European Journal of Pharmacology 653 (2011) 1–7

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

Synthesis and characterization of [^{3}H]-SN56, a novel radioligand for the σ_1 receptor

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ARTICLE INFO

Article history:
Received 22 May 2010
Received in revised form 30 September 2010
Accepted 6 October 2010
Available online 2 December 2010

Keywords:
Radioligand binding assay
σ Receptor

ABSTRACT

The study of the binding characteristics of σ ligands *in vivo* and *in vitro* requires radiolabeled probes with high affinity and selectivity. The radioligand presently used for *in vitro* studies of the σ_1 receptor, [^{3}H](+)-pentazocine, has significant limitations; it is difficult to synthesize, has limited chemical stability, and can be problematic to obtain. Evaluation of a series of novel 2(3H)-benzothiazolone compounds revealed SN56 to have sub-nanomolar and preferential affinity for the σ_1 subtype, relative to σ_2 and non-sigma, binding sites. The goal of this study was to characterize the binding of [^{3}H]-SN56 to σ_1 receptors isolated from rat brain. Standard *in vitro* binding techniques were utilized to 1) determine the specificity and affinity of binding to σ_1 receptors, 2) confirm that [^{3}H]-SN56 labels sites previously identified as σ_1 by comparing binding to sites labeled by [^{3}H](+)-pentazocine, and 3) characterize the kinetics of binding. The results indicate that [^{3}H]-SN56 exhibits 1) specific, saturable, and reversible binding to the σ_1 receptor, with $B_{max} = 340 \pm 10$ fmol/mg and $K_d = 0.069 \pm 0.0074$ nM, 2) competitive displacement by classical sigma compounds, yielding σ_1 K_i values consistent with those reported in the literature, and 3) binding kinetics compatible with a 90 min incubation, and filtration for separation of free and bound radioligand. The results of these studies suggest that [^{3}H]-SN56 may serve as a viable alternative to [^{3}H](+)-pentazocine in radioligand binding assays.

1. Introduction

The σ receptor was first identified as an opioid receptor subtype based on behavioral studies of morphine-like drugs in dogs (Martin et al., 1976). Subsequent *in vitro* binding data revealed that this site represents a new class of non-opioid receptor (Tam, 1983). To date, two subtypes of σ receptors (σ_1 and σ_2) have been identified based on differences in ligand selectivity, tissue distribution, and molecular characterization. The σ_1 receptor has been cloned from multiple species (Hanner et al., 1996; Kekuda et al., 1996; Mei and Pasternak, 2001; Pan et al., 1998; Seth et al., 1997, 1998) and a significant number of ligands with high affinity and selectivity for it are available. The σ_2 receptor is less well characterized; it has not been cloned, and few specific ligands have been described.

The σ_1 receptor is involved in numerous physiological processes and disease states, and *in vivo* and *in vitro* studies indicate that modulation of σ_1 receptors using σ specific ligands can affect these systems (Cobos et al., 2008; Guitart et al., 2004; Hashimoto and Ishiwata, 2006; Maurice and Su, 2009). Consequently, the σ_1 receptor is recognized as a potential medication development target and efforts to identify novel selective compounds are ongoing. While the σ_2 receptor may also represent a feasible drug development target, further research in this area will require the discovery of additional selective ligands for this subtype. The focus of the current work is therefore limited to the characterization of σ_1 binding.

DeCosta et al. (1989) first described [^{3}H](+)-pentazocine, a highly selective radioprobe for σ_1 receptors. Subsequent studies demonstrated that [^{3}H](+)-pentazocine labeled a single class of sites in guinea-pig brain that correlated with the profile observed following labeling with the prototypic σ_1 probe [^{3}H](+)-3-PPP (Bowen et al., 1993). [^{3}H](+)-Pentazocine exhibited low levels of non-specific binding and high affinity for σ_1 receptors ($K_d = 4.8 \pm 0.4$ nM), with >700 fold preference for the σ_1 over the σ_2 subtype (Bowen et al., 1993). [^{3}H](+)-Pentazocine does however exhibit shortcomings, including poor chemical stability, that limit its usefulness in routine studies.

Efforts to design new σ_1 specific ligands have produced a limited number of radioprobes useful for exploring the pharmacology of the σ_1 receptor. Unfortunately, like [^{3}H](+)-pentazocine, each of the proposed new radioprobes exhibit limtations. Therefore, we sought to characterize the performance of SN56, a novel σ_1 selective, 2(3H)-benzothiazolone compound, as a tritiated radioligand for use in σ_1 competition binding experiments. SN56 exhibited sub-nanomolar affinity ($K_{i\sigma 1} = 0.56$ nM) and >1000 fold selectivity for the σ_1 subtype relative to σ_2 and at least 350 times greater affinity for the σ_1 receptor

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doi:10.1016/j.ejphar.2010.10.099

versus a battery of common receptors and transporters (Yous et al., 2005). These binding characteristics coupled with a simple and economical synthetic scheme suggested [^{3}H]-SN56 may provide a viable alternative to [^{3}H](+)-pentazocine in competition binding studies of the σ_1 receptor.

