Published in final edited form as:

Eur J Pharmacol. 2015 September 5; 762: 118–126. doi:10.1016/j.ejphar.2015.05.026.

Characterization of pulmonary sigma receptors by radioligand binding

John R. Lever^{a,b,*}, Tyler P. Litton^{a,b}, and Emily A. Ferguson-Cantrell^{a,b}

^aDepartment of Radiology, University of Missouri, Columbia, MO 65212, USA

^bHarry S. Truman Memorial Veterans' Hospital, 800 Hospital Drive, Columbia, MO 65201, USA

Abstract

This study establishes the expression of appreciable populations of sites on mouse lung membranes that exhibit radioligand binding properties and pharmacology consistent with assignment as sigma₁ and sigma₂ receptors. Specific binding of the sigma₁ receptor radioligand [³H](+)-pentazocine reached steady state within 6 h at 37 °C. Saturation studies revealed high affinity binding to a single class of sites (K_d 1.36 ± 0.04 nM; B_{max} 967 ± 11 fmol / mg protein). Inhibition studies showed appropriate sigma₁ receptor pharmacology, including higher affinity for (+)-*N*-allylnormetazocine with respect to the (–)-enantiomer, and positive allosteric modulation of dextromethorphan binding by phenytoin. Using [³H]1,3-di(2-tolyl)guanidine in the presence of (+)-pentazocine to assess sigma₂ receptor binding, steady state was achieved within 2 min at 25 °C. Cold saturation studies revealed one high affinity, low capacity binding site (K_d 31.8 ± 8.3 nM; B_{max} 921 ± 228 fmol / mg protein) that displayed sigma₂ receptor pharmacology. A very low affinity, high capacity interaction also was observed that represents saturable, but not sigma receptor specific, binding. A panel of ligands showed rank order inhibition of radioligand binding appropriate for the sigma₂ receptor, with ifenprodil displaying the highest apparent affinity. In vivo, dextromethorphan inhibited the specific binding of a radioiodinated sigma₁ receptor ligand in lung with an ED₅₀ of 1.2 μmol / kg, a value near the recommended dosage for the drug as a cough suppressant. Overall, the present work provides a foundation for studies of drug interactions with pulmonary sigma₁ and sigma₂ receptors in vitro and in vivo.

Keywords

Sigma receptor; Pulmonary pharmacology; Mouse; Radioligand binding

*Corresponding author: John R. Lever, Department of Radiology, University of Missouri - Columbia M292 Medical Sciences Building, One Hospital Drive, Columbia, Missouri 65212 USA leverj@health.missouri.edu (J. R. Lever) Tel.: +1-573-814-6000 ext. 53686; fax: +1-573-814-6551.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Two sigma receptor subtypes, sigma₁ and sigma₂, have been characterized by radioligand binding studies in mammalian brain (DeHaven-Hudkins et al., 1992; Kornhuber et al., 1996; Kovács and Larson, 1995; Lever et al., 2006; Sjøby et al., 2002), and peripheral tissues including heart, liver, kidney, spleen and testes (Walker et al., 1990; Hellewell et al., 1994; DeHaven-Hudkins et al., 1994; Maurice et al., 2002; Monassier and Bousquet, 2002; Matsumoto et al., 2007). By contrast, scant information is available regarding sigma receptor binding sites in lung. Specifically, the sigma₁ receptor subtype has been cloned, and transcripts have been detected in rat (Seth et al., 1998) and mouse (Langa et al., 2003) lung. Maruo and colleagues (2000) reported binding of [³H](+)-pentazocine, a selective sigma₁ receptor radioligand, to lung membranes from mouse and guinea pig. However, radioligand affinity (K_d), maximal binding density (B_{max}), and a pharmacological profile of the sites labeled by [³H](+)-pentazocine were not included. In vivo studies using selective sigma₁ receptor-binding radioligands also indicate the presence of pulmonary sites in rodents (Collier et al., 2007; Lever et al., 2012; 2014). In fact, Brown et al. (2004) have suggested that agonist actions at sigma₁ receptors in guinea pig lung may be partly responsible for the antitussive effects of aerosolized dextromethorphan. Interestingly, evidence points to the hallucinogen *N,N*-dimethyltryptamine as an endogenous sigma₁ receptor agonist that is synthesized, in large measure, by the lung enzyme indolethylamine-*N*-methyltransferase (Fontanilla et al., 2009; Frecska et al., 2013; Chu et al., 2015).

The sigma₂ receptor has yet to be cloned, and very little is known about its expression and properties in normal pulmonary tissue. Radioligand binding studies of sigma₂ sites in lung membranes have not been reported, and single cell suspensions derived from mouse lung show only minimal binding of a fluorescent sigma₂ receptor ligand (Kashiwagi et al., 2007). On the other hand, the sigma₂ receptor may well be associated with progesterone receptor membrane component 1 (PGRMC1) (Xu et al., 2011), a protein that is detectable by immunoblot analysis in normal human lung (Mir et al., 2012). Both the sigma₂ / PGRMC1 and the sigma₁ receptors are elevated in lung cancer tumors and cell lines, and have been identified as diagnostic markers and therapeutic targets (Mach et al., 2013; van Waarde et al., 2014). Furthermore, sigma₁ receptors serve as protein chaperones that modulate oxidative / endoplasmic reticulum stress in lung and other tissues, help in cell management of misfolded proteins, and influence cell death and survival (Hayashi and Su, 2007; Hayashi, 2015; Pal et al., 2012; Su et al., 2010). In the present study, we characterized sigma₁ receptor binding in membranes from mouse lung, and established the presence of a substantial density of sites having the pharmacological properties of the sigma₂ receptor. We also defined the in vivo binding of dextromethorphan, a widely used over-the-counter cough suppressant, to sigma₁ receptors in mouse lung, spleen, heart and brain which lends support to potential roles for peripheral sigma receptors in the activity of certain drugs.

2. Materials and methods

2.1. Drugs, chemicals and solutions

Haloperidol, (+)-pentazocine, 1,3-di(2-tolyl)guanidine (DTG), dextromethorphan hydrobromide, (–)-cocaine hydrochloride, (+)- and (–)-*N*-allylnormetazocine (NANM,

SKF-10,047), ifenprodil (+)-tartrate, phenytoin, BD1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine) dihydrochloride, and AG-205 (Z-2-[[1-(4-chlorophenyl)-1H-tetrazol-5-yl]thio]-1-(1,2,3,4,4a,9b-hexahydro-2,8-dimethyl-5H-pyrido[4,3-b]indol-5-yl)ethanone) were supplied by Sigma-Aldrich, Inc. (St. Louis, MO). Batches of [³H]DTG (47.6 – 53.3 Ci / mmol) and [³H](+)-pentazocine (34.8 – 36.6 Ci / mmol) were purchased from PerkinElmer, Inc. (Waltham, MA). [¹²⁵I]E-IA-DM-PE-PIPZE (*E-N*-1-(3'-iodoallyl)-N'-4-(3'',4''-dimethoxyphenethyl)-piperazine; ca. 2000 Ci / mmol) was prepared as previously described (Lever et al., 2012). Other chemicals and solvents were obtained at the best grades available.

