Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 525 (2005) 8–17
www.elsevier.com/locate/ejphar

[^{3}H]*N*-[4-(3,4-dihydro-6,7-dimethoxyisoquinolin-2(1H)-*yl*) butyl]-2-methoxy-5-methylbenzamide: A novel sigma-2 receptor probe

Jinbin Xu [a], Zhude Tu [a], Lynne A. Jones [a], Suwanna Vangveravong [a], Kenneth T. Wheeler [c], Robert H. Mach [a,b,*]

[a] *Department of Radiology, Washington University School of Medicine, 510 S. Kingshighway Blvd., St. Louis, MO 63110, USA*
[b] *Department of Cell Biology and Physiology, Washington University School of Medicine, 510 S. Kingshighway Blvd., St. Louis, MO 63110, USA*
[c] *Department of Radiology, Wake Forest University School of Medicine, Medical Center Blvd, Winston-Salem, NC 27157, USA*

Received 12 May 2005; received in revised form 8 August 2005; accepted 21 September 2005
Available online 10 November 2005

Abstract

N-[4-(3,4-dihydro-6,7-dimethoxyisoquinolin-2(1H)-*yl*)butyl]-2-methoxy-5-methyl-benzamide (RHM-1) and *N*-[2-(3,4-dihydro-6,7-dimethoxyisoquinolin-2(1H)-*yl*)ethyl]-2-methoxy-5-methylbenzamide (RHM-2), two conformationally flexible benzamide analogues, were radiolabeled with tritium (specific activity = 80 Ci/mmol) and the binding of [^{3}H]RHM-1 and [^{3}H]RHM-2 to sigma-2 (σ_2) receptors was evaluated in vitro. [^{3}H]RHM-1 was found to have a higher affinity for σ_2 receptors compared to [^{3}H]RHM-2 and [^{3}H]1,3-di-*o*-tolylguanidine ([^{3}H]DTG). [^{3}H]RHM-1 had a dissociation constant (K_d) of 0.66 ± 0.12 nM in rat liver membrane homogenates, which was 30-fold higher than that of [^{3}H]RHM-2 (K_d = 19.48 ± 0.51 nM). The lower affinity of [^{3}H]RHM-2 can be attributed to its faster K_{off} rate since both radioligands have similar K_{on} rates. Competitive binding assays were also conducted using a panel of compounds with known affinity for σ_2 receptors. The pharmacologic profile of [^{3}H]RHM-1 was in agreement with that of [^{3}H]DTG. The results of this study indicate that [^{3}H]RHM-1 is a useful ligand for studying σ_2 receptors in vitro.

Keywords: σ_2 receptor; Radioligand binding; Benzamide; Breast tumor

1. Introduction

The sigma (σ) receptors are a distinct family of proteins capable of binding to a wide variety of drugs (Martin et al., 1976; Walker et al., 1990). Based on photoaffinity labeling studies with [^{3}H] azido-1,3-di-*o*-tolylguanidine ([^{3}H]azido-DTG), two different types of σ receptors have been defined, σ_1 and σ_2 receptors (Hellewell et al., 1994). The σ_1 receptor has a molecular weight of 25 kDa, and the gene encoding this receptor has been cloned from guinea pig, human, mouse, and rat (Hanner et al., 1996; Seth et al., 1997; Kekuda et al., 1996; Mei and Pasternak, 2001). The gene for the σ_1 receptor contains four exons and three introns and is approximately 7 kbp long. The σ_2 receptor has not been cloned, but biochemical studies indicate that this protein has a molecule weight of 21.5 kDa. Colocalization of σ_1 and σ_2 receptors has been reported in many normal tissues and numerous drugs have been found to bind to σ_1 and σ_2 receptors with similar affinity (McCann et al., 1994; Hellewell et al., 1994).

A σ_1 receptor splice variant missing exon 3 does not bind the σ_1-selective radioligand, [^{3}H](+)-pentazocine but retains affinity for the σ_1/σ_2 nonselective ligand, [^{3}H]DTG (Wang et al., 2004). Based on this observation, it was suggested that the σ_2 receptor may be the product of alternative gene splicing of σ_1 receptor, namely the $\sigma_{1\beta}$ receptor. However, in vitro binding studies conducted on tissues obtained from a σ_1 receptor knockout mouse demonstrated that although [^{3}H](+)-pentazocine binding was significantly reduced in brain tissues, binding of σ_1/σ_2 radioligand [^{3}H]DTG was unaffected (Langa et al., 2003). These data suggest that the σ_2 receptor is encoded by different gene and may not simply represent a slice variant of the σ_1 receptor.

Although the biological function of the σ receptors is not known, there is a large body of evidence to support that the σ_1 receptor plays an important role in central nervous system

* Corresponding author. Division of Radiological Sciences, Mail Box 8225, Washington University School of Medicine, 510 S. Kingshighway Blvd., St. Louis, MO 63110, USA. Tel.: +314 362 8538; fax: +314 362 0039.
E-mail address: rhmach@mir.wustl.edu (R.H. Mach).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.09.063

function. A number of in vitro binding and in vivo behavioral studies have suggested that neuroactive steroids are endogenous ligands for the σ_1 receptor (Su et al., 1988; Monnet et al., 1995; Maurice et al., 1999). Ligands binding to the σ_1 receptor also modulate the release of neurotransmitters, and several of these compounds have shown promise as antipsychotic, antidepressants, and drugs blocking the reinforcing effect of psychostimulants (Takebayashi et al., 2002; Su and Hayashi, 2003; Matsumoto et al., 2003; Guitart et al., 2004).

Much less is known about the biological function of the σ_2 receptor. However, a number of studies have reported that σ_2 receptors over expressed in a wide variety of human and murine tumor cells grown in cell culture (Bem et al., 1991; Vilner et al., 1995; Mach et al., 1997). Furthermore, we have previously reported that the density of σ_2 receptors is 10-fold higher in proliferative versus quiescent mouse mammary adenocarcinoma cells in vitro (Mach et al., 1997; Al-Nabulsi et al., 1999) and in vivo (Wheeler et al., 2000). Based on these data, we have proposed that the σ_2 receptor may serve as a receptor-based biomarker of the proliferative status of solid tumors. Recent studies have also shown that σ_2 receptor ligands can induce apoptosis in tumor cells, which raises the possibility that σ_2 selective ligands may be useful as anticancer or chemosensitizing agents (Crawford and Bowen, 2002; Matsumoto et al., 2004). A potential role of the σ_2 receptor in regulating tumor cell proliferation and apoptosis has led to a renewed interest in understanding the biological function of this receptor.

[^{3}H](+)-pentazocine is a selective ligand for in vitro studies of the σ_1 receptor. The radioligand most often used to study σ_2 receptors in vitro is [^{3}H]DTG. However, this radioligand binds with equal affinity to σ_1 and σ_2 receptors (Walker et al., 1990; Hellewell et al., 1994). In vitro binding studies aimed at measuring the density of σ_2 receptors in tissue or tumor membrane homogenates requires the addition of unlabeled (+)-pentazocine to the assay in order to mask the binding of [^{3}H]DTG to σ_1 receptors (Hellewell et al., 1994). Furthermore, the relatively low affinity of [^{3}H]DTG to σ_2 receptors ($K_d \sim 25$ nM) requires the use of a relatively high concentration of this compound in the binding assay. Therefore, the development of a novel radioligand with high affinity and specificity for σ_2 receptors would represent an important improvement in the methodology used to assay σ_2 receptors in vitro.

