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Comparison of binding parameters of σ_1 and σ_2 binding sites in rat and guinea pig brain membranes: novel subtype-selective trishomocubanes

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Abstract

Comparisons of binding parameters of [^{3}H](+)-pentazocine and [^{3}H]1,3-di-*o*-tolylguanidine (DTG) at σ binding sites in guinea pig and rat brain membranes demonstrated that [^{3}H](+)-pentazocine binds to a single high-affinity site, whereas [^{3}H]DTG binds to two high-affinity sites in both species. The K_d values of the radioligands were similar in both types of membranes. However, the density of σ_1 sites in guinea pig was significantly higher than that of rat. Novel trishomocubanes were tested for their affinities at σ_1 and σ_2 binding sites in guinea pig brain membranes using [^{3}H](+)-pentazocine and [^{3}H]DTG as the radioligands. *N*-(4-Phenylbutyl)-3-hydroxy-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecane (ANSTO-14) showed the highest affinity for the σ_1 site (K_i = 9.4 nM) and 19-fold σ_1/σ_2 selectivity, as a result of increasing the alkyl chain between the cubane moiety and the aromatic ring. *N*-(3′-Fluorophenyl)methyl-3-hydroxy-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecane (ANSTO-19), displayed the highest affinity for σ_2 sites (K_i = 19.6 nM) and 8-fold σ_2/σ_1 selectivity due to a fluoro substitution in the *meta* position of the aromatic ring. These represent structurally novel lead compounds, especially for the development of selective σ_2 receptor ligands.

Keywords: σ Binding site; Trishomocubane; (+)-Pentazocine; 1,3-Di-*o*-tolylguanidine; Brain membrane

1. Introduction

Martin et al. (1976) originally postulated the σ binding site as an opiate receptor which mediated the hallucinogenic effects of nalorphine-like agonists and the psychotomimetic effects of benzomorphans in man, and delirium in dog. Since then the σ binding site has been redefined a number of times (Su, 1982; Quirion et al., 1987; Walker et al., 1990; Tam and Zhang, 1988; Weissman et al., 1988). The concept of σ subtypes (σ_1 and σ_2), was first proposed on the basis of the marked differences in affinity for (+)-benzomorphans and molecular weight for σ sites labelled by photoaffinity probes in guinea pig brain and pheochromocytoma (PC12) cells (Hellewell and Bowen, 1990; Walker et al., 1990; Quirion et al., 1992). Hellewell and Bowen (1990) also concluded that the σ_1 site, unlike the σ_2 site, displays restricted stereospecificity for (+)-isomers of benzomorphans, morphinans and other opiates. Rothman et al. (1991), suggested that the σ_2 site may be associated with calcium channels on the basis of the modulatory effects of inorganic calcium channel blockers such as Cd^{2+} on σ_1 and σ_2 sites. A hypothesis suggesting a role for σ_2 binding sites in motor functions is also supported by several lines of evidence (Walker et al., 1990, 1993; Hemstreet et al., 1993).

The relative lack of σ-subtype selective, particularly σ_2 ligands, has limited the investigation of the binding, pharmacology and functional significance of σ binding sites, as most of the σ ligands display significant cross-reactivity at other binding sites such as opioid, dopamine, muscarinic and phencyclidine receptors (Tam and Cook, 1984; Walker et al., 1990; Hudkins and DeHaven-Hudkins, 1991; Hudkins et al., 1994a,b). Hashimoto and London (1993), and Hashimoto et al. (1994) reported that the pharmacological profile of [^{3}H]ifenprodil binding was highly correlated with σ_2 sites, not σ_1 sites, and also suggested that ifenprodil would be a useful tool for studying σ receptor subtypes. However, patch-clamp studies mentioned by these authors also revealed that the potent actions of ifenprodil and its analogues block NMDA channels which

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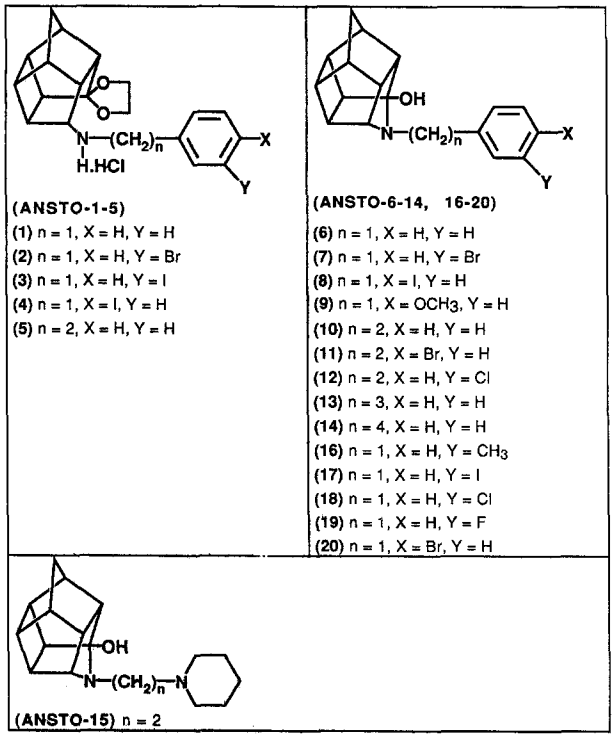