2. Materials and methods

2.1. Materials

Reagents and starting materials for the synthesis of SN56 were obtained from commercial suppliers and were used without purification. Precoated silica gel GF Uniplates from Analtech (Newark, DE) were used for thin-layer chromatography (TLC). Column chromatography was performed on silica gel 60 (Sorbent Technologies, Atlanta, GA). ^{1}H and ^{13}C NMR spectra were obtained on a Bruker APX400 (Billerica, MA) at 400 and 100 MHz, respectively. High resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-Tof Micro mass spectrometer with a lock spray source (Milford, MA). Mass spectra (MS) were recorded on a Waters Acquity Ultra Performance LC (Milford, MA) with ZQ detector in ESI mode. Elemental analysis (C, H, N) was performed on a Perkin-Elmer CHN/SO Series II Analyzer (Waltham, MA). Chemical names were generated using ChemDraw Ultra (CambridgeSoft, version 10.0, Cambridge, MA).

[^{3}H](+)-Pentazocine (29 Ci/mmol) was purchased from Perkin-Elmer (Boston, MS). (+)-Pentazocine, (−)-pentazocine, haloperidol, 1,3-di-o-tolylguanidine (DTG), bovine serum albumin (BSA) fraction V, sucrose, sodium chloride, tris(hydroxymethyl)aminomethane, 1N hydrochloric acid solution, and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO). Bio–rad Protein Assay reagent was purchased from Bio–rad (Hercules, CA). Ecoscint scintillation fluid and Brandel GF/B filter papers, 2.25×12.25 in. were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Synthesis of [^{3}H]-SN56

The design strategy for generating [^{3}H]-SN56 involved replacing a bromine atom on the aromatic ring of SN56 with a tritium atom (Fig. 1). The preparation of the brominated precursor 4 is described below. Compounds 1 and 2 were prepared according to previously described procedures with minor modifications (Ucar et al., 1998; Yous et al., 1994). Selective bromination of the 6-propylbenzo[d]thiazol-2(3H)-one 2 at the C-4 position was effected with bromine in acetic acid at room temperature. The bromo derivative was alkylated with 2-(hexamethyleneimino)ethylchloride in the presence of potassium carbonate in DMF to yield 4. Compound 4 was radiolabeled with tritium (30 Ci/mmol) by AmBios Labs, Inc. (Newington, CT).

2.2.1. 6-Propionylbenzo[d]thiazol-2(3H)-one (1)

Dimethylformamide (5.96 ml, 76.73 mmol) was added slowly to aluminium chloride (35.5 g, 264.6 mmol) with vigorous stirring. After 15 min, 2-hydroxybenzothiazole (5.4 g, 40 mmol) was added and the mixture was heated to 45 °C. After 15 min, propionyl chloride (3.46 ml, 39.7 mmol) was added and the reaction mixture was heated to 85 °C for 3 h. The hot mixture was then poured on ice; the crude product was collected by filtration, and washed with water. The solid was dissolved in ethyl acetate and the solvent was washed with water and brine, dried and evaporated. The residue was recrystallized from toluene/dioxane (2/1) to give 2.96 g (54%) of 6-propionylbenzo[d]thiazol-2(3H)-one as a white solid. ^{1}H NMR (DMSO-d_6): δ 12.23 (br s, 1H), 8.20 (s, 1H), 7.87 (d, J=8.4 Hz, 1H), 7.17 (d, J=8.3 Hz, 1H), 2.97 (q, J=7.1 Hz, 2H), 1.06 (t, J=7.1 Hz, 3H). ^{13}C NMR (DMSO-d_6): δ 198.64, 170.46, 140.13, 131.21, 126.74, 123.69, 123.04, 111.17, 30.93, 8.22. MS (ESI) m/z 206 (M^+-1).

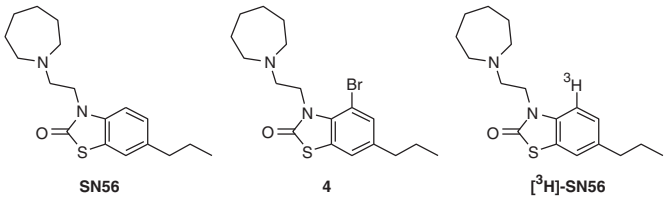

Fig. 1. Structure of SN56, [^{3}H]-SN56 and its bromo precursor 4.