Our group has previously reported a number of compounds having a high affinity and selectivity for σ_2 receptors compared to their affinity for σ_1 receptors (Mach et al., 1999, 2001, 2002, 2004). Two of these compounds are the conformationally flexible benzamide analogs, *N*-[4-(3,4-dihydro-6,7-dimethoxyisoquinolin-2(1H)-*yl*)butyl]-2-methoxy-5-methyl-benzamide (RHM-1) and *N*-[2-(3,4-dihydro-6,7-dimethoxyisoquinolin-2(1H)-*yl*)ethyl]-2-methoxy-5-methylbenzamide (RHM-2), which have been shown to have a high affinity and selectivity for σ_2 receptors (Mach et al., 2004). Their high σ_2 receptor affinity ($K_i < 15$ nM) and selectivity (σ_2/σ_1 ratio > 300) suggests that they may be useful for assessing the σ_2 receptor status of solid tumors and normal tissues when they are radiolabeled. Therefore, RHM-1 and RHM-2 were radiolabeled with tritium (specific activity = 80 Ci/mmol), and the binding of [^{3}H]RHM-1 and [^{3}H]RHM-2 to σ_2 receptors of rat liver and breast tumor homogenates were evaluated in vitro. [^{3}H]RHM-1 was found to have a higher affinity for σ_2 receptors compared to [^{3}H]RHM-2 and [^{3}H]DTG. The results of our studies demonstrate that [^{3}H]RHM-1 is a useful radioligand for assessing the σ_2 receptor status of tumors and normal tissues.

2. Materials and Methods

2.1. Precursor synthesis and radiolabeling

The tritiated compounds were synthesized by American Radiolabeled Chemicals, Inc. (St. Louis, MO) via *O*-alkylation of the corresponding phenol precursor (Tu et al., 2005); the specific activity of the radioligands was 80 Ci/mmol. The chemical structures of [^{3}H]RHM-1 and [^{3}H]RHM-2, which vary in the length of the carbon spacer between the amide nitrogen and the tertiary amine group, are shown in Fig. 1. [^{3}H]DTG (58.1 Ci/mmol) was purchased from PerkinElmer (Boston, MA).

2.2. Drugs and preparation

Chemical reagents and the standard compounds were purchased from Sigma (St. Louis, MO) and Tocris (Ellisville, MO). Novel compounds used in this study were synthesized by our group. *N*,*N*-Dimethylformamide (DMF), dimethyl sulfoxide (DMSO) or ethanol were used to dissolve the various compounds (Table 3); different concentrations were then achieved by diluting stock solutions with a solution containing 50 mM Tris–HCl, 150 mM NaCl and 100 mM EDTA at pH 7.4.

2.3. Membrane homogenate preparation

Membrane homogenates were prepared from the livers of male Sprague–Dawley rats (300–350 g). Animals were anesthetized with 3% isoflurane/oxygen and sacrificed by decapitation. The livers were removed, frozen on dry ice immediately and stored at −80 °C until used. Before homogenization, the livers were allowed to thaw slowly on ice. Tissue homogenization was carried out at 4 °C using a Potter–Elvehjem tissue grinder at a concentration of 1 g of tissue/ml of 50 mM Tris–HCl at pH 8.0. The crude membrane homogenate was

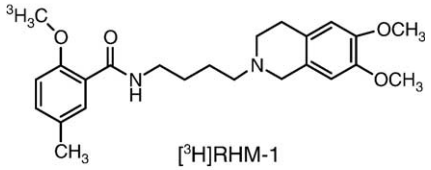

Fig. 1. Chemical structures of [^{3}H]RHM-1 and [^{3}H]RHM-2.

then transferred to a 50 ml centrifuge tube and resuspended to a concentration of 0.2 g of tissue/ml of 50 mM Tris–HCl. Additional homogenization was accomplished using an Ultra-Turrax T8 polython homogenizer (IKA Works, Inc., Wilmington, NC). The final homogenate was then centrifuged for 10 min at 1000×*g*, the pellet discarded and the supernatant mixed by vortexing. Aliquots were stored at −80 °C until use. The protein concentration of the suspension was determined using the DC protein assay (Bio-Rad, Hercules, CA) and averaged ~10 mg of protein/ml of stock solution. Membrane homogenates from ~1 g MDA-MB-435 human breast tumor xenografts and ~1 g EMT-6 mouse breast tumor xenografts were prepared as described for the liver membrane homogenates.

2.4. Sigma-2 receptor binding assay

2.4.1. Kinetic studies

The kinetic analysis of [^{3}H]RHM-1 (~2 nM), [^{3}H]RHM-2 (~4 nM) and [^{3}H]DTG (~16 nM) binding to rat liver homogenates (~2 mg/ml) was carried out to measure the association (K_{on}) and dissociation (K_{off}) rates. These values were then used to calculate the K_d values of each radioligand. Association and dissociation curves were obtained by recording the amount of the radioligand bound specifically (B_s) as a function of time (t) at a constant concentration of both the radioligand and the receptor. The specific binding was defined as the difference between the total (B_t) and the nonspecific (B_{ns}) binding, where the nonspecific binding of the radioligand was obtained in the presence of 10 μM DTG for [^{3}H]RHM-1 and [^{3}H]RHM-2 or 10 μM haloperidol for [^{3}H]DTG. For each kinetic analysis, three independent experiments were performed.

The values for receptor density (B_{max}) and the observed association rate constant (K_{ob}) were determined by fitting the curve of specific binding vs. time using the following equation:

$$B_s = B_{max} \cdot (1 - e^{-K_{ob}t}).$$

Dissociation experiments were carried out by adding 10 μM DTG to the reaction mixture after equilibrium was achieved and measuring the specific binding of the radioligand as a function of time. K_{off} was determined by fitting the curve of bound radioligand vs. time using the following equation:

$$B_s = B_{max} \cdot e^{-K_{off}t}.$$

The values for K_{on} and K_d were calculated using the formulas below, where L is the concentration of the radioligand being used in the assay (McGonigle and Molinoff, 1989).

$$K_{on} = \frac{K_{ob} - K_{off}}{[L]}$$

$$K_d = \frac{K_{off}}{K_{on}}.$$

The data analysis and curve fitting were performed with the KaleidaGraph software purchased from Synergy (Reading, PA).

