



Published in final edited form as:

*Curr Protoc Pharmacol.* ; 71: 1.34.1–1.34.21. doi:10.1002/0471141755.ph0134s71.

## SIGMA RECEPTOR BINDING ASSAYS

UYEN B. CHU and ARNOLD E. RUOHO

Department of Neuroscience, School of Medicine and Public Health, University of Wisconsin – Madison, Madison, WI 53706

### Abstract

Sigma receptors belong to a class of small molecule-regulated, primarily endoplasmic reticulum (ER) membrane-associated receptors, of which there are two subtypes: the Sigma-1 receptor (S1R) and the Sigma-2 receptor (S2R). Both S1R and S2R bind to a number of drugs including antipsychotic, haloperidol, and the opioid analgesic, (+)-pentazocine. Sigma receptors are implicated in multiple disease pathologies associated with the nervous system including diseases affecting motor control such as Amyotrophic Lateral Sclerosis (ALS) and Alzheimer's disease. This unit describes methods for the pharmacological characterization of S1R and S2R using radioligand-binding assays. In the first section, radioligand saturation binding assay to determine receptor densities and competitive inhibition assays to characterize affinities of novel compounds are presented for S1R using the selective S1R ligand, [<sup>3</sup>H]-(+)-pentazocine. The second section describes radioligand saturation binding assay and competitive inhibition assays for the S2R using a non-selective S1R and S2R ligand, [<sup>3</sup>H]-1,3-di(2-tolyl)guanidine ([<sup>3</sup>H]-DTG).

### Keywords

Sigma-1 receptor; sigma-2 receptor; saturation of radioligand binding assays; competitive inhibition of radioligand assays; [<sup>3</sup>H]-(+)-pentazocine; [<sup>3</sup>H]-(+)-DTG; haloperidol

## INTRODUCTION

Identification of the S1R and its differentiation from G protein-coupled opioid receptors (Walker et al., 1990) was initially based on radioligand binding (Kavanaugh et al., 1989; Su, 1982). Over several decades, a large number of synthetic and endogenous small molecules both agonists and antagonists have been reported to bind to S1R and S2R (Abate et al., 2010; Abate et al., 2011; Ablordeppey et al., 2000). Several of these have been useful in radiolabeled forms to identify S1Rs and S2Rs biochemically (Fontanilla et al., 2008; Pal et al., 2008; Pal et al., 2007). However, the biochemical and functional characterization of sigma receptors present unique challenges given their paucity and membrane environment.

Cloning of the S1R (Hanner et al., 1996; Kekuda et al., 1996) identified a 223 amino acid protein (~25 kDa as assessed by SDS PAGE analysis) with two transmembrane sequences that place the N- and C- termini on the same side of the membrane. The S1R occurs in homo

Conflicts of Interest

The authors declare that there are no conflicts of interest.

oligomeric states that likely regulate its functions (Chu et al., 2013; Gromek et al., 2014). The S1R knockout mouse was developed in 2003 and showed no overt phenotype which suggests a compensatory mechanism that may be mediated by other proteins including the S2R (Langa et al., 2003). Interestingly, the S1R knockout mice failed to respond to *N,N*-dimethyltryptamine (DMT), a hallucinogen, in a behavioral assay measuring motor coordination (Fontanilla et al., 2009). In addition, the S1R knockout mouse swam faster than wild-type animals (Mavlyutov et al., 2010) although the precise molecular mechanism(s) are unclear. These results corroborated with previous work suggesting a role of the S1R in coordinator motor response including earlier observations that sigma receptors are involved in dystonia and motor activities in response to drugs of abuse such as cocaine and methamphetamine (Hayashi et al., 2010; Kaushal and Matsumoto, 2011; Menkel et al., 1991; Robson et al., 2012; Ujike et al., 1996). The S2R has yet to be cloned but selective radiolabeled photoaffinity approaches using various mammalian tissues (including the S1R knockout tissues) and cultured cells have identified this receptor as an 18-20 kDa protein (Bowen et al., 1989; Hellewell and Bowen, 1990; Hellewell et al., 1994).

The S1R has been functionally characterized as a molecular chaperone (Hayashi and Su, 2007) with an impressive list of multitasking functions including chaperoning of many types of voltage gated ion channels (Kourrich et al., 2013) and G-protein coupled receptors (Navarro et al., 2013). The goal of the methods outlined below is to provide practical approaches for identification and quantification of S1Rs and S2Rs in membrane preparations and in intact cells. Detailed protocols for characterization of the Sigma receptors and the small molecule compounds that bind to these receptors using radioligand binding are described in Chapter 1 and photoaffinity labeling and immunofluorescence strategies are presented in Chapter 8.

The radioligand saturation-binding assay to assess the S1R characteristics in membranes can be performed using [<sup>3</sup>H]-(+)-pentazocine, a selective S1R ligand (see **Basic Protocol 1**). These experiments can be performed with tissue membrane homogenates, cell membranes, and/or *E.coli* expressed and purified protein to either compare the levels of S1R expression in these samples and/or characterize the ligand binding function of purified proteins that carry mutations.

The inhibition of [<sup>3</sup>H]-(+)-pentazocine binding assay is mainly used to determine the inhibition constant ( $K_i$ ) of potential S1R ligands (see **Basic Protocol 2**). These assays are performed with a single concentration of [<sup>3</sup>H]-(+)-pentazocine at a concentration near its  $K_D$  and increasing concentrations of non-radioactive ligand.

It should be mentioned that while there are a number of selective S2R ligands that have been developed over the past two decades, the standard method to assess S2R activities is the utilization of radioactive [<sup>3</sup>H]-DTG, a nonselective S1R and S2R ligand, in the presence of nonradioactive (+)-pentazocine to mask S1R binding sites. The radioligand saturation-binding assay to assess the S2R activity and densities in tissues and/or cells is presented in **Section II, Basic Protocol 3**.

The inhibition of [<sup>3</sup>H]-DTG binding assay is mainly used to determine the inhibition constant ( $K_I$ ) of potential S2R ligands (**Section II, Basic Protocol 4**). These assays are performed with a single concentration [<sup>3</sup>H]-DTG at a concentration near its  $K_D$  for the S2R and an increasing concentrations of non-radioactive ligand in the presence of (+)-pentazocine to mask S1R binding sites.

## SECTION I: RADIOLIGAND BINDING ASSAYS FOR SIGMA-1 RECEPTOR

S1R are highly expressed in liver tissues of many species. The use of guinea pig liver (GPL) membranes for S1R research has provided useful data in assessing the *in vitro* characteristics of S1R ligands and radioligands. S1R protein levels are highest in GPL compared to other tissues and from other biological sources such as cells. The preparation of tissue membranes is outlined in **Support Protocol 1** and can readily be used for preparation of mouse and other species liver membrane preparations (Fontanilla et al., 2008; Fontanilla et al., 2009; Pal et al., 2007). **Basic Protocol 1** describes the radioligand saturation-binding assay with a selective S1R ligand, [<sup>3</sup>H]-(+)-pentazocine, to determine  $B_{max}$  and  $K_D$ . **Basic Protocol 2** describes the inhibition of [<sup>3</sup>H]-(+)-pentazocine to determine  $K_I$  of novel compounds.

### BASIC PROTOCOL 1: RADIOLIGAND SATURATION-BINDING ASSAYS TO DETERMINE $B_{max}$ AND $K_D$ AT S1R

Saturation-binding studies of S1R binding (**Basic protocol 1**) describes the direct assessment of S1R to determine receptor densities ( $B_{max}$ ) and the dissociation equilibrium constant ( $K_D$ ) of radioligands for that site. [<sup>3</sup>H]-(+)-pentazocine ([<sup>3</sup>H]-(+)-PTZ) is the preferred radioligand since it is a selective S1R ligand.

**Materials**—*Assay Buffer* (see Reagents and Solutions section)

0.5 µg/µL GPL membranes (see Support Protocol 1) diluted in *Assay Buffer*

[<sup>3</sup>H]-(+)-pentazocine, 34 Ci/mmol (Perkin Elmer, Akron, OH)

100 µM haloperidol (Sigma-Aldrich, St. Louis, MO) dissolved in *Assay Buffer*

1 mL 96-well deep well polypropylene plates (e.g., USA Scientifics, Orlando, FL)

Glass fiber filter papers (Whatman GF/B, Whatman, Maidstone, UK)

0.5% Polyethyleneimine (Morris et al.) (Sigma-Aldrich, St. Louis, MO)

Filtration system (Brandel Cell Harvester, Gaithersburg, MD)

10 mL scintillation vials (e.g., Fisher Scientific, Waltham, MA)

Scintillation fluid (e.g., Ultima Gold, Perkin Elmer, Waltham, MA)

Scintillation counter (e.g., Packard model 1600CA, Packard Instrument Co., Downers Grove, IL)

Graphpad Prism (Graphpad Software, San Diego, CA)

**Procedures**

1. Prepare sufficient protein samples at a concentration of 0.4 mg/mL in *Assay Buffer*. Each well contains 80 μL of protein samples (or 0.032 mg) which will be added to binding reaction to a final volume of 100 μL. It should be noted that 0.032 mg protein per sample was determined empirically in our laboratory to give the best results for guinea pig liver membranes. SIR densities vary among different tissues and cells and researchers are advised to use more or less protein accordingly.