For in vitro binding studies, stock (10 mM) and serial dilutions of AG-205 were prepared in DMSO, with final assay concentrations of DMSO = 1%. Stock (1 – 10 mM) and serial dilutions of other non-radioactive ligands were prepared in water or Tris-HCl buffer (50 mM; pH 8.0, 25 °C), except for haloperidol and (+)-pentazocine where water containing 1% ethanol and 0.1% acetic acid was employed. Tritiated radioligand stock solutions and serial dilutions were prepared in Tris-HCl buffer (50 mM; pH 8.0, 25 °C). The [³H]-radioactivity was measured at an efficiency of 44% with an automated beta counter (Wallac 1409; Turku, Finland) using OptiPhase[®] HiSafe 2 liquid scintillation cocktail (PerkinElmer, Inc.).

For in vivo studies, solutions of [¹²⁵I]E-IA-DM-PE-PIPZE, dextromethorphan and BD1063 were prepared in sterile bacteriostatic saline (0.9% NaCl, 0.9% benzyl alcohol; w/v) from Hospira, Inc. (Lake Forest, IL). The radioligand formulation also contained 2% ethanol. The [¹²⁵I]-radioactivity was measured with 78% efficiency using an automated gamma counter (Wallac Wizard 1480; Turku, Finland).

2.2. Animals

Adult male CD-1[®] mice were obtained from Charles River Laboratories International, Inc. (Wilmington, MA). Animals were acclimated for one to two weeks prior to study in group-housing on a 12 h light-dark cycle in temperature and humidity controlled quarters with unrestricted access to standard chow and water. Studies were performed in compliance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and with prior approvals from the Institutional Animal Care and Use Committees of the University of Missouri and the Harry S. Truman Memorial Veterans' Hospital.

2.3. Pulmonary membrane preparation

Mice (25 – 35 g) were euthanized by cervical dislocation. Whole lungs, including trachea and bronchi, were excised, weighed and rinsed with Tris-HCl buffer (50 mM; pH 7.4, 25 °C). Tissues were processed immediately, or were stored frozen at –80 °C. Fresh or thawed pulmonary tissues were placed in 10 volumes of Tris-HCl buffer (50 mM; pH 7.4, 25 °C), and then homogenized using a Polytron[®] (Brinkmann Instruments, Inc.; Westbury, NY) on setting 6 (3 × 45 s). The homogenate was centrifuged at 48,000 g for 15 min at 4 °C. Pellets were suspended in 10 volumes of buffer, homogenized as above, and then incubated at 37 °C for 30 min. After centrifugation (48,000 g, 15 min; 4 °C), pellets were suspended in 20 volumes of pH 7.4 Tris buffer, and divided into aliquots (12 ml) that were stored at –80 °C

until use. Protein concentrations were determined by the bicinchoninic acid assay, against a bovine serum albumin standard curve, using a commercially available kit (Pierce Biotechnology, Rockford, IL). Preparations were typically 1.5 mg protein / ml, and membranes were diluted using pH 8.0 Tris-HCl (50 mM; 25 °C) as needed for binding assays.

2.4. Sigma₁ and sigma₂ receptor binding in vitro

Assays using lung membranes were performed using minor modifications of methods previously described for sigma receptor studies in other tissues (Bowen et al., 1993; DeHaven-Hudkins et al., 1992; Hellewell et al., 1994; Mach et al., 1995; Lever et al., 2006). In all cases, assays were terminated by addition of ice-cold assay buffer (5 ml) and rapid filtration (Brandel, Inc., Gaithersburg, MD) through glass fiber filters (GF/B) that had been pretreated for 1 h with polyethyleneimine (0.5%). Tubes and filter papers were washed (3 × 5 ml) with cold assay buffer. Filter papers were dried under vacuum, extracted with cocktail for at least 24 h, and then counted for radioactivity.

Association kinetics for sigma₁ receptor binding were determined in duplicate glass tubes containing 0.15 mg protein and either 0.7 nM or 3.0 nM [³H](+)-pentazocine in Tris-HCl (50 mM; pH 8.0, 25 °C) with final volumes of 1.0 ml / tube. Parallel sets of tubes containing haloperidol (1.0 μM) were used to define non-specific binding. Assays were initiated by addition of radioligand, and then incubated for various intervals from 15 min to 8 h at 37 °C in a shaking water bath. Additional samples were included at the 8 h time point to vary the protein from 0.075 – 0.30 mg / tube. Sigma₁ receptor saturation binding isotherms at 37 °C were generated using 0.20 mg protein / tube, six concentrations of [³H](+)-pentazocine ranging from 0.35 – 35.4 nM, 1.0 μM haloperidol to define non-specific binding, and an incubation time of 6 h. Competition binding assays for sigma₁ receptors were conducted in a final volume of 1.0 ml at 37 °C for 6 h using 0.20 mg protein / tube and 1.0 nM [³H](+)-pentazocine, with non-specific binding defined by 1.0 μM haloperidol. Competing ligands were used at ten concentrations that were equally spaced on log scale and centered on the anticipated K_i value. Each assay was performed in duplicate, and replicated three to six times. For dextromethorphan, allosteric modulation of sigma₁ receptor binding also was studied by including phenytoin (50 μL, 20 mM) in NaOH vehicle (0.15 M), or the NaOH vehicle (50 μL, 0.15 M) alone, as previously described for studies of central sigma₁ sites (DeHaven-Hudkins et al., 1993; Cobos et al., 2005).

Association kinetics for sigma₂ receptor binding were determined from 2 min to 2 h at 25 °C using 0.25 mg protein and 3.0 nM [³H]DTG in the presence of (+)-pentazocine (500 nM) to mask sigma₁ receptor binding. Final volumes were kept at 0.5 ml / tube, Tris-HCl (50 mM; pH 8.0, 25 °C) was used as the assay buffer, and haloperidol (10.0 μM) defined non-specific binding. At the 2 h time point, the protein per tube was varied from 0.13 – 0.50 mg. Homologous competitive binding of DTG (0.32 – 10,000 nM) to sigma₂ receptors was performed at 25 °C for 1 h using 0.20 mg protein / tube, 3.0 nM [³H]DTG / 500 nM (+)-pentazocine in pH 8 Tris-HCl buffer, and 10.0 μM haloperidol to define non-specific binding. Heterologous competitive binding data for sigma₂ receptors also were obtained under those assay conditions, using ten concentrations of test ligand centered on the

anticipated K_i value. Competition binding assays were performed in duplicate, and replicated three to six times.