2.4.2. Scatchard analysis

Membrane homogenates were diluted with 50 mM Tris–HCl buffer, pH 8.0 and incubated with the radioligand in a total volume of 150 μl at 25 °C in 96 well polypropylene plates (Fisher Scientific, Pittsburgh, PA). The incubation time was 60 min for [^{3}H]RHM-1 and [^{3}H]RHM-2, and 120 min for [^{3}H]DTG. For the [^{3}H]DTG binding assay; the σ_1 sites were masked with 1 μM (+)-pentazocine. The amount of protein added to each well was: rat liver membrane homogenates, ~300 μg; MDA-MB-435 human breast tumor membrane homogenates, ~100 μg; EMT-6 mouse breast tumor membrane homogenates, ~100 μg. The concentrations of the radioligand ranged from 0.001 to 100 nM. The reactions were terminated by the addition of 150 μl of cold wash buffer (10 mM Tris–HCl, 150 mM NaCl, pH 7.4, at 4 °C) using a 96 channel transfer pipette (Fisher Scientific, Pittsburgh, PA), and the samples harvested and filtered rapidly to 96 well fiber glass filter plate (Millipore, Billerica, MA) that had been presoaked with 100 μl of 50 mM Tris–HCl buffer, pH 8.0 for 1 h. Each filter was washed with 200 μl of ice-cold wash buffer for a total of three washes and added with 150 μl of scintillation fluid. A Wallac 1450 MicroBeta liquid scintillation counter (Perkin Elmer, Boston, MA) with a counting efficiency of ~19% was used to quantitate the bound radioactivity. Nonspecific binding was determined from samples which contained 10 μM cold RHM-1, DTG or haloperidol. The equilibrium dissociation constant (K_d) and maximum number of binding sites (B_{max}) were determined by a linear regression analysis of the transformed data using the method of Scatchard (Scatchard, 1949).

Data from saturation radioligand binding studies was transformed to determine the Hill coefficient, n_H, defined as:

$$\log \frac{B_s}{B_{max} - B_s} = \log K_d + n_H \log L$$

(Hill, 1910; McGonigle and Molinoff, 1989). L is the concentration of radioligand. n_H, the Hill slope, was determined from the Hill plot of $\log \frac{B_s}{B_{max} - B_s}$ versus $\log L$.

2.4.3. Competitive binding

Rat liver membrane homogenates (~300 μg protein) were diluted with 50 mM Tris–HCl buffer, pH 8.0 and incubated in a total volume of 150 μl with the radioligand at 25 °C in 96 well plates. The incubation time was 60 min for [^{3}H]RHM-1 and 120 min for [^{3}H]DTG. The σ_1 sites were masked in the presence of 1 μM (+)-pentazocine to determine the σ_2 receptor binding characteristics of [^{3}H]DTG,. The final concentration of the radioligand in each assay was ~1 nM for [^{3}H]RHM-1 and ~5 nM for [^{3}H]DTG. Inhibitor concentrations ranging from 0.1 nM to 10 μM were added to acquire the inhibition curves. After the reaction was completed, the samples were harvested, washed three times, and the bound radioactivity counted and analyzed as described above. Nonspecific binding was determined from samples that contained 10 μM of cold haloperidol.

Data from the competitive inhibition experiments were modeled using nonlinear regression analysis to determine the

concentration of inhibitor that inhibits 50% of the specific binding of the radioligand (IC_{50} value). The competition curves were modeled for a single site using the following equation:

$$B_s = B_0 - \left[(B_0^* I)/(\mathrm{IC}_{50} + I)\right]$$

where B_s is the amount of the radioligand bound specifically to the membrane homogenates (i.e. $B_s = B_t - B_{ns}$, where B_t is the total bound radioactivity and B_{ns} is the nonspecific binding of the radiotracer), B_0 is the amount of the radioligand bound in the absence of the competitive inhibitor, I is the concentration of the competitive inhibitor and the IC_{50} is the concentration of competitive inhibitor that blocks 50% of the total specific binding. The values for B_{ns} and B_0 were constrained using experimentally derived values. Competitive inhibition constants (K_i values) were calculated from the IC_{50}

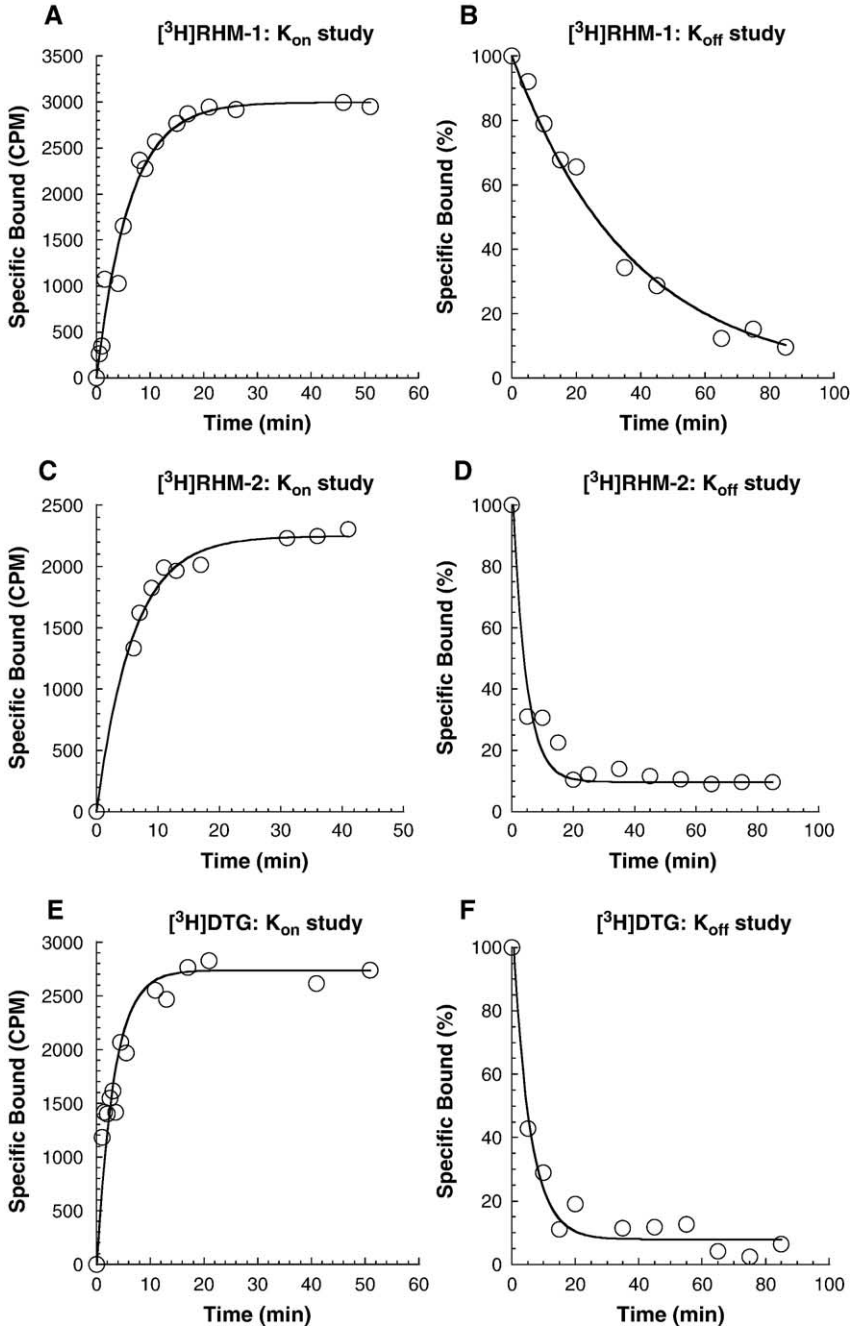

Fig. 2. Kinetic analysis of the binding of [^{3}H]RHM-1, [^{3}H]RHM-2 and [^{3}H]DTG in σ_2 receptors of rat liver homogenates. A, C, E: Representative association experiments of [^{3}H]RHM-1, [^{3}H]RHM-2 and [^{3}H]DTG, respectively. The data are shown as the counts per minute (cpm) of the radioligand specifically bound as a function of the incubation time (min). The curves were fitted by nonlinear regression. B, D, F: Representative dissociation experiments of [^{3}H]RHM-1, [^{3}H]RHM-2 and [^{3}H]DTG, respectively. The data are shown as the percent of the radioligand specifically bound as a function of time (min) after the addition of 10 μM of DTG (B, D) or haloperidol (F). The curves were fitted by nonlinear regression.