Fig. 1. Chemical structure of trishomocubanes.

could complicate functional studies. The inhibition constant (K_i) of BIMU-1 (endo-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazole-1-carboxamide hydrochloride) for σ_2 sites was reported to be 32 nM by Bonhaus et al. (1993). However, BIMU-1 also has high affinitiy for 5-HT_3 and 5-HT_4 receptors.

Numerous studies of the chemistry of various polycyclic hydrocarbons (cubanes) have been conducted in the past half-century. However, research on the biological activities of these compounds have been largely neglected. Since the discovery of its antiviral activity, amantadine was found to be beneficial to patients with Parkinson's disease (Schwab et al., 1969). Amantadine also interacts with the σ binding sites at therapeutic levels (K_i = 20.25 μM, Kornhuber et al., 1993). Oliver et al. (1991) reported the synthesis and pharmacological properties of a series of novel D_3-trishomocubanes. Promising anticataleptic activities were observed for some members of the series, while only weak to mild anticholinergic activities were noted. Anti-parkinsonian properties of these pentacycloundecylamines were attributed to their possible effects on dopaminergic systems, but interaction with σ sites was not explored.

We recently reported small series of trishomocubanes (Kassiou et al., 1996; Fig. 1) which have shown high affinity for σ binding sites and no cross-reactivity with other binding sites and receptors such as dopamine, opioid, phencyclidine, NMDA (*N*-methyl-D-aspartate) and serotonin. However, the selectivity for the two σ subtypes had not been resolved. In this work, we report comparative studies of binding properties of [^{3}H](+)-pentazocine and [^{3}H]DTG at the σ_1 and σ_2 binding sites in rat and guinea pig brain membranes, and a full characterisation of a new series of novel trishomocubanes (pentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecylamines and 4-azahexacyclo-[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecanes) on σ subtypes in guinea pig brain membranes. Some members of this series display high affinity and moderate selectivity for σ_2 sites. A trend of structure-activity relationship for σ_2 sites is also discussed which represents a new direction for development of novel high-affinity, high-selectivity σ_2 ligands.

2. Materials and methods

2.1. Materials

[^{3}H](+)-Pentazocine, [Ring-1,3-^{3}H] (two different preparations with specific radioactivities of 31.6 and 38.3 Ci/mmol of [^{3}H](+)-pentazocine were used) and [^{3}H]DTG, [5-^{3}H](1,3-di-*o*-tolylguanidine di-[*p*-Ring-^{3}H]-) (35.2 Ci/mmol) were purchased from Dupont/New England Nuclear (Boston, MA, USA). Trishomocubanes (pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecylamines and 4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecanes) were synthesised at the Australian Nuclear Science and Technology Organisation, Radiopharmaceuticals Division (Menai, NSW, Australia). (+)-Pentazocine, DTG, dextrorphan D-tartrate and dextromethorphan hydrobromide were purchased from Research Biochemicals Incorporated (Natick, MA, USA), haloperidol from Sigma Chemical Co. (St. Louis, MO, USA), and 1-amino adamantane from Fluka AG (CH-9470, Buchs, Switzerland). Trishomocubanes were dissolved in DMSO (dimethyl sulphoxide), subsequent dilutions from stock solutions, and other chemicals were made up in fresh assay buffer (Tris-HCl, 50 mM, pH 8.0 at room temperature for σ_2 or pH 8.2 at room temperature for σ_1 assay). Haloperidol and (+)-pentazocine were dissolved in Tris HCl buffer (50 mM, pH 7.4 at room temperature) with the help of 1–2 drops of glacial acetic acid. Trishomocubanes, ANSTO-(1–5) were expressed as hydrochloride salts and the rest, ANSTO-(6–20), as free bases.

2.2. Membrane preparations

Male Sprague-Dawley rats (350–500 g) and female Hartley-Albino guinea pigs (500–750 g) were used. The membrane preparation was as previously described by DeHaven-Hudkins et al. (1992, 1993).