2.5. Sigma₁ receptor binding in vivo

Dextromethorphan inhibition of [¹²⁵I]E-IA-DM-PE-PIPZE binding to sigma₁ receptors was determined using eight groups of non-fasted mice, with n = 5 for each experimental condition, as previously described for other inhibitors (Lever et al., 2014a,b). Each animal received 2.5 μCi of radioligand in 0.9% sterile saline (0.1 ml) containing 2% ethanol by tail vein injection. A control group received saline vehicle (0.1 ml) i.p. 1 min prior to i.v. administration of radioligand. Treatment groups received dextromethorphan, 0.32 to 100 μmol / kg, by i.p. injection in saline (0.1 ml) 1 min prior to i.v. radioligand. Non-specific binding was defined by a group that received BD1063 (5.0 μmol / kg) in saline (0.1 ml) by i.v. injection 5 min prior to i.v. radioligand. Animals were euthanized by cervical dislocation 30 min after radioligand administration, and whole brain, heart, lung and spleen were harvested, weighed and counted for radioactivity. The percent injected dose (%ID) / g wet tissue was calculated by comparison to standard dilutions of the ID.

2.6. Data analysis and statistics

Non-linear curve-fitting computer programs Prism 6.0c (GraphPad Software, Inc.; La Jolla, CA) and Radlig 6.0 (KELL Suite, Biosoft, Inc., Ferguson, MO) were employed for analysis of in vitro binding data. Hot and cold saturation binding isotherms were analyzed using program Radlig that corrects for free radioligand depletion. Kinetic and IC₅₀ data were analyzed using program Prism. K_i values were calculated from IC₅₀ values by the Cheng and Prusoff (1973) relationship using the measured K_d of the radioligand. Fitting of IC₅₀ specific binding data was performed using an unconstrained sigmoidal regression algorithm (Prism 6.0c), with AG-205 sigma₁ receptor binding being an exception where top and bottom plateaus were constrained to 100% and 0%, respectively.

Comparisons of two means were done using Student's unpaired *t*-test. Three or more means were compared using ANOVA at the $\alpha = 0.05$ level. *P*-values < 0.05 were considered significant. The *F*-ratio test was used to compare goodness-of-fit between one- and two-site models, and to test experimental Hill slopes against a theoretical value of 1.0 by comparison of the variable-slope, four-parameter fits against a three-parameter model having the Hill slope fixed. Correlations were investigated by Pearson analysis. Curve fitting of in vivo specific binding was performed using an unconstrained sigmoidal regression algorithm (Prism 6.0c).

3. Results

3.1. Sigma₁ receptor binding in mouse lung membranes

Kinetic studies showed very slow association of [³H](+)-pentazocine to binding sites in pulmonary membranes at 37 °C (*t*_{1/2} ~188 min, Fig. 1A). At a 0.7 nM concentration, specific binding reached steady state by 6 h, and remained constant through at least the 8 h time point. From 6 – 8 h, specific radioligand binding was 87% of the total binding using 1.0 μM haloperidol to define the non-specific component. Total binding represented < 6% of the

[³H](+)-pentazocine added, indicative of pseudo-first order kinetics. Data from the 3.0 nM concentration were similar, but steady state was reached a bit faster, by 5 h, as would be expected. In both instances, curves were fit as one-phase exponential associations (r^2 0.95). Data sets, each having two concentrations, were globally fit to derive shared best-fit estimates ($n = 3$, means $\pm$ S.E.M.) for k_{on} ($0.0018 \pm 0.00053 \text{ min}^{-1} \text{ nM}^{-1}$), k_{off} ($0.00089 \pm 0.00013 \text{ min}^{-1}$) and K_d ($0.61 \pm 0.24 \text{ nM}$). The specific binding of [³H](+)-pentazocine proved directly proportional ($r^2 = 0.99$) to the amount of membrane protein employed over the range (0.08 – 0.30 mg / tube) investigated (data not shown).

Using a 6 h incubation time at 37 °C, [³H](+)-pentazocine displayed saturable, high-affinity binding to a single class of sites in mouse lung membranes (Fig. 1B). The measured equilibrium dissociation constant (K_d) was $1.40 \pm 0.06 \text{ nM}$, and the maximal density of binding sites (B_{max}) was $951 \pm 18 \text{ fmol / mg protein}$ ($n = 4$, means $\pm$ S.E.M.). These results were replicated in an independent set of four experiments that showed no significant differences (t -test) from the first set. The pooled data gave a K_d of $1.36 \pm 0.04 \text{ nM}$ and a B_{max} of $967 \pm 11 \text{ fmol / mg protein}$ ($n = 8$).

Inhibitory potencies of well-characterized sigma receptor ligands against [³H](+)-pentazocine at 37 °C in lung membranes using a 6 h incubation period are given in Table 1. Competition binding curves are shown in Fig. 2A. The putative sigma₂ / PGRMC1 ligand, AG-205, was also included to gain knowledge of its potential sigma receptor subtype selectivity. Specific [³H](+)-pentazocine binding for the competition studies was > 85% of the total binding. Test compounds inhibited radioligand binding in monophasic, concentration-dependent fashion consistent with a single class of lung binding sites. Full inhibition was noted for all compounds except AG-205, where approximately 10% residual specific binding was noted at 0.02 M, the highest concentration used (point not shown in Fig 2A). Pseudo-Hill slopes (n_H) did not differ significantly from unity (F -ratio test, $P > 0.05$). Potency, as relative K_i values, was haloperidol, (+)-pentazocine > ifenprodil, (+)-NANM >> DTG, dextromethorphan >> (–)-cocaine, (–)-NANM >> AG-205. A robust Pearson rank order correlation ($r = 0.99$, $P = 0.0001$; Fig. 2B) exists between these data and the pK_i values reported for six of the inhibitors against [³H](+)-pentazocine binding in Swiss Webster mouse brain membranes (Kovács and Larson, 1995).

In additional studies, phenytoin (1.0 mM) caused a 5-fold shift to higher apparent affinity for the sigma₁ receptor agonist dextromethorphan (Fig. 3). An IC_{50} of $22.2 \pm 4.8 \text{ nM}$ (Hill slope 1.2 ± 0.2) was observed in the presence of phenytoin, while an IC_{50} of $109 \pm 10.4 \text{ nM}$ (Hill slope 1.3 ± 0.2) was observed when only the vehicle (50 μ L, 0.15 M NaOH) was included in the assay. The IC_{50} value for dextromethorphan in the presence of NaOH vehicle was not significantly different from that given in Table 1 (t -test, $P > 0.05$). By contrast, the IC_{50} value for dextromethorphan in the presence of phenytoin and vehicle was significantly enhanced with respect to both of the control IC_{50} values ($P < 0.0001$, ANOVA).

