RE-EVALUATION OF POTENTIAL HUMAN SIGMA-1 RECEPTOR LIGANDS UTILIZING A RELIABLE AND SENSITIVE SCREENING ASSAY

by

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ABSTRACT

RE-EVALUATION OF POTENTIAL HUMAN SIGMA-1 RECEPTOR LIGANDS UTILIZING A RELIABLE AND SENSITIVE SCREENING ASSAY

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Sigma receptors are distinct from opioid, NMDA, dopaminergic, and other known neurotransmitter or hormone receptor families. Two subtypes, Sigma-1 and Sigma-2, have been characterized pharmacologically, but only the Sigma-1 receptor has been cloned. Sigma-1 receptors' distribution and role as amplifiers of signal transduction have been characterized, but their precise physiological functions remain elusive. In order to rapidly screen compounds that interact with Sigma-1 receptors, human breast adenocarcinoma (MCF-7) cells, which do not express drug-sensitive Sigma-1 receptors, were selected as the host cell line for stably expressing the cloned human Sigma-1 receptor. The Sigma-1 radioligand [^{3}H](+)-pentazocine binds with a high affinity (K_D (s.d.) = 3.7 ± 0.87 nM) to a high density (B_{max} (s.d.) = 109 ± 23.7 pmol/mg) of receptors in cells stably transfected with the human Sigma-1 receptor, but not in untransfected cells. Known Sigma-1 receptor ligands, haloperidol and BD1063, bind with the expected low nanomolar affinities, and the stereoisomers of SKF-10047

have the expected selectivities. Utilizing this reliable assay system, approximately two dozen neurosteroids, benzomorphans, butyrophenones, D_4 dopamine receptor-selective ligands, various typical and atypical antipsychotic drugs, and drugs of abuse were tested for their affinities for the Sigma-1 receptor. In addition to explaining some noteworthy findings in previous reports, these results supported some, but not all prior Sigma-1 receptor ligand affinity studies utilizing whole tissue models.

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CHAPTER I

INTRODUCTION

Sigma receptors were first classified as members of the opiate receptor family to account for the psychotomimetic effects exhibited by racemic benzomorphans in the chronic spinal dog (Martin et al., 1976). However, the ineffectiveness of opioid receptor antagonists, naloxone and naltrexone, against behaviors induced by N-allylnormetazocine ((±)SKF-10047) later distinguished its identity as a non-opioid receptor (Su 1982, Vaupel 1983). The differences in the enantioselectivity of (±)SKF-10047 for benzomorphans and opioid receptors further supported this clarification (Su 1982). Subsequently, additional confusion also arose over the identity of Sigma receptor sites apart from PCP sites on NMDA receptors due to the similar affinities of several less selective ligands to both sites (Quirion et al., 1987, Zukin et al., 1984). This was clarified when the availability of more selective drugs for either the PCP sites or the Sigma receptor sites identified the binding sites as distinct (Tam 1983, Tam and Cook 1984).

Two subtypes of the unique non-opioid, non-NMDA sigma receptors, Sigma-1 and Sigma-2 have so far been identified (Bouchard et al., 1997, Leitner et al., 1994, Quirin et al., 1992). Since its first cloning from the guinea pig liver by Hanner et al. in 1996, the Sigma-1 receptor clones have been characterized from human (Kekuda et al.,

1996), mouse (Seth et al., 1997), and rat (Seth et al., 1998). Sigma-2 receptors have yet to be cloned and are less well understood. The gene encoding for the Sigma-1 receptor contains 223 amino acids and is unique among other mammalian proteins while exhibiting 35% identity and 60% homology to a yeast C8-C7 sterol isomerase (Hanner et al., 1996). Although the exact structure of the Sigma-1 receptor is still speculative, a putative transmembrane region on exon 2 of the gene has been proposed (Hanner et al., 1996, Seth et al., 1997, Kekuda et al., 1996). Experiments by Aydar and colleagues, however, have raised the possibility of an existing second transmembrane region on the Sigma-1 receptor (Aydar et al., 2002).

Sigma-1 receptors are diverse in their localization in both the central nervous system and the peripheral nervous system. At the subcellular level, Sigma-1 receptors are largely associated with mitochondrial membranes and the endoplasmic reticulum (Moebius 1993). At the cellular level, Sigma-1 receptors can be found within neurons and oligodendrocytes (Alonso et al., 2000, Palacios et al., 2003). High densities of this receptor can be found in various limbic structures including the olfactory bulb, hypothalamus, hippocampus, cerebral cortex, and in other brain areas such as the brainstem, cerebellum, and sensory regions (Alonso et al., 2000). Outside of the nervous system, Sigma-1 receptors can be found, among many places, in the heart, kidneys, testes, ovaries, lungs, intestines, muscles, and the liver (see Walker et al., 1990 for review).

Along with their widespread distribution in the body, Sigma-1 receptors are also reported to bind to a remarkably large and diverse class of ligands with moderate to

high affinity. These ligands include (+)-benzomorphans, morphinans, psychotropic drugs, anticonvulsants, addictive drugs, and endogenous neurosteroids. Progesterone, which exhibits the highest affinity for Sigma-1 receptors among neurosteroids, raised considerable interests due to its presence in the body's natural physiology.

With its prevalent spread in the body and its promiscuous binding to various compounds, Sigma-1 receptors have been implicated in playing a possible role in the etiology and therapy of a wide range of psychiatric disorders. These potential range from the development of new antidepressants, antiamnesics, and antipsychotics that would be devoid of the negative side-effects seen with dopaminergic drugs to the treatment of Alzheimer's disease, stroke, and traumatic injuries (see Maurice et al., 1999, Su and Hayashi 2003, Hayashi and Su 2004 for review). Clearly, these important potential implications in medical treatments call for a better knowledge of the relationship between the Sigma-1 receptor and its ligands. In the past decades, however, conflicting affinity values of various Sigma-1 receptor ligands, primarily due to the use of different *in vivo* and *in vitro* conditions and the use of different living systems and varying parameters, have made better understanding difficult. Using MCF-7 cells, which are naturally devoid of drug-sensitive Sigma-1 receptors, we made a stable cell line expressing the cloned human Sigma-1 receptor and then established an efficient and reliable system for screening Sigma-1 receptor ligands. Representative drugs from various structural classes were tested for their binding affinity, and the absolute affinity values (K_i) are reported here.

CHAPTER II

MATERIALS AND METHODS

Reagents

All drugs and reagents were purchased from Sigma-Aldrich Chemical Company and Fluka (St. Louis, MO). Cell culture supplies were purchased from Thermo Fisher Scientific (Logan UT). The [^{3}H](+)-pentazocine (NET-1056, 36.6 Ci/mmol) was purchased from Dupont NEN.

Establishment of a stable cell line

MCF-7 cells (American Type Cell Culture, HTB-22) were cultured in 150 cm^2 flasks (Sarstedt 83.1812) in Dulbecco's Modified Eagle's Medium (DMEM; Fisher Scientific SH30003.02) supplemented with 10% Bovine Calf Serum (Fisher Scientific 30072.03), 100 μg/ml non-essential amino acids (Hyclone SH3023801), 2mM L-glutamine (Sigma G8540), and 10 μg/L Bovine Insulin (Sigma-Aldrich 11070.73.8). Cells were kept in an incubator with 5% CO_2 and 95% air and 95% humidity at 37 ^oC. The full length coding region of the cloned human Sigma-1 receptor DNA (ACCESSION BC004899) was obtained from American Type Culture Collection (MGC-3851) and its integrity was confirmed by sequencing. The full length Sigma-1 receptor was digested and subcloned into a pcDNA3.1 (Invitrogen, CA) vector, which was then transfected into MCF-7 cells using a calcium phosphate precipitation method (Invitrogen, CA).