No effects of prolonged storage (> 3 months) of the membranes at −70°C on σ receptor density or affinity for radioligands were observed as the results showed that the σ sites were remarkably stable over a long period of freezing. On the day of the assay, membrane aliquots were

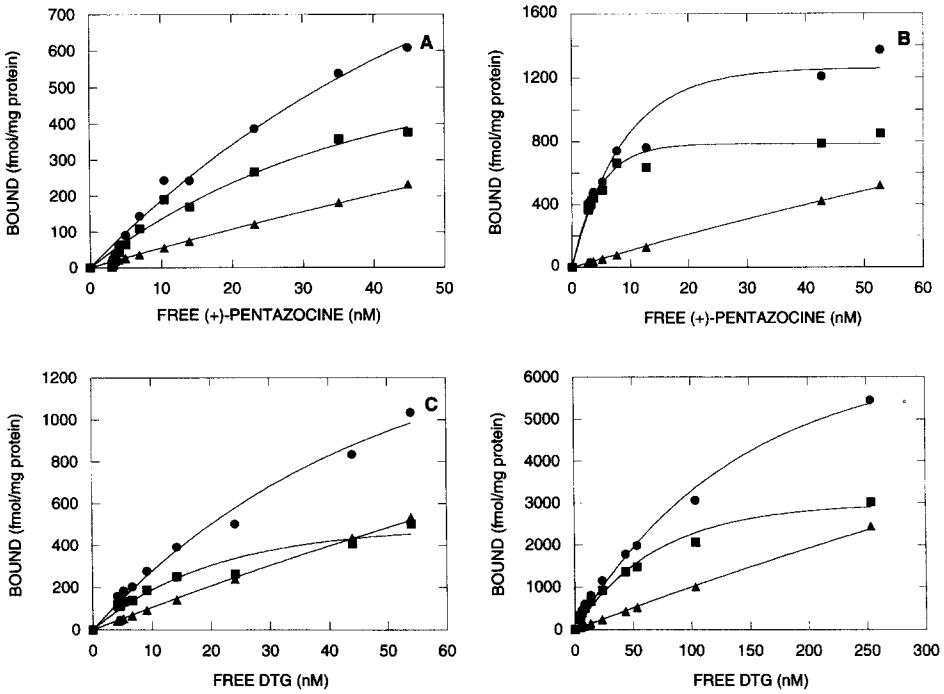

Fig. 2. Representative binding curves (●, total binding; ■, specific binding; ▲, non-specific binding) of [^{3}H](+)-pentazocine and [^{3}H]DTG in male Sprague-Dawley rat (A,C) and female Hartley-Albino guinea pig (B,D), brain membranes, respectively. Assays were carried out using the conditions described in Methods: a fixed concentration of 3 nM of radioligand was incubated with 15 concentrations of unlabelled ligand ranging from 0.01 to 1000 nM. Non-specific binding was determined using 10 μM haloperidol. Data were collected from the LIGAND program then fitted by KaleidaGraph, Version 3.0, Abelbeck Software. For regression values, see Results). Panel A: [^{3}H](+)-Pentazocine (rat). Panel B: [^{3}H](+)-Pentazocine (guinea pig). Panel C: [^{3}H]DTG (rat). Panel D: [^{3}H]DTG (guinea pig).

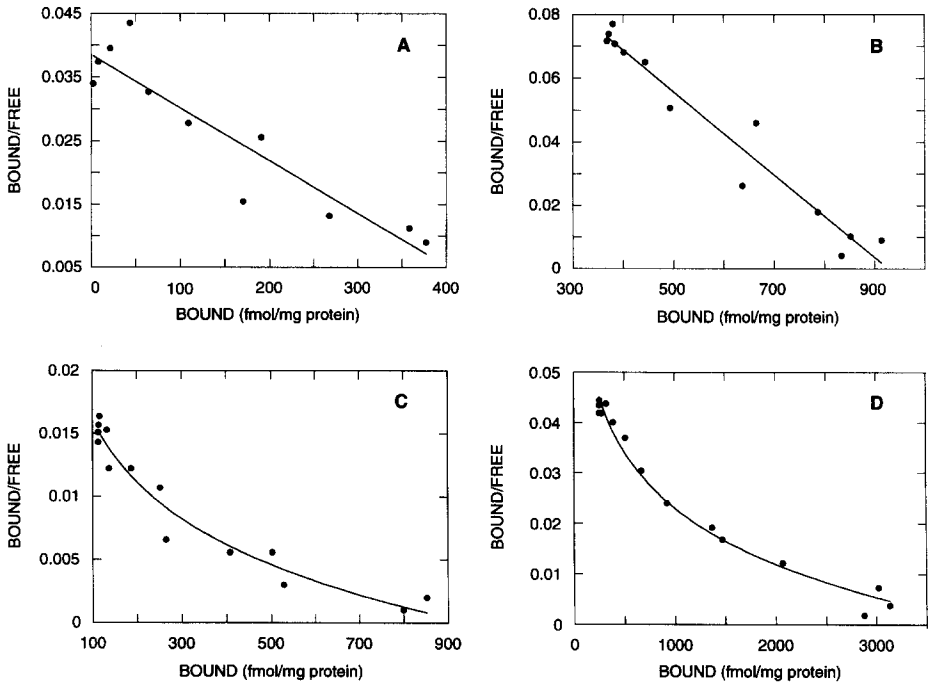

Fig. 3. Representative Scatchard plots of [^{3}H](+)-pentazocine and [^{3}H]DTG binding in male Sprague-Dawley rat (A,C) and female Hartley Albino guinea pig (B,D) brain membranes, respectively. Data were fit to both a one-site and two-site model in order to determine which gave the best fit by the LIGAND program. Panel A: [^{3}H](+)-Pentazocine (rat), $r = 0.93$. Panel B: [^{3}H](+)-Pentazocine (guinea pig), $r = 0.98$. Panel C: [^{3}H]DTG (rat), $r = 0.97$. Panel D: [^{3}H]DTG (guinea pig), $r = 0.99$.

