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Short communication

A 96-well filtration method for radioligand binding analysis of σ receptor ligands

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ABSTRACT

σ receptors represent a potential drug target for numerous therapeutic indications including cancer, depression, psychostimulant abuse, and stroke. Most published radioligand binding studies for σ receptors utilize a low throughput method employing a "cell harvester." Higher throughput methods are required to facilitate efficient screening of large numbers of novel compounds. In this study, a series of reference compounds was analyzed with a new medium-throughput 96-well filtration method and the results were compared to those obtained using the conventional cell harvester-based method. The 96-well assay utilized rat liver membranes for the determination of both known σ receptor subtypes (σ_1 and σ_2) because this tissue contains high densities of both subtypes and fulfills criteria required for reliable use with the 96-well format. The new method gave comparable K_i values for reference ligands analyzed in parallel with samples prepared in rat brain membranes and processed on the traditional cell harvester. For σ_1 receptors, equivalent affinity values were observed for both methods/tissues. For σ_2 receptors, approximately 2-fold higher affinities were observed for most compounds in liver, as compared to brain membranes, but excellent correlation with brain-derived values was maintained. To further demonstrate the utility of the new method it was used to screen a novel series of 2(3H)-benzothiazolone compounds, resulting in the identification of several analogues with nanomolar affinity and greater than 50-fold specificity for σ_1 versus σ_2 receptors.

1. Introduction

Two subtypes of σ receptors are currently recognized, σ_1 and σ_2; these subtypes can be distinguished by differences in ligand selectivity, tissue distribution and molecular properties [1,2]. Because σ receptors are recognized as potential therapeutic and radioprobe targets, research to ascribe *in vitro* and *in vivo* activities to the respective subtypes is a major focus of σ receptor research [3–7]. Consequently, to facilitate these studies, efforts to synthesize and identify novel subtype selective agonist and antagonist compounds are ongoing.

Radioligand binding assays serve a critical role in screening novel σ ligands, but the use of conventional cell harvester-based methods significantly limits assay throughput. 96-well filtration offers the potential to increase throughput and reduce costs for routine radioligand binding assays. Previous reports of the use of 96-well filtration methodologies for the analysis of σ receptor binding are limited [8–12]. Therefore, to support routine use of the 96-well filtration, we sought to confirm that results obtained using our proposed method would produce results equivalent to the more established cell harvester-based method.

Rat liver was used as the source of σ receptors for these assays. Previous reports show that rat brain and rat liver homogenates yield similar binding affinities for σ_1 ligands [13–15] and rat liver has already been established as the preferred tissue for σ_2 binding studies [2]. Receptor expression levels of 2 pmol/mg or greater are required for detection with tritiated ligands and the typical sample sizes of 2–100 μg total protein per well used in 96-well filtration assays [16–18]. Rat liver P_2 contains densities of both subtypes of σ receptors that exceed this requirement [13,19,20], making it a suitable receptor source for the proposed assay platform.

Extending on earlier work by Ucar et al. [21], Yous et al. [22] reported a structure-binding affinity study for a small series of benzothiazolone compounds with high affinity and specificity for σ receptors. SN56 (3-(2-(azepan-1-yl)ethyl)-6-propylbenzo[d]thiazol-2(3H)-one) was identified as a new σ receptor specific ligand with nanomolar affinity and unprecedented selectivity for the σ_1 versus the σ_2 subtype and versus a battery of non-σ receptors and neurotransmitter transporters [22]. In the present report, in addition to evaluating a series of reference compounds using the 96-well format, an expanded series of novel

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2(3H)-benzothiazolone compounds were analyzed for binding to σ receptors to further validate the 96-well filtration method for routine use in the screening of novel compounds.