Individual clones were established over a period of weeks on the basis of G418 (2 mg/ml; InvivoGen ant-gn-5) selection. The expression levels of the Sigma-1 receptor in individual clones were quantified by saturation isotherm binding using the Sigma-1 selective radioligand [^{3}H](+)-pentazocine (Perkin-Elmer, sp. radioactivity 36.6-37.7 Ci/mmol).

Preparation of cell membranes

A flask of cells at full confluency were used for four racks consisting of 48 tubes each. Cells were detached from culture flasks by a 10 min incubation in Ca^{2+} and Mg^{2+} free Dulbecco's Phosphate Buffered Saline (Fisher Scientific 55-031-PB) and supplemented with 5 mM EDTA (Sigma Aldrich E6511) at 37 ^{o}C. Cells were subsequently pelleted by centrifugation at 850xg with 4 volumes (v/v) of Earle's balanced saline solution (EBSS; Fisher Scientific SH30014.03) for 10 minutes. Following decantation of the supernatant, the membrane pellet was lysed in a cold pH 7.4 buffer containing 5 mM Tris and 5 mM $MgCl_2$. The membrane solution was then homogenized firmly with a Dounce glass-glass homogenizer and centrifuged at 35,000xg for 60 min. After centrifugation and decantation of the lysis buffer, the membrane pellet was re-suspended in cold binding buffer (50 mM Tris, pH = 7.4 at 4°C) and washed by re-centrifugation after trituration with a polypropylene Pasteur pipette. The resulting washed pellet was re-suspended in warmed binding buffer (50 mM Tris pH 8.0 at 37°C) immediately before use in the binding experiments.

Receptor binding assay and data analysis

In all reported binding studies, each assay tube contained 50 µl of the drug of interest, 100 µl of [^{3}H](+)-pentazocine, 100 µl of membrane suspension, and 750 µl of 50 mM Tris binding buffer at pH 8.0. Protein concentration in each tube ranged from 5-30 µg/mL and was measured in triplicates using the bicinchonic acid (BCA) protein reagent (Pierce, IL). Color changes were monitored by a microplate reader (EL-312e Bio-tek) and a bovine serum albumin standard curve was generated from duplicate standard dilutions ranging from 100 µg/ml to 1,000 µg/ml. In the binding affinity assays, non-specific binding was defined by 5 µM haloperidol or 5 µM BD1063. All binding reactions were allowed 180 minutes to reach equilibrium at 37 ^{o}C with moderate shaking and were terminated by the addition of ice-cold wash buffer (10 mM Tris pH= 8.0 at 2 ^{o}C) during rapid filtration through Whatman GF/C filters (Brandel FPD-205) pre-soaked in 0.3 % polyethyleneimine. Filter-bound radioactivity was quantified by a liquid scintillation analyzer (Packard, Tri-Carb 2300T12). For competition binding experiments, membranes were equilibrated with a fixed concentration of [^{3}H](+)-pentazocine (~1 nM) and increasing concentrations of the competing ligand. The IC_{50} values were determined by non-linear regression curve fitting models with a 95% confidence interval using GraphPad's Prism version 4.0. The inhibition constant (K_i) values were calculated from IC_{50} values according to the Cheng-Prusoff equation ($Ki=IC_{50}/(1+[ligand]/K_D)$ (Cheng and Prusoff, 1973). The statistical measures of curve fitting were the F-test, the run test and a correlation coefficient (r2). All experiments on individual drugs were repeated at least three times.

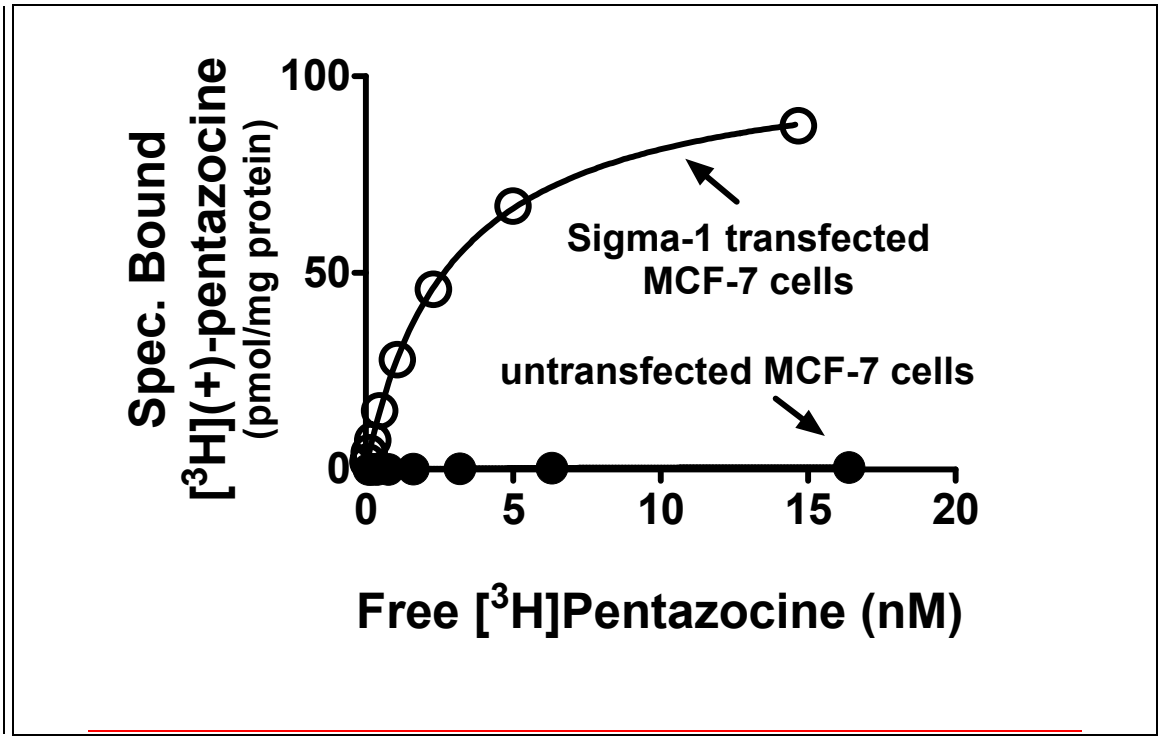
CHAPTER III

RESULTS

Characterization of [^{3}H](+)-Pentazocine Binding to Membranes Prepared from MCF-7 Cells Stably Expressing the Cloned Human Sigma-1 Receptor

The presence of Sigma-1 receptors in the untransfected and transfected MCF-7 cells was examined using [^{3}H](+)-pentazocine as the radioligand. [^{3}H](+)-pentazocine binds with high affinity and high selectivity for Sigma-1 receptors (Quirion et al., 1992; Hellewell et al., 1994). Initially, the parameters of the assay condition were investigated. Binding buffer in the range of 10-50 mM Tris at pH 7.2-8.0 were tested in temperatures from 25-37 ^{o}C for 90-180 min. As determined, 50 mM Tris buffer at pH 8.0 under incubation temperature of 37 ^{o}C and incubation time of 180 minutes were the optimal conditions for the binding to reach equilibrium. In order to determine the receptor density, saturation isotherm studies were performed using [^{3}H](+)-pentazocine ligand concentrations ranging from 0.125 nM to 16 nM. Nonspecific binding was characterized by 10 μM unlabeled haloperidol in the presence of [^{3}H](+)-pentazocine. The total binding increased in a time-dependent fashion and the binding reached equilibrium by 3 h when incubated at 37 ^{o}C with continuous shaking at ~40 rpm. The nonspecific binding was linear and was always found to be <1% of total binding under equilibrium binding conditions (data not shown). As shown in *Figure 1*, the binding revealed no specific binding of [^{3}H](+)-pentazocine to the untransfected MCF-7 cells. On the other hand, a high saturable expression of Sigma-1 receptors in transfected MCF-7 cells was observed as expected. The affinity and the receptor

density of the transfected MCF-7 cells were determined to be K_D (s.d.) = 3.7 ± 0.87 nM and B_{max} (s.d.) = 109 ± 23.7 pmol/mg, respectively.