2.2.2. 6-Propylbenzo[d]thiazol-2(3H)-one (2)

Triethylsilane (4.75 ml, 29.75 mmol) was added to a stirred solution of 1 (2.5 g, 12.06 mmol) in trifluoroacetic acid (13 ml). The mixture was stirred vigorously for 2 h at room temperature. The trifluoroacetic acid was removed by evaporation and the residue was purified by chromatography on a silica gel column using petroleum ether/ethyl acetate (9:1) as the eluent to give 2.06 g (88%) of 6-propylbenzo[d]thiazol-2(3H)-one as a white solid. ^{1}H NMR (DMSO-d_6): δ 11.76 (br s, 1H), 7.33 (s, 1H), 7.05 (d, J=8.1 Hz, 1H), 6.99 (d, J=8.1 Hz, 1H), 2.50 (t, J=7.4 Hz, 2H), 1.56–1.50 (m, 2H), 0.84 (t, J=7.3 Hz, 3H). ^{13}C NMR (DMSO-d_6): δ 169.99, 136.68, 134.23, 126.57, 123.24, 122.12, 111.22, 36.87, 24.30, 13.54. MS (ESI) m/z 194 (M^++1).

2.2.3. 4-Bromo-6-propylbenzo[d]thiazol-2(3H)-one (3)

Bromine (0.45 ml, 8.75 mmol) was added slowly to a solution of 2 (1.5 g, 7.76 mmol) in acetic acid (10 ml). The mixture was stirred 15 h at room temperature, poured into water and extracted with ethyl acetate (3×30 ml). The combined organic layers were washed with a 10% solution of potassium carbonate followed by brine. The solution was dried over sodium sulfate and evaporated under vacuum. The residue was purified by chromatography on a silica gel column using petroleum ether/ethyl acetate (9:1) as the eluent to give 0.5 g (24%) of 4-bromo-6-propylbenzo[d]thiazol-2(3 H)-one as a white solid. ^{1}H NMR ($CDCl_3$): δ 9.29 (s, 1H), 7.26 (s, 1H), 7.12 (s, 1H), 2.56 (t, J=7.6 Hz, 2H), 1.62 (m, 2H), 0.93 (t, J=7.2 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 170.45, 139.35, 132.22, 129.34, 124.44, 121.24, 103.90, 37.37, 24.48, 13.54. MS (ESI) m/z 270 (M^+−1), 272 (M^++1).

2.2.4. 3-(2-(Azepan-1-yl)ethyl)-4-bromo-6-propylbenzo[d]thiazol-2(3H)-one hydrochloride (4)

$NaHCO_3$ (0.51 g, 6.09 mmol) and 2-(hexamethyleneimino) ethylchloride hydrochloride (0.80 g, 4.06 mmol) were added, with mechanical stirring, to a solution of 3 (0.55 g, 2.03 mmol) in anhydrous DMF (15 ml). The reaction mixture was heated to 80 °C for 1 h. After cooling, the mixture was poured into 80 ml of water, extracted with ethyl acetate (3×60 ml), and the combined organic layers were washed with brine and dried. The solvent was removed in vacuo, and the residue was chromatographed on a silica gel column using ethyl acetate/petroleum ether (4:6) as the eluent. 3-(2-(azepan-1-yl)ethyl)-4-bromo-6-propylbenzo[d]thiazol-2(3H)-one was isolated as a hydrochloride salt (white solid, 0.44 g, 49%) by addition of HCl/dioxane. ^{1}H NMR (DMSO-d_6): δ 10.81 (br s, 1H), 7.60 (s, 1H), 7.46 (s, 1H), 4.72 (t, J=7.2 Hz, 2H), 3.47–3.24 (m, 6H), 2.56 (t, J=7.5 Hz, 2H), 1.86 (br s, 4H), 1.67–1.55 (m, 6H), 0.88 (t, J=7.2 Hz, 3H). ^{13}C NMR (DMSO-d_6): δ 170.54, 138.87, 132.87, 132.63, 125.62, 121.79, 103.79, 56.73, 55.89, 43.14, 37.04, 28.58, 27.22, 24.51, 13.81. Anal. calcd for $C_{18}H_{26}$ $BrClN_2OS$: C, 49.83; H, 6.04; N, 6.46. Found: C, 50.06; H, 5.93; N, 6.47. HRMS calcd for $C_{18}H_{26}N_2$-OSBr $[M+H]^+$ 397.0949, found 397.0945.

A synopsis of the synthetic route of the synthesis of the brominated [^{3}H]-SN56 precursor is provided in Fig. 2.