values using the Cheng and Prussoff equation (Cheng and Prussoff, 1973):

$$K_i = \frac{IC_{50}}{1 + L_t/K_d}$$

where the K_d values of [^{3}H]RHM-1 and [^{3}H]DTG derived from rat liver homogenate experiments are 0.66 nM and 30.73 nM, respectively, and L_t is the concentration of radioligand used in each assay.

Data from competitive radioligand binding studies was transformed to determine the pseudoHill coefficient, n'_H, defined as:

$$\log \frac{B_s}{B_0 - B_s} = n'_H \log I - n'_H \log IC_{50}.$$

(Hill, 1910; McGonigle and Molinoff, 1989). n'_H, which is the negative of the Hill slope, was readily determined from the plot of $\log \frac{B_s}{B_0 - B_s}$ versus log I.

3. Results

3.1. Radiochemical synthesis

The chemical structures of [^{3}H]RHM-1 and [^{3}H]RHM-2 are shown in Fig. 1. The specific activity of both radioligands is 80 Ci/mmol, with a radiochemical purity of >98%, determined by high performance liquid chromatography (HPLC). The complete details of the synthesis and radiolabeling procedures will be published elsewhere. The primary difference between these two radioligands is the four carbon spacer for [^{3}H]RHM-1 and the two carbon spacer for [^{3}H]RHM-2. Because of this structural difference, [^{3}H]RHM-2 has a lower log P value than [^{3}H]RHM-1 (Tu et al., 2005). Their affinities and selectivity for σ_2 receptors, were found to be similar based on the results of the competitive binding assays using [^{3}H]DTG as the radioligand (Mach et al., 2004).

3.2. Kinetics experiments in rat liver membrane homogenates

As an initial step, we characterized the kinetic properties of [^{3}H]RHM-1, [^{3}H]RHM-2 and [^{3}H]DTG binding to the σ_2 receptors in rat liver homogenates. Both association and dissociation experiments were carried out with [^{3}H]RHM-1, [^{3}H]RHM-2 and [^{3}H]DTG (Fig. 2). The values of the association rates (K_{on}) and dissociation rates (K_{off}) and dissociation constants (K_d) calculated from the kinetic studies (K_{off}/K_{on}) are summarized in Table 1. [^{3}H]RHM-1 and [^{3}H]RHM-2 have similar association constants, while their dissociation constants are quite different. [^{3}H]RHM-1 dissociates slowly from σ_2 receptors, whereas [^{3}H]RHM-2 dissociates very fast from σ_2 receptors (Fig. 2). The more rapid dissociation of [^{3}H]RHM-2 results in a lower affinity for [^{3}H]RHM-2 (K_d=15.22 nM) than for [^{3}H]RHM-1 (K_d=0.72 nM). These data suggest that the four carbon spacer of [^{3}H]RHM-1 provides a better match for the ligand binding domain of the σ_2 receptor, thereby decreasing the reversibility of the receptor–ligand complex. Although the dissociation constant of [^{3}H]RHM-2 is similar to that of [^{3}H]DTG, [^{3}H]RHM-2 possesses a better σ_2 receptor selectivity than [^{3}H]DTG, which binds with equal affinity to both σ_1 and σ_2 receptors (Dehaven-Hudkins et al., 1996).

Table 1
Summary of the binding kinetics of [^{3}H]RHM-1, [^{3}H]RHM-2 and [^{3}H]DTG to the σ_2 receptors on rat liver membrane homogenates[a]

Radioligand	K_{off}, min^{-1} (±S.E.M.)	K_d, nM (±S.E.M.)	K_{on}, nM^{-1} min^{-1} (±S.E.M.)
[^{3}H]RHM-1	0.027±0.004	0.72±0.28	0.05±0.01
[^{3}H]RHM-2	0.144±0.003	15.22±4.34	0.01±0.003
[^{3}H]DTG	0.21±0.012	38.5±4.15	0.0054±0.0006

[a] The values are the mean±S.E.M. of duplicate samples from 3 independent experiments.

3.3. Saturation experiments in rat liver membrane homogenates

Direct saturation binding studies were carried out using [^{3}H]RHM-1, [^{3}H]RHM-2 and [^{3}H]DTG with σ_2 membrane homogenates of rat liver. The saturation curve and Scatchard plots are shown in Fig. 3. The K_d, B_{max} and n_H values of the receptor–radioligand binding of [^{3}H]RHM-1, [^{3}H]RHM-2 and [^{3}H]DTG are summarized in Table 2. The K_d values for [^{3}H]RHM-1 and [^{3}H]RHM-2 are in good agreement with those obtained from the kinetics studies (Table 1). The mean of n_H values for all the three radioligands were found to be close to unity, and indicates that the receptor binding of these radioligands is to one site and displays non-cooperative binding. The B_{max} values determined from the [^{3}H]RHM-1 and [^{3}H]RHM-2 binding experiments are consistent with that found for [^{3}H]DTG under conditions where unlabeled (+)-pentazocine (1 μM) was added to mask the binding of [^{3}H]DTG to σ_1 receptors. Therefore, we can reasonably conclude that [^{3}H]RHM-1 and [^{3}H]RHM-2 are binding to the same population of receptors as [^{3}H]DTG when the σ_1 receptors are masked by (+)-pentazocine. Of the two radioligands, [^{3}H]RHM-1 has a higher affinity for σ_2 receptors (K_d=0.66 nM) than [^{3}H]RHM-2 (K_d=19.48 nM). The σ_2 receptor affinity of both ligands is higher than that of [^{3}H]DTG (K_d=30.73 nM), the traditional radioligand used in σ_2 receptor binding studies. These data suggest that both [^{3}H]RHM-1 and [^{3}H]RHM-2 can potentially replace [^{3}H]DTG as novel σ_2 receptor probes.