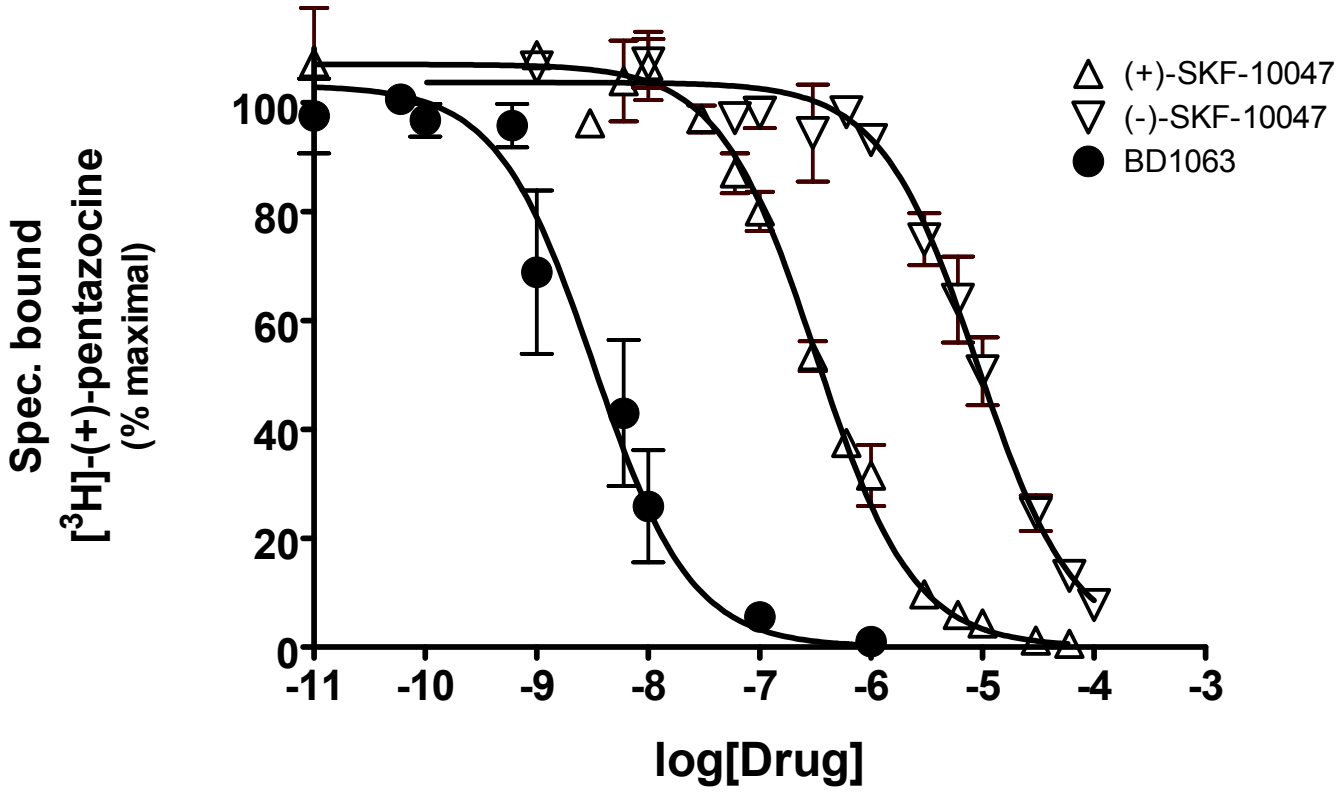


Description:

Figure 1. [^{3}H]-(+)-pentazocine saturation isotherm binding to a clonal human MCF-7 cell line stably expressing the human Sigma-1 receptor. The average affinity (K_D) and receptor density (B_{max}) values (n = 3) are: 3.7 ± 0.87 nM and 109 ± 23.7 pmoles/mg protein. No specific [^{3}H]-(+)-pentazocine binding was detected in untransfected MCF-7 cells, indicating the absence of drug-sensitive endogenous Sigma-1 or opioid receptors.

Competitive displacement of [^{3}H](+)-pentazocine binding

To further verify the identity of the transfected Sigma-1 receptors, several Sigma-1 reference compounds were first examined for their ability to displace the specific binding of [^{3}H](+)-pentazocine to the transfected Sigma-1 receptor. Several Sigma-1 selective ligands inhibited the binding of [^{3}H](+)-pentazocine to transfected MCF-7 cell membranes with high affinity (*Fig. 2*). The K_i values, derived from the IC_{50} for the capacity of Sigma-1 selective ligands to inhibit [^{3}H]-(+)-pentazocine binding are given in *Table 1*.



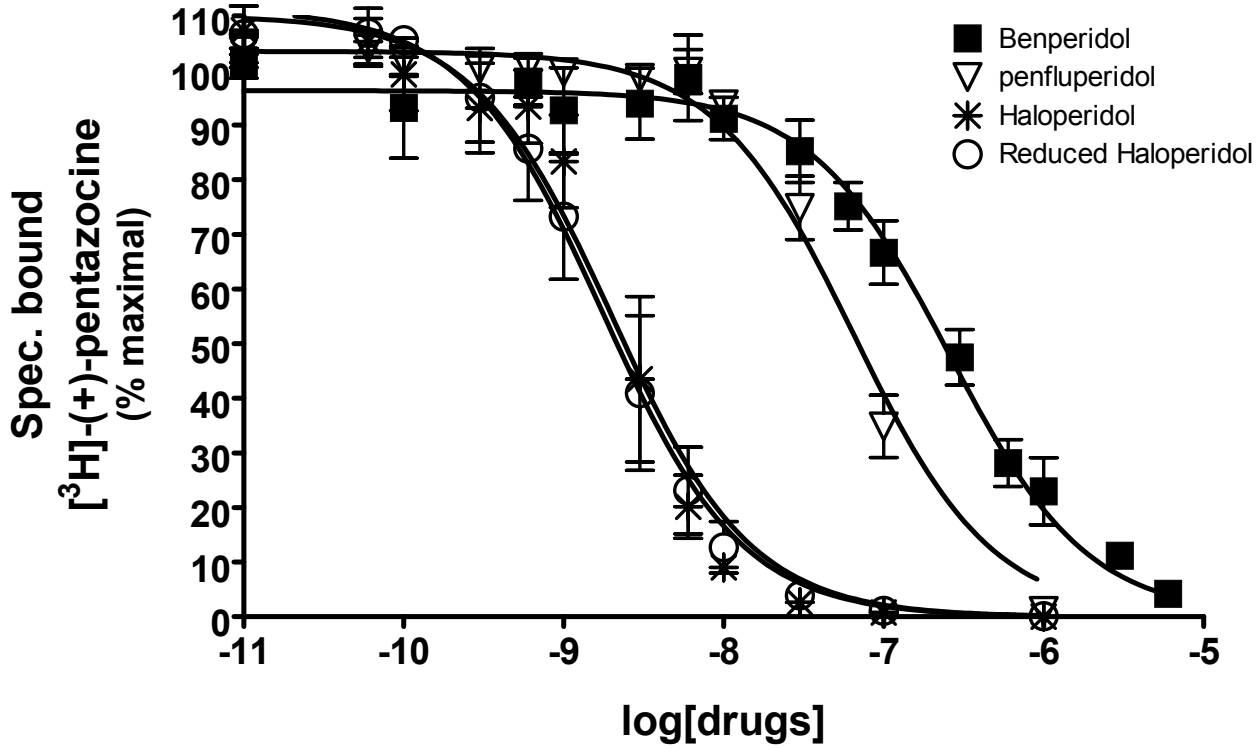
Description:

Figure 2. Displacement of [^{3}H]-(+)-pentazocine binding by a Sigma-1 selective antagonist (BD1063) and by steroisomers of a prototypical benzomorphan agonist (+)-SFK10,047 and (-)-SKF10,077. Binding studies utilized cell homogenates derived from membranes of MCF-7 cells stably expressing the human Sigma-1 receptor. The affinity values (K_i) are listed in Table 1.