3.2. Sigma₂ receptor binding in mouse lung membranes

Sigma₂ receptor assays with [³H]DTG in vitro using lung membranes were conducted at 25 °C in the presence of non-radioactive (+)-pentazocine (500 nM) to preclude binding of the

radioligand to sigma₁ sites (cf. Lever et al., 2006). The association of [³H]DTG was rapid (Fig 4A). A high level of specific binding, 70% of total, was reached by 2 min and maintained for at least 90 min (Fig. 2A). Experiments designed to extract discrete rate constants were not performed. Specific binding increased linearly ($r^2 = 0.97$) over the protein range tested (0.12 – 0.50 mg / tube; data not shown).

Homologous competition studies (Fig. 4B), using 3.0 nM [³H]DTG / 500 nM (+)-pentazocine in a 60 min assay, revealed saturable binding that was best fit (*F*-ratio test, $P < 0.05$) to a two-site model using program Radlig 6.0. The K_d for the sole high affinity site was 31.8 ± 8.3 nM, and the B_{max} was 921 ± 228 fmol / mg protein ($n = 5$, mean $\pm$ S.E.M.). A low affinity, high capacity site was also observed, with large coefficients of variation for K_d (4877 ± 3194 nM) and B_{max} (22080 ± 9205 fmol / mg protein). Using program Prism 6.0c, the two-site binding model did not converge, and the same data showed an excellent fit ($r^2 = 0.99$) to a one-site model with a K_d of 41.5 ± 6.0 nM and B_{max} of 1068 ± 149 fmol / mg protein.

Inhibitory potencies for selected sigma receptor ligands against 3.0 nM [³H]DTG / 500 nM (+)-pentazocine were then determined at 25 °C in lung membranes using a 60 min incubation period. Inhibition curves are shown in Fig. 5A, and the binding parameters are given in Table 1. The measured K_d of 31.8 nM was used with the Cheng and Prusoff (1973) relationship to derive the K_i values. Radioligand binding was fully inhibited in monophasic, concentration-dependent fashion. Pseudo-Hill slopes were not significantly different from unity (*F*-ratio test, $P > 0.05$). The rank order of potency was ifenprodil > DTG, haloperidol > AG-205 > (+)-pentazocine, dextromethorphan, (–)-NANM >> (+)-NANM, (–)-cocaine. The K_i values for five of these inhibitors against [³H]DTG / (+)-pentazocine (100 nM) in Swiss Webster mouse brain membranes have also been reported (Kovács and Larson, 1995). A strong Pearson rank order correlation ($r = 0.99$, $P < 0.0001$) was found between the pK_i values for the pulmonary and brain binding data (Fig. 5B).

3.3. Sigma₁ receptor binding in vivo

Dose-response data for dextromethorphan inhibition of [¹²⁵I]E-IA-DM-PE-PIPZE specific binding to sigma₁ receptors is shown in Fig. 5. Six treatment groups received dextromethorphan, 0.32 to 100 μmol / kg (i.p.) spaced at half-log intervals, 1 min prior to administration of radioligand (2.5 μCi, i.v.). Total binding was determined using a vehicle treated control group. Non-specific binding was defined by a group that received the selective sigma₁ receptor antagonist BD1063 (5.0 μmol / kg, i.v.) 5 min prior to the radioligand. Specific radioligand binding to sigma₁ receptors in a given tissue sample was obtained by subtracting the average non-specific radioligand uptake that was separately determined for that tissue.

[¹²⁵I]E-IA-DM-PE-PIPZE exhibited good levels of specific binding in vivo to sigma₁ receptors in lung (68%), as well as brain (85%), heart (50%) and spleen (66%). Dextromethorphan inhibition of specific binding was dose-dependent (Fig. 6). The data were fit well ($r^2 = 0.99$) by sigmoidal curves that were used to calculate the dosage required for 50% occupancy (ED₅₀). Dextromethorphan exhibited an ED₅₀ of 1.2 μmol / kg in lung, and was also a potent inhibitor of sigma₁ receptor binding in heart (ED₅₀ 1.2 μmol / kg). The

ED₅₀ values were somewhat lower for spleen (3.5 $\mu\text{mol} / \text{kg}$) and whole brain (4.4 $\mu\text{mol} / \text{kg}$).

4. Discussion

We identified sites having the pharmacological characteristics of σ_1 and σ_2 receptors in mouse lung membranes using the radioligand binding techniques previously employed for studies of brain and other peripheral organs (Bowen et al., 1993; DeHaven-Hudkins et al., 1996, 1994, 1992; Hellewell et al., 1994; Lever et al., 2006). Binding of the selective σ_1 receptor agonist [^3H](+)-pentazocine to pulmonary membranes reached steady state within 5 – 6 h at 37 °C, with specific binding > 85% of total binding. [^3H](+)-Pentazocine also exhibits slow kinetics for binding to membranes from guinea pig (DeHaven-Hudkins et al., 1992) and human brain (Kornhuber et al., 1996). The estimated association rate constant in lung tissue ($0.0018 \text{ min}^{-1} \text{ nM}^{-1}$) is near to the k_{on} ($0.0019 \text{ min}^{-1} \text{ nM}^{-1}$) reported for [^3H](+)-pentazocine binding to human frontal cortex membranes, where steady state was reached in 8 h at 37 °C (Kornhuber et al., 1996). By contrast, [^3H]DTG binding to σ_2 sites of lung was fast, with steady state achieved within 2 min at 25 °C. Studies were conducted in the presence of non-radioactive (+)-pentazocine to mask radioligand binding to σ_1 sites. Fast kinetics are also observed for [^3H]DTG binding to membranes from brain and other tissues (DeHaven-Hudkins et al., 1996; Kovács and Larson, 1995; Lever et al., 2006).

Saturation studies provided a K_d of 1.36 nM and a B_{max} of 967 fmol / mg protein for [^3H](+)-pentazocine binding to mouse lung membranes. A higher affinity, K_d of 0.61 nM, was calculated from the estimated rate constants. A comparable difference between the kinetic and thermodynamic values for [^3H](+)-pentazocine was reported by Dehaven Hudkins et al. (1992) using guinea pig brain membranes, and was attributed to errors in the measurement of slow kinetics. For reference, Kovács and Larson (1995) reported a K_d of 1.3 nM and a B_{max} of 640 fmol / mg protein for [^3H](+)-pentazocine binding to brain membranes from Swiss Webster mouse. A lower affinity, $K_d = 9 \text{ nM}$, and site density, $B_{\text{max}} = 368 \text{ fmol} / \text{mg}$ protein, were observed for mouse brain by Matsumoto and colleagues (2001). Thus, σ_1 receptor density in mouse lung is equal to or higher than in brain. Northern blot analysis indicates the converse, with σ_1 receptor gene expression 8-fold lower in mouse lung compared to brain (Langa et al., 2003). Various factors that might lead to discordant relationships between protein levels and gene expression have been reviewed by Vogel and Marcotte (2012).