3.4. Competitive profile of standard and novel compounds in rat liver membrane homogenates

A series of σ_1, σ_2, dopaminergic, serotonergic and NMDA ligands, whose σ_2 receptor affinities have been well documented, were tested to characterize the pharmacological profile of [^{3}H]RHM-1. In Table 3, compounds 1a and 2f are σ_2 specific compounds previously reported by our group (Mach et al., 2002). Compounds SV239-1, SV246-1, SVW-III-22-1 and SVW-III-24-1 are new compounds synthesized in our lab; the synthesis of these compounds will be reported elsewhere. Competitive binding assays on this panel of compounds were performed using both [^{3}H]RHM-1 and [^{3}H]DTG. The K_i values

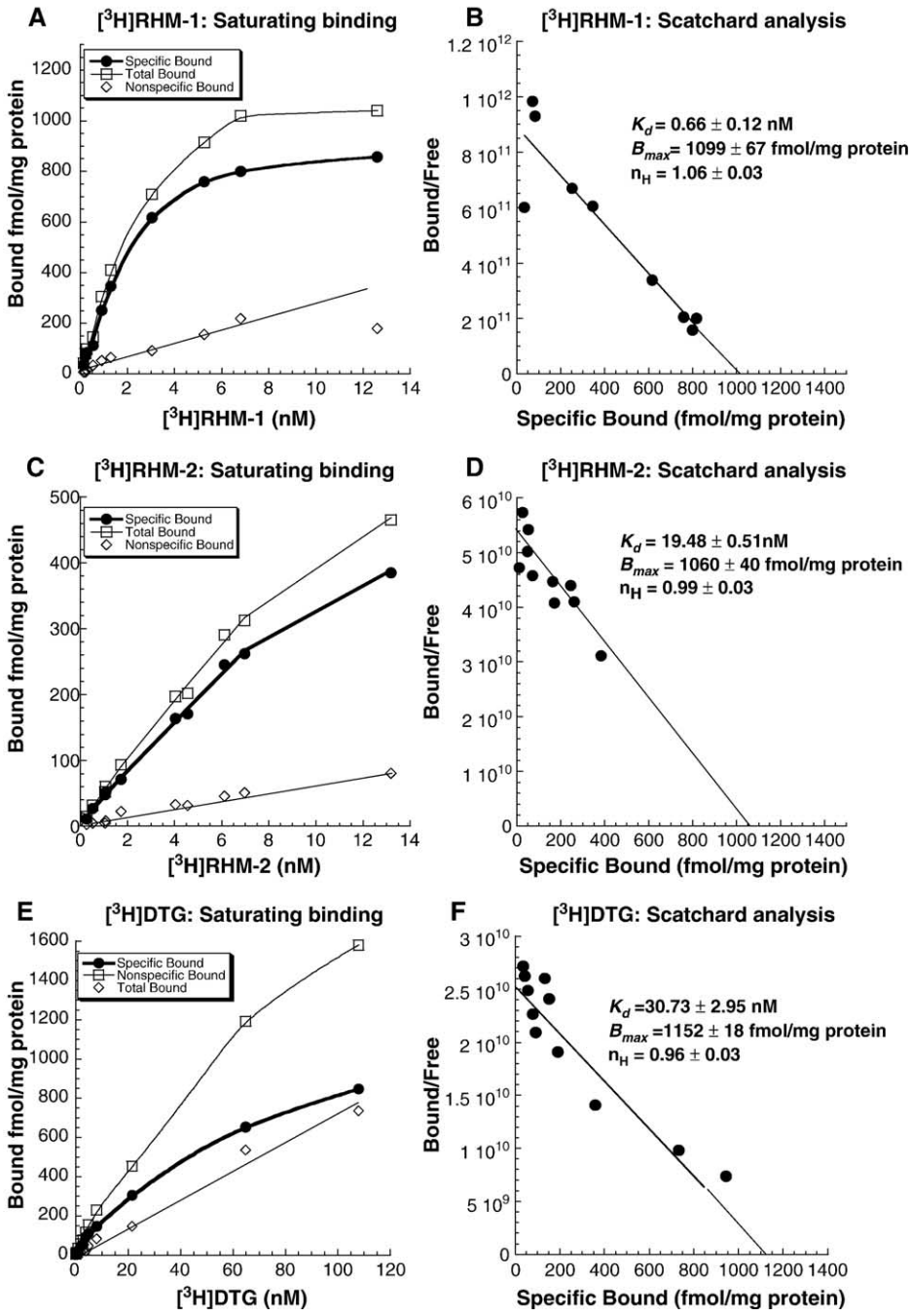

Fig. 3. Scatchard analysis of [^{3}H]RHM-1, [^{3}H]RHM-2 and [^{3}H]DTG binding to the σ_2 receptors in rat liver membrane homogenates. A, C, E: Representative saturation binding experiments which show the total bound, non-specific bound and specific bound. B, D, F: Representative Scatchard plots which were used to determine K_d and B_{max} values.

for inhibiting [^{3}H]RHM-1 binding to σ_2 receptors are consistent with the K_i values for inhibiting [^{3}H]DTG binding to σ_2 receptors (Table 3). Furthermore, the data points were consistently distributed along the line of unity when a plot of the K_i values obtained with [^{3}H]RHM-1 and [^{3}H]DTG was made (Fig. 4). These data indicate that [^{3}H]RHM-1 binds to σ_2 receptors with the same pharmacological profile as that of [^{3}H]DTG. Competitive binding curves for some of the σ_2 selective ligands and the σ_1 selective ligand, (+)-pentazocine, were also generated. As shown in Fig. 5, the affinity rank order was 2f > ifenprodil > 1a > DTG > haloperidol > SM21 ≫ (+)-pentazocine. With the exceptions of MDL72222, 2f and DTG, the mean of n'_H values for competitive radioligand binding with [^{3}H]RHM-1 and [^{3}H]DTG were found to range from 0.6 to unity for standard or novel compounds tested (Table 3).

3.5. Saturation experiments in mouse and human membrane homogenates

Overexpression of σ_2 receptors has been observed for many human and rodent tumor cell lines growing under cell culture conditions (e.g. Bem et al., 1991; Vilner et al., 1995). Therefore,

Table 2
Summary of the Scatchard analyses for [^{3}H]RHM-1, [^{3}H]RHM-2 and [^{3}H]DTG binding to the σ_2 receptors of rat liver membrane homogenates [a]

Radioligand	Rat liver		
	K_d (nM±S.E.M.)	B_{max} (fmol/mg protein±S.E.M.)	n_H (±S.E.M.)
[^{3}H]RHM-1	0.66±0.12	1099±67	1.06±0.03
[^{3}H]RHM-2	19.48±0.51	1060±40	0.99±0.03
[^{3}H]DTG	30.73±2.95	1152±18	0.96±0.03

[a] The values are the mean±S.E.M. of duplicate samples from 3 independent experiments.

a series of in vitro binding studies were conducted using EMT-6 mouse mammary adenocarcinoma and MDA-MB-435 human breast tumor xenografts grown in BALB/C mice. Tissue homogenates were prepared from these tumors, and Scatchard analyses were conducted to investigate the binding of [^{3}H]RHM-1 to the σ_2 receptors in membrane homogenates isolated from the two breast tumors. The saturation binding curves and Scatchard plots (Fig. 6) indicate that [^{3}H]RHM-1 binds with a high affinity to both EMT-6 mouse breast tumors (K_d=3.45 nM) and MDA-MB-435 human breast tumors (K_d=4.47 nM). These K_d values were not as high as the K_d values obtained for [^{3}H]RHM-1 using rat liver homogenates (Table 1). However, such a difference in the K_d values between tumors and normal tissues has been previously reported by others for [^{3}H]DTG (Vilner et al., 1995). The different K_d values implies that σ_2 receptors may display a different three dimensional conformation in rat liver versus tumor membranes, thus the binding characteristics differ even for the same radioligand. The σ_2 receptor densities (B_{max}) of both tumors were also found to be high; B_{max}=1820 fmol/mg of protein for MDA-MB-435 human breast tumors, B_{max}=2290 fmol/mg of protein for EMT-6 mouse breast tumors, respectively. Hill coefficients obtained from both tumors were close to unity, n_H=1.01 for MDA-MB-435 human breast tumors and n_H=1.03 for EMT-6 mouse breast tumors. All of these data suggest that [^{3}H]RHM-1 is a novel probe for assessing the σ_2 receptor density of tumors in vitro.