Example calculations for an assay containing 48 wells are shown below:

$$(1) \quad 48 \text{ wells} \times 80 \text{ } \mu\text{L/well} = 3840 \text{ } \mu\text{L} \text{ or approximately } 4000 \text{ } \mu\text{L} \text{ (4 mL) of 0.4 mg/mL membranes is needed}$$

$$(2) \quad 4 \text{ mL} \times 0.4 \text{ mg/mL} = (10 \text{ mg/mL}) \times \underline{\quad V \quad} \text{ } \mu\text{L}$$

$V = 0.16 \text{ mL or } 160 \text{ } \mu\text{L}$  of 10 mg/mL GPL membranes added to 3840 μL of *Assay Buffer* to make 4 mL of 0.4 mg/mL

2. Prepare 10X stock concentrations of [<sup>3</sup>H]-(+)-pentazocine following **Table 1**. The concentrations should range from 3 to 3000 nM [<sup>3</sup>H]-(+)-pentazocine (final concentrations ranging from 0.3 – 300 nM). We recommend using several concentrations above and below the  $K_D$  of [<sup>3</sup>H]-(+)-pentazocine for the SIR which we have previously determined to be ~ 10 nM.
3. Determine the actual [<sup>3</sup>H]-(+)-pentazocine in the reaction mixture, count 10 μL of each 10X stock concentrations and determine the measured radioligand concentration in assay using the specific activities of [<sup>3</sup>H]-(+)-pentazocine of 34 Ci/mmol (see **Table 2**).
4. Set up binding assays in a 96-well plate below. For one saturation binding experiment, we use 48 wells or ½ of a 96-well plate.

	Total binding (μL)	Non-specific (μL)
Assay buffer	10	-
10X [ <sup>3</sup> H]-(+)-pentazocine (concentrations vary)	10	10
100 μM haloperidol	-	10
GPL (0.4 mg/ml)	80	80
<b>Total</b>	100	100

For each concentration of [<sup>3</sup>H]-(+)-pentazocine in “total binding” prepare a “non-specific” sample by adding 10 μM haloperidol. As an example, use wells A1 to B12 for “total binding” and C1 –

D12 for “non-specific.” It is recommended that a ligand with a chemical structure different from that of the radioligand be used to define nonspecific binding.

5. Incubate the 96-well plate for 90 min at 37°C.  
*Note:* The approximately time to reach equilibrium has been determined to be 60 minute for the sigma-1 receptor (Ramachandran et al., 2007).
6. Soak a glass fiber filter paper in 0.5% PEI at room temperature.
7. Terminate the reaction by rapid filtration with *Assay Buffer* through GF/B filters using a Brandel cell harvester. Rinse three times with ~500 µL of ice-cold *Assay Buffer*.
8. Remove glass filter paper discs and insert in liquid scintillation vials. Allow the filter paper to dry at room temperature for 1 hour before adding 3 mL of scintillation fluid into each vial and incubate scintillation vials overnight at room temperature.
9. Determine the bound radioactivity (in DPM) the following day using the scintillation counter.

#### Data Analyses

10. Calculate the average of triplicate total and nonspecific binding values for each concentration of [<sup>3</sup>H]-(+)-pentazocine tested and convert DPM into pmol/mg protein (see **Equation 1**), results are displayed in **Table 3**.

$$\text{Bound (pmol/mg)} = \frac{\left\{ \frac{\left[ \frac{\text{DPM}}{2.22 \times 10^{12} \text{ DPM/Ci}} \right]}{\left[ \text{Sp. activity of PTZ in } \frac{\text{Ci}}{\text{mmol}} \right]} \right\} \times 10^9 \text{ pmol/mmol}}{\text{mg proteins}}$$

11. Determine the “specific binding” by subtracting “nonspecific binding” (in pmol/mg) from “total binding” (see **Table 4**).
12. Using nonlinear regression analysis, fit the specific binding data to **Equation 2** below:

$$B = \frac{L \times B_{max}}{L + K_D}$$

where L and K<sub>D</sub> are the radioligand concentration and dissociation constant, respectively, and B<sub>max</sub> is the maximum binding (B<sub>max</sub> is the receptor densities).

Our laboratory uses Graphpad Prism 5.0 to perform data analysis and graphing the results. An example showing saturation binding of [<sup>3</sup>H]-(+)-pentazocine is shown in **Figure 1**. Bound [<sup>3</sup>H]-(+)-pentazocine

data are plotted against measured concentrations of [ $^3\text{H}$ ]-(+)-pentazocine. Both the  $B_{\text{max}}$  and  $K_{\text{D}}$  can be obtained from the software package.

## BASIC PROTOCOL 2: INHIBITION OF [ $^3\text{H}$ ]-(+)-PENTAZOCINE TO DETERMINE INHIBITION BINDING CONSTANTS ( $K_{\text{i}}$ ) OF POTENTIAL SIGMA-1 RECEPTOR LIGANDS

**Basic Protocol 2** describes inhibition of [ $^3\text{H}$ ]-(+)-pentazocine with an unlabeled ligand to determine its inhibition constant ( $K_{\text{i}}$ ) (Chu et al., 2011; Fontanilla et al., 2008; Fontanilla et al., 2009; Pal et al., 2007). The biological source use for this example is GPL membranes since guinea pig liver expresses high levels of S1R.

### Materials—*Assay Buffer* (see **Reagents and Solutions** section)

0.5  $\mu\text{g}/\mu\text{L}$  GPL membranes (see Support Protocol 1) diluted in *Assay Buffer*

[ $^3\text{H}$ ]-(+)-pentazocine (Perkin Elmer, Akron, OH)

Nonradioactive test compounds/inhibitors

100  $\mu\text{M}$  haloperidol (Sigma-Aldrich, St. Louis, MO) dissolved in *Assay Buffer*

1 mL 96-well deep well polypropylene plates (e.g., USA Scientifics, Orlando, FL)

Glass fiber filter papers (Whatman GF/B, Whatman, Maidstone, UK)

0.5% Polyethyleneimine (Morris et al.) (Sigma-Aldrich, St. Louis, MO)

Filtration system (Brandel Cell Harvester, Gaithersburg, MD)

10 mL scintillation vials (e.g., Fisher Scientific, Waltham, MA)

Scintillation fluid (e.g., Ultima Gold, Perkin Elmer, Waltham, MA)

Scintillation counter (e.g., Packard model 1600CA, Packard Instrument Co., Downers Grove, IL)

Graphpad Prism (Graphpad Software, San Diego, CA)

### Procedures

1. Prepare sufficient protein samples at a concentration of 0.4 mg/mL in *Assay Buffer*. Each well contains 80  $\mu\text{L}$  of protein samples (or 0.032 mg) which will be added to binding reaction to a final volume of 100  $\mu\text{L}$ . It should be noted that 0.032 mg proteins per sample was determined empirically in our laboratory to give the best results for guinea pig liver membranes. S1R densities vary in different tissue and cell types, researchers are advised to adjust the protein concentrations according when another biological source is used.

Example calculations for an assay containing 48 wells are shown below

(1)  $48 \text{ wells} \times 80 \text{ } \mu\text{L/well} = 3840 \text{ } \mu\text{L}$  or approximately 4000  $\mu\text{L}$  (4 mL) of 0.4 mg/mL membranes is needed

(2)  $4 \text{ mL} \times 0.4 \text{ mg/mL} = (10 \text{ mg/mL}) \times \underline{\quad V \quad} \mu\text{L}$

$V = 0.16 \text{ mL}$  of 10 mg/mL GPL membranes added to 3.840 mL of *Assay Buffer* to make 4 mL of 0.4 mg/mL

2. Prepare 100 nM [ $^3\text{H}$ ]-(+)-pentazocine.

We have previously determined the  $K_D$  of (+)-pentazocine for the S1R to be approximately 10 nM. It is ideal to use the concentration of the radioligand equal to or slightly above the  $K_D$ . To determine the actual [ $^3\text{H}$ ]-(+)-pentazocine in the reaction mixture, count 10  $\mu\text{L}$  of the 100 nM [ $^3\text{H}$ ]-(+)-pentazocine (10X stock) and determine the measured radioligand concentration in assay taking into account the specific activities of [ $^3\text{H}$ ]-(+)-pentazocine (for this example, the specific activity of 100 nM [ $^3\text{H}$ ]-(+)-pentazocine was 34 Ci/mmol and the conversion factor is  $2.22 \times 10^{12}$  DPM/Ci).

3. Prepare stock concentrations of inhibitors following **Table 6**. It is suggested that researchers prepare a stock 1.00E-2 M (or 100 mM) of inhibitor and use this master stock to prepare 1.00E-3 M (or 1 mM) and 3.00E-4 M (or 0.3 mM). These are used to prepare subsequent dilutions for the binding assay.

4. Set up binding assays in a 96-well plate as shown below in quadruplicates. For one competition experiment, we use 48 wells or  $\frac{1}{2}$  of the 96-well plate which allows for one quadruplicate set (4 wells) of “total binding,” one quadruplicate set (4 wells) of “non-specific binding” (4 wells), and 10 concentrations of inhibitor (40 wells) for a total of 48 wells.

	Total binding ( $\mu\text{L}$ )	Non-specific ( $\mu\text{L}$ )	Inhibitor ( $\mu\text{L}$ )
GPL (0.4 mg/ml)	80	80	80
100 nM [ $^3\text{H}$ ]-(+)-pentazocine	10	10	10
100 $\mu\text{M}$ haloperidol	-	10	-
10X Inhibitor (concentrations vary, see Table 6)	-	-	10
Assay buffer	10	-	-
<b>Total</b>	100	100	100

5. Incubate the 96-well plate for 90 min at 37°C.

*Note:* Time to reach equilibrium depends on the physical and chemical properties of the inhibitor. We typically incubate the assay for 90 min but researchers are advised to make adjustments appropriately (see Unit 1.3 for background on estimation of time to reach steady state).