2. Materials and methods

2.1. Chemicals and reagents

[^{3}H](+)-Pentazocine (specific activity = 29 Ci/mmol) and [^{3}H]di-o-tolylguanidine (DTG) (specific activity = 53.3 Ci/mmol) were purchased from Perkin Elmer (Boston, MS). (+)-Pentazocine, (−)-pentazocine, (+)-*N*-allylnormetazocine hydrochloride, 1,3-di-o-tolylguanidine, haloperidol, progesterone, dextromethorphan hydrobromide, rimcazole dihydrochloride monohydrate, sucrose, NaCl, dimethylsulfoxide (DMSO) and tris(hydroxymethyl)aminomethane (Tris), were purchased from Sigma–Aldrich (St. Louis, MO). NE100 (4-methoxy-3-(2-phenylethoxy)-*N*,*N*-dipropylbenzeneethanamine hydrochloride), BD1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride), and fluvoxamine maleate were obtained from Tocris Bioscience (Ellisville, MO). AC927 (N-phenethylpiperidine oxalate) was provided by Dr. Andrew Coop from the University of Maryland (Baltimore, MD). SN56 and the RB compound series (see Table 2) were provided by the laboratory of Dr. Christopher McCurdy from the University of Mississippi (University, MS). Coomassie Protein Assay reagent, 1 N hydrochloric acid, glacial acetic acid, Ecoscint, Microscint 20, Brandel GF/B filter papers, 2.25 × 12.25″, and Unifilter-96 GF/B filter plates were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Membrane preparation

Rat brain P_2 and rat liver P_2 fractions were prepared as described previously from frozen tissues obtained from Pel-Freeze (Rogers, AR) [23]. Tissue preparations were aliquoted in 1 ml portions and stored at −80 °C. The Bradford assay was used to quantitate protein concentration using Bio-Rad Protein Assay reagent (Hercules, CA).

2.3. Competition binding assays

Binding assays utilized optimized buffer and incubation conditions that are consistent with those reported in the literature for the analysis of σ receptor binding [20,24,25]. Stock solutions of test ligands were prepared in DMSO or deionized water at 5 or 25 mM. Dilutions of reference ligands for competition studies were made with assay buffer (50 mM Tris, pH 8). Dilutions of 2(3H)-benzothiazolone analogues were prepared in 1 mM HCl. The use of 1 mM HCl for dilution of 2(3H)-benzothiazolone analogues was required to reduce binding of these compounds to glass tubes or polypropylene microplates and had no effect on the final pH of the samples or on total binding relative to samples prepared in assay buffer alone (data not shown).

Assays with rat brain were processed using a Brandel R48 harvester (Gaithersburg, MD), and assays with rat liver were processed using a Connectorate 96-well harvester (Dietikon, Switzerland). For compounds assayed with brain homogenate, 400 μg of rat brain P_2 membrane was added to a glass test tube containing test ligand and radioligand in assay buffer in a final volume of 0.5 ml. For compounds analyzed with rat liver homogenate, 40 μg of rat liver P_2 membrane was added to a polypropylene plate (catalogue number 07-200-697, Fisher Scientific) containing test ligand and radioligand in assay buffer in a total volume of 0.25 ml. Assays for σ_1 receptors used a final concentration of 5 nM [^{3}H](+)-pentazocine. Labeling of σ_2 was effected with either 3 nM [^{3}H]DTG for brain membranes, or 5 nM [^{3}H]DTG for liver membranes; these samples also contained 300 nM (+)-pentazocine (to block σ_1 receptors). Non-specific binding was determined by the addition of haloperidol to a final concentration of 10 μM. Samples were incubated for 120 min at 25 °C for all assays. Following incubation, samples were filtered and washed. Samples processed on the Brandel cell harvester were washed 3 times with 3 ml of 10 mM Tris, pH 8. Samples processed by 96-well filtration were washed 5 times with 0.2 ml of 10 mM Tris, pH 8. Prior to use, GF/B filter papers and Unifilter GF/B filter plates were soaked in 0.5% polyethyleneimine (PEI) for 30 min to reduce non-specific binding.

For the determination of binding affinities, each test compound was assayed at 11 concentrations varying from 0.001–10 μM. Samples were prepared and processed in duplicate for each binding curve and triplicate determinations of binding curves were made for each compound. Following washing, filters processed on the Brandel harvester were transferred to scintillation vials and 3 ml scintillation cocktail was added to each sample. Filters were allowed to soak in cocktail for a minimum of 10 h prior to counting on a Beckman LS6500 scintillation counter (Brea, CA). Samples processed by 96-well filtration were counted on a Perkin Elmer Microbeta2 2450 microplate counter (Waltham, MA), in the Unifilter plates, following a 2 h incubation at room temperature with 40 μl Microscint-20 cocktail per well.