Table 3
Summary of the σ_2 affinity of a series of ligands with known binding characteristics obtained from competitive binding studies with [^{3}H]RHM-1 and [^{3}H]DTG using rat liver membrane homogenates [a]

Drugs	[^{3}H]RHM-1		[^{3}H]DTG	
	K_i (nM±S.E.M.)	n'_H (±S.E.M.)	K_i (nM±S.E.M.)	n'_H (±S.E.M.)
1a	8±0.86	0.89±0.003	4.85±1.06	0.60±0.02
2f	2.27±0.75	0.66±0.03	1.64±1.01	0.40±0.05
DTG	17.19±6.51	0.88±0.1	27.73±6.55	0.51±0.05
Haloperidol	37.78±2.70	0.82±0.05	47.24±12.76	0.87±0.08
Ifenprodil	3.47±0.56	0.84±0.06	2.70±0.60	0.66±0.06
(+)-Pentazocine	3267±853	–	1440±264	–
SM21	83.72±19.42	0.79±0.06	104.35±9.82	0.84±0.06
3-Tropanylindole-3-Carboxylate	4573±1012	–	3868±219	–
Dextromethorphan	4427±757	–	2863±290	–
GR113808	99.77±24.65	0.92±0.12	137.24±10	0.97±0.06
GR55562	5431±2420	–	5666±1271	–
Ketanserin	1761±613	–	2358±219	–
MDL72222	12.45±1.97	0.55±0.05	11.69±0.53	0.70±0.1
Pindolol	1642±454	–	1970±551	–
Way100635	4120±508	–	3615±748	–
RHM-1 (cold standard)	7.59±1.29	0.73±0.03	6.03±1.06	0.70±0.04
SV239-1	46.78±5.60	0.91±0.11	66.11±20.69	0.73±0.07
SV246-1	114.04±7	0.73±0.07	81.04±15.19	0.62±0.02
SVW-III-22-1	26.29±2.6	0.76±0.06	30.41±4.30	0.73±0.12
SVW-III-24-1	12.63±1.75	0.86±0.02	15.92±1.13	0.93±0.09

[a] Mean K_i values±S.E.M. were calculated from the IC_{50} values obtained in competitive binding experiments. The IC_{50} values were determined by a one site fit of the competitive curve, and converted to equilibrium dissociation constants (K_i values) using the Cheng and Prussoff (1973) equation, where the K_d values of [^{3}H]RHM-1 and [^{3}H]DTG for rat liver homogenates are 0.66 nM and 30.73 nM, respectively.

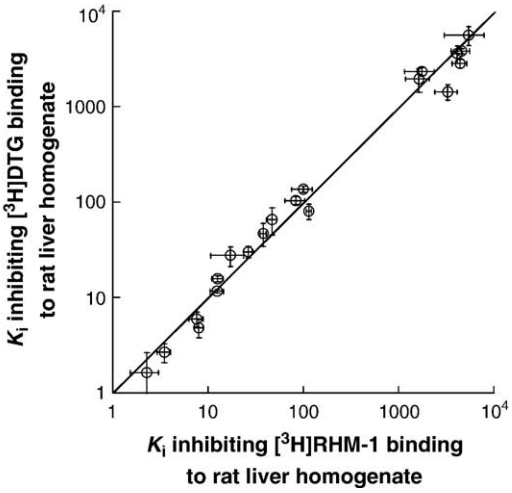

Fig. 4. Comparison of the K_is for [^{3}H]RHM-1 and [^{3}H]DTG obtained from competitive binding assays using rat liver membrane homogenates. The solid line represents the line of unity. The data for the two K_is are in good agreement.

4. Discussion

The goal of the current study was to evaluate two tritiated conformationally flexible benzamide analogs, [^{3}H]RHM-1 and [^{3}H]RHM-2, as potential ligands for measuring σ_2 receptor

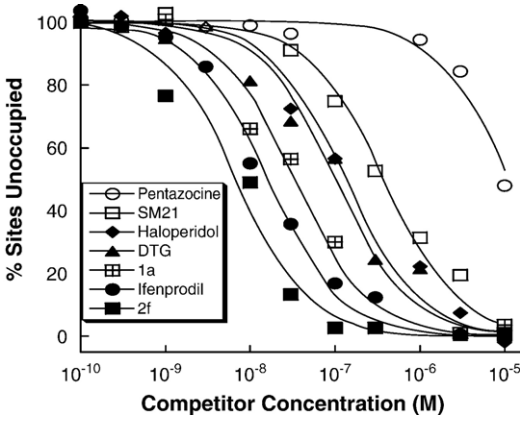

Fig. 5. Representative competitive binding data for inhibition of the [^{3}H]RHM-1 binding to σ_2 receptors in rat liver membrane homogenates of several known σ_2 ligands and a σ_1 selective ligand, (+)-pentazocine.