Listed for comparison are values of previously reported K_i values of these ligands from binding assays utilizing whole tissue homogenates. The Sigma-1 receptor selective antagonist BD1063 was found to be a high affinity Sigma-1 receptor selective inhibitor of [^{3}H]-(+)-pentazocine binding (K_i = 3.1 nM). In addition, characteristic of the Sigma-1 receptor, the positive enantiomer of the benzomorphan Sigma-1 receptor agonist, SKF-10047, showed higher affinity than the negative enantiomer. Similar to reference values from previous studies (Tam and Cook 1984), (+)-SKF-10047 was ~28 fold more potent towards Sigma-1 receptors than (-)-SKF-10047. Sigma-1 receptors are known to differ from opioid receptors in their rank order affinity for (+) vs (-) benzomorphans (Su 1982). The rank order of affinity for the Sigma-1 reference compounds derived from [^{3}H]-(+)-pentazocine binding are: 4-PPBP > BD1063 > IPAB >> PRE-084 > BMY14802 > (+)-SKF-10047 > (-)-SKF-10047.

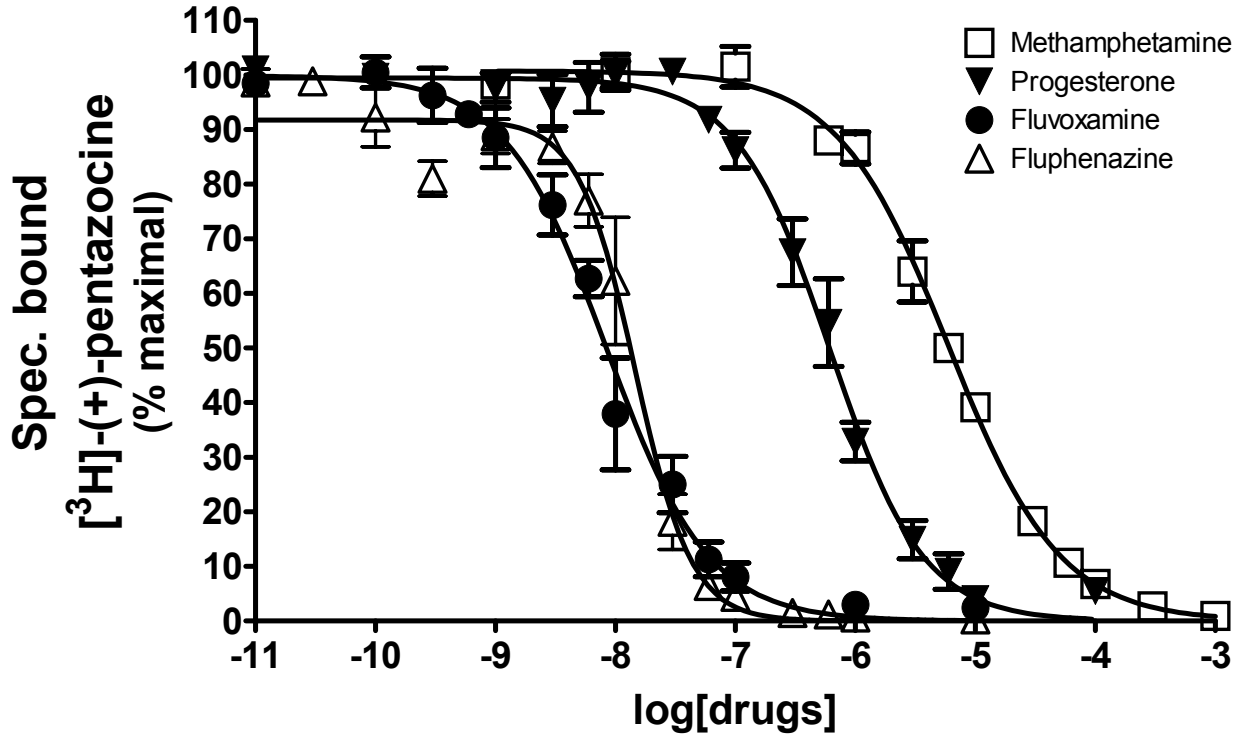
In the dynamic screening of putative Sigma-1 receptor ligands, many drugs of distinct structural classes were examined. Shown in *Figure 3, 4, and 5* are some of the compounds that were tested. The true affinity values (K_i) are given in *Table 1.* Values from several previous

studies, if available, are also given for a side-by-side comparison. Because haloperidol is the prototypical Sigma-1 receptor antagonists (Matsuno et al., 1997, Maurice and Privat 1997), it, its human metabolites and several other butyrophenone class antipsychotics drugs were included in the screening. Haloperidol, trifluperidol, bromperidol, and chlorohaloperidol were all found to bind Sigma-1 receptors with high affinity (very low K_i values in the range of 1 nM to 4 nM). Reduced haloperidol (metabolite II), which binds to cloned D_2 receptors with significantly lower affinity than haloperidol (Bowen et al., 1990), exhibited a similar K_i value compared with the binding of haloperidol to the Sigma-1 receptor, as has been reported previously by Vilner et al., 2000 using a human neuroblastoma cell line that expressed both Sigma-1 and Sigma-2 receptors. Haloperidol metabolite I binds with a moderate nanomolar affinity while haloperidol metabolite III, which was unable to inhibit [^{3}H](+)-pentazocine binding, has no affinity for the Sigma-1 receptor. Other butyrophenone class drugs exhibited a wide range of K_i values from 54 nM to 2,240 nM



Description:

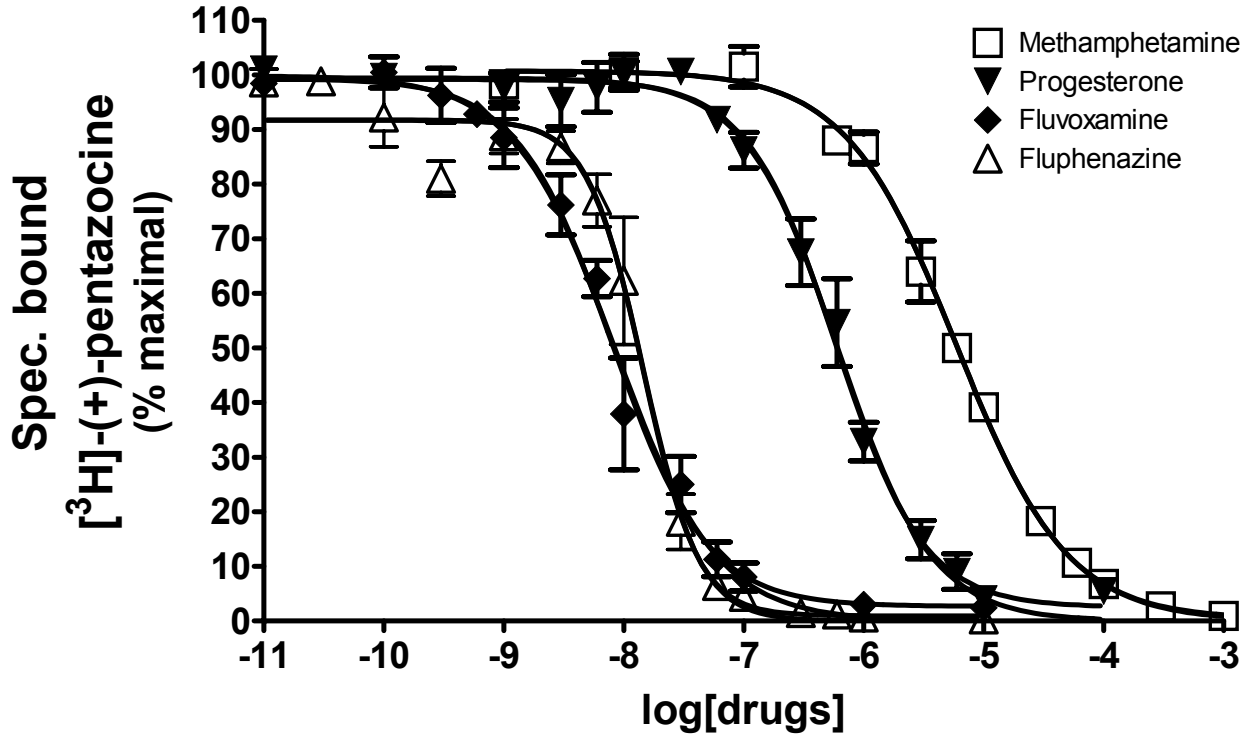
Figure 3. Displacement of [^{3}H]-(+)-pentazocine binding by butyrophenones and reduced haloperidol. Butyrophenone class compounds binds to the Sigma-1 receptor with varying degrees affinity. Reduced haloperidol exhibits similar binding as haloperidol. The affinity values (K_i) are listed in Table 1.



Description:

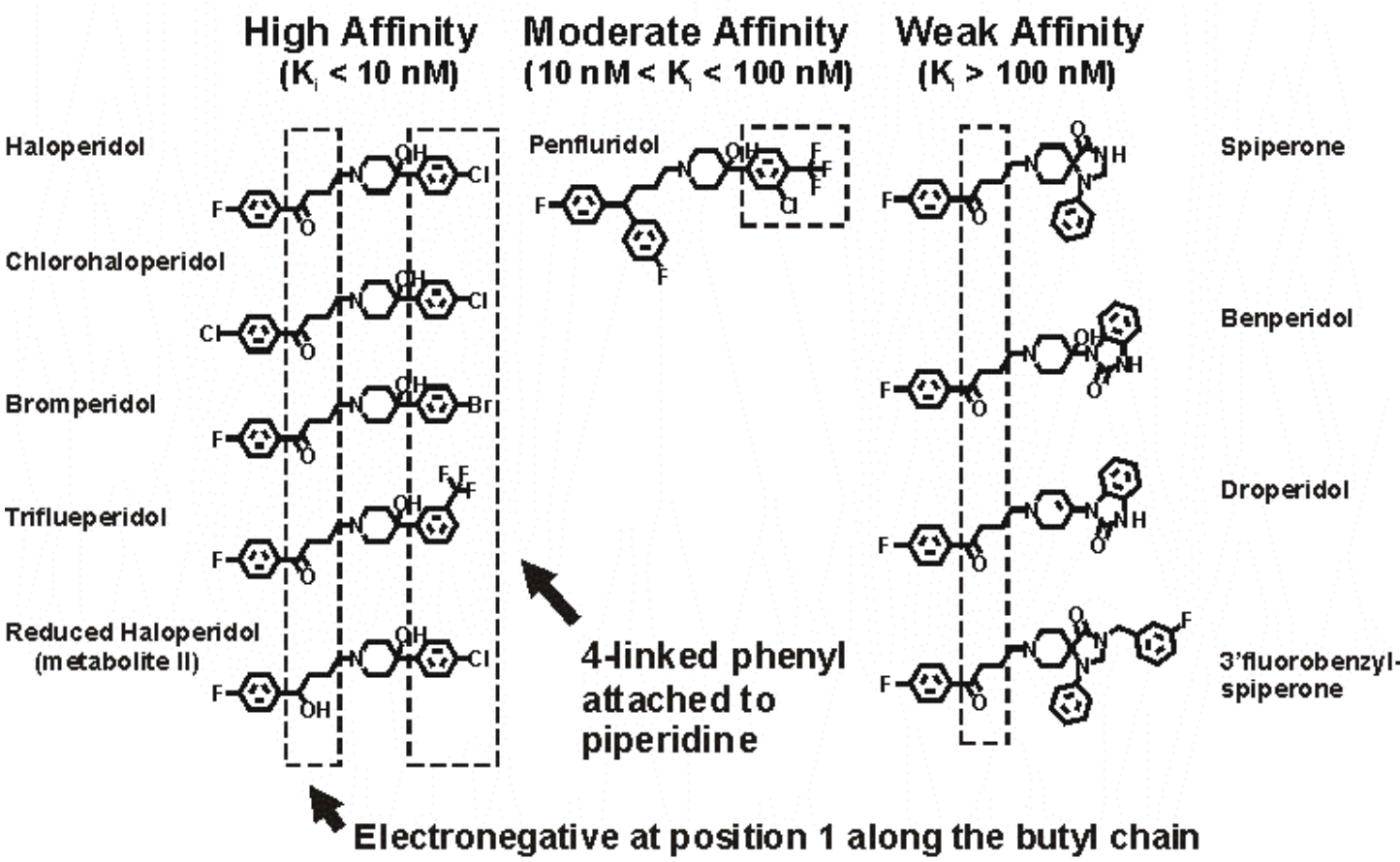
Figure 4. Displacement of [^{3}H]-(+)-pentazocine binding by D_4-selective compounds. Several dopamine D_4-selective drugs also bind to Sigma-1 receptors with a range of affinities. The rank order for these drug affinities is: RBI-257 >> L745,870 >> PD168,077 > NGD94-1. The affinity values (K_i) are listed in Table 1.

Other compounds screened for binding to the cloned Sigma-1 receptor (some shown in Figure 5) include common typical antipsychotics, pimozide and fluphenazine, which both inhibit [^{3}H](+)-pentazocine binding with a nanomolar affinity. Clozapine, an atypical antipsychotic commonly used to treat schizophrenia, showed no affinity to the Sigma-1 receptor. The antidepressant fluvoxamine exhibited a low affinity with a K_i ~7 nM. Known dopamine D_4 receptor-selective drugs, L745, 870 (Kulagowski et al., 1996), RBI-257 (Kula et al., 1999), NGD 94-1 (Tallman et al., 1997), and PD 168,077 (Glase et al., 1997) were also screened for possible binding to the Sigma-1 receptor. The K_i values vary widely in the range of 2.5 nM to 5,161 nM. Of the three drugs of abuse screened, methamphetamine, a common drug of abuse, bound with very modest affinity (K_i = 5248 nM). Progesterone, a hormone of interest as a putative endogenous Sigma-1 receptor ligand (Su et al., 1988), was found to have a K_i value of 468 nM, while β-estradiol exhibited no detectable binding.