6. Soak a glass fiber filter paper in 0.5% PEI at RT.
7. Terminate the reaction by rapid filtration with *Assay Buffer* through GF/B filters using a Brandel cell harvester. Rinse three times with ~500  $\mu$ L of ice-cold *Assay Buffer*.
8. Remove glass filter paper discs and insert in liquid scintillation vials. Allow the filter paper to dry at room temperature for 1 hour before adding 3 mL of scintillation fluid into each vial and incubate scintillation vials overnight at room temperature.
9. Determine the bound radioactivity (in DPM) the following day using the scintillation counter.

### Data Analyses

10. Inhibition binding data are typically expressed simply as radioligand binding (CPM or DPM) or % specific binding against a logarithmic molar concentrations of inhibitor. Data should be fit with an unrestricted Hill slope (see Unit 1.3 for background).
11. We typically report our results as % specific binding and these values can be determined using **Equation 3**

$$\% \text{ Specific binding} = \frac{\text{Bound} - \text{Nonspecific binding}}{\text{Total binding (no drug)}} \times 100$$

12. To determine the  $K_I$  values for tested compounds, we fit the data to a “one-site competition” function in GraphPad Prism (see **Figure 2**). From the  $IC_{50}$  value (concentration of inhibitor causing a 50% reduction in radioligand specific binding), a  $K_I$  (inhibition constant; the concentration of inhibitor that would occupy 50% of the receptors in the absence of radioligand) is calculated using the Cheng-Prusoff Equation (**Equation 4**).

$$K_I = \frac{IC_{50}}{\left(1 + \frac{[L]}{K_d}\right)}$$

$IC_{50}$  = inhibition concentration at 50%, which is determined from the graph [L] = concentrations of [ $^3$ H]-(+)-pentazocine, we use 10 nM (researchers should count a small aliquot of radioactive [ $^3$ H]-(+)-pentazocine to determine the actual concentration)  $K_D$  = equilibrium dissociation constant of [ $^3$ H]-(+)-pentazocine, we use 10 nM which



can be determined in a saturation binding of [<sup>3</sup>H]-(+)-pentazocine (see **Basic Protocol 1**).

## SECTION II: RADIOLIGAND BINDING ASSAYS FOR SIGMA 2 RECEPTORS

Section II first describes a saturation equilibrium binding of [<sup>3</sup>H]-1,3-Di-*o*-tolyguanidine ([<sup>3</sup>H]-DTG) to directly assess the receptor densities ( $B_{max}$ ) and dissociation constant ( $K_D$ ) for this receptor (**Basic Protocol 3**) in different tissues and cell types. **Basic Protocol 4** outlines a competitive inhibition of [<sup>3</sup>H]-DTG to determine the dissociation constant ( $K_I$ ) of compounds that bind to the S2R. DTG is a non-selective ligand for both the S1R and S2R thus requiring the addition of non-radioactive (+)-pentazocine, a selective S1R ligand, to each reaction to mask the S1R sites. The S2R protein levels in RL are 3 folds higher than the S1R protein level (e.g. the ratio of S2R to S1R is approximately 75% to 25% respectively (unpublished data). In addition, the absolute levels of the S2R is highest in RL as compared to other tissues/cells thus RL is a preferred biological source of S2R for testing potential S2R compounds.

### BASIC PROTOCOL 3: RADIOLIGAND SATURATION-BINDING ASSAYS TO DETERMINE $K_D$ and $B_{max}$ AT S2R

**Basic Protocol 3** describes the direct assessment of S2R levels using saturation equilibrium binding of [<sup>3</sup>H]-DTG. It should be noted that the emphasis of this protocol is on the measurement of S2R, data are presented for [<sup>3</sup>H]-DTG binding to both S1R and S2R.

**Materials**—*Assay Buffer* (see Reagents and Solutions section)

0.5 µg/µL GPL membranes (see Support Protocol 1) diluted in *Assay Buffer*

[<sup>3</sup>H]-DTG, 40 Ci/mmol (Perkin Elmer, Akron, OH)

100 µM haloperidol (Sigma-Aldrich, St. Louis, MO) dissolved in *Assay Buffer*

Nonradioactive 1 µM (+)-pentazocine (Sigma-Aldrich, St. Louis, MO) prepared in *Assay Buffer*

1 mL 96-well deep well polypropylene plates (e.g., USA Scientifics, Orlando, FL)

Glass fiber filter papers (Whatman GF/B, Whatman, Maidstone, UK)

0.5% Polyethyleneimine (Morris et al.) (Sigma-Aldrich, St. Louis, MO)

Filtration system (Brandel Cell Harvester, Gaithersburg, MD)

10 mL scintillation vials (e.g., Fisher Scientific, Waltham, MA)

Scintillation fluid (e.g., Ultima Gold, Perkin Elmer, Waltham, MA)

Scintillation counter (e.g., Packard model 1600CA, Packard Instrument Co., Downers Grove, IL)

Graphpad Prism (Graphpad Software, San Diego, CA)

### Procedures

1. Prepare sufficient protein samples at a concentration of 0.4 mg/mL in *Assay Buffer*. Each well contains 70  $\mu$ L of protein samples (or 0.028 mg) which will be added to binding reaction to a final volume of 100  $\mu$ L. It should be noted that 0.028 mg proteins per sample was determined empirically in our laboratory to give the best results for rat liver membranes. S2R densities vary among different tissues and cells and researchers are advised to use more or less proteins accordingly.

Example calculations for an assay containing 72 wells are shown below:

$$(1) \quad 72 \text{ wells} \times 70 \text{ } \mu\text{L/well} = 5040 \text{ } \mu\text{L} \text{ or round up to } 5200 \text{ } \mu\text{L} (5.2 \text{ mL}) \text{ of } 0.4 \text{ mg/mL membranes is needed}$$

$$(2) \quad 5.2 \text{ mL} \times 0.4 \text{ mg/mL} = (10 \text{ mg/mL}) \times \underline{\quad V \quad} \text{ } \mu\text{L}$$

$V = 0.208 \text{ mL}$  of 10 mg/mL RL membranes added to 4.992 mL of *Assay Buffer* to make 5.2 mL of 0.4 mg/mL

2. Prepare 10X stock concentrations of [ $^3\text{H}$ ]-DTG following the examples shown in **Table 9**. The concentrations should range from 3 to 3000 nM [ $^3\text{H}$ ]-DTG (final concentrations ranging from 0.3 – 300 nM). We recommend using several concentrations above and below the  $K_D$  of [ $^3\text{H}$ ]-DTG for the S2R which we have determined to be 35 – 60 nM.
3. To determine the actual [ $^3\text{H}$ ]-DTG in the reaction mixture, count 10  $\mu$ L of each 10X stock concentration and determine the measured radioligand concentration in assay using the specific activities of [ $^3\text{H}$ ]-DTG of 40 Ci/mmol (see **Table 10**).
4. Set up binding assays in a 96-well plate below. For one saturation binding experiment, we use 72 wells or  $\frac{3}{4}$  of a 96-well plate.

	Total binding ( $\mu$ L)	S2R Binding ( $\mu$ L)	Non-specific ( $\mu$ L)
RL membranes (0.4 mg/ml)	70	70	70
10X [ $^3\text{H}$ ]-DTG (concentrations see Table 9)	10	10	10
1 $\mu$ M (+)-pentazocine	-	10	10
100 $\mu$ M haloperidol	-	-	10
Assay buffer	20	10	-
<b>Total</b>	100	100	100

As an example, use A1 to B12 for “total binding,” C1 – D12 for “S2R binding,” and E1 – F12 for “non-specific.” It is recommended that a ligand with a chemical structure different

from that of the radioligand be used to define nonspecific binding and haloperidol is a standard molecule used since it binds to both S1R and S2R.

5. Incubate the 96-well plate for 90 min at 37°C.  
*Note:* The approximately time to reach equilibrium has been determined to be 60 minute for the S2R (Ramachandran et al., 2007).
6. Soak 2 glass fiber filter paper in 0.5% PEI at RT.
7. Terminate the reaction by rapid filtration with *Assay Buffer* through GF/B filters using a Brandel cell harvester. Rinse three times with ~500  $\mu$ L of ice-cold *Assay Buffer*.
8. Remove glass filter paper discs and insert in liquid scintillation vials. Allow the filter paper to dry at room temperature for 1 hour before adding 3 mL of scintillation fluid into each vial and incubate scintillation vials overnight at room temperature.
9. Determine the bound radioactivity (in DPM) the following day using the scintillation counter.

#### Data Analyses

10. Calculate the average of triplicate total and nonspecific binding values for each concentration of [<sup>3</sup>H]-DTG tested and convert DPM into pmol/mg protein (see **Basic Protocol 1, Equation 1**). To simplify the display of data, only specific binding values are presented (**Table 11**).
11. Using nonlinear regression analysis, fit the specific binding data to **Equation 2 (Basic Protocol 1)**.

Our laboratory uses Graphpad Prism 5.0 to perform data analysis and graphing the results. An example showing saturation binding of [<sup>3</sup>H]-DTG is shown in **Figure 3**. Bound [<sup>3</sup>H]-DTG data are plotted against measured increasing concentrations of measured free [<sup>3</sup>H]-DTG. Both the  $B_{\max}$  and  $K_D$  can be obtained from the software program.

#### BASIC PROTOCOL 4: INHIBITION OF [<sup>3</sup>H]-1,3-DI-O-TOLYLGUANIDINE TO DETERMINE INHIBITION BINDING CONSTANTS ( $K_I$ ) OF POTENTIAL S2R LIGANDS

**Basic Protocol 4** describes inhibition of [<sup>3</sup>H]-DTG with an unlabeled ligand to determine its inhibition constant ( $K_I$ ). The biological source use for this example is RL membranes since rat liver expresses the highest levels of S2R as compared to S1R.