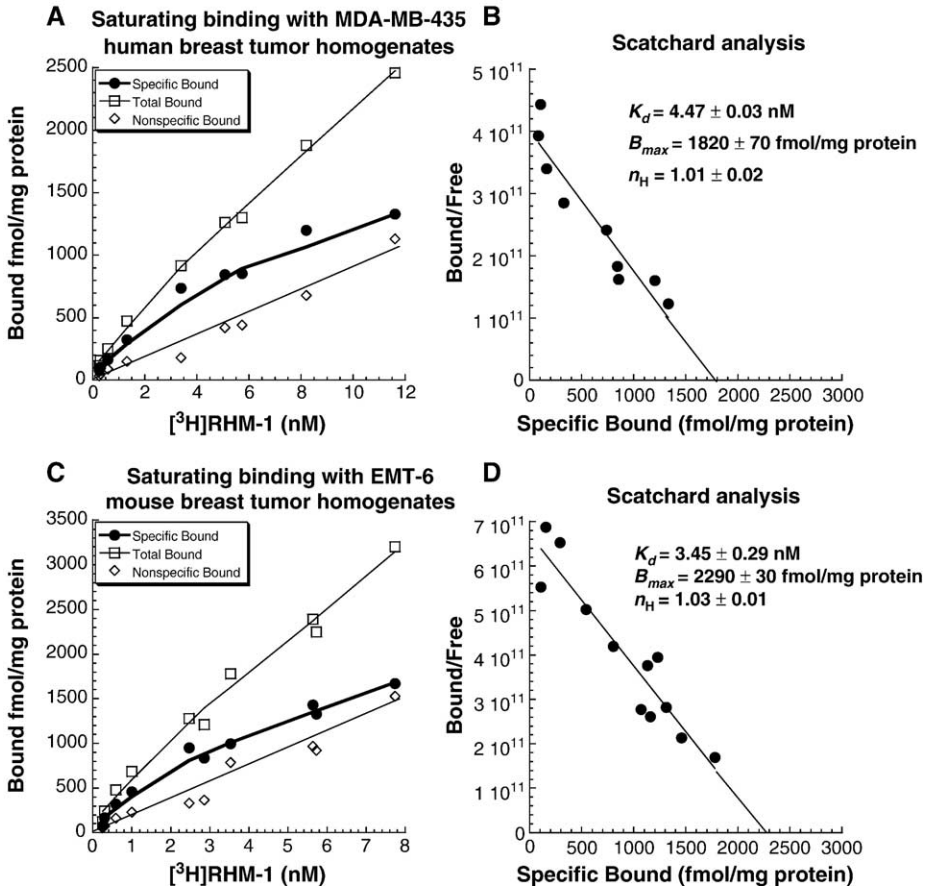

Fig. 6. Scatchard analysis of the [^{3}H]RHM-1 binding to the σ_2 receptors in membrane homogenates from MDA-MB-435 human breast tumor xenografts and EMT-6 mouse breast tumor xenografts.

function in vitro. Our previous studies have shown that both compounds have a high selectivity for σ_2 receptors and similar K_i values for displacing [^{3}H]DTG from σ_2 receptors in vitro (Mach et al., 2004). Although [^{3}H]DTG is the traditional radioligand for studying σ_2 receptors in vitro, this ligand has a relatively low σ_2 receptor affinity (~25 nM) and poor selectivity for σ_2 receptors. A tritiated ligand having a higher σ_2 receptor affinity and better selectivity for σ_2 receptors would represent a significant improvement over the ligands currently available for quantifying σ_2 receptors in vitro.

The main structural difference between [^{3}H]RHM-1 and [^{3}H]RHM-2 is the carbon spacer between the amide nitrogen and the tertiary amine group (Fig. 1). The two carbon spacer group of [^{3}H]RHM-2 results in a lower log *P* and a slightly lower amount of nonspecific binding relative to the four carbon homologue, [^{3}H]RHM-1 (Fig. 3). However, [^{3}H]RHM-2 has a much faster dissociation rate (K_{off}) compared to that of [^{3}H]RHM-1 (Fig. 2, Table 1). Since the difference in the level of nonspecific binding of [^{3}H]RHM-1 and [^{3}H]RHM-2 is small relative to the difference in the K_{off} and K_d values of the two tritiated ligands, it is clear that [^{3}H]RHM-1 is the better of the two ligands for measuring σ_2 receptor density in vitro. There is also a discrepancy between the data from the direct binding studies reported here and the competitive binding data reported data reported previously (Mach et al., 2004). Although RHM-1 and RHM-2 have similar K_i values, 10.3 nM and 13.3 nM, and RHM-2 possess a higher selectivity (σ_1/σ_2 ratio=783) than RHM-1 (σ_1/σ_2 ratio=300) (Mach et al., 2004), the results of the direct binding studies shows [^{3}H]RHM-1 has a K_d of 0.66 nM, while [^{3}H]RHM-2 holds a K_d of 19.48 nM. The direct binding data indicates that the selectivity of [^{3}H]RHM-1 is >4000-fold higher for σ_2 receptors (0.66 nM) versus σ_1 receptors (3078 nM) (Mach et al., 2004).

The dissociation rate of [^{3}H]RHM-2 was found to be similar to that of [^{3}H]DTG (Table 1); however, [^{3}H]RHM-2 was found to have a much lower level of nonspecific binding than that of [^{3}H]DTG (Fig. 3). The results of competitive binding assays comparing [^{3}H]RHM-1 and [^{3}H]DTG with a diverse panel of compounds with known σ_2 receptor pharmacology demonstrated a high level of agreement between the two ligands (Fig. 4, Table 3). These data further support that [^{3}H]RHM-1 and [^{3}H]DTG label the same sites in rat liver membranes. Given the results of our in vitro studies, the rank order of ligands described here for quantifying σ_2 receptors in vitro is [^{3}H]RHM-1>[^{3}H]RHM-2>[^{3}H]DTG.

To date, the σ_1 receptor has been cloned (Hanner et al., 1996; Seth et al., 1997; Kekuda et al., 1996; Mei and Pasternak, 2001) and a σ_1 receptor knockout mouse model has been reported (Langa et al., 2003). Consequently, the biological and pharmacological properties of the σ_1 receptors are understood better than the biological and pharmacological properties of the σ_2 receptors that have not been cloned. In vitro binding studies

with [^{3}H]DTG in the presence of unlabeled (+)-pentazocine to mask σ_1 sites have shown that σ_2 receptors are expressed in the liver, kidneys and central nervous system (Guitart et al., 2004). It also has been shown that σ_2 receptors are overexpressed in both human and murine tumors (Vilner et al., 1995), and that the σ_2 receptor is a reliable biomarker of the proliferative status of solid tumors (Mach et al., 1997; Al-Nabulsi et al., 1999; Wheeler et al., 2000). Therefore, radioligands having a high affinity and selectivity for the σ_2 receptor should be useful probes for the detection and assessment of the proliferative status of solid tumors both in vitro and in vivo. In addition, a radioligand having a higher affinity and selectivity than that of [^{3}H]DTG should be useful in isolating, purifying and characterizing the σ_2 receptor protein. We believe that the binding properties of [^{3}H]RHM-1 reported here indicate that it is the best radioligand with which to study the properties of the σ_2 receptor in vitro.

In conclusion, we have evaluated two novel probes having a high affinity and selectivity for σ_2 receptors and compared each with the traditional σ_2 receptor probe, [^{3}H]DTG. The results of our study indicate that [^{3}H]RHM-1, which possesses a subnanomolar affinity and a >4000-fold selectivity for σ_2 receptors, is a superior ligand to [^{3}H]DTG for measuring σ_2 receptor density and function using in vitro binding assays.

Acknowledgements

This research was funded by grants CA 102869 from the National Institutes of Health and DAMD17-01-0446 from the Department of Defense. We thank Dr. Carolyn Anderson and her group for assistance with MicroBeta counting experiments. We appreciate Dr. Wenhua Chu for coordinating the custom radiolabeling of these ligands.

References

Al-Nabulsi, I., Mach, R.H., Wang, L.-M., Wallen, C.A., Keng, P.C., Sten, K., Childers, S.R., Wheeler, K.T., 1999. Effect of ploidy, recruitment, environmental factors, and tamoxifen on the expression of sigma-2 receptors in proliferating and quiescent tumour cells. Br. J. Cancer 81, 925–933.

Bem, W.T., Thomas, G.E., Mamone, J.Y., Homan, S.M., Levy, B.K., Johnson, F. E., Coscia, C.J., 1991. Overexpression of σ receptors in nonneural human tumors. Cancer Res. 55, 6558–6562.

Cheng, Y.C., Prussoff, W.H., 1973. Relationships between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzyme reaction. Biochem. Pharmacol. 22, 3099–3108.

