM) of test and reference (mecamylamine) compounds. After incubation for 2 min, the assay buffer is collected and the amount of $^{86}Rb^{+}$ in the buffer is determined by liquid scintillation counting. $^{86}Rb^{+}$ remaining in the cells is also measured: cells are lysed in 1 ml of 100 mM NaOH per well, then the lysate is collected and its $^{86}Rb^{+}$ content is determined by liquid scintillation counting. For the cells in each well, the total amount of $^{86}Rb^{+}$ loaded (cpm) is calculated as the sum of the $^{86}Rb^{+}$ content in the assay supernatant and in the lysate. $^{86}Rb^{+}$ efflux into the assay supernatant is expressed as a percentage of the total $^{86}Rb^{+}$ loaded (i.e., supernatant $^{86}Rb^{+}$ content plus lysate $^{86}Rb^{+}$ content). Maximal stimulated $^{86}Rb^{+}$ efflux is then defined as the efflux in the presence of 100 µM (-)-nicotine (adjusted to 100%); basal efflux is that measured in the absence of agonist (adjusted to 0%). Typically, basal $^{86}Rb^{+}$ efflux ranges from 3% to 6%, and maximal stimulated efflux is approximately 45%, of the total amount of $^{86}Rb^{+}$ loaded.

For agonists, baseline- and nicotine-normalized data [% $^{86}Rb^{+}$ effux, with baseline efflux being 0% and 100 µM (-)-nicotine-stimulated efflux being 100%] for each concentration of test compound or (-)-nicotine are plotted as a function of the logarithm of the molar concentration of the drug. Non-linear regression of the normalized data is performed in Prism 4.0 (GraphPad Software) using the built-in three parameter logistic model (*i.e.*, sigmoidal concentration-response) describing agonist-stimulated activation of one receptor population:

$$y = bottom + [(top-bottom)/(1 + 10^{x-logEC50})]$$

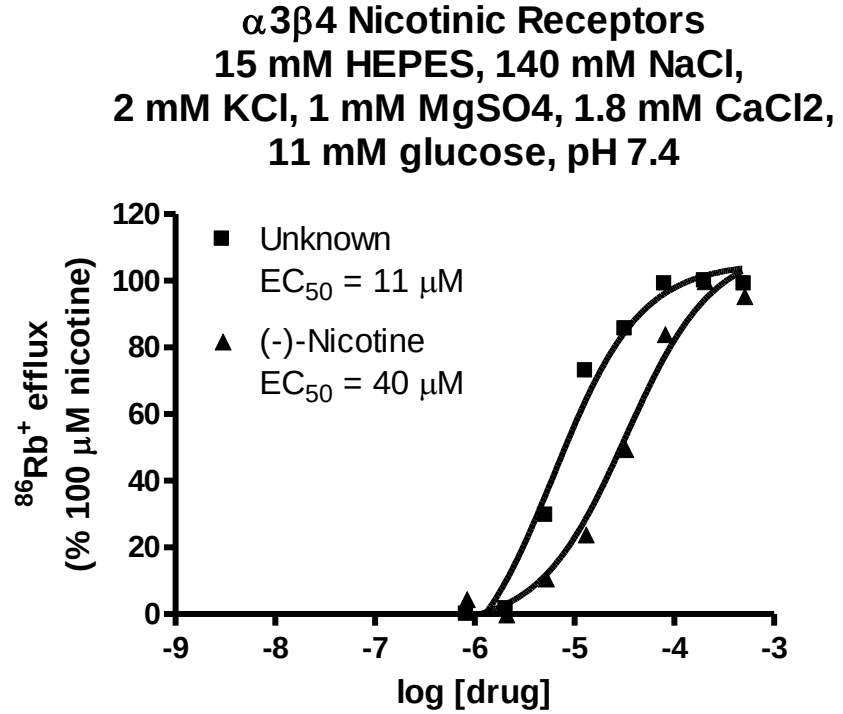
where bottom equals the basal $^{86}Rb^{+}$ efflux (0%) and top equals the maximum $^{86}Rb^{+}$ efflux stimulated by (-)-nicotine (100%). The log EC_{50} [*i.e.*, the log of the drug concentration that increases $^{86}Rb^{+}$ efflux by 50% of the maximum value observed for the test compound or (-)-nicotine] is thus estimated from the data, and the EC_{50} (agonist potency) is obtained. To obtain an estimate of the relative efficacy of the test compound (Rel. E_{max}), its best-fit top is compared to and expressed as a ratio of that for (-)-nicotine (Rel. E_{max} of the reference agonist is 1.00).