Description:

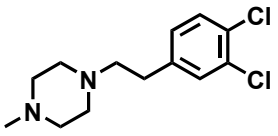
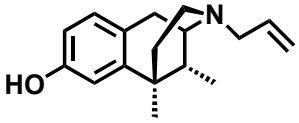
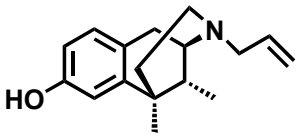
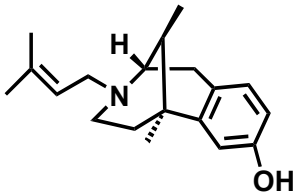
Figure 5. Displacement of [^{3}H]-(+)-pentazocine binding by other distinct Sigma-1 receptor ligands. Several chemicals of different structural classes also compete with [^{3}H]-(+)-pentazocine binding with high to moderate affinity. Not all data are shown. The affinity values (K_i) are listed in Table 1.

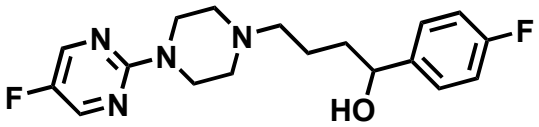
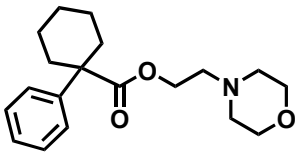
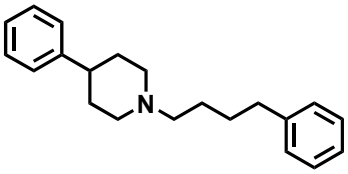
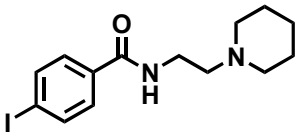
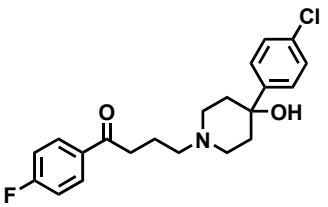


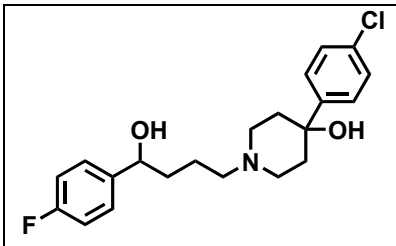
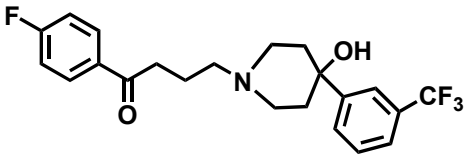
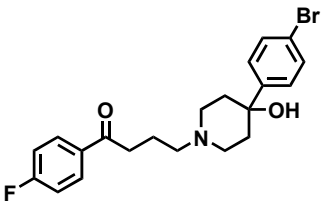
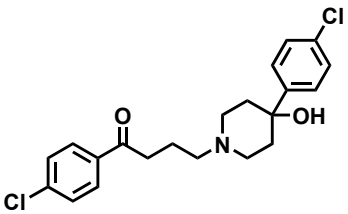
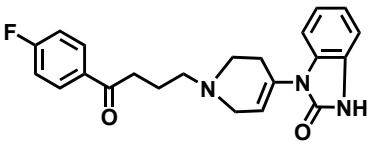
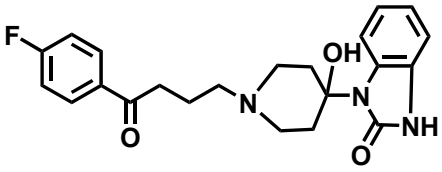
Description:

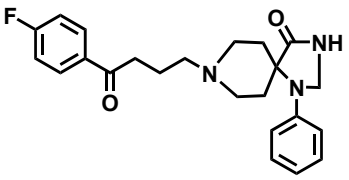
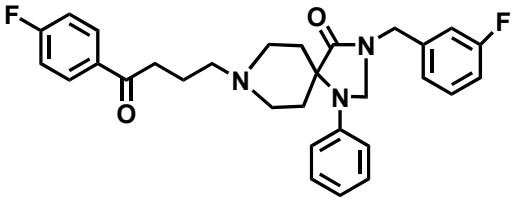
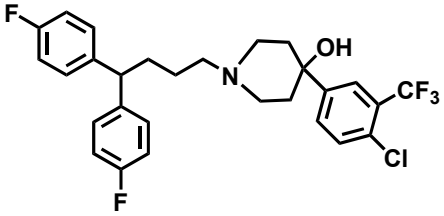
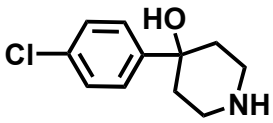
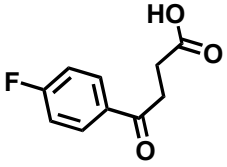
Figure 6. Structure-Affinity Relationships of Butyrophenone Interactions with Sigma-1 receptors. The combination of a 4-linked phenyl attached to piperidine and an electronegative moiety at position 1 along the butyl chain in part determines the affinity strength of the ligands to the Sigma-1 receptor.

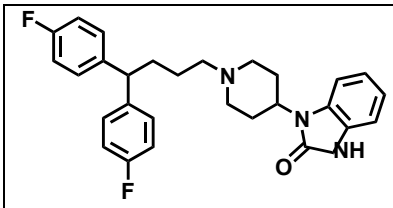
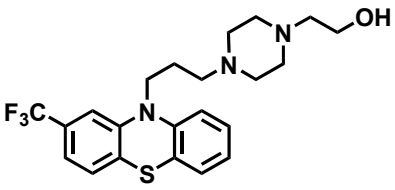
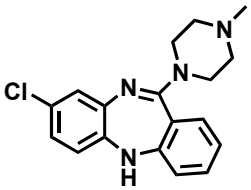
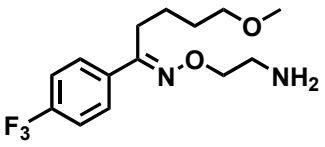
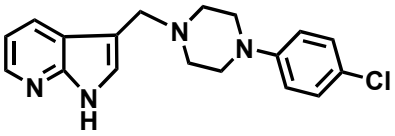
Table 1. Affinity (K_i) values of various ligands for the cloned Sigma-1 receptor. Values are given in units of nanomolar (nM) concentrations. Standard deviations (std.) are listed next to the values.Average K_i values from available past studies using whole tissues are listed for comparison.

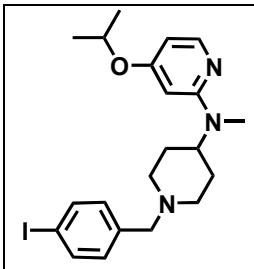
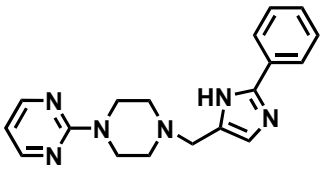
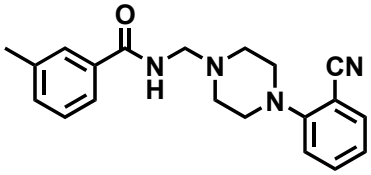
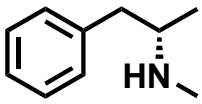
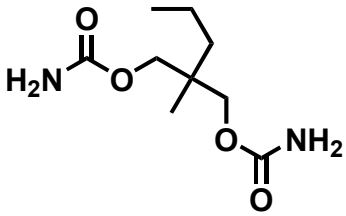
Drugs	K_i values from cloned hSigma-1 in MCF-7	K_i values from whole tissues	Average K_i values and std. of whole tissues
Sigma-1 Reference			
BD1063 	3.1 ± 2.4	9.15[I]	9
(+)-SKF-10047 	250.7 ± 79.1	19.4[A] 48[D] 713[B] 41[L]	205 ± 339
(-)-SKF-10047 	7132.0 ± 2550.8	84.9[A] 1800[D] 5133[B] 1970[L]	2247 ± 2104
(+)-[3H]-pentazocine 	3.7 ± 0.9	17[B] 4.59[A] 1[L]	8 ± 8