2.4. Data analysis

The competition binding data were analyzed with GraphPad Prism software (San Diego, CA) using a one-site nonlinear regression model to determine the concentration of ligand that inhibits 50% of the specific binding of the radioligand (IC_{50} value). K_i values were calculated from the IC_{50} using the Cheng–Prusoff equation [26]. To compare binding data from conventional binding experiments to the 96-well filtration method, correlation plots were generated with GraphPad Prism, using a two-tailed fit with the assumption that data were sampled from Gaussian populations (Pearson *r*). For comparison of individual K_i values obtained using rat liver versus rat brain, a two-tailed *t*-test was performed using InStat software (San Diego, CA).

3. Results and discussion

3.1. Binding affinities of reference ligands

σ receptor binding affinities for individual reference compounds using both the new 96-well method and conventional cell harvester method as reported in the literature and as determined in this study are shown in Table 1. Overall, values obtained for σ binding in rat brain P_2 (using the conventional cell harvester method) from this study were similar to values reported in the literature, where measurements were made with similar experimental conditions in either rat or guinea-pig brain fractions (see legend to Table 1). Likewise, similar σ_1 binding affinities were observed for samples analyzed in this study with liver P_2 (using the 96-well method) versus brain P_2 for all reference compounds with the exception of those compounds with low affinity: progesterone, dextromethorphan and rimcazole; these compounds showed statistically significant higher affinities in liver as compared to brain (progesterone, $P < 0.001$; dextromethorphan, $P < 0.005$; rimcazole, $P < 0.001$), a pattern that is consistent with previous observations reported by Klouz et al. [14,15]. At σ_2 receptors, all compounds tested showed higher affinity in liver versus brain, with most compounds displaying an approximately 2-fold higher affinity in liver compared to brain; the difference was statistically significant, for six of the ten compounds tested (DTG, $P < 0.005$; haloperidol, $P < 0.001$; NE100, $P < 0.001$; BD1063, $P < 0.001$; AC927, $P < 0.005$; and fluvoxamine, $P < 0.005$). Fig. 1 shows a correlation plot for σ_1 binding in rat liver

Table 1
Summary of experimentally determined binding affinities (K_i) vs. literature values for reference compounds.

Compound	K_i at σ_1 (nM)			K_i at σ_2 (nM)		
	Literature	Experimental		Literature	Experimental	
		Brain/Brandel	Liver/96-well		Brain/Brandel	Liver/96-well
(+)-Pentazocine	6.7 ± 1.2[a]	8.65 ± 0.4	13.3 ± 1.8	1361 ± 134[a]	1414 ± 207	1067 ± 94
(−)-Pentazocine	44.0 ± 1.2[a]	57.2 ± 1.9	47.6 ± 3.8	108 ± 6[a]	108 ± 4.4	58.1 ± 4.0
(+)-SKF10,047	28.7 ± 2.8[a]	287 ± 36	288 ± 24	33,654 ± 9409[a]	ND	>10,000
DTG	74.3 ± 13.9[a]	57.4 ± 3.3	55.9 ± 3.9	61.2 ± 13.4[a]	43.3 ± 0.6	24.0 ± 2.3
Haloperidol	1.9 ± 0.3[a]	3.9 ± 0.5	3.3 ± 0.6	79.8 ± 20.6[a]	155 ± 2	57.2 ± 2.4
NE100	1.54 ± 0.26[b]	2.8 ± 0.5	2.0 ± 0.5	84.6 ± 32.9[b]	95.5 ± 1.0	36.2 ± 1.1
BD1063	9.15 ± 1.28[c]	15.0 ± 2.1	33.4 ± 4.1	449 ± 11[c]	928 ± 40	462 ± 10.4
AC927	30 ± 2[d]	61.2 ± 5.6	74.3 ± 2.1	138 ± 18[d]	384 ± 34	94.3 ± 3.1
Fluvoxamine	36[e]	64.0 ± 5.2	74.3 ± 9.4	8439[e]	4818 ± 204	2254 ± 206
Progesterone	338 ± 8[f]	234 ± 4.4	99.7 ± 3.1	NR	>10,000	>10,000
Dextromethorphan	652 ± 33[g]	403 ± 22	214 ± 15	NR	>10,000	>10,000
Rimcazole	867 ± 185[g]	2565 ± 36	594 ± 61	NR	1568 ± 154	719 ± 72
SN56	0.56[h]	1.7 ± 0.1	1.6 ± 0.1	>1000[h]	627 ± 115	248 ± 15