**Materials**—*Assay Buffer* (see **Reagents and Solutions** section)

0.5  $\mu$ g/ $\mu$ L GPL membranes (see Support Protocol 1) diluted in *Assay Buffer*

0.3  $\mu$ M or 300 nM [<sup>3</sup>H]-DTG (Perkin Elmer, Akron, OH)

1  $\mu\text{M}$  (+)-pentazocine (Sigma-Aldrich, St. Louis, MO) dissolved in *Assay Buffer*

Nonradioactive test compounds/inhibitors

100  $\mu\text{M}$  haloperidol (Sigma-Aldrich, St. Louis, MO) dissolved in *Assay Buffer*

1 mL 96-well deep well polypropylene plates (e.g., USA Scientifics, Orlando, FL)

Glass fiber filter papers (Whatman GF/B, Whatman, Maidstone, UK)

0.5% Polyethyleneimine (Morris et al.) (Sigma-Aldrich, St. Louis, MO)

Filtration system (Brandel Cell Harvester, Gaithersburg, MD)

10 mL scintillation vials (e.g., Fisher Scientific, Waltham, MA)

Scintillation fluid (e.g., Ultima Gold, Perkin Elmer, Waltham, MA)

Scintillation counter (e.g., Packard model 1600CA, Packard Instrument Co., Downers Grove, IL)

Graphpad Prism (Graphpad Software, San Diego, CA)

### Procedures

1. Prepare sufficient protein samples at a concentration of 0.4 mg/mL in *Assay Buffer*. Each well contains 70  $\mu\text{L}$  of protein samples (or 0.028 mg) which will be added to binding reaction to a final volume of 100  $\mu\text{L}$ . It should be noted that 0.028 mg proteins per sample was determined empirically in our laboratory to give the best results for rat liver membranes. S2R densities vary in different tissue and cell types, researchers are advised to adjust the protein concentrations according when another biological source is used.

Example calculations for an assay containing 48 wells are shown below:

$$(1) \quad 48 \text{ wells} \times 70 \mu\text{L/well} = 3360 \mu\text{L} \text{ or approximately } 3500 \mu\text{L} \\ (3.5 \text{ mL}) \text{ of } 0.4 \text{ mg/mL membranes is needed}$$

$$(2) \quad 3.5 \text{ mL} \times 0.4 \text{ mg/mL} = (10 \text{ mg/mL}) \underline{\quad V \quad} \mu\text{L}$$

$V = 0.14 \text{ mL}$  of 10 mg/mL RL membranes added to  
3.360 mL of *Assay Buffer* to make 3.5 mL of 0.4  
mg/mL

2. Prepare 0.3  $\mu\text{M}$  [ $^3\text{H}$ ]-DTG.

We have previously determined the  $K_D$  of [ $^3\text{H}$ ]-DTG for the S2R to be approximately 30 - 50 nM. It is ideal to use the concentration of the radioligand equal to or slightly above the  $K_D$ . To determine the actual [ $^3\text{H}$ ]-DTG in the reaction mixture,

count 10  $\mu\text{L}$  of each 0.3  $\mu\text{M}$  [ $^3\text{H}$ ]-DTG and determine the measured radioligand concentration in assay using the specific activities of [ $^3\text{H}$ ]-DTG (for this example, the specific activity of 300 nM [ $^3\text{H}$ ]-DTG was 40 Ci/mmol and the conversion factor is  $2.22 \times 10^{12}$  DPM/Ci).

3. Prepare stock concentrations of inhibitors following **Table 5**. It is suggested that researchers prepare a stock 1.00E-2 M (or 100 mM) of inhibitor and use this master stock to prepare 1.00E-3 M (or 1 mM) and 3.00E-4 M (or 0.3 mM). These are used to prepare subsequent dilutions for the binding assay.
4. Set up binding assays in a 96-well plate as shown below in quadruplicates. For one competition experiment, we use 48 wells or  $\frac{1}{2}$  of the 96-well plate which allows for one quadruplicate set (4 wells) of “total binding,” one quadruplicate set (4 wells) of “non-specific binding” (4 wells), and 10 concentrations of inhibitor (40 wells) for a total of 48 wells.

	Total binding ( $\mu\text{L}$ )	Non-specific ( $\mu\text{L}$ )	Inhibitor ( $\mu\text{L}$ )
<b>RL membranes (0.4 mg/ml)</b>	70	70	70
<b>0.30 <math>\mu\text{M}</math> or 300 nM [<math>^3\text{H}</math>]-DTG</b>	10	10	10
<b>100 <math>\mu\text{M}</math> haloperidol</b>	-	10	-
<b>1 <math>\mu\text{M}</math> (+)-pentazocine</b>	10	10	10
<b>10X Inhibitor (concentrations vary)</b>	-	-	10
<i>Assay buffer</i>	10	-	-
<b>Total</b>	100	100	100

5. Incubate the 96-well plate for 90 min at 37°C.
 

*Note:* Time to reach equilibrium depends on the physical and chemical properties of the inhibitor. We typically incubate the assay for 90 min but researchers are advised to make adjustments appropriately (see Unit 1.3 for background on estimation of time to reach equilibrium).
6. Soak a glass fiber filter paper in 0.5% PEI at RT.
7. Terminate the reaction by rapid filtration with *Assay Buffer* through GF/B filters using a Brandel cell harvester. Rinse three times with  $\sim 500$   $\mu\text{L}$  of ice-cold *Assay Buffer*.
8. Remove glass filter paper discs and insert in liquid scintillation vials. Allow the filter paper to dry at room temperature for 1 hour before adding 3 mL of scintillation fluid into each vial and incubate scintillation vials overnight at room temperature.

9. Determine the bound radioactivity (in DPM) the following day using the scintillation counter.

### Data Analyses

10. Inhibition binding data are typically expressed simply as radioligand binding (CPM or DPM) or % specific binding against a logarithmic molar concentrations of inhibitor. Data should be fit with an unrestricted Hill slope (see Unit 1.3 for background).
11. We typically report our results as % specific binding and these values can be determined using **Equation 3** (see **Basic Protocol 2**).
12. To determine the  $K_I$  values for tested compounds, we fit the data to a “one-site competition” function in GraphPad Prism (see **Basic Protocol 2, Figure 2 for an example**). From the  $IC_{50}$  value (concentration of inhibitor causing a 50% reduction in radioligand specific binding), a  $K_I$  (inhibition constant; the concentration of inhibitor that would occupy 50% of the receptors in the absence of radioligand) is calculated using the Cheng-Prusoff Equation (see **Basic Protocol 2**). We use 30 nM as the concentration of [ $^3H$ ]-DTG and 30 nM as the  $K_D$  for S2R.

## SUPPORT PROTOCOL 1: PREPARATION OF MEMBRANE FRACTIONS FROM TISSUES

S1R and S2R are ubiquitously expressed in many different tissues throughout the body. Although originally found in brain tissues, S1R and S2R are also abundant in liver tissues which are the main tissue membranes used in our laboratory to characterize novel ligands for both receptor subtypes. S1R expression is higher in guinea pig livers compared to rat livers (approximately 80% of [ $^3H$ ]-DTG binding is attributed to S1R in guinea pig livers). In contrast, rat expresses higher levels of S2R (approximately 75% of [ $^3H$ ]-DTG binding in rat liver is attributed to S2R). Described below are ways to prepare crude membranes from these tissues for use in radioligand-binding studies and for photolabeling experiments (**Chapter 8**). The S1R activities (as assessed both by [ $^3H$ ]-(+)-pentazocine) in guinea pig liver membranes are stable for up to one year. On the contrary, the half-life of the S2R (as assessed by [ $^3H$ ]-DTG binding) is three months. Researchers are advised to prepare fresh membranes as needed.

### Materials—*Homogenization Buffer* (see **Reagents and Solutions** section)

Fresh or frozen liver tissues (Pel Freez Biologicals, Rogers, AR)

Sharp dissecting scissor

Tissue disrupter (e.g., Brinkmann Polytron homogenizer)

Tissue homogenizer (e.g., Potter-Elvehjem Grinding Chambers and Pestles)

Hi-speed centrifuge and centrifuge tubes or an equivalent centrifuge (e.g., Beckman L7 Ultracentrifuge with 45Ti rotor)

Bradford Protein Assay Reagent (Thermo Scientific, Waltham, MA)

Liquid nitrogen

### Procedures

1. Weigh ~10 – 15 g frozen/fresh liver tissue and thaw on ice in *Homogenization Buffer* at a buffer to tissue ratio of 5 mL/g liver tissue.
2. Mince liver tissue on ice using dissecting scissors.
3. Homogenize liver tissue by four 10-sec bursts using a Brinkmann Polytron on setting 6 (setting 10 is maximum).
4. Sediment the homogenate by centrifuging for 10 min at 17,000  $\times g$  and 4°C.
5. Pour supernatant from step 4 into a separate tube taking care not to disrupt the pellet and centrifuge for 60 min at 100,000  $\times g$  and 4°C.
6. Discard the resultant supernatant and resuspend the pellet from step 5 in 10 mL of *Homogenization Buffer*/g tissue and homogenize tissue pellet using a Potter-Elvehjem Pestles on setting 5 until membranes are homogenous (smooth milky suspension without the presence of detectable particulate).
7. Determine the protein concentration using Bradford Protein Assay Reagent according to manufacturer's protocol.
8. Snap-freeze liver membranes in 1 mL aliquots at a stock concentrations of 10 mg/mL and store at -80°C.

## REAGENTS AND SOLUTIONS

Use deionized distilled water in all recipes and protocol steps.