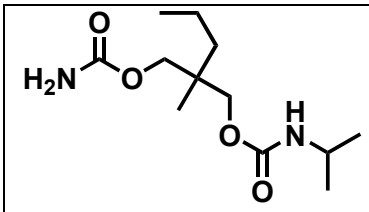
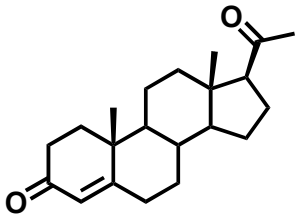
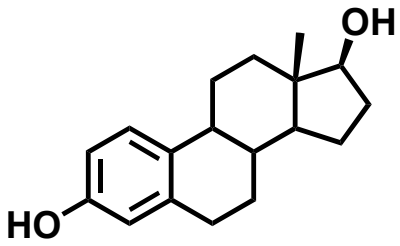
BMY14802 	172.6 ± 58.2	306 [B] 66 [M]	186 ± 170
PRE-084 	84.63	9[N]	
4-PPBP 	0.53	1.14[H]	1.1
IPAB 	4.66	2.57[C] 13.1[J]	8 ± 7
Butyrophenone Antipsychotics and Metabolites			
Haloperidol 	1.7 ± 0.8	3.12 [A] 0.2 [L] 1.47[G] 4 [D] 3[M] 10.2[B] 2.7[R]	4 ± 3
Reduced haloperidol (metabolite II)	1.5 ± 0.8	22 [M] 5.1 [R]	14 ± 12

			
Trifluperidol 	3.3 ± 0.1	12^{M}	12
Bromperidol 	1.2 ± 0.4	----------	----------
Chlorohaloperidol 	1.5 ± 0.7	----------	----------
Droperidol 	2240.6 ± 863.8	----------	----------
Benperidol 	240.9 ± 64.1	----------	----------

Spiperone 	1053.7 ± 579.0	1090 [D]	1090
3'-fluorobenzylspiperone 	146.6 ± 39.5	----------	----------
Penfluridol 	53.8 ± 25.2	----------	----------
Haloperidol metabolite I 	128 ± 20.6	362 [M] 326[R]	344 ± 25
Haloperidol metabolite III 	>10,000	>10,000[M]	>10,000
Antipsychotics/antidepressants			
Pimozide	159.0 ± 57.4	144[D] 508[O] 1,555[M]	736 ± 733

			
Fluphenazine 	10 ± 8	17[D] 62[O] 109 [M]	63 ± 46
Clozapine 	>10,000	11400[D] >10,000[M]	>10,000
Fluvoxamine 	7 ± 3	36[E]	36
D4 DopamineReceptor Selective Ligands			
L 745, 870 	63.0 ± 0.27	----------	----------
RBI-257	2.5 ± 0.6	----------	----------

			
NGD 94-1 	5160.7 ± 2670.2	----------	----------
PD 168, 077 	2611.7 ± 268.6	----------	----------
Drugs of abuse			
Methamphetamine 	5247.7 ± 1044.3	2160[F]	2160
Meprobamate 	>1,000,000	----------	----------
Carisoprodol	613300	----------	----------

			
Endogenous neurosteroids			
Progesterone 	468 ± 139	268[G] 24.6[A] 338[L]	210± 165
β-estradiol 	>10,000	----------	----------

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CHAPTER IV

DISCUSSION

Studies of Sigma-1 receptor binding have been carried out primarily with the use of whole animal tissues. Because most Sigma-1 receptor ligands, including the radioligands, are non-selective and bind to other receptors expressed by the whole tissues, this method has proven to be an often inaccurate and inconsistent way of ascertaining the true binding affinity of Sigma-1 ligands to the receptor. More recently, independent studies have shifted to using Sigma-1 receptor cDNA transfection into a variety of clonal cell lines for the purpose of using Sigma-1 expressing cell membranes for binding assays. A complication that often results from this approach is that careful steps need to be taken to account for what are thought to be the endogenous Sigma-1 receptors in the clonal cell lines (Mei and Pasternak 2001, Ganapathy 1999). In an effort to generate a reliable binding assay for screening various putative ligands to the Sigma-1 receptor, we stably expressed the cloned human Sigma-1 receptor into the well

characterized human mammary carcinoma cell line MCF-7, which in its natural state, lacks innate Sigma-1 receptor binding sites and has low background (non-specific) binding for the somewhat selective Sigma-1 radioligand [^{3}H](+)-pentazocine. Consistent with the binding data from other groups (Vilner et al., 1995, Seth et al., 1998, Ganapathy et al., 1999, Brent et al., 1995), our results indicate no specific binding of [^{3}H](+)-pentazocine to the untransfected MCF-7 cells. The Sigma-1 receptor transfected MCF-7 cells, in contrast, exhibited a high and dose-dependent saturable binding (K_D=3.7 ± 0.87 nM), and the receptor protein density (B_{max}=109 ± 23.7 pmoles/mg) was determined to be within the range expected for Sigma-1 receptors (Seth et al., 1998, Mei and Pasternak, 2001).

Out of the diverse number of ligands screened, the K_i values of several well-established Sigma-1 selective ligands using this assay system follow similarly with the reported values found in literature. These results demonstrate the successful establishment of a rapid and reliable assay for Sigma-1 receptor ligands. Included in the 36 drugs screened were the antipsychotic drugs of the butyrophenone class, which exhibit a wide range of affinities (K_i values from 54 nM to 2,240 nM) for the Sigma-1 receptor. This large discrepancy in binding can be partly explained by looking at the structure-to-affinity relationship of Butyrophenone-class drugs to the Sigma-1 receptor (*Figure 6*). As shown, high affinity binding to the Sigma-1 receptor is associated with the presence of both an electronegative moiety at position one along the butyl chain and a 4-linked phenyl attached to the piperidine. Haloperidol and chlorohaloperidol, for example, bind with high affinity to the Sigma-1 receptor due to the presence of both the electronegative moiety and the 4-linked phenyl. Spiperone and benperidol, on the other hand, have only the position one electronegativity and consequently bind only with a weak affinity. Penfluridol, interestingly, binds with a moderate affinity while possessing only the 4-linked phenyl, suggesting that the

phenyl group plays a more significant role in binding to the Sigma-1 receptor than the electronegative moiety at position one. It is interesting to note that 3'-fluorobenzylspiperone, although highly electronegative near the piperdine, binds only with a weak affinity, demonstrating that the electronegativity near the piperdine is not alone sufficient for high-affinity binding. The 4-linked phenyl is necessary for high affinity binding of Butyrophenone-class compounds to the Sigma-1 receptor, while the electronegativity at position one along the butyl chain also plays some role in the binding.

Furthermore, along with the notable differences in binding affinities among butyrophenone antipsychotics, the structure-to-affinity relationship points to the lack of association between antipsychotic treatments and the Sigma-1 receptor. Droperidol and haloperidol, for example, are both potent antipsychotics but differ greatly in their binding to the Sigma-1 receptor, suggesting that the antipsychotic effects are not mediated by the Sigma-1 receptor. In addition, haloperidol and reduced haloperidol (a haloperidol metabolite) bind to the Sigma-1 receptor with an almost identical affinity, yet haloperidol is a highly potent neuroleptic while reduced haloperidol has no antipsychotic activity, further suggesting that Sigma-1 receptors do not contribute to the activity of butyrophenone antispychotics in the treatment of psychosis. Indeed, no current reports have conclusively demonstrated a correlation between the efficacies of butyrophenone drugs in treating psychosis and its affinity for the Sigma-1 receptor (for review, see Volz and Stoll 2004).