2.3. Membrane preparation

Crude P_2 rat brain homogenates were prepared from male, Sprague Dawley rats (150–200 g) purchased from Harlan (Indianapolis, IN) as described previously (Matsumoto et al., 1995). All procedures involving

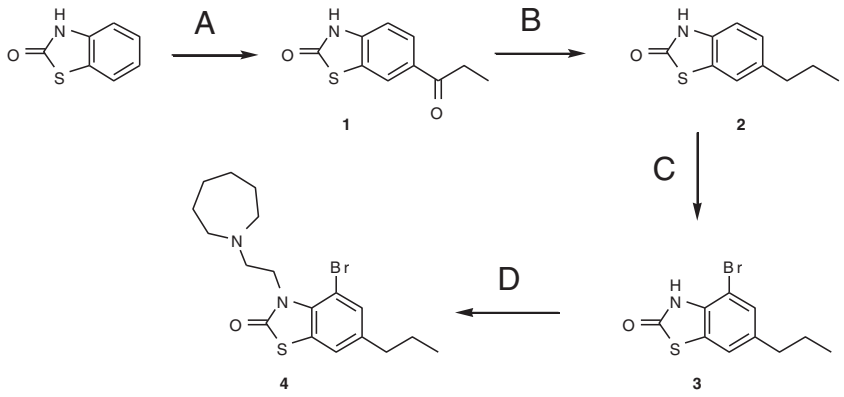

Fig. 2. Synthesis of brominated [^{3}H]-SN56 precursor 4: A) propionyl chloride, $AlCl_3$, 85 °C; B) $(C_2H_5)_3SiH$, CF_3COOH, rt; C) Br_2, CH_3COOH, rt; d) 2-(hexamethyleneimino)ethylchloride hydrochloride, K_2CO_3, DMF, 80 °C.

live animals were performed as approved by the Institutional Animal Care and Use Committee at the locations where the assays were performed. Briefly, unanesthetized rats were sacrificed by decapitation; brains minus cerebellum were harvested and maintained in ice cold 10 mM Tris–HCl/0.9% NaCl until processed. Tissues were homogenized with a Potter-Elvehjem homogenizer (5–10 strokes with motor driven Teflon pestle) in ice-cold 10 mM Tris–sucrose buffer (0.32 M sucrose in 10 mM Tris–HCl, pH 7.4) using 10 ml buffer per gram of tissue × ~3 g tissue/batch. Homogenates from multiple batches were combined and centrifuged for 10 min at 1000 × *g*, at 4 °C. Supernatants were decanted, combined and centrifuged for 15 min at 31,000 × *g*, at 4 °C. To reduce levels of bound endogenous ligand(s), the material from centrifugation at 31,000 × *g* was washed as follows: 1) pellets were re-suspended in 10 mM Tris–HCl, pH 7.4 using 3 ml buffer per gram of tissue, 2) the resulting suspension was incubated for 30 min at 25 °C, 3) following incubation, the suspension was centrifuged for 15 min at 31,000 × *g*, at 4 °C. The resulting pellets were re-suspended in Tris–HCl, pH 7.4 buffer at a final concentration of 1 g of tissue per 1.53 ml buffer. Tissue preparations were aliquoted in 1 ml portions and stored at −80 °C. The Bradford assay was used to quantitate protein concentration (Bradford, 1976).

2.4. Radioligand binding assays

Initial optimization of assay conditions was performed to maximize total binding and minimize non-specific binding. Parameters examined included evaluation of the buffer composition and pH, ratio of radioligand to membrane concentration, and determination of ligand and ligand concentration for defining non-specific binding. The following optimized conditions were used for subsequent studies reported below: 0.5 ml final sample volume, 90 min sample incubation at 25 °C, Tris–HCl pH 8.0 (assay buffer), and 10 μM haloperidol (to define non-specific binding). Assay termination was effected by vacuum filtration through glass fiber filters on a 24 position Brandel cell harvester. Prior to use, filters were presoaked for 30 min in 0.5% polyethyleneimine to reduce non-specific binding. Following the initial filtration step, filters were washed in triplicate with 5 ml ice-cold 10 mM Tris–HCl, pH 8. The conditions determined from the preliminary studies were consistent with those reported in the literature for the analysis of σ_1 receptor binding using [^{3}H](+)–pentazocine (Bowen et al., 1993; Hellewell et al., 1994; Matsumoto et al., 1996). It should be noted that it was necessary to prepare [^{3}H]-SN56 spiking solutions in 1 mM HCl, to prevent non-specific binding of the radioligand to glass and plasticware, which was problematic with solutions prepared in the assay buffer. This requirement is not unprecedented and the small amount of acid has no impact on the final pH of the assay sample (Bylund and Toews, 1993).

2.4.1. Association and dissociation assays

Association and dissociation studies were conducted to confirm that the binding kinetics of [^{3}H]-SN56 were appropriate for a 1–2 h incubation and processing by filtration. Kinetic studies were performed with 0.8 nM [^{3}H]-SN56 and 100 μg membrane. For determination of association rates, samples were incubated for times ranging from 5 min to 2 h prior to filtration. For determination of dissociation rates, membranes were incubated for 120 min with [^{3}H]-SN56 prior to the addition of 100 μM (final concentration) haloperidol, followed by filtration at times ranging from 30 min to 4 h from the addition of haloperidol. The assays were performed in duplicate and repeated three times.

