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2(3*H*)-benzoxazolone and 2(3*H*)-benzothiazolone derivatives: Novel, potent and selective σ_1 receptor ligands

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Abstract

A series of original 2(3*H*)-benzoxazolone and 2(3*H*)-benzothiazolone derivatives were evaluated for their affinity at σ_1 and σ_2 receptor subtypes in competition binding experiments, using [^{3}H](+)-pentazocine or [^{3}H]1,3-di-*o*-tolyl-guanidine (DTG) in the presence of 100 nM (+)-*N*-allylnormetazocine (NANM) in guinea-pig brain membranes. Several of these derivatives showed preferential selectivity for σ_1 binding sites. Compound **1** [3-(1-piperidinoethyl)-6-propylbenzothiazolin-2-one] emerged as a potent σ_1 receptor ligand ($K_i = 0.6$ nM) and displayed a moderate selectivity over the σ_2 receptor subtype (K_i for σ_2/K_i for $\sigma_1 = 29$). Compounds **2** [3-(1-piperidinopropyl)-6-propanoylbenzothiazolin-2-one] and **3** [3-(1-piperidinopropyl)-6-propanoylbenzoxazolin-2-one] still showed rather high affinities for σ_1 binding sites with K_i values of 2.3 and 8.5 nM, respectively. On the contrary, they had 87- and 58-fold less affinity at σ_2 receptors, respectively. Unlike their potent affinity for σ binding sites, these compounds had negligible affinity for μ-, δ- and κ-opioid receptors, 5-HT$_2$, dopamine D$_2$, and muscarinic M$_2$ receptors. σ Receptor ligands may affect neuronal transmission and display, in animal models, antipsychotic, cognitive, motor, neuroprotective and anticonvulsant activity. Therefore, on the basis of these findings, these novel σ receptor ligands were assayed, in mice, in three tests: maximal electroshock, subcutaneous pentylenetetrazol and rotarod neurotoxicity. Compound **1**, administered intraperitoneally, was the most effective against maximal electroshock-induced seizures and was devoid of significant neurotoxic effects.

Keywords: σ Receptor; 2(3*H*)-Benzoxazolone; 2(3*H*)-Benzothiazolone; Anticonvulsant; [^{3}H](+)-Pentazocine

1. Introduction

σ Binding sites were first defined and classified by Martin et al. (1976) as being an opioid receptor subtype. Later on, it was reported that these sites were identical to phencyclidine (PCP) receptors (Zukin et al., 1984; Mendelsohn et al., 1985; Sircar et al., 1986). However, further investigations demonstrated that σ receptors were distinct from opioid (Su, 1982; Tam, 1983) and PCP receptors (Largent et al., 1984; Gundlach et al., 1985; McLean and Weber, 1988; Walker et al., 1990). It is now well established that σ receptors are heterogeneous. At least two distinct σ receptor subtypes have been pharmacologically characterized (Hellewell and Bowen, 1990; Itzhak and Stein, 1991; Quirion et al., 1992) and designated σ_1 and σ_2. Recently, a purported σ_1 receptor subtype has been purified and cloned from the guinea-pig liver (Hanner et al., 1996). Its sequence shows significant similarities with sterol C_8–C_7 isomerases from fungi. This enzyme is crucial for the biosynthesis of ergosterol which is the fungal equivalent of cholesterol.

Ligands displaying preferential affinity for the σ_1 receptor subtype are the (+)-benzomorphans such as (+)-pentazocine and (+)-*N*-allylnormetazocine (NANM) whereas haloperidol and 1,3-di-*o*-tolyl-guanidine (DTG) exhibit high affinity for both receptor subtypes (Walker et al., 1990; Quirion et al., 1992). Several authors have described novel compounds displaying high affinity for the σ_1 (Chaki et al., 1994; Hudkins et al., 1994; Matsuno et al., 1996) or for the σ_2 receptor subtype (Bowen et al., 1995). The development of a large variety of compounds of diverse chemical structures, displaying high affinities

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and selectivity for σ binding sites, has contributed to the identification of σ receptor properties and to the understanding of their possible physiological role and of their involvement in pathological conditions. σ Receptors seem to be implicated in neuroprotection (Contreras et al., 1991) by modulation of *N*-methyl-D-aspartate receptor complex (Monnet et al., 1992; Yamamoto et al., 1995), regulation of movement and postural tone (Walker et al., 1988; Matsumoto et al., 1990), modulation of immune functions (Su et al., 1988) and display antipsychotic activity (Deutsch et al., 1988; Snyder and Largent, 1989; Gilligan et al., 1994). Moreover, dextromethorphan, carbetapentane, and caramiphen which bind to σ_1 receptor subtype with high affinity (Walker et al., 1990; Karbon et al., 1991) protected rats against maximal electroshock-induced seizures, an effect which was potentiated in the presence of the anticonvulsant drug phenytoin and not associated with any cholinergic activity (Tortella et al., 1988). Additionally, it has been reported that phenytoin enhances the binding affinity of (+)-NANM and dextromethorphan for the σ_1 receptor subtype (DeHaven-Hudkins et al., 1993).