In addition to the butyrophenone antipsychotics screened were the typical antipsychotics fluphenazine and pimozide, which are both considered drugs with more extrapyramidal side-effect liability than haloperidol. Their respective K_i's, 10 nM and 159 nM to the Sigma-1 receptor are similar to those derived from binding studies using [^{3}H](+)-SKF-10047 with guinea

pig brain homogenates (Tam and Cook 1984), but rather large differences were reported (see refs O and M). Of significance also in the psychiatric drugs tested are the selective serotonin reuptake inhibitor, fluvoxamine and the atypical antipsychotic, clozapine. Our reported K_i value of 7 nM for the binding of fluvoxamine to the Sigma-1 receptor is comparable (~5-fold less) to the published K_i value of 36 nM reported by Narita et al., 1996. This nanomolar affinity value supports the view that Sigma-1 receptor may play a role in the pharmacological effects of this selective serotonin reuptake inhibitor. Clozapine, on the other hand, was found to exhibit no specific binding to the Sigma-1 receptor. Hashimoto et al., 2005 first reported the improvement of PCP-induced cognitive deficits in rats through the use of a 2 week subcutaneous administration of clozapine. Subsequently, in another similar study, Hashimoto et al., 2005 demonstrated, cognitive improvements by fluvoxamine. In this study, the cause of this improvement was linked to the Sigma-1 receptor when subcutaneous injections of fluvoxamine with the Sigma-1 receptor antagonist NE-100 prevented the cognitive improvements (Hashimoto et al., 2005, Hashimoto et al., 2006). Our reports demonstrated that clozapine has no binding capability to the Sigma-1 receptor as has been reported by Tam and Cook 1984 and Matsumoto et al., 2000. This finding indicates that the improvement of PCP-induced cognitive deficits in rats by clozapine does not involve the Sigma-1 receptor. The high affinity of fluvoxamine to the Sigma-1 receptor would suggest that fluvoxamine may play a role in the alleviation of PCP-induced cognitive deficits in rats, but more studies will be necessary to establish the differences in clozapine and fluvoxamine's mechanism of action in achieving the cognitive improvements.

Our study is the first report of the binding affinities of several dopamine D_4-selective ligands to the Sigma-1 receptor. Interestingly, L 745,870 and RBI-257 both bind to Sigma-1 receptors with nanomolar affinities. In binding studies for the purpose of assessing potential

dopamine D_4 selective radioligands, Kula et al., 1999 observed an unsaturable binding with rising concentrations of [^{125}I] RBI-257 in the rat brain. Furthermore, autoradiography studies indicated limited displacement of another radioactively labeled dopamine D_4-selective ligand, [^{3}H] PNU-101958, by either RBI-257 or L745,870 (Kula et al., 1999). This low potency of displacement of [^{3}H]PNU-101958 by RBI-257 and L745, 870 and the ineffectiveness of [^{125}I]RBI-257 to be used for visualization of dopamine D_4 receptors in brain tissues may in part be explained by our discovery of the coincidentally high binding affinities of RBI-257 (K_i ~2.5 nM) and L745, 870 (K~63.0 nM) to the Sigma-1 receptor.

Also of significance, Sigma-1 receptors are thought to represent a common pathway for many different drugs of abuse such as opioids (Mei and Pasternak 2002), cocaine (Romieu et al., 2003, Su and Hayashi 2001), and methamphetamine (Nguyen et al., 2005). In our study, we looked at three different types of drugs of abuse. Out of the three, methamphetamine revealed a moderate K_i of 5.2 μM to the Sigma-1 receptor. This value is consistent (< 3-fold different) with the report by Nguyen et al., 2005 of a micromolar range of methamphetamine binding in whole P2 rat brains. Other reports have also implicated Sigma-1 receptor antagonists in attenuating methamphetamine-induced behavioral sensitization (Takahashi et al., 2000, Ujike et al., 1992) and up-regulation of [^{3}H](+)-pentazocine binding in the frontal cortex, cerebellum, and substantia nigra of rats exposed to methamphetamine (Ithzak, 1993). Normal therapeutic dosing of methamphetamine results in a peak blood concentration of around 0.02 mg/L, which corresponds to 134 nM, and is a concentration that would have negligible effect on the Sigma-1 receptor (Logan 2002). Toxic levels of blood methamphetamine concentration, however, range from approximately 0.2 to 5.0 μg/ml, and fatal levels of methamphetamine concentration in the blood exceed 10 μg/ml (Inoue et al., 2006). The molar equivalents of these levels are

approximately 1.34 μM to 33.5 μM and 67 μM, respectively. At these concentrations, there would be significant binding to the Sigma-1 receptor and would suggests that the Sigma-1 receptor could possibly play a role in methamphetamine toxicity. Further studies will need to be undertaken to understand the molecular actions of methamphetamine on the Sigma-1 receptors in order to develop a possible treatment for methamphetamine-related brain deterioration.

We also report here the binding affinity of progesterone to the Sigma-1 receptor. Our K_i value of 468 nM is several fold greater than the K_i value of 55 nM reported by Ganapathy et al. using [^{3}H](+)-pentazocine as the radioligand with HeLa cells which are transfected with Sigma-1 receptor cDNA from the Jurkat Human T Lymphocyte cells, a cell line with intrinsic drug-binding Sigma-1 receptors (Ganapathy et al., 1999). Other independent studies have reported K_i values of 338 nM (Hanner et al., 1996), 24.6 nM (McCann et al., 1994), and 268 nM (Su et al., 1988) using whole tissue models. At this point, the identity and the physiological significance of progesterone as an endogenous Sigma-1 receptor ligand still remain controversial because its high nanomolar affinity may not allow much actual binding to occur within the body (see Bermack et al., 2005 for review).

In summary, a reliable and efficient assay system was established using MCF-7 cell stably expressing the cloned human Sigma-1 receptor. Drugs of completely distinct chemical profiles were screened and their unambigous affinity values (K_i) were reported. In addition to discovering new Sigma-1 ligands, some results from previous studies were re-examined. Our data supports the findings of some, but not all prior Sigma-1 receptor-ligand binding studies.

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BIOGRAPHICAL INFORMATION

Ivan was born in Chicago but spent most of his childhood in Taiwan. Calling Arlington, Texas his second home since coming to the U.S. in the fifth grade, Ivan chose to attend the University of Texas at Arlington in order to stay close to his family. Following his interest in genetics, Ivan joined Dr. Pawel Michalak's evolutionary genomics lab in his second semester at UT-Arlington. In Dr. Michalak's lab, Ivan investigated genes and pathways that lead to reproductive incompatibility involved in the evolutionary process of species differentiation. His work there resulted in a poster presentation, an oral presentation, and a published paper titled "Gene expression polymorphism in *Drosophila* populations." Wishing to explore the field of neuroscience, Ivan next joined Dr. John Schetz's neuropharmacology laboratory in the summer of 2005. During his time in Dr. Schetz's lab, Ivan worked on the enigmatic Sigma-1 receptor and produced the present Honors Thesis along with two papers that are currently in the process of submission. Ivan's third extensive research experience was in Dr. Masashi Yanagisawa's molecular genetics lab at the University of Texas Southwestern Medical Center where his primary duty was to characterize the newly de-orphaned GPR41 receptor. His work in the summer had resulted in a poster at the end of the Summer Undergraduate Research Program. Ivan is very grateful for the

wonderful opportunities that his mentors had given him. He aspires to become a physician-scientist in the future.