Crawford, K.W., Bowen, W.D., 2002. Sigma-2 receptor agonists activate a novel apoptotic pathway and potentate antineoplastic drugs in breast tumor cell lines. Cancer Res. 62, 313–322.

Dehaven-Hudkins, D.L., Daubert, J.D., Sawutz, D.G., Tiberio, L., Baine, Y., 1996. [^{3}H] 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. Immunopharmacology 35, 27–39.

Guitart, X., Codony, X., Monroy, X., 2004. Sigma receptors: biology and therapeutic potential. Psychopharmacology 174, 301–319.

Hanner, M., Moebius, F.F., Flandorfer, A., Knaus, H.-G., Striessnig, J., 1996. Expression of the mammalian sigma-1 binding site. Proc. Natl. Acad. Sci. U. S. A. 93, 8072–8077.

Hellewell, S.B., Bruce, A., Feinstein, G., Orringer, J., Williams, W., Bowen, W. D., 1994. Rat liver and kidney contain high densities of sigma-1 and sigma-2 receptors: characterization by ligand binding and photoaffinity labeling. Eur. J. Pharmacol., Mol. Pharmacol. Sect. 268, 9–18.

Hill, A.V., 1910. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. J. Physiol. (Lond) 40, iv–vii.

Kekuda, R., Prasad, P.D., Fei, Y.-J., Leibach, F.H., Ganapathy, V., 1996. Cloning and functional expression of the human type 1 sigma receptor. Biochem. Biophys. Res. Commun. 229, 553–558.

Langa, F., Codony, X., Tovar, V., et al., 2003. Generation and phenotypic analysis of sigma receptor type I (sigma 1) knockout mice. Eur. J. Neurosci. 18, 2188–2196.

Mach, R.H., Smith, C.R., Al-Nabulsi, I., Whirrett, B.R., Childers, S.R., Wheeler, K.T., 1997. Sigma-2 receptors as potential biomarkers of proliferation in breast cancer. Cancer Res. 57, 156–161.

Mach, R.H., Wu, L., West, T., Whirrett, B.R., Childers, S.R., 1999. The analgesic tropane analogue (±)-SM 21 has a high affinity for σ_2 receptors. Life Sci. 64, 131–137.

Mach, R.H., Yang, B., Wu, L., Kuhner, R.J., Whirrett, B.R., West, T., 2001. Synthesis and sigma receptor binding affinities of 8-azabicyclo[3.2.1]octan-3α-yl and 9-azabicyclo[3.3.1]nonan-3α-yl phenylcarbamates. Med. Chem. Res. 10, 339–355.

Mach, R.H., Vangveravong, S., Huang, Y., Yang, B., Blair, J.B., Wu, L., 2002. Synthesis of *N*-substituted 9-azabicyclo[3.3.1]nonan-3α-yl phenylcarbamate analogs as sigma-2 receptor ligands. Med. Chem. Res. 11, 380–398.

Mach, R.H., Huang, Y., Freeman, R.A., Wu, L., Vangveravong, S., Luedtke, R. R., 2004. Conformationally-flexible benzamide analogues as dopamine D3 and σ_2 receptor ligands. Bioorg. Med. Chem. Lett. 14, 195–202.

Maurice, T., Phan, V.-L., Urani, A., Kamei, H., Noda, Y., Nabeshima, T., 1999. Neuroactive steroids as endogenous effectors for the σ_1 receptor: pharmacological evidence and therapeutic opportunities. Jpn. J. Pharmacol. 81, 125–155.

Martin, W.R., Eades, C.G., Thompson, J.A., Huppler, R.E., Gilbert, P.E., 1976. The effects of morphine and nalorphine like drugs in the nondependent and morphine-dependent chronic spinal dog. J. Pharmacol. Exp. Ther. 197, 517–532.

Matsumoto, R.R., Liu, Y., Lerner, M., Howard, E.W., Brackett, D.J., 2003. σ Receptors: potential medications development target for anti-cocaine agents. Eur. J. Pharmacol. 469, 1–12.

Matsumoto, R.R., Gilmore, D.L., Pouw, B., Bowen, W.D., Williams, W., Kausar, A., Coop, A., 2004. Novel analogs of the σ_2 receptor ligand BD1008 attenuate cocaine-induced toxicity in mice. Eur. J. Pharmacol. 492, 21–26.

McCann, D.J., Weissman, A.D., Su, T.P., 1994. Sigma-1 and sigma-2 sites in rat brain: comparison of regional ontogenetic and subcellular patterns. Synapse 17, 182–189.

McGonigle, P., Molinoff, P.B., 1989. Quantitative aspects of drug–receptor interactions. In: Siegel, Agranoff, Albers, Molinoff (Eds.), Basic Neurochemistry. Raven Press, New York.

Mei, J., Pasternak, G.W., 2001. Molecular cloning and pharmacological characterization of the rat sigma1 receptor. Biochem. Pharmacol. 62, 349–355.

Monnet, F.P., Mahe, V., Robel, P., Baulieu, E.-E., 1995. Neurosteroids, via σ receptors, modulate the [^{3}H]norepinephrine release evoked by *N*-methyl-D-aspartate in the rat hippocampus. Proc. Natl. Acad. Sci. U. S. A. 92, 3774–3778.

Scatchard, G., 1949. The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. 51, 662–672.

Seth, P., Leibach, F.H., Ganapathy, V., 1997. Cloning and structural analysis of the cDNA and the gene encoding the murine type 1 sigma receptor. Biochem. Biophys. Res. Commun. 241, 535–540.

Su, T.P., Hayashi, T., 2003. Understanding the molecular mechanism of sigma-1 receptors: towards a hypothesis that sigma-1 receptors are intracellular amplifiers for signal transduction. Curr. Med. Chem. 10, 2073–2080.

Su, T.P., London, E.D., Jaffe, J.H., 1988. Steroid binding at sigma receptors suggests a link between endocrine, neurotransmitter and immune systems. Science 240, 219–221.

Takebayashi, M., Hayashi, T., Su, T.P., 2002. Nerve growth factor-induced neurite sprouting in PC12 cells involves sigma-receptors: implications for antidepressants. J Pharmacol. Exp. Ther. 303, 1227–1237.

Tu, Z., Dence, C.S., Ponde, D.E., Jones, L.A., Wheeler, K.T., Welch, M.J., Mach, R.H., 2005. Carbon-11 labeled σ_2 receptor ligands for imaging breast cancer. Nucl. Med. Biol. 32, 423–430.

Vilner, B.J., John, C.S., Bowen, W.D., 1995. Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines. Cancer Res. 55, 408–413.

Walker, J.M., Bowen, W.D., Walker, F.O., Matsumoto, R.R., de Costa, B.R., Rice, K.C., 1990. Sigma receptors: biology and function. Pharmacol. Rev. 42, 355–402.

Wang, L.M., Bowen, W.D., Mach, R.H., Wheeler, K.T., Morton, K.A., 2004. Subcellular localization of sigma-1β receptor protein in CHO cells. Proc. Am. Assoc. Cancer Res. 262.

Wheeler, K.T., Wang, L.M., Wallen, C.A., et al., 2000. Sigma-2 receptors as a biomarker of proliferation in solid tumors. Br. J. Cancer 82, 1223–1232.