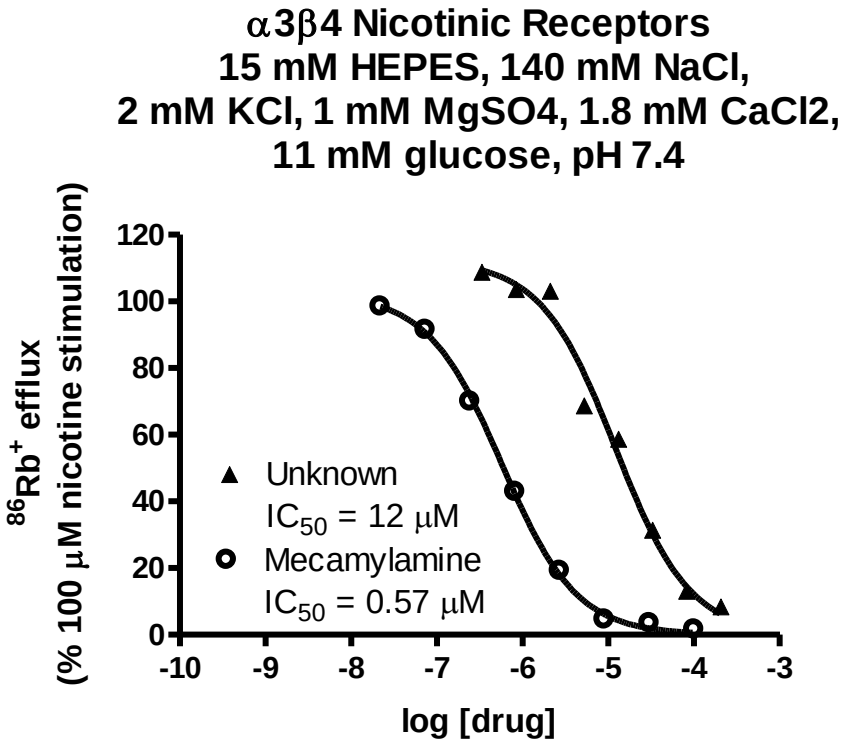
For antagonists, non-specific $^{86}Rb^{+}$ efflux is measured in the presence of 100 µM mecamylamine and is set to 0%; non-specific (-)-nicotine-stimulated $^{86}Rb^{+}$ efflux (i.e., efflux in the absence of antagonist) is set to 100%. Normalized data for each concentration of test compound or mecamylamine are plotted as a function of the logarithm of the molar concentration of the drug. Non-linear regression of the normalized data is performed in Prism 4.0 (GraphPad Software) using the built-in three parameter logistic model (*i.e.*, sigmoidal concentration-response) describing agonist-stimulated activation of one receptor population:

$$y = bottom + [(top-bottom)/(1 + 10^{x-logIC50})]$$

where bottom equals the basal $^{86}Rb^{+}$ efflux (0%) and top equals the $^{86}Rb^{+}$ efflux stimulated by (-)-nicotine (100%). The log IC_{50} [*i.e.*, the log of the drug concentration that decreases (-)-nicotine-stimulated $^{86}Rb^{+}$ efflux by 50% of the control value (i.e., that measured in the absence of antagonist) is thus estimated from the data, and the IC_{50} (antagonist potency) is obtained.

Figure 27. Representative $^{86}Rb^{+}$ efflux concentration-response isotherms for reference compounds and NIMH PDSP investigator-submitted unknowns are shown. Top panel: a representative agonist assay; bottom panel: a representative antagonist assay.





Multidrug Resistance Transporter (MDR)

Assay Buffer: Dulbecco's PBS, 10 mM glucose

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

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. Moreover, calcein is highly fluorescent, but calcein-AM is not.

The assay is based upon the principal that calcein-AM, but not free calcein, is transported from cells by MDR. In cells that express MDR, lipophilic calcein-AM that crosses the plasma membrane will be pumped from the cells before it can be converted to calcein and 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.