Table 1
Comparisons of binding parameters (K_d and B_{max}) of [^{3}H](+)-pentazocine and [^{3}H]DTG on σ binding sites in male Sprague-Dawley rat and female Hartley-Albino guinea pig brain membranes

Radioligand		Membrane preparation	
		Rat	Guinea pig
[^{3}H](+)-Pentazocine			
	K_d (nM)	2.46 ± 0.37	3.38 ± 0.46
	B_{max} (fmol/mg protein)	124 ± 33	823 ± 21
[^{3}H]DTG			
σ_1	K_d (nM)	9.81 ± 2.49	16.96 ± 6.34
	B_{max} (fmol/mg protein)	263 ± 62	997 ± 179
σ_2	K_d (nM)	76.93 ± 20.12	91.15 ± 27.52
	B_{max} (fmol/mg protein)	1 185 ± 390	2 816 ± 894
σ_2/σ_1	B_{max} (fmol/mg protein)	4.5	2.8

Data from Table 1 are the means ± S.E.M. from 3–8 separate experiments performed in quadruplicate. In all experiments, the K_d and B_{max} values were calculated using the LIGAND program. The results were obtained from the best fits which were determined by a Run test showing a non-serial correlation ($P > 0.05$), plus the presence of a significant F-test ($P < 0.05$) between the more complex and the next simplest fit.

thawed, resuspended in fresh Tris buffer (Tris-HCl, 50 mM, pH 7.4 at room temperature) and centrifuged 3 times at 22 000 × g for 20 min at 4°C. The final pellets were resuspended in fresh assay buffer and stored on ice until use.

2.3. [^{3}H](+)-Pentazocine and [^{3}H]DTG radioreceptor assays

The binding to σ sites of [^{3}H](+)-pentazocine was performed largely as described by Hudkins et al. (1994a), and DeHaven-Hudkins et al. (1992), and [^{3}H]DTG by Weber et al. (1986), and Rothman et al. (1991). Briefly, each assay tube contained radioligand at a final concentration of approximately 2 nM for [^{3}H](+)-pentazocine or 10 nM for [^{3}H]DTG, tissue suspension (approximately 0.75 mg of protein per tube), various concentrations of test compounds and the assay buffer in a final volume of 1 ml. Non-specific binding was determined using 10 μM haloperidol. The selectivity for σ_2 sites of the test compounds was determined by labelling with [^{3}H]DTG and masking σ_1 with 0.5 μM (+)-pentazocine. After incubation at 37°C for 150 min in the [^{3}H](+)-pentazocine assay or at 25°C for 90 min in the [^{3}H]DTG assay, the reaction was terminated by rapid filtration using a Brandel 24-well cell harvester (Brandel, Gaithersburg, MD, USA) over Whatman GF/B glass fiber filters that were presoaked in a solution of 0.5% polyethylenimine at room temperature for at least 2 h prior to use. Filters were washed with three 2.5-ml volumes of ice-cold assay buffer. Following addition of scintillation cocktail, Emulsifier-Safe (Packard Instrument B.V.-Chemical Operations, Groningen, Nether-

Table 2
Inhibitory constants and Hill coefficients of the trishomocubanes for [^{3}H](+)-pentazocine, σ_1 and [^{3}H]DTG, σ_2 in female Hartley-Albino guinea pig brain membranes