### Homogenization Buffer

10 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.4

0.32 M sucrose

1 mM MgSO<sub>4</sub>

10 µg/mL leupeptin

1 µg/mL pepstatin A

5 µg/mL soybean trypsin inhibitor

1 mM phenylmethylsulfonyl fluoride (PMSF)

0.5 mM EGTA

## Assay Buffer

50 mM Tris-HCl pH 8.0

## COMMENTARY

### Background Information

Receptor-binding assays are straightforward and powerful techniques for determining ligand-receptor interactions. The three main types of binding assays include saturation, inhibition and kinetic. Saturation binding measures the direct interaction between a radioligand that binds with high affinity (nanomolar concentrations) to a receptor and expresses as dissociation constant ( $K_D$ ). In addition, maximum binding of the radioligand ( $B_{max}$ ) can inform researchers of the receptor densities in the tissue being studied. A second method for determining affinity of compounds for a receptor is compete a fixed concentration of the radioligand off using increasing concentrations of an inhibitor. The affinity for the nonradiolabel inhibitor can be derived from the Cheng-Prusoff correction and expresses as inhibition constant ( $K_I$ ). This method relies on an assumption that the binding site for the radioligand and the inhibitor share some overlapping regions. Finally, kinetic binding studies (not cover in this unit) measure the binding of radiolabel ligand at various time points to determine association and dissociation rates. For more information about the basic principles of receptor binding assays, see Unit 1.3.

Sigma-1 and sigma-2 receptors represent two distinct binding sites in mammalian tissues. [ $^3\text{H}$ ]-(+)-pentazocine was established as the radioligand used in the detection of sigma-1 receptor (de Costa et al., 1989) while the accepted radioligand binding techniques for measuring sigma-2 receptor uses [ $^3\text{H}$ ]-DTG in the presence of 100 nM of unlabeled (+)-pentazocine (Quirion et al., 1992) (Hellewell and Bowen, 1990; Hellewell et al., 1994; Walker et al., 1992). While a number of radiolabeled compounds have been developed in recent years (Mach and Wheeler, 2009), from a historical stand point [ $^3\text{H}$ ]-(+)-pentazocine and [ $^3\text{H}$ ]-DTG remain reliable radioligand for the pharmacological assessment of S1R and S2R respectively.

### Critical Parameters

**Saturation binding of radioligand assays**—In our experience, it takes at least 90 min at 37°C for [ $^3\text{H}$ ]-(+)-pentazocine to reach equilibrium in tissue membranes, we recommend keeping incubation time to at least 90 min. Although it is possible to use a different compound to measure non-specific binding, in our laboratory haloperidol has been a reliable ligand for non-specific binding measurements.

**Inhibition of radioligand**—Assuming the inhibition of radioligand binding follows a one-site model, 10 - 12 concentrations of test compound inhibitor should be sufficient to accurately measure the  $\text{IC}_{50}$  value for only one site and 20 if there is evidence of a biphasic interaction. We recommend to use the concentration range spanning at least 100-fold above and below the anticipated  $\text{IC}_{50}$ .



**Preparation of tissue membranes**—We recommend preparing membranes from fresh tissues when possible, in the case where fresh tissues are not available frozen tissues can be purchased from vendors such as Pel Freez (see **Support Protocol 1**). When working with fresh tissues harvested from rodents and/or other organisms, consult institutional guidelines for proper method of euthanization. While the S1R binding site in liver membranes is highly stable at  $-80^{\circ}\text{C}$ , in our experience, the half-life of S2R binding site (as measured by both  $[^3\text{H}]\text{-DTG}$  binding and  $[^{125}\text{I}]\text{-IAF}$  photolabeling (see Chapter 8)) in tissue membranes is about three months, researchers are advised to prepare fresh membranes as needed.

**Data analysis**—data analyses are outlined in each Basic Protocol, refer to Unit 1.3 for detail theory on receptor pharmacology.

## Troubleshooting

General issues with saturation radioligand-binding assays		
Issue	Possible Explanations	Solutions
Linearity of binding curve	1. Insufficient incubation time 2. Radioligand concentrations too low	1. Increase incubation time 2. Increase radioligand concentrations
No binding	Receptor concentrations too low due receptor degradation	Prepare new membranes

General issues with inhibition of radioligand-binding assays		
Issue	Possible Explanations	Solutions
Incomplete inhibition of radioligand	Narrow concentration range of inhibitor	Widen the concentration range of inhibitor

## Anticipated Results

**Radioligand binding assays**—**Figure 1** shows a sample saturation binding of  $[^3\text{H}]\text{-}(+)\text{-pentazocine}$  to the S1R in GPL membranes. **Figure 2** shows sample results for inhibition of  $[^3\text{H}]\text{-}(+)\text{-pentazocine}$  with a test compound in GPL membranes. **Figure 3** shows saturation binding of  $[^3\text{H}]\text{-DTG}$  to the S1R and S2R in RL membranes. We did not show an example of competitive inhibition of  $[^3\text{H}]\text{-DTG}$  with test compounds but researchers should expect to see similar a binding curve that that reported in **Figure 3**. A table of  $K_i$  of compounds that bind to both S1R and S2R is summarized in **Table 8**.

## Time Considerations

**Radioligand binding assays**—Saturation binding assay and inhibition of radioligand binding assays typically require approximately one to three hours to complete if all reagents are prepared in advance. For inhibition of radioligand binding assays the time to complete an experiment may vary depending on the number of compounds tested at one time. We advise limitation of the number of compounds tested to a maximum of three as the number of samples may get too large. While the experiment takes one to three hours to complete, the data are obtained on the second day.

**Preparation of tissue membranes**—The time required to prepare tissue membranes depends on whether the tissue is fresh or frozen. For one to two tissue samples, it typically requires approximately two hours to prepare.

## Acknowledgements

This work is supported by NIH MH065503 (AER), NIH NS075820 (AER), Edwin and Dorothy Gamewell UW McPherson Retina Research Foundation Professorship (AER) and a Pharmaceutical Manufacturer's Post Doctoral Fellowship (UBC).

## References

- Abate C, Elenewski J, Niso M, Berardi F, Colabufo NA, Azzariti A, Perrone R, Glennon RA. Interaction of the sigma(2) receptor ligand PB28 with the human nucleosome: computational and experimental probes of interaction with the H2A/H2B dimer. *ChemMedChem*. 2010; 5:268–273. [PubMed: 20077462]
- Abate C, Niso M, Lacivita E, Mosier PD, Toscano A, Perrone R. Analogues of sigma receptor ligand 1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperazine (PB28) with added polar functionality and reduced lipophilicity for potential use as positron emission tomography radiotracers. *J Med Chem*. 2011; 54:1022–1032. [PubMed: 21229979]
- Aboloripeye SY, Fischer JB, Glennon RA. Is a nitrogen atom an important pharmacophoric element in sigma ligand binding? *Bioorg Med Chem*. 2000; 8:2105–2111. [PubMed: 11003156]
- Bowen WD, Hellewell SB, McGarry KA. Evidence for a multi-site model of the rat brain sigma receptor. *Eur J Pharmacol*. 1989; 163:309–318. [PubMed: 2542066]
- Chu UB, Hajipour AR, Ramachandran S, Ruoho AE. Characterization of Interactions of 4-nitrophenylpropyl-N-alkylamine with Sigma Receptors. *Biochemistry*. 2011; 50:7568–7578. [PubMed: 21790129]
- Chu UB, Ramachandran S, Hajipour AR, Ruoho AE. Photoaffinity labeling of the sigma-1 receptor with N-[3-(4-nitrophenyl)propyl]-N-dodecylamine: evidence of receptor dimers. *Biochemistry*. 2013; 52:859–868. [PubMed: 23324054]
- de Costa BR, Bowen WD, Hellewell SB, Walker JM, Thurkauf A, Jacobson AE, Rice KC. Synthesis and evaluation of optically pure [3H]-(+)-pentazocine, a highly potent and selective radioligand for sigma receptors. *FEBS Lett*. 1989; 251:53–58. [PubMed: 2568952]
- Fontanilla D, Hajipour AR, Pal A, Chu UB, Arbabian M, Ruoho AE. Probing the steroid binding domain-like I (SBDLI) of the sigma-1 receptor binding site using N-substituted photoaffinity labels. *Biochemistry*. 2008; 47:7205–7217. [PubMed: 18547058]
- Fontanilla D, Johannessen M, Hajipour AR, Cozzi NV, Jackson MB, Ruoho AE. The hallucinogen N,N-dimethyltryptamine (DMT) is an endogenous sigma-1 receptor regulator. *Science*. 2009; 323:934–937. [PubMed: 19213917]
- Gromek KA, Suchy FP, Meddaugh HR, Wrobel RL, LaPointe LM, Chu UB, Primm JG, Ruoho AE, Senes A, Fox BG. The oligomeric states of the purified sigma-1 receptor are stabilized by ligands. *J Biol Chem*. 2014; 289:20333–20344. [PubMed: 24847081]
- Hanner M, Moebius FF, Flandorfer A, Knaus HG, Striessnig J, Kempner E, Glossmann H. Purification, molecular cloning, and expression of the mammalian sigma1-binding site. *P.N.A.S.* 1996; 93:8072–8077. [PubMed: 8755605]
- Hayashi T, Justinova Z, Hayashi E, Cormaci G, Mori T, Tsai SY, Barnes C, Goldberg SR, Su TP. Regulation of sigma-1 receptors and endoplasmic reticulum chaperones in the brain of methamphetamine self-administering rats. *J Pharmacol Exp Ther*. 2010; 332:1054–1063. [PubMed: 19940104]
- Hayashi T, Su TP. Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. *Cell*. 2007; 131:596–610. [PubMed: 17981125]
- Hellewell SB, Bowen WD. A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain. *Brain Res*. 1990; 527:244–253. [PubMed: 2174717]