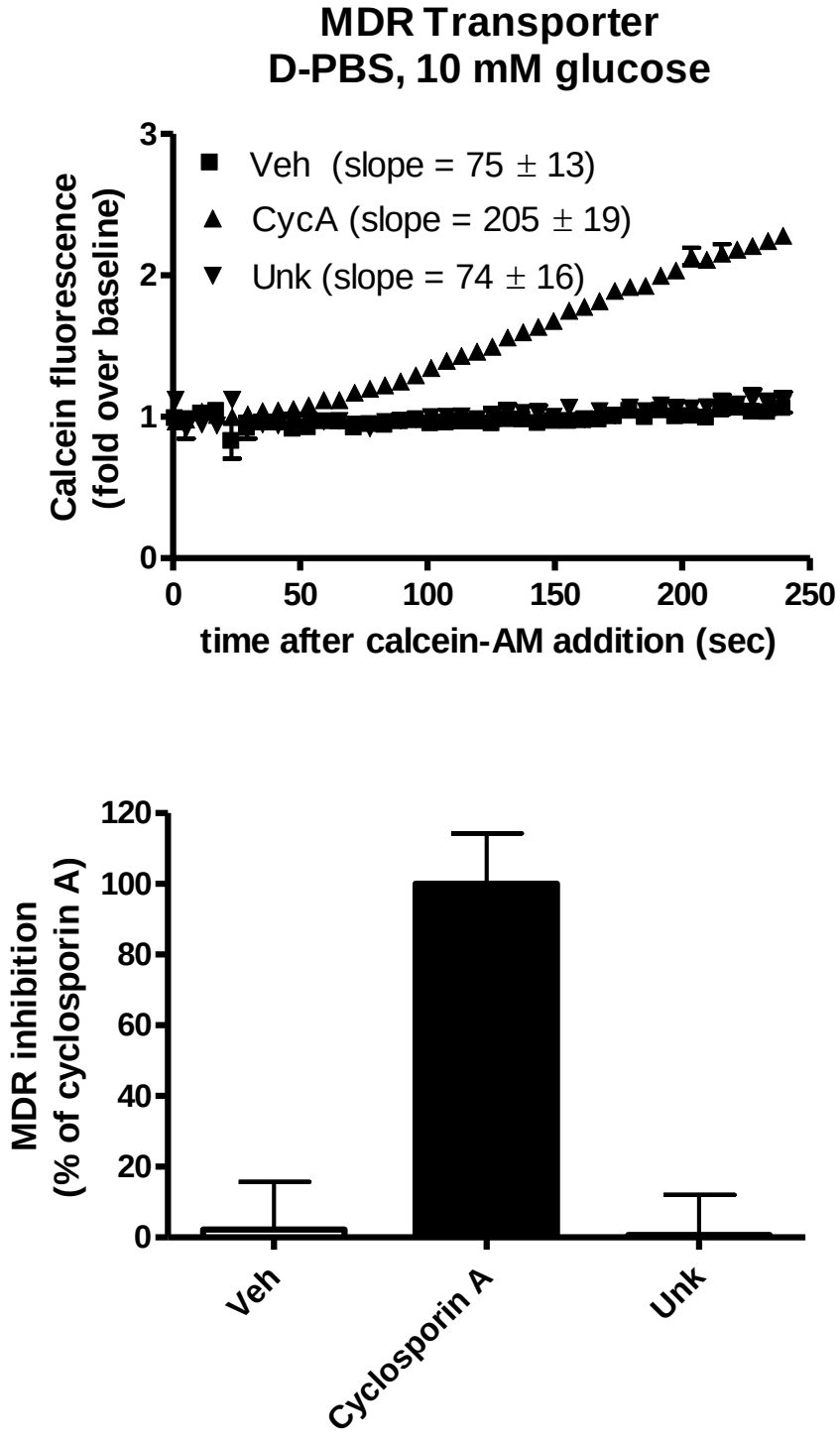
The assay monitors the time-dependent increase in calcein fluorescence in live cells in 96 well plates. This is carried out using a FlexStation II fluorimeter (Molecular Devices). Cells are seeded into glass-bottom 96-well plates one day before assay (80,000 cells per well). On the day of the assay,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

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

Similarly, the assay provides a means for assessing not only interactions with MDR but also partitioning across cell membranes and thus hydrophobicity.