NR = not reported.
[a] Bowen [24]. Rat brain P_2 with 5 nM [^{3}H](+)-pentazocine for σ_1 and 3 nM [^{3}H]DTG with 1 μM dextrallorphan for σ_2.
[b] Chaki [41]. Guinea-pig brain. *Note*: Reported value is IC_{50}.
[c] Matsumoto [23]. Guinea-pig brain P_2 with 3 nM [^{3}H](+)-pentazocine for σ_1. Rat liver P_2 with 3 nM [^{3}H]DTG with 1 μM dextrallorphan for σ_2.
[d] Matsumoto [42]. Rat brain P_2 with 3 nM [^{3}H](+)-pentazocine for σ_1 and 3 nM [^{3}H]DTG with 300 nM (+)-pentazocine for σ_2.
[e] Narita [43]. Rat brain P_1/P_2 with 5 nM [^{3}H](+)-pentazocine for σ_1 and 5 nM [^{3}H]DTG with 1 μM (+)-pentazocine for σ_2.
[f] Hanner [13]. Guinea-pig brain P_3 with 0.3–0.5 nM [^{3}H](+)-pentazocine.
[g] Klouz [14]. Rat brain P_2/P_3, described as "synaptosomal" with 2–3 nM [^{3}H](+)-pentazocine.
[h] Yous [22]. Guinea-pig brain P_2 with 3 nM [^{3}H](+)-pentazocine for σ_1 and 3 nM [^{3}H]DTG with 100 nM (+)-normetazocine for σ_2.

P_2 versus rat brain P_2 as determined in this study (Pearson $r = 0.97$). Fig. 2 shows a correlation plot for σ_2 binding in rat liver P_2 versus rat brain P_2 as determined in this study (Pearson $r = 0.98$).

Because the σ_1 receptor appears well conserved among species and tissues, the choice of liver as a source of σ_1 receptors was not expected to greatly impact affinity measurements [13,27–31]. Moreover, binding studies with different crude membrane preparations, subcellular fractions and solubilized extracts derived from brain and liver from rat and guinea-pig, indicate that affinities of ligands for σ_1 remain essentially constant despite the varied sources of receptors [13–15,19,20,32–34]. However, the cell biology of σ_1 receptors is still being elucidated and it is unknown whether variations in lipid or protein partners exist between tissues that could impact ligand binding, making a systematic comparison necessary.

Our observation that most reference ligands tested bound with higher affinity to σ_2 receptors isolated from liver compared to brain was unexpected. Published studies show that σ_2 receptors reside in lipid rafts [35,36], and differences in binding of [^{3}H]DTG (with σ_1 blocking) have been observed in partially solubilized rafts isolated from the rat liver P_2 fraction using different detergents ($K_d \sim 23$ nM in 20 mM CHAPS versus $K_d \sim 170$ nM in 1% Triton X-100), but fully solubilized preparations in these same detergents showed equivalent binding affinities [35,36]. This suggests that the binding affinity of σ_2 can be influenced by manipulating the composition of lipids and/or proteins associated with it. This observation

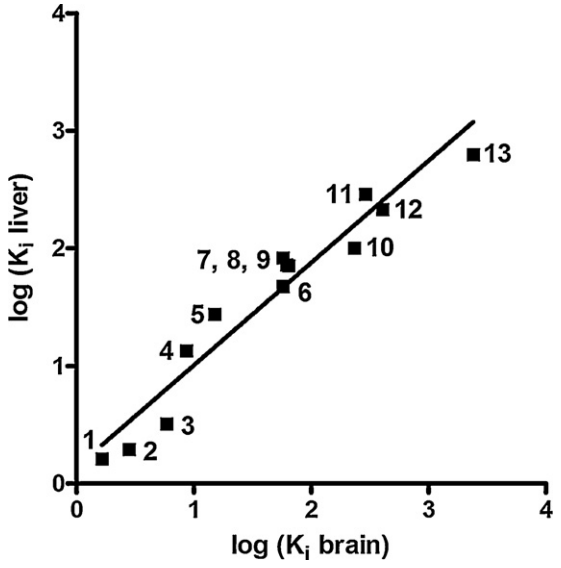

Fig. 1. Correlation plot for binding of σ reference ligands to σ_1 receptors in rat liver versus rat brain P_2 membranes. Plot shows least squares regression line. Correlation plots yielded Pearson r value = 0.97. [1, SN56; 2, NE100; 3, haloperidol; 4, (+)-pentazocine; 5, BD1063; 6, (−)-pentazocine; 7, DTG; 8, AC927; 9, fluvoxamine; 10, progesterone; 11, (+)-SKF10,047; 12, dextromethorphan; 13, rimcazole].