- Hellewell SB, Bruce A, Feinstein G, Orringer J, Williams W, Bowen WD. Rat liver and kidney contain high densities of sigma 1 and sigma 2 receptors: characterization by ligand binding and photoaffinity labeling. *Eur J Pharmacol.* 1994; 268:9–18. [PubMed: 7925616]
- Kaushal N, Matsumoto RR. Role of sigma receptors in methamphetamine-induced neurotoxicity. *Curr Neuropharmacol.* 2011; 9:54–57. [PubMed: 21886562]
- Kavanaugh MP, Parker J, Bobker DH, Keana JF, Weber E. Solubilization and characterization of sigma-receptors from guinea pig brain membranes. *J Neurochem.* 1989; 53:1575–1580. [PubMed: 2552016]
- Kekuda R, Prasad PD, Fei YJ, Leibach FH, Ganapathy V. Cloning and functional expression of the human type 1 sigma receptor (hSigmaR1). *Biochem Biophys Res Commun.* 1996; 229:553–558. [PubMed: 8954936]
- Kourrich S, Hayashi T, Chuang JY, Tsai SY, Su TP, Bonci A. Dynamic interaction between sigma-1 receptor and Kv1.2 shapes neuronal and behavioral responses to cocaine. *Cell.* 2013; 152:236–247. [PubMed: 23332758]
- Langa F, Codony X, Tovar V, Lavado A, Gimenez E, Cozar P, Cantero M, Dordal A, Hernandez E, Perez R, Monroy X, Zamanillo D, Guitart X, Montoliu L. Generation and phenotypic analysis of sigma receptor type I (sigma 1) knockout mice. *Eur J Neurosci.* 2003; 18:2188–2196. [PubMed: 14622179]
- Mach RH, Wheeler KT. Development of molecular probes for imaging sigma-2 receptors in vitro and in vivo. *Cent Nerv Syst Agents Med Chem.* 2009; 9:230–245. [PubMed: 20021357]
- Mavlyutov TA, Epstein ML, Andersen KA, Ziskind-Conhaim L, Ruoho AE. The sigma-1 receptor is enriched in postsynaptic sites of C-terminals in mouse motoneurons. An anatomical and behavioral study. *Neuroscience.* 2010; 167:247–255. [PubMed: 20167253]
- Menkel M, Terry P, Pontecorvo M, Katz JL, Witkin JM. Selective sigma ligands block stimulant effects of cocaine. *Eur J Pharmacol.* 1991; 201:251–252. [PubMed: 1686761]
- Morris DI, Speicher LA, Ruoho AE, Tew KD, Seamon KB. Interaction of forskolin with the P-glycoprotein multidrug transporter. *Biochemistry.* 1991; 30:8371–8379. [PubMed: 1679346]
- Navarro G, Moreno E, Bonaventura J, Brugarolas M, Farre D, Aguinaga D, Mallol J, Cortes A, Casado V, Lluís C, Ferre S, Franco R, Canela E, McCormick PJ. Cocaine inhibits dopamine D2 receptor signaling via sigma-1-D2 receptor heteromers. *PLoS one.* 2013; 8:e61245. [PubMed: 23637801]
- Pal A, Chu UB, Ramachandran S, Grawoig D, Guo LW, Hajipour AR, Ruoho AE. Juxtaposition of the steroid binding domain-like I and II regions constitutes a ligand binding site in the sigma-1 receptor. *The Journal of biological chemistry.* 2008; 283:19646–19656. [PubMed: 18467334]
- Pal A, Hajipour AR, Fontanilla D, Ramachandran S, Chu UB, Mavlyutov T, Ruoho AE. Identification of regions of the sigma-1 receptor ligand binding site using a novel photoprobe. *Molecular Pharmacology.* 2007; 72:921–933. [PubMed: 17622576]
- Quirion R, Bowen WD, Itzhak Y, Junien JL, Musacchio JM, Rothman RB, Su TP, Tam SW, Taylor DP. A proposal for the classification of sigma binding sites. *Trends Pharmacol Sci.* 1992; 13:85–86. [PubMed: 1315463]
- Ramachandran S, Lu H, Prabhu U, Ruoho AE. Purification and characterization of the guinea pig sigma-1 receptor functionally expressed in *Escherichia coli*. *Protein expression and purification.* 2007; 51:283–292. [PubMed: 16962337]
- Robson MJ, Noorbakhsh B, Seminerio MJ, Matsumoto RR. Sigma-1 receptors: potential targets for the treatment of substance abuse. *Curr Pharm Des.* 2012; 18:902–919. [PubMed: 22288407]
- Steinman RM, Mellman IS, Muller WA, Cohn ZA. Endocytosis and the recycling of plasma membrane. *The Journal of cell biology.* 1983; 96:1–27. [PubMed: 6298247]
- Su TP. Evidence for sigma opioid receptor: binding of [<sup>3</sup>H]SKF-10047 to etorphine-inaccessible sites in guinea-pig brain. *J Pharmacol Exp Ther.* 1982; 223:284–290. [PubMed: 6290634]
- Ujike H, Kuroda S, Otsuki S. sigma Receptor antagonists block the development of sensitization to cocaine. *Eur J Pharmacol.* 1996; 296:123–128. [PubMed: 8838447]
- Walker JM, Bowen WD, Goldstein SR, Roberts AH, Patrick SL, Hohmann AG, DeCosta B. Autoradiographic distribution of [<sup>3</sup>H](+)-pentazocine and [<sup>3</sup>H]1,3-di-o-tolylguanidine (DTG) binding sites in guinea pig brain: a comparative study. *Brain Res.* 1992; 581:33–38. [PubMed: 1323368]

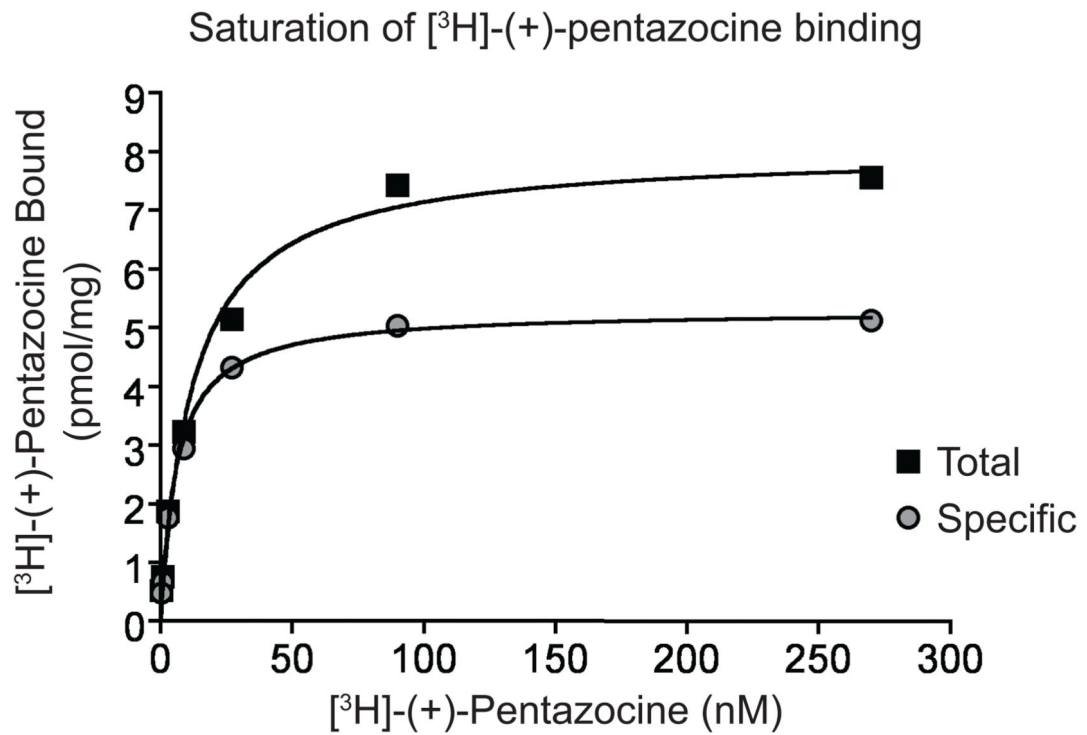
Walker JM, Bowen WD, Walker FO, Matsumoto RR, De Costa B, Rice KC. Sigma receptors: biology and function. *Pharmacol Rev.* 1990; 42:355–402. [PubMed: 1964225]