Trishomocubanes	K_i (nM) [a,b]				σ_1/σ_2
	[^{3}H](+)-Pentazocine (σ_1)	n_H	[^{3}H]DTG (σ_2)	n_H	
ANSTO-					
1	67 ± 11	0.97 ± 0.02	864 ± 258	0.88 ± 0.06 *	0.08
2	17 ± 1	0.78 ± 0.08	208 ± 36	0.96 ± 0.05	0.08
3	124 ± 10	1.08 ± 0.10	285 ± 46	0.68 ± 0.08 *	0.44
4	72 ± 10	0.95 ± 0.11	246 ± 9	0.86 ± 0.07	0.29
5	15 ± 2	0.96 ± 0.05	608 ± 2	0.59 ± 0.14	0.02
6	103 ± 25	0.80 ± 0.05 *	51 ± 8	0.40 ± 0.06 *	2.02
7	208 ± 13	0.98 ± 0.09	40 ± 22	0.56 ± 0.03 *	5.20
8	81 ± 4	0.85 ± 0.04	246 ± 46	1.11 ± 0.08	0.33
9	103 ± 1	0.83 ± 0.04	136 ± 19	0.57 ± 0.00 *	0.76
10	20 ± 4	0.83 ± 0.11	307 ± 18	0.66 ± 0.04 *	0.06
11	10 ± 1	0.92 ± 0.16	166 ± 32	0.89 ± 0.06	0.06
12	21 ± 2	0.91 ± 0.05	153 ± 35	0.71 ± 0.15	0.14
13	21 ± 2	0.90 ± 0.05	238 ± 7	0.61 ± 0.01 *	0.09
14	9 ± 3	0.92 ± 0.10	171 ± 17	0.67 ± 0.11	0.05
15	355 ± 24	0.85 ± 0.05	3 108 ± 142	0.47 ± 0.04 *	0.11
16	97 ± 6	0.82 ± 0.03	108 ± 6	0.50 ± 0.06 *	0.90
17	169 ± 10	0.93 ± 0.02	54 ± 18	0.53 ± 0.04 *	3.13
18	186 ± 8	0.89 ± 0.02	30 ± 1	0.40 ± 0.01 *	6.20
19	152 ± 1	0.79 ± 0.01	20 ± 4	0.33 ± 0.00 *	7.60
20	86 ± 3	0.81 ± 0.02 *	176 ± 32	0.76 ± 0.07	0.49
Amantadine	14 040 ± 3 210	1.02 ± 0.03	26 210 ± 1 670	1.25 ± 0.07	0.54

[a] Data from Table 2 are the means ± S.E.M. from 3–5 separate experiments performed in quadruplicate. [b] The K_i and n_H values were calculated using the LIGAND program. Asterisks indicate n_H significantly different from unity with $P < 0.01$.

lands), samples were allowed to equilibrate overnight. The amount of bound radioactivity was determined by liquid scintlillation spectrometry using a Packard 1500 Tri-Carb Liquid Scintillation Analyser (Packard Instrument Co., Downers Grove, IL, USA) with a counting efficiency for tritium of 57%. Each concentration of test compounds was tested in quadruplicate. Protein concentrations of the samples were determined by the method described by Lowry et al. (1951). For saturation studies, a fixed concentration of 3 nM of radioligand was incubated with 15 concentrations of unlabelled ligand ranging from 0.01 to 1000 nM. For inhibition studies of the reference and test compounds, at least 10–12 concentrations of the studied compounds were used to achieve consistent results, typically the concentration range was 1 nM to 20 μM. Saturation binding data were fitted using KaleidaGraph, Version 3.0, Abelbeck Software. The K_d and B_{max} values of the radioligands, and the K_i and n_H values of the test and reference compounds were calculated by non-linear regression analysis using the LIGAND program (Munson and Rodbard, 1980; McPherson, 1983). Differences of n_H values from unity were tested using paired t-tests on 3–7 independent experiments. Significant differences were noted for $P < 0.01$ to account for use of multiple t-tests.

3. Results

Fig. 2 shows the binding curves for total, specific and non-specific binding of [^{3}H](+)-pentazocine and [^{3}H]DTG in rat and guinea pig brain membranes (see Panel A, B, C, and D, respectively). Saturation and apparent K_d appeared similar to those fitted by the LIGAND program (see below). Specific binding of the σ_1 assay was greater than 90%, and greater than 70% for the σ_2 assay at the concentrations of tritiated ligands used for saturation and displacement assays (2 nM for [^{3}H](+)-pentazocine, 10 nM for [^{3}H]DTG). Specific binding was pH- and time-dependent. Maximum binding in the σ_1 assay occurred at pH 8.2 at 37°C and reached in 2–2.5 h, whereas maximum specific binding for the σ_2 assay was observed at pH 8.0 at room temperature and reached in 1.5 h (data not shown).