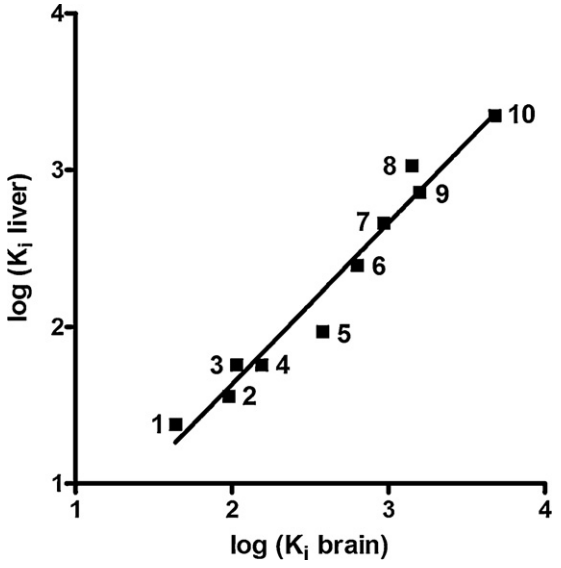

Fig. 2. Correlation plot for binding of σ reference ligands to σ_2 receptors in rat liver versus rat brain P_2 membranes. Plot shows least squares regression line. Correlation plots yielded Pearson r value = 0.98. [1, DTG; 2, NE100; 3, (−)-pentazocine; 4, haloperidol; 5, AC927; 6, SN56; 7, BD1063; 8, (+)-pentazocine; 9, rimcazole; 10, fluvoxamine].

Table 2
Summary of binding affinities (K_i) for 2(3H)-benzothiazolone compounds.

Compound	R	Linker length (n)	Ring size (m)	K_i (nM)		$K_i\ \sigma_2/K_i\ \sigma_1$
				σ_1	σ_2	
RB65	—H	1	0	578 ± 41	8264 ± 500	14
RB67		1	3	9.7 ± 0.6	716 ± 30	74
RB2		2	2	4.1 ± 0.3	177 ± 26	43
RB4		3	2	3.2 ± 0.02	101 ± 14	31
RB6		4	2	7.0 ± 0.3	2.5 ± 0.3	0.4
RB8		5	2	7.5 ± 0.6	2.4 ± 0.4	0.3
SN56	—$(CH_2)_2CH_3$	1	2	1.6 ± 0.1	270 ± 4.7	168
RB10		2	2	1.4 ± 0.1	17.2 ± 1.0	13
RB14		3	2	6.1 ± 1.2	4.3 ± 0.3	0.7
RB16		4	2	4.6 ± 0.4	1.6 ± 0.1	0.3
RB18		5	2	6.3 ± 0.9	2.3 ± 0.2	0.4
RB20	—$(CH_2)_3CH_3$	2	2	2.2 ± 0.4	15.3 ± 0.9	7
RB34		3	2	1.9 ± 0.2	4.4 ± 0.3	2.4
RB24		5	2	12.0 ± 0.7	4.1 ± 0.6	0.3
RB75	—$COCH_2CH_3$	1	0	116 ± 15	4787 ± 101	41
RB74		1	2	4.5 ± 0.2	2181 ± 127	483
RB26		2	2	3.7 ± 0.3	305 ± 7.0	83
RB28		3	2	10.3 ± 0.9	30.3 ± 2.0	3.0
RB30		4	2	12.2 ± 1.1	8.3 ± 0.8	0.7
RB32		5	2	10.4 ± 0.1	1.1 ± 0.1	0.1
RB36	—$CO(CH_2)_2CH_3$	2	2	2.6 ± 0.4	104 ± 1.9	39
RB38		3	2	4.8 ± 0.1	21.6 ± 4.2	5
RB40		4	2	16.3 ± 0.6	5.7 ± 0.5	0.4
RB70		5	2	10.8 ± 0.4	2.3 ± 0.3	0.2

contrasts with the σ_1 receptor which appears to maintain consistent binding affinity for ligands in different subcellular fractions, soluble extracts, in purified form, and in lipid reconstituted purified forms [13,19,33,37].