Author Manuscript

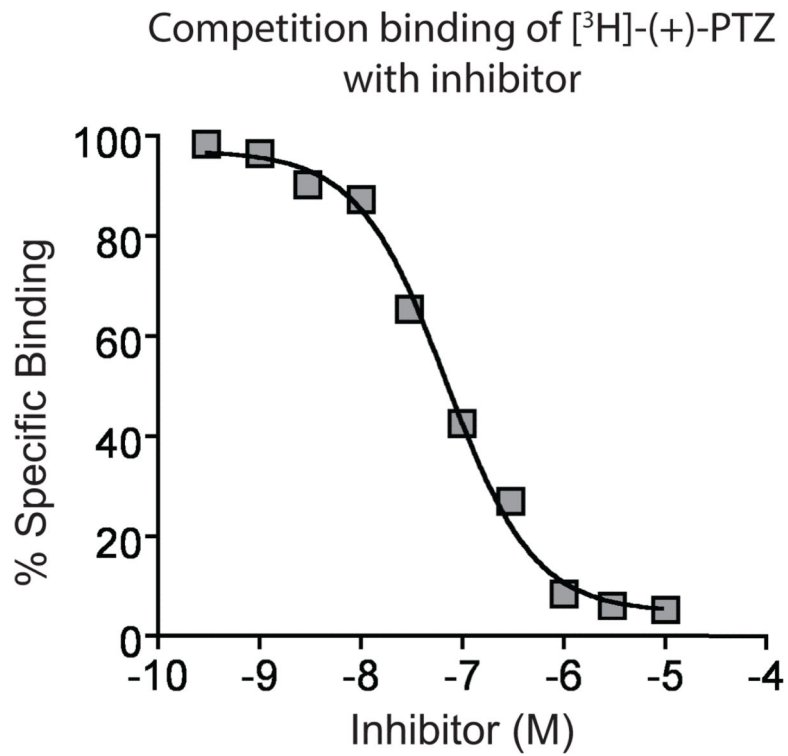
Author Manuscript

Author Manuscript

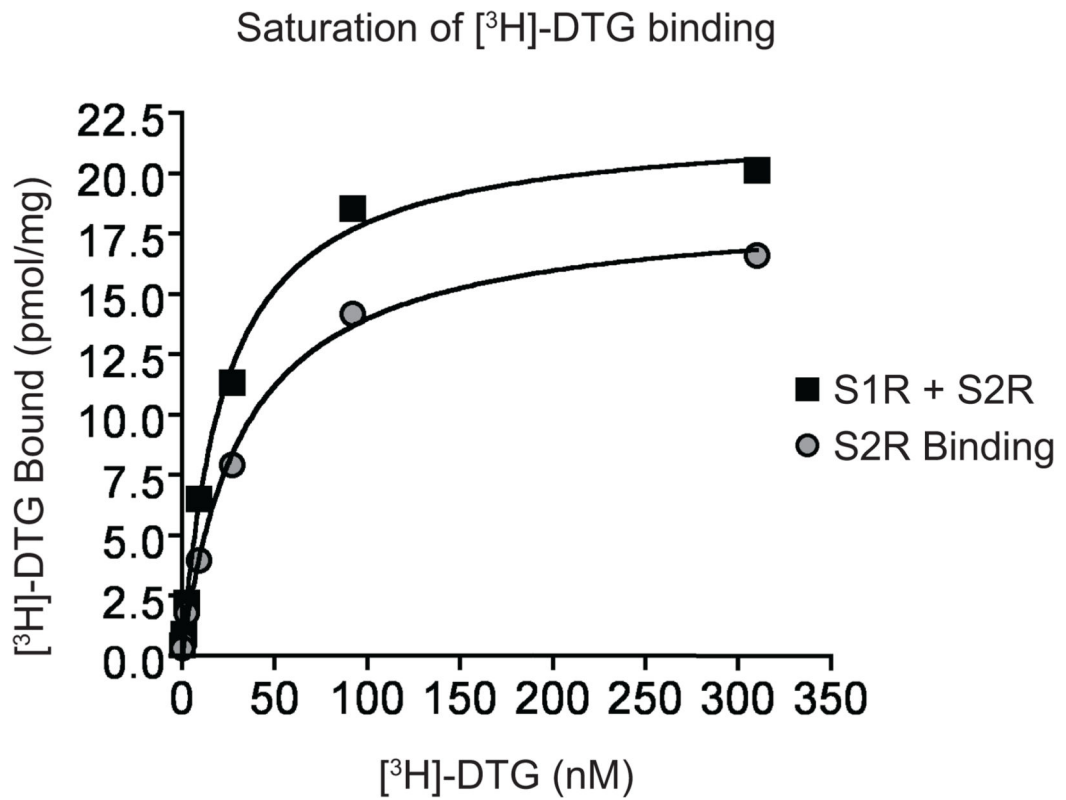
Author Manuscript



**Figure 1. Sample saturation binding of [<sup>3</sup>H]-(+)-pentazocine**  
A representative saturation binding of [<sup>3</sup>H]-(+)-pentazocine to assess activities and S1R protein levels in GPL membranes. Total refers to total radioactivity bound and Specific refers to Total binding subtract nonspecific binding.



**Figure 2. Sample inhibition of [<sup>3</sup>H]-(+)-pentazocine binding to the S1R**  
A representative inhibition of [<sup>3</sup>H]-(+)-pentazocine to determine  $K_I$  of a test compound. Points are mean of quadruplicates. Percent specific binding is calculated using **Equation 3** (see text).



**Figure 3. Sample saturation binding of [<sup>3</sup>H]-DTG to both S1R and S2R**  
A representative saturation-binding of [<sup>3</sup>H]-DTG to assess S1R and S2R proteins in RL membranes. S1R + S2R binding is defined as total [<sup>3</sup>H]-DTG bound while S2R binding is determined by subtracting the counts from the plus (+)-pentazocine conditions from the total [<sup>3</sup>H]-DTG binding (without (+)-pentazocine (see **Table 11** for data).

**Table 1**Sample dilution scheme of [<sup>3</sup>H]-(+)-pentazocine for saturation-binding experiments

Dilution #	Final [ <sup>3</sup> H]-(+)-PTZ concentration <sup>I</sup> (nM)	10X stock [ <sup>3</sup> H]-(+)-PTZ concentrations (nM)	Dilution factor	Dilution volumes
1	300 nM	3000 nM	10	102 μL stock + 898 μL of Assay Buffer
2	100 nM	1000 nM	28	34 μL of stock + 966 μL of Assay Buffer
3	30 nM	300 nM	10	100 μL of dilution #1 + 900 μL of Assay Buffer
4	10 nM	100 nM	10	100 μL of dilution #2 + 900 μL of Assay Buffer
5	3 nM	30 nM	10	100 μL of dilution #3 + 900 μL of Assay Buffer
6	1 nM	10 nM	10	100 μL of dilution #4 + 900 μL of Assay Buffer
7	0.3 nM	3 nM	10	100 μL of dilution #5 + 900 μL of Assay Buffer

<sup>2</sup> The stock concentration of [<sup>3</sup>H]-(+)-pentazocine is supplied at 34 Ci/mmol (e.g., Perkin Elmer). The calculated concentrations of this stock [<sup>3</sup>H]-(+)-pentazocine is counted and determined to be approximately 29.4 μM (1/34 Ci/mmol × 1000) which is the concentrations used to prepare the 10X stocks radioligand.

<sup>I</sup> Final radioligand concentration is the concentrations of [<sup>3</sup>H]-(+)-pentazocine in the binding assay.



**Table 2**Sample conversion of total [<sup>3</sup>H]-(+)-pentazocine counts into nanomolar concentrations in assay

Targeted [ <sup>3</sup> H]-(+)-PTZ concentration (nM)	Measured total radioactivity (DPM)	Measured total [ <sup>3</sup> H]-(+)-PTZ (mmol)	Measured [ <sup>3</sup> H]-(+)-PTZ concentration in assay (nM)
300	2037960	2.70E-08	270.0
100	679420	9.00E-09	90.0
30	204684	2.71E-09	27.1
10	66382	8.79E-10	8.8
3	21047	2.79E-10	2.8
1	6573	8.71E-11	0.9
0.3	2234	2.96E-11	0.3

The first column displays targeted concentrations of [<sup>3</sup>H]-(+)-pentazocine calculated from the stock vial (34 Ci/mmol or ~29.4 μM). Second column contains radioactivity counts in DPM of the targeted concentration of [<sup>3</sup>H]-(+)-pentazocine. Third column shows radioactivity values transformed from DPM into millimoles of radioligand (Note: the conversion factor used was  $2.22 \times 10^{12}$  DPM per Ci). The fourth column shows the actual concentrations of [<sup>3</sup>H]-(+)-pentazocine as calculated from the DPM values obtained.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

**Table 3**

Sample transformation of bound radioactivity data from DPM into pmol/mg protein

Measured [ <sup>3</sup> H]-(+)-PTZ concentration (nM)	Average bound		
	Bound (DPM)	Bound (pmol)	Bound (pmol/mg protein)
270.0	18260	0.241918389	7.559949656
90.0	17936	0.237625861	7.425808161
27.1	12433	0.164719131	5.14747284
8.8	7799	0.103325384	3.228918256
2.8	4559	0.060400106	1.887503312
0.9	1866	0.024721781	0.772555644
0.3	1253	0.016600424	0.518763249

The first column displays measured [<sup>3</sup>H]-(+)-pentazocine calculated from counting an aliquot of each [<sup>3</sup>H]-(+)-pentazocine concentrations (see **Table 2** for detail calculations). The second column shows the mean from triplicates in DPM and the third column displays transformation of the DPM values into radioactivity bound expressed as pmol [Note: radioactivity bound is determined using the conversion factor  $2.22 \times 10^{12}$  DPM per Ci and the specific activity of [<sup>3</sup>H]-(+)-pentazocine of 34 Ci/mmol]. The last column shows bound radioligand levels normalized to the total protein added to the assay which is 0.032 mg per well.