2.4.2. Saturation binding assays

For the determination of K_d and B_{max} by saturation binding, ten concentrations ranging from 0.01 to 0.8 nM of [^{3}H]-SN56 were tested per experiment with 100 μg membrane per sample. Non-specific binding was determined by the addition of haloperidol, at a 100 μM final concentration. Samples for the determination of total and non-specific binding for each experiment were run concurrently and filtered simultaneously.

2.4.3. Competition binding assays

For the determination of K_i for established σ ligands by competition binding, aliquots of membrane were incubated with [^{3}H]-SN56 and varying concentrations of test ligands. The following test compounds were evaluated: DTG, haloperidol, (+)-pentazocine, (−)-pentazocine. For each test compound, 10 concentrations were incubated with 0.7–0.8 nM [^{3}H]-SN56 with 100 μg membrane per sample. Non-specific binding was determined by the addition of haloperidol, 100 μM final concentration. Samples for the determination of total and non-specific binding for each experiment were run concurrently and filtered simultaneously.

2.5. Scintillation counting and data analysis

Following washing, filters were transferred to scintillation vials and 5 ml scintillation cocktail was added. Filters were allowed to soak in cocktail for a minimum of 10 h prior to counting.

The data were analyzed using GraphPad Prism software (San Diego, CA). Saturation binding data were fit using nonlinear regression to a one site model. Association kinetics data were fit using linear regression of the plot of $\ln(B_e - B/B_e)$ versus time, where B_e = radioligand bound at equilibrium, and B = radioligand bound at time t; the slope of the plot yielded k_{obs}. The association rate constant (k_{+1}) was calculated using the pseudo first-order method from the equation $k_{+1} = (k_{obs} - k_{-1})/[L]$, where [L] = radioligand concentration. Dissociation kinetics data were fit using linear regression of the

plot of $\ln(B/B_0)$ versus time, where B_0 = specific radioligand bound at time of addition of haloperidol, and B = specific radioligand bound at time t. For competition binding data, K_i values were calculated from experimentally determined IC_{50} values using the Cheng–Prusoff equation using the K_d for [^{3}H]-SN56 determined from the saturation binding experiments (0.069 nM).

3. Results

3.1. Basic binding parameters

At near saturating conditions, non-specific binding of [^{3}H]-SN56 remained constant in the presence of 25 to 200 μg membrane, suggesting that the observed non-specific binding is due primarily to radioligand binding to the filter. Total binding was linear from 50 to 200 μg membrane (Fig. 3).

3.2. Association and dissociation kinetics

From the association studies, $k_{obs} = 0.080\ min^{-1}$ and $k_{+1} = 9.05 \times 10^7\ min^{-1}\ M^{-1}$. From the dissociation studies, $k_{-1} = 0.0076\ min^{-1}$ and $t^{1/2} = 91$ min. The K_d calculated from k_{-1}/k_{+1} was 0.084 nM. The low dissociation rate permits the use of filtration for the separation of free from bound radioligand (Bylund et al., 2004), while the association rate supports 90 min incubations for attaining steady-state binding.

3.3. Saturation binding

Fig. 4 shows the results of the saturation binding study of [^{3}H]-SN56. The binding affinity of [^{3}H]-SN56 was $K_d = 0.069 \pm 0.007$ nM which represents a 70 fold higher affinity than reported for [^{3}H](+)-pentazocine ($K_d = 4.8$ nM) (Bowen et al., 1993). Receptor density (B_{max}) as determined by saturation binding with [^{3}H]-SN56 was 340 ± 10 fmol/mg.

3.4. Competition binding

Tabulated values of K_is determined in this study using [^{3}H]-SN56 versus values reported by Bowen et al. (1993) using [^{3}H](+)-pentazocine are shown in Table 1. In Fig. 5, a comparison of binding profiles of the sites labeled by [^{3}H]-SN56 versus [^{3}H](+)-pentazocine is shown in a correlation plot of K_i values obtained experimentally versus values reported by Bowen et al. (1993). For the group of hallmark σ ligands tested, there was a significant correlation between the affinities obtained using the novel versus conventional radioligand. Of particular note is the higher affinity of (+)-pentazocine as compared to (−)-pentazocine in the assays, a stereoselectivity pattern that is consistent with binding to σ_1 receptors.

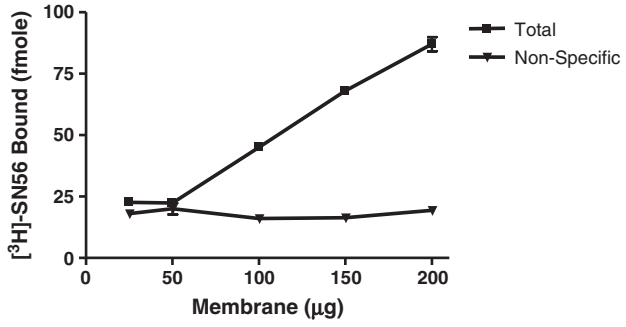

Fig. 3. Total and non-specific binding of [^{3}H]-SN56 to rat brain membranes. Samples contained 0.7 nM [^{3}H]-SN56, in a total volume of 0.5 ml. Data points represent the mean of three independent determinations of duplicate samples at each concentration.