Cold saturation studies of [^3H]DTG, in the presence of (+)-pentazocine, revealed one high affinity, low capacity site on pulmonary membranes having a K_d of 31.8 nM and B_{max} of 921 fmol / mg protein. This is in line with typical [^3H]DTG affinities of 20 – 40 nM observed for σ_2 sites. For illustration, σ_2 receptor parameters for [^3H]DTG binding in mouse brain have been reported as $K_d = 17.8 \text{ nM}$, $B_{\text{max}} = 823 \text{ fmol} / \text{mg}$ protein by Kovács and Larson (1995), and $K_d = 43 \text{ nM}$, $B_{\text{max}} = 788 \text{ fmol} / \text{mg}$ protein by Matsumoto et al. (2001). Hence, radioligand affinities and σ_2 receptor site densities are similar for mouse lung and brain. We also observed a low affinity, high capacity lung binding site for [^3H]DTG that exhibits a K_d of 4877 nM and a B_{max} of 22080 fmol / mg protein. This two-

site model was preferred by program Radlig, while program Prism favored a high affinity, one-site model. This divergence is not particularly surprising from a mathematical perspective, since the site 2 parameters display considerable variability, and the calculated K_d of 5 μM is approaching the highest concentration of DTG used in the assay, 10 μM . From a scientific perspective, we accepted the two-site model because a similar low affinity site for [^3H]DTG binding has been reported in guinea pig brain membranes (Basile et al., 1994; Lever et al., 2006). Binding persists after the brain membranes have been boiled (Basile et al., 1994), and the interaction has been described as saturable but nonspecific. Furthermore, lung tissue is well known to accumulate some lipophilic organic bases, including amphetamines, imipramine and methadone, by multicomponent, saturable processes that depend more on pK_a and lipophilicity than on interactions with discrete biological recognition sites (Anderson et al., 1974; Audi et al., 1998; Boer, 2003; Foth et al., 1995).

The pharmacology of radioligand binding to the putative pulmonary sigma receptors was established by determining inhibitory potencies for a panel of sigma ligands including haloperidol, ifenprodil, DTG, dextromethorphan and the (+)- and (–)-enantiomers of *N*-allylnormetazocine (NANM, SKF-10,047). In all cases, the ligands fully inhibited radioligand binding in monophasic, concentration-dependent fashion consistent with a single class of high affinity sites. Appropriate enantioselectivity was noted for the benzomorphans (+)- and (–)-NANM (Walker et al., 1990; Hellewell et al., 1994). Pulmonary sites labeled by [^3H](+)-pentazocine displayed a σ_1 receptor profile, with much higher affinity for (+)-NANM than (–)-NANM. The reverse enantioselectivity, a trait of σ_2 receptors, was observed for sites labeled by [^3H]DTG in the presence of (+)-pentazocine. The affinity of (–)-NANM was similar for both pulmonary sites, as noted for other tissues (Walker et al., 1990; Hellewell et al., 1994).

Ligand potency against [^3H](+)-pentazocine in lung membranes (haloperidol, (+)-pentazocine $\gg$ DTG, dextromethorphan) proved consistent with σ_1 receptor pharmacology (Cobos et al., 2005; Lever et al., 2006). Ligands with high affinity for σ_2 sites, ifenprodil, haloperidol and DTG (Hashimoto and London, 1993; DeHaven-Hudkins et al., 1996), showed the highest affinities for pulmonary sites labeled by [^3H]DTG / (+)-pentazocine. Explicit comparisons of relative inhibitory potency at the putative pulmonary σ_1 and σ_2 receptors with mouse brain data from Kovács and Larson (1995) gave robust Pearson rank order correlations. The pulmonary data also agreed well with the ligand binding profiles previously determined for σ_1 and σ_2 receptors in other peripheral tissue preparations. The pulmonary σ_1 receptor K_i values were close to those reported for brain, liver and kidney, while σ_2 receptor K_i values were about two-fold lower. The σ_2 values were in tightest agreement with those reported for ligand binding to Jurkat human T lymphocyte cells (DeHaven-Hudkins et al., 1996). We also demonstrated positive allosteric modulation of dextromethorphan binding to the pulmonary sites labeled by [^3H](+)-pentazocine using the anticonvulsant phenytoin, which is a hallmark of σ_1 receptor pharmacology (DeHaven-Hudkins et al., 1993; Cobos et al., 2005).

To augment the inhibition studies, (–)-cocaine and the σ_2 / PGRMC1 ligand AG-205 were tested. (–)-Cocaine was selected because some behavioral effects of the drug are mediated, in part, by agonist actions at central σ_1 and σ_2 receptors (Matsumoto et

al., 2007, 2002, 2001; Lever et al., 2014a, 2014b). (–)-Cocaine showed the same 2000 nM apparent affinity for the pulmonary sites labeled by [³H](+)-pentazocine as reported for mouse brain (Matsumoto et al., 2002), and also displayed the same decided preference for binding to sigma₁ over sigma₂ receptors. While it is possible that pulmonary sigma₁ receptors have a weak interaction with the drug, particularly in the case of inhaled crack cocaine, positron emission tomography studies of carbon-11 labeled (–)-cocaine show no pulmonary accumulation in human beings (Volkow et al., 1992). AG-205, a small molecule ligand for PGRMC1 (Ahmed et al., 2010), was studied because it inhibits the binding of a radioiodinated sigma₂ receptor ligand to cancer cell membranes and proved instrumental in establishing the sigma₂ receptor binding site as part of the PGRMC1 protein complex (Xu et al., 2011). AG-205 binding to sigma₁ receptors does not seem to have been reported, and our pulmonary data indicate a 14-fold higher apparent affinity for sigma₂ sites, $K_i = 1263$ nM, over sigma₁ sites, $K_i = 17790$ nM. To investigate pulmonary sigma₁ receptor binding as a possible mediator of drug uptake and retention in vivo, we examined occupancy by dextromethorphan, a widely used over-the-counter cough suppressant. Dextromethorphan, and its active metabolite dextrorphan, are moderately high affinity sigma₁ receptor agonists and weak *N*-methyl-D-aspartate glutamatergic receptor antagonists (Werling et al., 2007a). Brown and colleagues (2004) suggested that dextromethorphan acts, in part, through lung sigma₁ receptors to modulate cough in a guinea pig model. We assessed dextromethorphan occupancy of sigma₁ receptors in mouse lung, spleen, heart and brain by inhibition of the uptake of [¹²⁵I]*E*-IA-DM-PE-PIPZE, a radioligand that selectively labels sigma₁ sites in vivo in mouse brain and peripheral organs (Lever et al., 2012; 2014a, 2014b).