Although this assay is excellent for initial screening, users should be aware that the assay has several drawbacks. i) The assay does not distinguish between MDR substrates and inhibitors. Both will give similar signals in the assay because they prevent the transport of calcein-AM. ii) Some compounds may give spurious results by inhibiting the esterases that convert calcein-AM to calcein. Finally (iii) activity depends on the cytoplasmic concentration of the compounds. For a compound that is an MDR substrate, this concentration depends on the rate of diffusion across the plasma membrane and the rate at which MDR pumps the compound 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.

Figure 28. Representative data from an MDR inhibition assay, wherein a reference compound (cyclosporin A) and a NIMH PDSP investigator-submitted unknown were assayed in parallel (25 µM final concentration).



IV. Membrane Fraction Preparation

The majority of assays performed by the NIMH PDSP staff employ transfected (primarly stably transfected) cell lines expressing mainly human recombinant receptors, monoamine transporters, or ion channels (ligand-gated and otherwise). A detailed description of the cell lines we use, the culture and sub-culture conditions, the appropriate media, etc. has been prepared by Ms. Hufeisen and can be found in the body of the Statement of Work. What follows here will be a detailed description of the processing of cells from the incubator to the wells of the assay plate.

For binding assays using stably transfected cell lines, cells seeded (using 5% dialyzed fetal bovine serum-supplemented medium) in 10-cm dishes (90% confluent) are incubated overnight in either serum-free medium or medium containing 1% dialyzed fetal bovine serum. The next day, the cells are scraped into the medium and pelleted by centrifugation (1000 x *g*, 10 min). The cell pellet is resuspended in chilled 50 mM Tris, pH 7.4 (4 degrees) and triturated gently with a P1000 pipette tip to effect hypotonic lysis. The suspension is then centrifuged at 21,000 x *g* for 20 min to yield a crude membrane fraction pellet. The 50 mM Tris is removed and cells are either frozen on dry ice and maintained at -80 degrees centigrade until assay or resuspended immediately in a desired volume of appropriate assay buffer.

For binding assays using transiently transfected cell lines, cells are transfected as described in the Statement of Work. Twenty-four hours after transfection, the cells are split at an appropriate ratio (typically 1:2 or 1:3) into 10-cm dishes using 5% dialyzed fetal bovine serum-supplemented medium. Then, after overnight growth (generally 90% confluence is reached), the cells are incubated overnight in medium supplemented with no or 1% dialyzed fetal bovine serum. The next day (72 hours after transfection), the cells are scraped and processed as described for stable transfectants.

For binding assays using tissue, crude membrane fractions are prepared from rodent (typically rat) brain or kidney (purchased from PelFreeze Biologicals). Frozen tissue (maintained at -80 degrees centigrade) is thawed on ice, and then homogenized on ice in 10 vol of 50 mM Tris, pH 7.4 containing protease inhibitor cocktail (Roche) using a Polytron homogenizer (3 pulses, each of 10 sec). The homogenate is centrifuged at 40,000 x *g* for 20 min, and then the resulting supernatant is decanted and replaced with the same buffer. Two to three additional rounds of homogenization-centrifugation are performed to ensure thorough homogenization and also to wash away endogenous ligands (particularly important for GABA assays). The final, washed pellet is resuspended in the same buffer, homogenized one last time, and aliquoted into eppendorf tubes such that one tube contains sufficient material for 24 wells of a binding assay plate (generally 2.5-12.5 mg membrane protein/eppendorf tube, or 100 to 500 µg of membrane protein per well of the binding assay plate). The crude membrane fractions are stored at -80 degrees centigrade until assay, which in most instances is within 3 days of the crude membrane fraction preparation.

V. Complete List of Research Publications Co-authored or Otherwise Supported by the NIMH PDSP. Detailed validated protocols for all assays can also be found in these peer-reviewed publications

1. **Abate C, Kolanos R, Dukat M, Setola V, Roth BL, and Glennon RA.** Interaction of chiral MS-245 analogs at h5-HT6 receptors. *Bioorg Med Chem Lett* 15: 3510-3513, 2005.
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