3.2. Binding affinities of 2(3H)-benzothiazolone analogues

σ receptor binding affinities determined in this study for individual test compounds are shown in Table 2. The parent compound structure is shown in Fig. 3 where R represents the appended alkyl or ketonyl chain, m represents ring size, and n represents linker chain length. Some general trends observed include: (1) a reversal in selectivity from σ_1 to σ_2, for azepine ring compounds ($m = 2$) with increasing linker arm length (from $n = 2$ to $n = 5$), reflecting marginal changes in affinity at σ_1 and significant increases in affinities for the σ_2 subtype, (2) reduced affinity for both σ subtypes when the azepine ring of SN56 was replaced with a pyrrolidine ring ($m = 0$), as demonstrated by RB65 and RB75, and (3) a dramatic decrease in σ_2 affinity with a marginal change in σ_1 affinity for linker length $n = 1$, with an azepine ring, and a change of the chain at position 6 from $(CH_2)_2CH_3$ to $COCH_2CH_3$, as demonstrated by SN56 and RB74. The $K_i\ \sigma_2/K_i\ \sigma_1$ value of 483 for RB74 versus 168 for SN56 represents an approximately 3-fold improvement in σ_1 selectivity, primarily due to an almost 8-fold decrease in affinity at σ_2 for RB74 versus SN56.

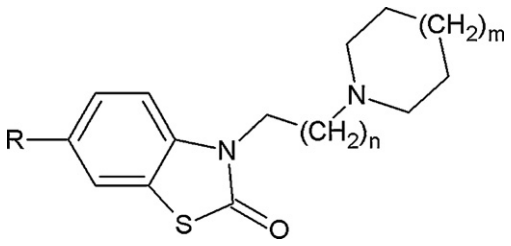

Fig. 3. Structure of 2(3H)-benzothiazolone analogs. R = H, $(CH_2)_2CH_3$, $(CH_2)_3CH_3$, $COCH_2CH_3$, or $CO(CH_2)_2CH_3$.

3.3. Alternative methods for determination of σ receptor binding

Several alternative methods for analyzing σ receptor binding have been reported in recent years [9,12,38–40]. Lee et al. [38] produced an MCF7 cell line expressing very high levels of cloned human σ_1 ($B_{max} = 109 \pm 24$ pmol/mg). This cell line was successfully used to analyze a wide range of compounds including 10 σ reference ligands [38]. Studies from the Wunsch group utilized cell lines with high endogenous expression levels of σ_1 or σ_2 (RPMI 8226 and RT-4 cells respectively) in conjunction with 96-well filtration; these studies were validated with σ reference ligands as well [39,40]. The 96-well format has also been successfully employed for a number of studies using guinea pig brain for σ_1 and rat liver for σ_2 with filtration through filtermats [9] or filterplates [8,10,12], but our efforts to filter rat brain homogenates were unsuccessful with the filterplates we examined. Each of these methods presents researchers options that may have distinct advantages depending on equipment and materials available in their laboratories and the goals of their studies.

4. Conclusion

In this study, a 96-well method for radioligand competition binding was evaluated for the determination of σ ligand binding affinities. The binding of reference ligands to rat liver P_2 σ membranes analyzed with the 96-well method demonstrated excellent correlation with values derived in rat brain P_2 membranes assayed using the conventional Brandel-based method for both σ receptor subtypes. These results suggest that 96-well filtration is a suitable alternative to Brandel filtration for the analysis of σ receptor radioligand binding. Our data also showed that σ_2 receptors derived from rat liver P_2 exhibit higher affinity for σ ligands than those isolated from rat brain P_2, a result that has not been previously reported. This result suggests caution should be exercised with respect to comparisons of $K_i\ \sigma_2/K_i\ \sigma_1$ selectivity ratios in the literature, which due to potential tissue specific differences in binding at σ_2 may be misleading when comparing compounds reported by different groups. Application of the assay to the analysis of a novel

series of 2(3H)-benzothiazolone compounds identified several new σ_1 selective analogues and provided structure-binding affinity data that distinguish structural features which confer subtype selectivity for this compound class.

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