Dextromethorphan proved particularly potent in lung and heart as compared to brain and spleen. The ED₅₀ values ranged from 1.1 – 4.4 μmol / kg, which corresponds to 0.4 – 1.6 mg / kg. Of note, the lung ED₅₀ of 0.40 mg / kg (i.p.) matches the 0.4 mg / kg oral dosage of dextromethorphan recommended every 4 h as a cough suppressant. Across tissues, sigma₁ receptors were fully occupied at the higher 31.6 – 100 μmol / kg (12 – 37 mg / kg) doses of dextromethorphan that are known to engender neuroprotection (Werling et al., 2007b). The control uptakes of [¹²⁵I]*E*-IA-DM-PE-PIPZE in lung and brain in vivo, as per cent injected dose / g, were almost equal, which supports the similar sigma₁ receptor densities determined for these tissues in vitro.

In summary, mouse lung contains substantial levels of sigma₁ and sigma₂ receptors that exhibit pharmacological properties that are virtually indistinguishable from those previously described for the sites in brain and other peripheral organs. Thus, pulmonary receptors are likely to participate in the various pharmacological actions of both endogenous and exogenous sigma receptor ligands.

Acknowledgments

We thank the National Institute on Drug Abuse (1RC1DA028477: Development of Anti-Cocaine Medications) and the University of Missouri Life Sciences Mission Enhancement program for partial support of this research. We also thank the University of Missouri Life Sciences Undergraduate Research Opportunity Scholars program for supporting TPL. The authors thank Lisa D. Watkinson and Terry L. Carmack for assistance with animal studies, and acknowledge resources and facilities provided by the Harry S. Truman Memorial Veterans' Hospital. This work does not represent the views of the U. S. Department of Veterans Affairs or the United States Government. Funding sources had no involvement in the decision to submit this article for publication.

References

- Anderson MW, Orton TC, Pickett RD, Eling TE. Accumulation of amines in the isolated perfused rabbit lung. *J. Pharmacol. Exp. Ther.* 1974; 189:456–466. [PubMed: 4151395]
- Audi SH, Dawson CA, Linehan JH, Krenz GS, Ahlf SB, Roerig DL. Pulmonary disposition of lipophilic amine compounds in the isolated perfused rabbit lung. *J. Appl. Physiol.* 1998; 84:516–530. [PubMed: 9475861]
- Basile AS, DeCosta B, Paul IA. Multiple [³H]DTG binding sites in guinea pig cerebellum: evidence for the presence of non-specific binding. *Eur. J. Pharmacol.* 1994; 252:139–146. [PubMed: 8157054]
- Boer F. Drug handling by the lungs. *Br. J. Anaesth.* 2003; 91:50–60. [PubMed: 12821565]
- Bowen WD, de Costa BR, Hellewell SB, Walker JM, Rice KC. [³H](+)-Pentazocine: a potent and highly selective benzomorphan-based probe for sigma-1 receptors. *Mol. Neuropharmacol.* 1993; 3:117–126.
- Brown C, Fezoui M, Selig WM, Schwartz CE, Ellis JL. Antitussive activity of sigma-1 receptor agonists in the guinea-pig. *Br. J. Pharmacol.* 2004; 141:233–240. [PubMed: 14691051]
- Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* 1973; 22:3099–3108. [PubMed: 4202581]
- 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]
- Cobos EJ, Baeyens JM, Del Pozo E. Phenytoin differentially modulates the affinity of agonist and antagonist ligands for σ_1 receptors of guinea pig brain. *Synapse.* 2005; 55:192–195. [PubMed: 15635593]
- Collier TL, Waterhouse RN, Kassiou M. Imaging sigma receptors: applications in drug development. *Curr. Pharm. Design.* 2007; 13:51–72.
- DeHaven-Hudkins DL, Fleissner LC, Ford-Rice FY. Characterization of the binding of [³H](+)-pentazocine to sigma recognition sites in guinea pig brain. *Eur. J. Pharmacol.* 1992; 227:371–378. [PubMed: 1359973]
- DeHaven-Hudkins DL, Ford-Rice FY, Allen JT, Hudkins RL. Allosteric modulation of ligand binding to [³H](+)-pentazocine-defined sigma recognition sites by phenytoin. *Life Sci.* 1993; 53:41–48. [PubMed: 8515681]
- DeHaven-Hudkins DL, Lanyon LF, Ford-Rice FY, Ator MA. σ recognition sites in brain and peripheral tissues. Characterization and effects of cytochrome P450 inhibitors. *Biochem. Pharmacol.* 1994; 47:1231–1239. [PubMed: 8161352]
- DeHaven-Hudkins DL, Daubert JD, Sawutz DG, Tiberio L, Baine Y. [³H]1,3-di(2-tolyl) guanidine binds to a σ_2 receptor on Jurkat cell membranes, but σ compounds fail to influence immunomodulatory events in human peripheral blood lymphocytes. *Immunopharmacol.* 1996; 35:27–39.
- 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]
- Foth H. Role of the lung in accumulation and metabolism of xenobiotic compounds implications for chemically induced toxicity. *Crit. Rev. Toxicol.* 1995; 25:165–205. [PubMed: 7612175]
- Frecka E, Szabo A, Winkelman MJ, Luna LE, McKenna DJ. A possibly sigma-1 receptor mediated role of dimethyltryptamine in tissue protection, regeneration, and immunity. *J. Neural. Transm.* 2013; 120:1295–1303. [PubMed: 23619992]
- Hashimoto K, London ED. Further characterization of [³H]ifenprodil binding to σ receptors in rat brain. *Eur. J. Pharmacol.* 1993; 236:159–163. [PubMed: 8319742]
- 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]
- Hayashi T. Sigma-1 receptor: The novel intracellular target of neuropsychotherapeutic drugs. *J. Pharmacol. Sci.* 2015; 127:2–5. [PubMed: 25704011]