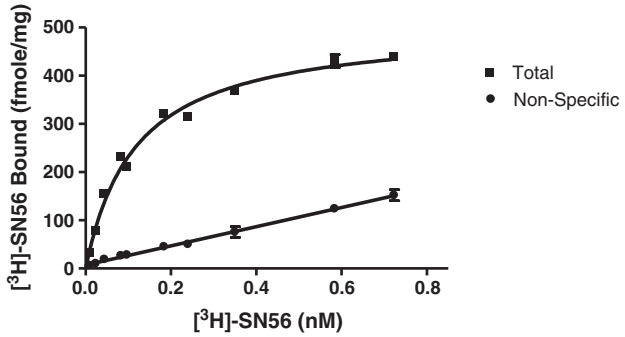

Fig. 4. Saturation curve for [^{3}H]-SN56 in rat brain membranes. Samples contained 100 μg membrane in a total volume of 0.5 ml. Data points represent the mean ± SEM of three independent determinations of duplicate samples at each concentration. $B_{max} = 340 \pm 10$ fmol/mg, $K_d = 0.069 \pm 0.007$ nM and $r^2 = 0.96$.

4. Discussion

Characterization of a series of novel 2(3H)-benzothiazolone compounds in σ receptor competition binding assays revealed SN56 (3-(2-(azepan-1-yl)ethyl)-6-propylbenzo[*d*]thiazol-2(3*H*)-one) to have sub-nanomolar affinity and >1000 fold selectivity for the σ_1 subtype relative to σ_2 (Yous et al., 2005). Binding of SN56 to non-σ binding sites was tested with a battery of receptors and transporters including, adrenergic α_1, adrenergic α_2, adrenergic β_1, adrenergic β_2, histamine H_1, histamine H_2, mu opioid, delta opioid, kappa opioid, dopamine D_1, dopamine D_2, serotonin $5HT_{2a}$, serotonin $5HT_3$, $GABA_A$, dopamine transporter, and serotonin transporters. Of the binding sites tested, only α_2 and H_1 showed affinities greater than 1 μM ($K_{i\alpha2} = 205$ nM, and $K_{iH1} = 311$ nM respectively) (Yous et al., 2005); however, the affinity of SN56 for the σ_1 receptor is approximately 350 times higher than its affinity for either of these receptors, indicating a favorable selectivity profile for the development of a radioprobe for use in radioligand binding studies.

In the present study, [^{3}H]-SN56 exhibited >95% specific binding to σ_1 in rat brain membranes at concentrations up to 10 times the K_d. However, non-specific binding of [^{3}H]-SN56 to the glass fiber filters used to separate bound from free radioligand was 25–35% at 10 times the K_d concentration, resulting in a final specific binding signal of 65–75% of total observed binding. [^{3}H]-SN56 exhibited saturable and reversible binding to a single high affinity site in rat membranes with a binding profile similar to that observed for [^{3}H](+)-pentazocine. The B_{max} observed for [^{3}H]-SN56 (340 ± 10 fmol/mg) was consistent with the range of values reported in the literature for rat brain labeled with [^{3}H]-BHDP, [^{3}H]-SA4503, or [^{3}H](+)-pentazocine (Ishiwata et al., 2003; Klouz et al., 2003). In addition, the K_i values of classical σ compounds in competition binding assays against [^{3}H]-SN56 were consistent with those reported in the literature against the well established σ_1 radioligand [^{3}H](+)-pentazocine (Bowen et al., 1993). The association and dissociation kinetics of [^{3}H]-SN56 were also shown to be amenable for filtration assays.

Yous et al. (2005) reported a K_i of 0.56 nM for SN56 versus [^{3}H](+)-pentazocine in P_2 membranes prepared from guinea pig brain. Similarly, we obtained a K_i of 0.38 nM versus [^{3}H](+)-pentazocine in P_2

Table 1
Summary of data from competition binding experiments.

Compound	K_i (nM)	
	Experimental	Literature[a]
DTG	41.6 ± 8.8	74.3 ± 14.9
Haloperidol	3.48 ± 1.05	1.9 ± 0.3
(+)-Pentazocine	5.70 ± 1.04	6.7 ± 1.2
(−6)-Pentazocine	77.0 ± 9.0	44.0 ± 1.2

[a] Bowen et al., 1993.