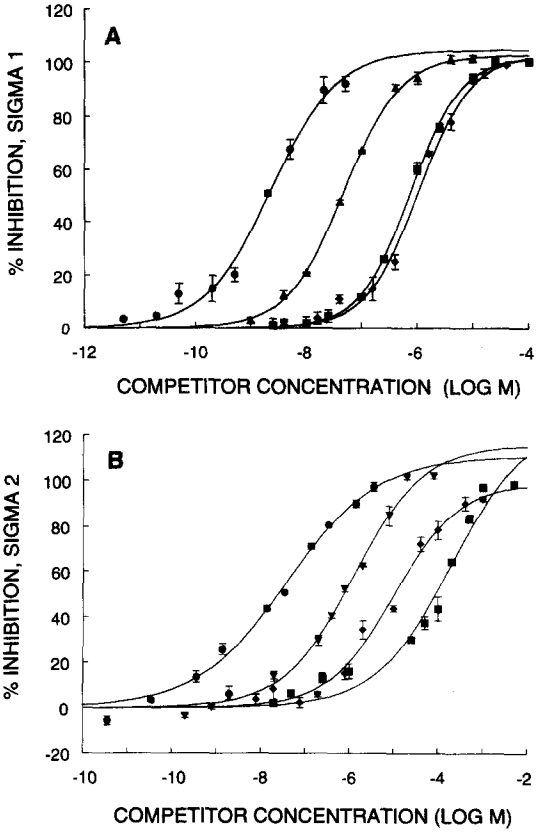

Fig. 4. Competitive inhibition of specific binding of [^{3}H](+)-pentazocine (σ_1 sites) and [^{3}H]DTG (σ_2 sites) in female Hartley-Albino guinea pig brain membranes by reference compounds. At least 10–12 concentrations of the test compounds were used in each assay, typically the concentration range was 1 nM to 20 μM. Data from Fig. 3 are the mean ± S.E.M. from 3–4 separate experiments for each compound performed in quadruplicate. A S.E.M. which does not appear on a particular point on the curve fit, is smaller than the symbol size. Panel A: 2 nM [^{3}H](+)-pentazocine was inhibited by: (●) haloperidol, (■) dextromethorphan, (♦) dextrorphan, (▲) DTG. Panel B: 10 nM [^{3}H]DTG was inhibited by: (●) haloperidol, (■) dextromethorphan, (♦) dextrorphan, (▼) (+)-pentazocine.

The results presented in Fig. 3 and Table 1 compare binding parameters of [^{3}H](+)-pentazocine and [^{3}H]DTG in female Hartley-Albino guinea pig and male Sprague-Dawley rat. Data were fit to both a one-site and two-site

Table 3
Inhibitory constants and Hill coefficients of the reference compounds for [^{3}H](+)-pentazocine, σ_1 and [^{3}H]DTG, σ_2 in female Hartley-Albino guinea pig brain membranes

Reference compounds	K_i (nM) [a,b]			
	[^{3}H](+)-Pentazocine	n_H	[^{3}H]DTG	n_H
Haloperidol	1.3 ± 0.5	0.97 ± 0.00	13.2 ± 1.0	0.64 ± 0.12
DTG	17 ± 1	0.98 ± 0.09	91 ± 28	0.98 ± 0.07
Dextrorphan	391 ± 29	1.06 ± 0.02	10156 ± 1147	0.73 ± 0.03 *
Dextromethorphan	270 ± 1	1.04 ± 0.07	82968 ± 9134	1.10 ± 0.08
(+)-Pentazocine	3.4 ± 0.5	0.90 ± 0.06	–	–

[a] Data from Table 3 are the means ± S.E.M. from 3–7 separate experiments performed in quadruplicate. [b] The K_i and n_H values were calculated using the LIGAND program. Asterisks indicate n_H significantly different from unity with $P < 0.01$.

model using the LIGAND program in order to determine which gave the best fit. In all experiments, the best fits were determined by a Run test showing a non-serial correlation ($P > 0.05$), plus the presence of a significant F-test ($P < 0.05$), between the more complex and the next simplest fit. [^{3}H](+)-Pentazocine bound to a single high-affinity site in both species (one-site fit is better than two-site fit, see Fig. 3A,B). The binding parameters in rat brain membranes were: $K_d = 2.5$ nM, $B_{max} = 124$ fmol/mg protein, $r = 0.93$, $n = 3$ (Fig. 3A). In guinea pig, the parameters were: $K_d = 3.4$ nM, $B_{max} = 823$ fmol/mg protein, $r = 0.98$, $n = 3$ (Fig. 3B). In both types of membranes, the K_d values of [^{3}H](+)-pentazocine were similar. However, guinea pig showed a 7-fold higher σ_1 density than rat. [^{3}H]DTG bound to two high-affinity sites (evidence of a curvilinear in both types of membranes, and two-site fit model was better than one-site fit, see Fig. 3C,D). The binding parameters in rat brain membranes were: $K_d = 9.8$ and 76.9 nM, for σ_1 and σ_2 sites respectively, corresponding $B_{max} = 263$ and 1185 fmol/mg protein, $n = 6$, $r = 0.97$ (Fig. 3C). In guinea pig, the parameters were: $K_d = 17.0$ and 91.2 nM, for σ_1 and σ_2 respectively, corresponding $B_{max} = 997$ and 2,816 fmol/mg protein, $n = 8$, $r = 0.99$ (Fig. 3D). In both types of membranes, the K_d values of [^{3}H]DTG were also similar, the ratios of σ_2/σ_1 density were also comparable (5 and 3 for rat and guinea pig, respectively).