- Hellewell SB, Bruce A, Feinstein G, Orringer J, Williams W, Bowen WD. Rat liver and kidney contain high densities of σ_1 and σ_2 receptors: characterization by ligand binding and photoaffinity labeling. *Eur. J. Pharmacol.* 1994; 268:9–18. [PubMed: 7925616]
- Kashiwagi H, McDunn JE, Simon PO Jr, Goedegebuure PS, Xu J, Jones L, Chang K, Johnston F, Trinkaus K, Hotchkiss RS, Mach RH, Hawkins WG. Selective sigma-2 ligands preferentially bind to pancreatic adenocarcinomas: applications in diagnostic imaging and therapy. *Mol. Cancer.* 2007; 6:48. [PubMed: 17631687]
- Kornhuber J, Schoppmeyer K, Bendig C, Riederer P. Characterization of [^3H]pentazocine binding sites in post-mortem human frontal cortex. *J. Neural Transm.* 1996; 103:45–53. [PubMed: 9026376]
- Kovács KJ, Larson AA. Discrepancies in characterization of sigma sites in the mouse central nervous system. *Eur. J. Pharmacol.* 1995; 285:127–134. [PubMed: 8566130]
- Langa F, Codony X, Tovar V, Lavado A, Giménez E, Cozar P, Cantero M, Dordal A, Hernández E, Pérez R, Monroy X, Zamanillo D, Guitart X, Montoliu L. Generation and phenotypic analysis of sigma receptor type I (σ_1) knockout mice. *Eur. J. Neurosci.* 2003; 18:2188–2196. [PubMed: 14622179]
- Lever JR, Gustafson JL, Xu R, Allmon RL, Lever SZ. σ_1 and σ_2 receptor binding affinity and selectivity of SA4503 and fluoroethyl SA4503. *Synapse.* 2006; 59:350–358. [PubMed: 16463398]
- Lever SZ, Xu R, Fan K-H, Fergason-Cantrell EA, Carmack TL, Watkinson LD, Lever JR. Synthesis, radioiodination and in vitro and in vivo sigma receptor studies of *N*-1-allyl-*N'*-4-phenethylpiperazine analogs. *Nucl. Med. Biol.* 2012; 39:401–414. [PubMed: 22172395]
- Lever JR, Miller DK, Green CL, Fergason-Cantrell EA, Watkinson LD, Carmack TL, Fan K-H, Lever SZ. A selective sigma-2 receptor ligand antagonizes cocaine-induced hyperlocomotion in mice. *Synapse.* 2014a; 68:73–84. [PubMed: 24123353]
- Lever JR, Miller DK, Fergason-Cantrell EA, Green CL, Watkinson LD, Carmack TL, Lever SZ. Relationships between cerebral sigma-1 receptor occupancy and attenuation of cocaine's motor stimulatory effects in mice by PD144418. *J. Pharmacol. Exp. Ther.* 2014b; 351:153–163. [PubMed: 25100754]
- Mach RH, Smith CR, Childers SR. Ibogaine possesses a selective affinity for σ_2 receptors. *Life Sci.* 1995; 57:PL57–PL62. [PubMed: 7603301]
- Maruo J, Yoshida A, Shimohira I, Matsuno K, Mita S, Ueda H. Binding of [^{35}S]GTP γ S stimulated by (+)-pentazocine sigma receptor agonist, is abundant in the guinea pig spleen. *Life Sci.* 2000; 67:599–603. [PubMed: 10993125]
- Mach RH, Zeng C, Hawkins WG. The σ_2 receptor: a novel protein for the imaging and treatment of cancer. *J. Med. Chem.* 2013; 56:7137–7160. [PubMed: 23734634]
- Matsumoto RR, Hewett KL, Pouw B, Bowen WD, Husbands SM, Cao JJ, Newman AH. Rimcazole analogs attenuate the convulsive effects of cocaine: correlation with binding to sigma receptors rather than dopamine transporters. *Neuropharmacol.* 2001; 41:878–886.
- Matsumoto RR, McCracken KA, Pouw B, Zhang Y, Bowen WD. Involvement of sigma receptors in the behavioral effects of cocaine: Evidence from novel ligands and antisense oligodeoxynucleotides. *Neuropharmacol.* 2002; 42:1043–1055.
- Matsumoto, RR.; Bowen, WD.; Su, TP. *Sigma Receptors: Chemistry, Cell Biology and Clinical Implications.* New York: Springer; 2007.
- Maurice T, Martin-Fardon R, Romieu P, Matsumoto RR. Sigma1 (σ_1) receptor antagonists represent a new strategy against cocaine addiction and toxicity. *Neurosci. Biobehav. Rev.* 2002; 26:499–527. [PubMed: 12204195]
- Monassier L, Bousquet P. Sigma receptors: from discovery to highlights of their implications in the cardiovascular system. *Fundam. Clin. Pharmacol.* 2002; 16:1–8. [PubMed: 11903506]
- Pal A, Fontanilla D, Gopalakrishnan A, Chae YK, Markley JL, Ruoho AE. The sigma-1 receptor protects against cellular oxidative stress and activates antioxidant response elements. *Eur. J. Pharmacol.* 2012; 682:12–20. [PubMed: 22381068]
- Seth P, Leibach F, Ganapathy V. Cloning and structural analysis of the cDNA and the gene encoding the murine type 1 sigma receptor. *Biochem. Biophys. Res. Commun.* 1997; 241:535–540. [PubMed: 9425306]

- Søby KK, Mikkelsen JD, Meier E, Thomsen C. Lu 28–179 labels a σ_2 -site in rat and human brain. *Neuropharmacology*. 2002; 43:95–100. [PubMed: 12213263]
- Su TP, Hayashi T, Maurice T, Buch S, Ruoho AE. The sigma-1 receptor chaperone as an inter-organellar signaling modulator. *Trends Pharmacol. Sci.* 2010; 31:557–566. [PubMed: 20869780]
- van Waarde A, Rybczynska AA, Ramakrishnan NK, Ishiwata K, Elsinga PH, Dierckx RA. Potential applications for sigma receptor ligands in cancer diagnosis and therapy. *Biochim. Biophys. Acta*. 2014 Aug 27. pii: S0005-2736(14)00304-6.
- Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat. Rev. Genet.* 2012; 13:227–232. [PubMed: 22411467]
- Volkow ND, Fowler JS, Wolf AP, Wang GJ, Logan J, MacGregor R, Dewey SL, Schlyer D, Hitzemann R. Distribution and kinetics of carbon-11-cocaine in the human body measured with PET. *J. Nucl. Med.* 1992; 33:521–525. [PubMed: 1552335]
- 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]
- Werling LL, Keller A, Frank JG, Nuwayhid SJ. A comparison of the binding profiles of dextromethorphan, memantine, fluoxetine and amitriptyline: treatment of involuntary emotional expression disorder. *Exp. Neurol.* 2007a; 207:248–257. [PubMed: 17689532]
- Werling LL, Lauterbach EC, Calef U. Dextromethorphan as a potential neuroprotective agent with unique mechanisms of action. *Neurologist*. 2007b; 13:272–293. [PubMed: 17848867]
- Xu J, Zeng C, Chu W, Pan F, Rothfuss JM, Zhang F, Tu Z, Zhou D, Zeng D, Vangveravong S, Johnston F, Spitzer D, Chang KC, Hotchkiss RS, Hawkins WG, Wheeler KT, Mach RH. Identification of the PGRMC1 protein complex as the putative sigma-2 receptor binding site. *Nat. Commun.* 2011; 22:380. [PubMed: 21730960]

Fig. 1.