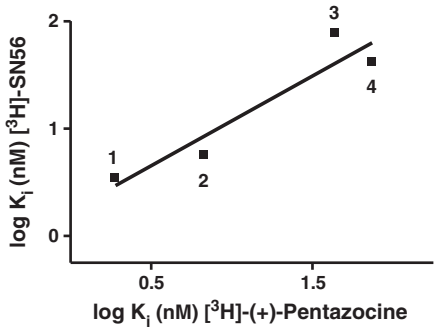

Fig. 5. Comparison of K_i values determined experimentally with [^{3}H]-SN56 versus values determined with [^{3}H](+)-pentazocine by Bowen et al. (1993). $r^2 = 0.89$. 1) 1haloperidol, 2) (+)-pentazocine, 3) (−)-pentazocine, 4) DTG.

membranes prepared from rat brain (data not shown). These values are ~5 fold higher than the affinity determined with saturation and kinetic studies of [^{3}H]-SN56 (~0.07 nM and ~0.08 nM respectively). We suspect that depletion of the non-labeled ligand results in an erroneously high value for the K_i of SN56 as determined by competition binding.

Systematic errors resulting from the use of high receptor concentrations may also contribute to errors in both the determination of K_d from saturation and kinetic experiments, and in the determination of K_is of un-labeled compounds. However, these errors are minimal, quantifiable, and in practice when [^{3}H]-SN56 is used for competition binding assays of σ_1 ligands, our preliminary results suggest they have no impact on our K_i determinations as compared to historical data for the compounds tested.

While most researchers are familiar with radioligand depletion due to excessive receptor concentration, depletion of the un-labeled ligand occurs when the affinity of the un-labeled compound greatly exceeds the affinity of the labeled compound (Chang et al., 1975; Goldstein and Barrett, 1987). Goldstein and Barrett (1987) used computer modeling to derive estimates of the error in the determination of K_i of ligands exhibiting higher affinities than the radioligands utilized in their measurement; the authors projected that for an un-labeled ligand with a true affinity 100 times greater than the radioligand (as in our case), 10% radioligand depletion would result in an experimentally determined K_d ~6 times higher than the true value. Thus, the ~5 fold difference between the affinity of SN56, as determined by competition binding, and the affinity of [^{3}H]-SN56 determined with saturation and kinetic studies may be explained by this phenomena.

Practical considerations dictated that we use relatively high receptor concentrations; this introduces systematic error that is quantifiable and within acceptable limits. When possible, experimental conditions for binding experiments should be chosen so that the receptor concentration is less than 10% of the K_d of the radioligand to minimize radioligand depletion (Chang et al., 1975). However, with a radioligand with sub-nanomolar affinity this would require multi-milliliter sample volumes. Our experiments required 100 μg of tissue to obtain adequate signal for precise detection. We chose 0.5 ml sample volumes because we intend to adapt this method to a higher throughput 96-well method where sample volumes are more limited than in test tube based binding determinations. To ensure that >90% of added radioligand was "free" (unbound) in competition binding experiments run under these conditions, we utilized high concentrations of [^{3}H]-SN56. While these conditions are not ideal, they are tolerated if required for detection and the error in values obtained with the method are known and within an acceptable range (Carter et al., 2007).

Carter et al. (2007) examined the effects of assay miniaturization using the human muscarinic M3 receptor expressed in CHO cells. This cell line expressed receptor at ~5 pmol/mg, and the novel radioligand tested, [^{3}H]-NMS (1-[N-methyl-^{3}H] scopolamine methyl chloride) had an affinity of 0.42 pM. In saturation binding studies, receptor concentrations were varied over a wide range, with membrane amounts from 5–50 μg in volumes from 50–1750 μl, and conditions where the ratio of receptor concentration ([receptor]) to K_d were from 0.42–~147. As the volume was decreased or the amount of membrane was increased in these studies, the calculated K_d and B_{max} increased. When no corrections were made for radioligand depletion, with [receptor]/K_d = 0.42 (the lowest ratio tested, corresponding to 5 μg in 1750 μl) the calculated K_d was approximately 2.3 times the "true" value, and with [receptor]/K_d = 1.25 the calculated K_d was approximately 2.8 times the true value. When the K_ds were recalculated, taking into account radioligand depletion, the resulting values were 2.1 and 3.9 times the true value, respectively.

The conditions chosen for our saturation studies utilized a [receptor]/K_d of ~1. No corrections were made for radioligand depletion because it was not possible to accurately assess what portion of non-specific binding was due to binding to membrane versus binding to filter. Based on Carter's work, we might expect our results to overestimate the K_d of [^{3}H]-SN56 by 2 to 3 fold.

Carter's studies for competition binding did not model the approach we utilized, with [receptor] = ~$K_{d[3H]\text{-}SN56}$ and concentration [^{3}H]-SN56 = ~10 times $K_{d[3H]\text{-}SN56}$, so useful comparisons to their data are not possible. However, as stated previously, the K_i values we derived for the σ ligands tested correlated well with values reported in the literature.

No significant difference in binding affinities was observed following labeling with [^{3}H](+)-pentazocine versus [^{3}H]-SN56 for the ligands tested. A larger pool of compounds will need to be screened to confirm that this relationship is maintained. However, compounds that show significant differences in affinity following labeling with the two different radioligands may provide valuable insight into differences in the binding of σ ligands from different chemical classes.