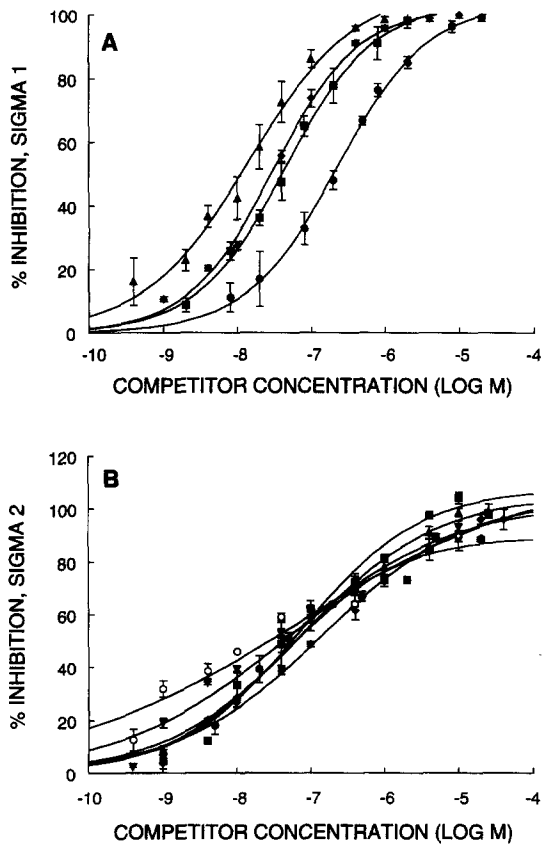

Fig. 5. Competitive inhibition of specific binding of [^{3}H](+)-pentazocine (σ_1 sites) and [^{3}H]DTG (σ_2 sites) in female Hartley-Albino guinea pig brain membranes by selected trishomocubanes. Selectivity of σ_2 sites was determined in the presence of (+)-pentazocine 0.5 mM. Data from Fig. 5 are the means ± S.E.M. from 3–7 separate experiments for each compound performed in quadruplicate. A S.E.M. which does not appear on a particular point on the curve fit, is smaller than the symbol size. Panel A: 2 nM [^{3}H](+)-pentazocine was inhibited by: (●) ANSTO-6, (■) ANSTO-10, (♦) ANSTO-13, (▲) ANSTO-14. Panel B: 10 nM [^{3}H]DTG was inhibited by: (●) ANSTO-6, (■) ANSTO-7, (♦) ANSTO-16, (▲) ANSTO-17, (▼) ANSTO-18, (○) ANSTO-19.

Inhibition curves of specific binding of [^{3}H](+)-pentazocine (Fig. 4A, σ_1 sites) and [^{3}H]DTG (Fig. 4B, σ_2 sites) in female Hartley-Albino guinea pig brain membranes by reference compounds are shown in Fig. 4. The K_i and n_H values were calculated using the LIGAND program and presented in Table 3.

Fig. 5 shows the competitive inhibition curves of specific binding of [^{3}H](+)-pentazocine (Fig. 5A, σ_1 sites) and [^{3}H]DTG in the presence of 0.5 μM (+)-pentazocine (Fig. 5B, σ_2 sites) in female Hartley-Albino guinea pig brain membranes by representative trishomocubanes. The series ANSTO-6, 10, 13, 14 are presented as σ_1-selective and the series ANSTO-6, 7, 16–19 as σ_2-selective compounds. The K_i and n_H values of the whole series, ANSTO-(1–20) were calculated using the LIGAND program and are presented in Table 2. The inhibitory constants and Hill coefficients of trishomocubanes, ANSTO-(1–20) for [^{3}H](+)-pentazocine (σ_1) and [^{3}H]DTG (σ_2), the selectivity ratio of σ_2/σ_1 were also calculated. The undecyclamine series, ANSTO-(1–5) displayed a trend of preferential selectivity for the σ_1 site, however the most potent σ_1 binding was exhibited by the dodecanes, series ANSTO-6, 10, 13, 14 which was a result of increasing the alkyl chain between the cubane moiety and the aromatic ring in their structures. ANSTO-14 displayed the highest affinity for the σ_1 site ($K_i = 9.4$ nM). The selectivity for the σ_2 site appeared in the dodecanes, series ANSTO-6, 7, 16–19 which showed a halogen *meta* substitution of the aromatic ring. ANSTO-19 displayed the highest affinity for the σ_2 site ($K_i = 19.6$ nM).