Panel A: Association kinetics for [³H](+)-pentazocine (0.7 nM, closed circles; 3.0 nM, open circles) binding to mouse lung membranes at 37 °C. Data shown are representative experiments performed in duplicate. Panel B: Saturation binding of [³H](+)-pentazocine (0.35 – 35 nM) to mouse lung membranes at 37 °C with a six h incubation and haloperidol (1.0 μM) to define non-specific binding. Open circles show specific radioligand binding, while the Rosenthal plot is depicted by closed squares on the inset. Data shown are from a

representative experiment performed in duplicate, and replicated eight times to give K_d 1.36 ± 0.04 nM and B_{max} 967 ± 11 fmol / mg protein.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2.

Panel A: Inhibition curves for sigma receptor ligands against [3 H](+)-pentazocine (1.0 nM) binding to sigma $_1$ sites in mouse lung membranes at 37 °C using haloperidol (1.0 μ M) to define non-specific binding and a six h incubation. Data are mean $\pm$ S.E.M. for four to six experiments, each performed in duplicate, except for AG-205 where n = 3. Panel B: Pearson correlation of ligand inhibitory potencies, as pK $_i$ values, determined in mouse lung membranes (Table 1) with data reported for inhibition of [3 H](+)-pentazocine binding to sigma $_1$ sites in mouse brain membranes (Kovács and Larson, 1995).

Fig. 3. Phenytoin (1.0 mM) causes a five-fold shift (arrow) to higher apparent affinity for the σ_1 receptor agonist dextromethorphan against $[^3\text{H}](+)\text{-pentazocine}$ in mouse lung membranes. Data are mean $\pm$ S.E.M. for four trials, each performed in duplicate.

Fig. 4.

Panel A: Association kinetics for [³H]DTG (3.0 nM) binding to mouse lung membranes at 25 °C using (+)-pentazocine (500 nM) to mask sigma₁ sites and haloperidol (10.0 μM) to define non-specific binding. Data shown is for a representative experiment performed in duplicate. Panel B: Homologous saturation isotherm for [³H]DTG (0.32 – 10000 nM) binding to mouse lung membranes at 25 °C with a 60 min incubation, 500 nM (+)-pentazocine to mask sigma₁ binding, and haloperidol (10.0 μM) to define non-specific binding. Open circles show specific radioligand binding, while the curvilinear Rosenthal

plot is depicted by closed squares on the inset. Data shown are from a representative experiment that was performed in duplicate, and replicated five times to give a site 1 K_d of 31.8 ± 8.3 nM with $B_{\max}$ 921 ± 228 fmol / mg protein and a site 2 K_d of 4877 ± 3194 nM with $B_{\max}$ 22080 ± 9205 fmol / mg protein (mean $\pm$ S.E.M.).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5.

Panel A: Inhibition curves for sigma receptor ligands against [³H]DTG (3.0 nM) / (+)-pentazocine (500 nM) binding to sigma₂ sites in mouse lung membranes at 25 °C using haloperidol (10.0 μM) to define non-specific binding and a 60 min incubation. Data are mean ± S.E.M. for four to six experiments, each performed in duplicate. Panel B: Pearson correlation of ligand inhibitory potencies, as pK_i values, determined in mouse lung membranes (Table 1) with data reported for inhibition of [³H]DTG / (+)-pentazocine binding to sigma₂ sites in mouse brain membranes (Kovács and Larson, 1995).

Fig. 6.

Dextromethorphan (0.32 – 100 µmol / kg; i.p.) dose dependently inhibits specific [¹²⁵I]E-IA-DM-PE-PIPZE binding to sigma₁ receptors at 30 min in mouse lung (A), heart (B), spleen (C) and brain (D). Data represent mean ± S.E.M., *n* = 4 – 5. Curves are the sigmoidal fits (*r*² = 0.99, Panels A – D) used to obtain the ED₅₀ values shown.

Table 1

Sigma receptor binding parameters and sigma₁ / sigma₂ subtype selectivity for a panel of ligands in mouse lung membranes.

Compound	^a Sigma ₁				^b Sigma ₂				Selectivity $\frac{K_i \text{Sigma}_2}{K_i \text{Sigma}_1}$
	IC ₅₀ (nM)	K _i (nM)	n _H		IC ₅₀ (nM)	K _i (nM)	n _H		
Ifenprodil	28.27 ± 3.26	16.38 ± 1.89	1.06 ± 0.11		2.40 ± 0.27	2.18 ± 0.24	0.88 ± 0.10		0.1
Haloperidol	1.54 ± 0.18	0.90 ± 0.11	0.89 ± 0.09		77.21 ± 8.26	70.05 ± 7.50	1.20 ± 0.25		78
DTG	129.8 ± 13.83	75.22 ± 8.01	1.21 ± 0.09		43.89 ± 6.99	39.82 ± 6.34	0.90 ± 0.16		0.5
Dextromethorphan	127.2 ± 12.4	71.72 ± 6.56	0.86 ± 0.12		4507 ± 714	4195 ± 664	1.22 ± 0.12		58
(+)-Pentazocine	2.24 ± 0.08	1.30 ± 0.05	1.07 ± 0.13		3944 ± 682	3588 ± 621	0.94 ± 0.10		2760
(-)-Cocaine	6570 ± 572	1962 ± 171	1.18 ± 0.19		65280 ± 7908	59450 ± 7213	0.93 ± 0.09		30
(+)-NANM	30.03 ± 2.38	16.38 ± 1.30	1.04 ± 0.10		20830 ± 2539	19040 ± 2321	1.63 ± 0.35		1162
(-)-NANM	3949 ± 616	2154 ± 336	1.48 ± 0.28		4623 ± 998	4224 ± 912	0.85 ± 0.09		2.0
AG-205	30870 ± 14600	17790 ± 8412	1.11 ± 0.12		1382 ± 355	1263 ± 325	1.06 ± 0.20		0.07

^a Conditions: 1.0 nM [³H](+)-pentazocine; 1.0 μM haloperidol defined non-specific binding; 6 h; 50mM Tris, pH 8.0, 37 °C; 0.2 mg protein / tube; n = 4 – 6 (mean ± S.E.M.) except for AG-205 where n = 3.

^b Conditions: 3.0 nM [³H]DTG / 500 nM (+)-pentazocine; 10.0 μM haloperidol defined non-specific binding; 60 min; 50mM Tris, pH 8.0, 25 °C; 0.2 mg protein / tube; n = 4 – 6 (mean ± SEM).