**Table 4**Sample saturation of [<sup>3</sup>H]-(+)-pentazocine binding data

Measured [ <sup>3</sup> H]-(+)-PTZ concentration (nM)	Bound [ <sup>3</sup> H]-(+)-pentazocine (pmol/mg)		
	Total	Nonspecific	Specific
270.0	7.56	2.43	5.13
90.0	7.43	2.39	5.04
27.1	5.15	0.82	4.33
8.8	3.23	0.28	2.95
2.8	1.88	0.11	1.77
0.9	0.77	0.09	0.68
0.3	0.52	0.04	0.48

Total binding is defined as [<sup>3</sup>H]-(+)-pentazocine bound while nonspecific binding is the counts obtained for samples that contain 10 μM haloperidol. Specific binding is defined here as total – nonspecific.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

**Table 5**Typical  $K_D$  and  $B_{max}$  values for both [ $^3H$ ]-(+)-pentazocine and [ $^3H$ ]-DTG binding

	[ $^3H$ ]-(+)-pentazocine		[ $^3H$ ]-DTG	
	$K_D$ (nM)	$B_{max}$ (pmol/mg)	$K_D$ (nM)	$B_{max}$ (pmol/mg)
<b>Guinea Pig Liver</b>	0.2	3.1	-	-
<b>Rat Liver</b>	-	-	150	11
<b>Recombinant SIR</b> *	35	10600	-	-

\*  $K_D$  and  $B_{max}$  of [ $^3H$ ]-(+)-pentazocine for recombinant SIR were obtained from Ramachandran *et al.* 2007.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

**Table 6**

Sample dilution scheme for a binding site inhibitor tested in a competition binding assay

Dilution #	Final unlabeled inhibitor concentration (M)	10X Stock unlabeled inhibitor concentrations (M)	Dilution factor	Dilution volumes
1	1.00E-05	1.00E-04	10	100 $\mu$ L of 1.00E-03 M stock + 900 $\mu$ L of Assay Buffer
2	3.00E-06	3.00E-05	10	100 $\mu$ L of 3.00E-04 M stock + 900 $\mu$ L of Assay Buffer
3	1.00E-06	1.00E-05	10	100 $\mu$ L of dilution #2 + 900 $\mu$ L of Assay Buffer
4	3.00E-07	3.00E-06	10	100 $\mu$ L of dilution #3 + 900 $\mu$ L of Assay Buffer
5	1.00E-07	1.00E-06	10	100 $\mu$ L of dilution #4 + 900 $\mu$ L of Assay Buffer
6	3.00E-08	3.00E-07	10	100 $\mu$ L of dilution #5 + 900 $\mu$ L of Assay Buffer
7	1.00E-08	1.00E-07	10	100 $\mu$ L of dilution #6 + 900 $\mu$ L of Assay Buffer
8	3.00E-09	3.00E-08	10	100 $\mu$ L of dilution #7 + 900 $\mu$ L of Assay Buffer
9	1.00E-09	1.00E-08	10	100 $\mu$ L of dilution #8 + 900 $\mu$ L of Assay Buffer
10	3.00E-10	3.00E-09	10	100 $\mu$ L of dilution #9 + 900 $\mu$ L of Assay Buffer

The final concentration of unlabeled inhibitor is the concentration in the well (column 2). The 10X stock unlabeled inhibitor concentrations is the stock concentration prior to dilution (column 3).

**Table 7**Sample transformation of competition binding against [<sup>3</sup>H]-(+)-PTZ

Final unlabeled inhibitor concentration (M)	Specific [ <sup>3</sup> H]-(+)-PTZ Bound (DPM)	% Specific Binding
1.00E-05	851	5.25
3.00E-06	950	6.06
1.00E-06	1250	8.51
3.00E-07	3521	27.10
1.00E-07	5420	42.64
3.00E-08	8210	65.47
1.00E-08	10886	87.37
3.00E-09	11253	90.37
1.00E-09	12011	96.57
3.00E-10	12296	98.91

The first column displays the concentrations of unlabeled inhibitor as determined by serial dilution (Table 6). The second column shows mean of quadruplicate counts (in DPM) measured. Values in the third column are derived using **Equation 3** where the nonspecific binding value was 209 DPM and the total binding was 12220 DPM.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

**Table 8**

Typical  $K_I$  values (nM) for reference compounds at sigma-1 and sigma-2 receptors.

	S1R	S2R
<b>BD1047</b>	0.93 <sup>1</sup>	47 <sup>1</sup>
<b>BD1063</b>	9.2 <sup>1</sup>	449 <sup>1</sup>
<b>Dimethyltryptamine</b>	14,700 <sup>2</sup>	21,700 <sup>2</sup>
<b>DTG</b>	35.5 <sup>3</sup>	39.9 <sup>3</sup>
<b>Fenpropimorph</b>	9.3 <sup>4</sup>	Not yet determined
<b>Haloperidol</b>	0.9 <sup>3</sup>	7.9 <sup>3</sup>
<b>(+)-Pentazocine</b>	1.6 <sup>3</sup>	728.4 <sup>3</sup>
<b>PRE-084</b>	44 <sup>5</sup>	13091 <sup>6</sup>
<b>Progesterone</b>	239 <sup>7</sup>	441 <sup>7</sup>
<b>Rimcazole</b>	85 <sup>8</sup>	386 <sup>8</sup>

<sup>1</sup>Matsumoto RR *et al.* (1995) EJP 280: 301-310.

<sup>2</sup>Fontanilla D *et al.* (2009) Science 323(5916): 934-937.

<sup>3</sup>Lever JR *et al.* (2006) Synapse 59: 350-358.

<sup>4</sup>Pal A *et al.* (2007) *Molecular Pharmacology* 72:921-933.

<sup>5</sup>Su TP *et al.* (1991) JPET 259(2): 543-550.

<sup>6</sup>Maurice T. *et al.* (1999) Br J Pharmacol 127(2): 335-342.

<sup>7</sup>Johannessen M *et al.* (2011) Am J Physiol Cell Physiol 300(2): C328-337.

<sup>8</sup>Husbands SM *et al.* (1997) J Med Chem 40:4340-4346.

**Table 9**Sample dilution scheme of [<sup>3</sup>H]-DTG for saturation-binding experiments

Dilution #	$I_{\text{Final}}$ [ <sup>3</sup> H]-DTG concentration (nM)	Stock [ <sup>3</sup> H]-DTG concentrations (nM)	Dilution factor	Dilution volumes
1	300 nM	3000 nM	8.3	120 $\mu$ L stock <sup>2</sup> + 880 $\mu$ L of Assay Buffer
2	100 nM	1000 nM	25	40 $\mu$ L of stock + 960 $\mu$ L of Assay Buffer
3	30 nM	300 nM	10	100 $\mu$ L of dilution #1 + 900 $\mu$ L of Assay Buffer
4	10 nM	100 nM	10	100 $\mu$ L of dilution #2 + 900 $\mu$ L of Assay Buffer
5	3 nM	30 nM	10	100 $\mu$ L of dilution #3 + 900 $\mu$ L of Assay Buffer
6	1 nM	10 nM	10	100 $\mu$ L of dilution #4 + 900 $\mu$ L of Assay Buffer
7	0.3 nM	3 nM	10	100 $\mu$ L of dilution #5 + 900 $\mu$ L of Assay Buffer

<sup>1</sup>Final radioligand concentration is the concentrations of [<sup>3</sup>H]-(+)-pentazocine in the binding assay.

<sup>2</sup>The stock concentration of [<sup>3</sup>H]-DTG is supplied at 40 Ci/mmol (e.g., Perkin Elmer). The calculated concentrations of this stock [<sup>3</sup>H]-DTG is counted and determined to be approximately 25  $\mu$ M (1/40 Ci/mmol  $\times$  1000) which is the concentrations used to prepare the 10X stocks radioligand.



**Table 10**

Sample conversion of total [<sup>3</sup>H]-DTG counts into nanomolar concentrations in assay

Targeted [ <sup>3</sup> H]-DTG concentration (nM)	Measured total radioactivity (DPM)	Measured total [ <sup>3</sup> H]-DTG (mmol)	Measured [ <sup>3</sup> H]-DTG concentration in assay (nM)
300	2337238	2.63E-08	310
100	693785.5	7.81E-09	92
30	204121.8	2.30E-09	27
10	67841.83	7.64E-10	9
3	20148.28	2.27E-10	2.7
1	6984	7.86E-11	0.8
0.3	2223	2.50E-11	0.25

The first column displays targeted concentrations of [<sup>3</sup>H]-DTG calculated from the stock vial (40 Ci/mmol or ~30 μM). Second column contains radioactivity counts in DPM of the targeted concentration of [<sup>3</sup>H]-DTG. Third column shows radioactivity values transformed from DPM into millimoles of radioligand (Note: the conversion factor used was  $2.22 \times 10^{12}$  DPM per Ci). The fourth column shows the actual concentrations of [<sup>3</sup>H]-DTG as calculated from the DPM values obtained.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

**Table 11**

Sample transformation of bound radioactivity data from DPM into pmol/mg protein

Measured [ <sup>3</sup> H]-DTG concentration (nM)	S1R + S2R Binding		S2R Binding	
	Specific Binding * (DPM)	Specific Binding * (pmol/mg)	Specific Binding * (DPM)	Specific Binding * (pmol/mg)
310	57234	20.14	47234	16.62
92	52722	18.55	40345	14.19
27	32299	11.36	22584	7.94
9	18545	6.52	11345	3.99
2.7	6280	2.21	5123	1.80
0.8	2676	0.94	1543	0.54
0.25	1253	0.44	938	0.33

The first column displays measured [<sup>3</sup>H]-DTG (see **Table 10** for detail calculations). Values in columns two and three represent specific binding\* of [<sup>3</sup>H]-DTG to both S1R and S2R. The second column shows the mean from triplicates in DPM and the third column displays transformation these counts into radioactivity bound normalized to proteins (pmol/mg). The fourth and fifth columns display specific binding\* of [<sup>3</sup>H]-DTG to only the S2R (the S1R sites were masked by the addition of (+)-pentazocine). Column four are mean from triplicates in DPM and the fifth column displays transformation of these counts into radioactivity bound normalized to proteins (pmol/mg). Note: radioactivity bound is determined using the conversion factor  $2.22 \times 10^{12}$  DPM per Ci, the specific activity of [<sup>3</sup>H]-DTG is 40 Ci/mmol, and total proteins equal 0.032 mg per well (see **Equation 1** for details).

\* Specific binding is defined as total bound – nonspecific binding (in the presence of 10 μM of haloperidol).