[^{3}H]-SN56 appears to provide similar binding characteristics compared to [^{3}H](+)-pentazocine with some noteworthy advantages. Synthetically, [^{3}H]-SN56 can be produced more easily and in significantly higher yields than [^{3}H](+)-pentazocine. The chemical stability of [^{3}H]-SN56 is also expected to be greater than that of [^{3}H](+)-pentazocine, which degrades over time, resulting in increased background levels.

A number of radiolabeled σ_1 ligands have been reported in the literature but none have been widely accepted as a replacement for [^{3}H](+)-pentazocine in competition binding studies. The two best candidates, SA4503 (Matsuno et al., 1996) and BHDP (Klouz et al., 2003), have been studied in rat brain membranes in tritiated and un-labeled forms and exhibit approximately 100 fold higher affinity for σ_1 versus σ_2 with no significant binding to other common receptors or transporters (Klouz et al., 2003; Matsuno et al., 1996). However, differences in relative expression of σ_1 versus σ_2 in disease states, tissues, or cell lines may compromise the accurate analysis of σ_1 binding with these radioligands since their σ_1/σ_2 selectivity ratios just meet the 100 fold difference generally accepted as the minimum difference required for discriminating receptor subtypes. Because [^{3}H]-SN56 displays a >1000 fold higher affinity for σ_1 versus σ_2, changes in σ expression would not impact measurements performed with this radioligand as much as with the other proposed alternatives.

In vitro binding studies with receptor specific radioligands have historically been important in the discovery and characterization of receptors, and continue to play a central role in the process of drug discovery. Application of this technology to the σ receptor has resulted in: 1) identification of σ receptors as a unique receptor type (Su, 1982; Tam, 1983), 2) confirmation that σ receptors have at least two subtypes (Bowen et al., 1989; Hellewell and Bowen, 1990), and 3) determination of its anatomical distribution (Hellewell et al., 1994; Jansen et al., 1991a,b; Walker et al., 1992). Radioligand binding studies continue to play a primary role in selecting σ ligands for *in vivo* testing because there are no widely accepted *in vitro* functional assays for σ_1 activation.

In addition to its service in the initial characterization of the σ receptor, radioligand binding data has contributed to the elucidation of 1) structural elements that define high affinity σ_1 ligands, and 2) specific amino acids of the σ_1 protein that are critical for selective, high affinity binding of known ligands (Ablordeppey et al., 2000, 2002; Glennon, 2005; Seth et al., 2001; Yamamoto et al., 1999). Studies correlating structure with binding affinity are ongoing; numerous examples of such labors have been reported in the literature, with the majority of effort focused on identifying subtype specific ligands (de Costa et al., 1992; Holl et al., 2009; Mesangeau et al., 2008; Zampieri et al., 2009).

σ_1 Radioligands have also demonstrated utility in radio-imaging studies; several examples of the successful use of the σ_1 radioligand [^{11}C-SA4503] as a positron emission tomography (PET) imaging agent have been reported in recent years (Ishikawa et al., 2007; Rybczynska et al., 2009; Toyohara et al., 2009). Displacement of [^{11}C]-SA4503 by the σ_1 active antidepressant fluvoxamine confirmed that fluvoxamine occupies σ_1 receptors in healthy human male volunteers (Ishikawa et al., 2007). Other studies showed reduced σ_1 receptor density in defined brain regions of Alzheimer's and Parkinson's patients (Toyohara et al., 2009). Also, supporting a role for σ_1 in cancer, Rybczynska et al. (2009) reported that steroid hormones that are known σ_1 ligands displace [^{11}C]-SA4503 from tumors in rats (Rybczynska et al., 2009). Thus, these studies suggest σ_1 radioligands may hold significant diagnostic value for psychiatric diseases and cancer.

Future advances in the field of σ receptor therapeutic development will require greater knowledge of the nature of the interaction of the σ receptor with its ligands and protein binding partners. Additional tools needed to further this knowledge include new subtype specific agonist and antagonist ligands, radioligands, and other affinity labels and probes. The development of other technologies, such as high throughput methods for the determination of binding affinities, and *in vitro* functional assays, will also hasten efforts to design and identify new selective σ ligands with potential therapeutic value.

In conclusion, the results of our studies suggest that [^{3}H]-SN56 possesses high affinity and selectivity for the σ_1 receptor, and appears to be a viable alternative for [^{3}H](+)-pentazocine in radioligand binding assays. Further, because [^{3}H]-SN56 has a >70 fold higher affinity for the σ_1 receptor than [^{3}H](+)-pentazocine, competition binding studies require less radioligand and membrane, resulting in significant efficiencies in resources when performing the assays. Thus, [^{3}H]-SN56 represents another valuable tool for the study of the σ_1 receptor and the development of σ_1 based therapeutics.

Acknowledgements

This study was supported by grants from the National Institute on Drug Abuse (DA023205 and DA013978).

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