4. Discussion

Comparison of binding parameters of [^{3}H](+)-pentazocine and [^{3}H]DTG radioreceptor assays at the σ binding sites in female Hartley-Albino guinea pig and male Sprague-Dawley rat brain membranes has shown that [^{3}H](+)-pentazocine bound to a single high-affinity site with a K_d of 2.5 nM and a B_{max} of 124 fmol/mg protein in rat, and a K_d of 3.4 nM and a B_{max} of 823 fmol/mg protein in guinea pig, whereas [^{3}H]DTG was demonstrated to bind to two high-affinity sites (two-site fit is better than one-site fit model) in both types of membranes. The results showed that the K_d values of [^{3}H](+)-pentazocine and of [^{3}H]DTG are similar in both types of membranes. However, the density of σ_1 sites in guinea pig is approximately

7 times higher than that of rat. The ratio of σ_2/σ_1 density is approximately 5 in rat compared to 3 in guinea pig brain membranes. These results support previous reports that rat brains possess a higher proportion of σ_2 sites than guinea pig brains (Hellewell and Bowen, 1990; Walker et al., 1990; Leitner et al., 1994). Guinea pig brain membranes were used for the inhibition studies because they represented only a slight difference in the ratio of σ_2/σ_1 binding sites (a ratio of 3), whereas rat brain appeared to have a higher ratio of σ_2/σ_1 binding sites (a ratio of 5). Screening σ_1 binding in rat brain membranes yielded a significant standard error in our laboratory, presumably because of the abundance of σ_2 sites.

A reference compound, amantadine and 20 compounds from a series of novel trishomocubanes, were tested for their affinities at two σ binding site subtypes in guinea pig brain membranes using [^{3}H](+)-pentazocine and [^{3}H]DTG as the radioligands. All reference compounds appeared to have comparable K_i and n_H values to those reported by Rothman et al. (1991), suggesting that the binding conditions used here resolved σ_1 and σ_2 sites as described by others.

The Hill coefficients of most of the trishomocubanes and reference compounds (see Tables 2 and 3) for σ_1 sites appeared to be approximately unity ($0.8 < n_H < 1.2$; paired t-tests, $P > 0.01$) which indicate that [^{3}H](+)-pentazocine binds only to a single site population. However, the Hill coefficients of several trishomocubanes and reference compounds for σ_2 sites (Tables 2 and 3) were significantly lower than unity (< 0.8; paired t-tests, $P < 0.01$) possibly suggesting the presence of negative cooperativity, or perhaps multiple binding site interactions. Boeynaems and Dumont (1975), and Rothman et al. (1991) suggested that negative cooperativity at σ_2 sites was due to an increase in the dissociation rate of a labelled ligand produced by the addition of an unlabelled ligand in which the unlabelled ligand binds to a site not occupied by the labelled ligand and therefore induces a conformational change in binding sites occupied by the labelled ligand, producing a configuration characterised by lower affinity and an increased dissociation rate. Not all σ_2 ligands showed Hill coefficients of less than unity. Moreover, this phenomenon was not related to the dissociation constants of the ligands, nor the relative potency for σ_1 or σ_2 sites. Thus, it is not clear whether the explanation of negative cooperativity holds for all compounds.

The data shown in Table 2 also revealed that all trishomocubanes displayed moderate-to-high affinity for σ_1 and σ_2 binding sites. Selectivity and affinity of trishomocubanes for the two subtypes were affected by various structural features. All pentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecylamines screened, compounds ANSTO-(1–5), contain a secondary amine and ketal functionality and displayed preferential selectivity for the σ_1 sites. The most potent binding was exhibited by ANSTO-2 which is substituted in the *meta* position of the aromatic ring with bromine ($K_i = 17.0$ nM) while ANSTO-5 resulting from extension of the unsubstituted aromatic ring and the amine functionality by one carbon displayed equal affinity ($K_i = 15.0$ nM). The σ_1/σ_2 ratios for ANSTO-2 and ANSTO-5 were 12 and 40, respectively. 4-Azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecane derivatives contain tertiary amine and hydroxyl functionality which are a result of ketal hydrolysis of pentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecylamine compounds. This series displayed a variety of trends for binding to the σ_1 and σ_2 sites. Compound ANSTO-14 displayed the highest affinity for the σ_1 site ($K_i = 9.4$ nM) which was a result of increasing the alkyl chain between the cubane moiety and the aromatic ring in the series of compounds ANSTO-6, 10, 13, 14. Binding to σ_2 site was affected by other modifications. The series of trishomocubanes ANSTO-6, 7, 16–19 were potent at the σ_2 site with affinity in the range of 107.6–19.6 nM. All compounds were substituted in the *meta* position of the aromatic ring, as this appears important for σ_2 binding, with differences in the type of substitution. The order of highest to lowest affinity was F > Cl > Br > I > H > CH_3. Compound ANSTO-19 therefore displayed the highest affinity for the σ_2 site ($K_i = 19.6$ nM) with a σ_2/σ_1 ratio of 7.8. It is interesting to note that no significant changes were observed for binding to the σ_1 site, with all compounds of this series having K_i of around 200–150 nM.

Detailed structure-activity analyses are in progress to evaluate parameters such as changes in conformation, steric interactions, and effects of electronegativity in this series to further elaborate structure-activity relationships and develop more potent and selective σ_2 ligands. The trishomocubanes presented here may prove useful for physiological studies of σ receptor subtypes because they display a considerable range of potency and selectivity for σ_1 and σ_2 binding sites with few non-specific interactions (Kassiou et al., 1996).

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