In this study, we report the synthesis and the binding profile to σ_1 and σ_2 receptor subtypes of a series of original 2(*3H*)-benzoxazolone and 2(*3H*)-benzothiazolone derivatives. Previously, Dalkara et al. (1988) have described the anticonvulsant properties of a series of 3-ethanone- and 3-ethanol-benzoxazolone derivatives. Therefore, on the basis of this report, the anticonvulsant activity of these novel compounds was evaluated by the National Institutes of Health Antiepileptic Drug Development Program. In this paper we report and analyze these data to see if there is a correlation between σ_1 affinity and in vivo anticonvulsant activity.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (300–400 g) and male Sprague–Dawley rats (250–300 g) obtained from Charles River (Calco, Como, Italy) were used. Animals were kept in air-conditioned rooms (22 ± 2°C and 60 ± 10% relative humidity) under a constant 12 h light:12 h dark cycle and received standard diet and tap water. All procedures followed the guidelines of the animal care and use committee of the University of Bologna (Bologna, Italy).

2.2. Drugs

Haloperidol, DTG, (+)-*N*-allylnormetazocine hydrochloride (NANM), methisergide maleate, [D-Ala^2,*N*-Me–Phe^4,Gly-ol^5]enkephalin (DAMGO), [D-Ala^2,D-Leu^5]enkephalin (DADLE), (±)-trans-U-50488 methanesulfonate (U-50488) and naloxone hydrochloride were purchased from Research Biochemicals International (Natick, MA, USA). [^{3}H](+)-pentazocine, [^{3}H]DTG, [^{3}H]spiroperidol, [^{3}H]ketanserin and [^{3}H]-*N*-methylscopolamine (NMS) were obtained from DuPont NEN (Milan, Italy). [^{3}H]diprenorphine was obtained from Amersham (Milan, Italy).

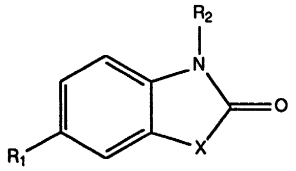
2.3. Compounds

3-(1-piperidinoethyl)-6-propylbenzothiazolin-2-one (**1**), 3-(1-piperidinopropyl)-6-propanoylbenzothiazolin-2-one (**2**), 3-(1-piperidinopropyl)-6-propanoylbenzoxazolin-2-one (**3**), 3-(1-piperidinoethyl)-6-pentanoylbenzoxazolin-2-one (**4**), 3-(1-piperidinopropyl)-6-pentanoylbenzoxazolin-2-one(**5**),3-(1-piperidinoethyl)-6-propanoylbenzothiazolin-2-one (**6**), 3-(1-piperidinoethyl)-6-pentanoylbenzothiazolin-2-one (**7**), 3-(1-pyrrolidinoethyl)-6-benzoylbenzothiazolin-2-one (**8**), 3,6-dipropanoylbenzothiazolin-2-one(**9**), 3-(1-piperidinoethyl)-6-propanoylbenzoxazolin-2-one (**10**), 3-(1-morpholinoethyl)-6-benzylbenzothiazolin-2-one (**11**), 3-(1-piperidinoethyl)-6-benzoylbenzothiazolin-2-one (**12**), 3-(dimethylaminoethyl)-6-benzoylbenzothiazolin-2-one (**13**), 3-(1-morpholinoethyl)-6-pentylbenzoxazolin-2-one (**14**), 3-(1-pyrrolidinoethyl)-6-benzoylbenzoxazolin-2-one (**15**), 3-(1-piperidinoethyl)-6-benzoylbenzoxazolin-2-one (**16**), 3-(1-pyrrolidinoethyl)-6-pentanoylbenzoxazolin-2-one (**17**), 3-(dimethylaminoethyl)-6-benzoylbenzoxazolin-2-one (**18**), 3-(1-pyrrolidinoethyl)-6-propanoylbenzothiazolin-2-one (**19**), 3-(1-pyrrolidinoethyl)-6-propanoylbenzoxazolin-2-one (**20**), 3-(dimethylaminoethyl)-6-pentanoylbenzothiazolin-2-one (**21**).

All these compounds were synthesized by standard procedures. They were characterized for purity by thin layer chromatography, and elemental microanalysis. Their structure was ascertained by proton and carbon-13 nuclear magnetic resonance. Their synthesis, analytical and spectroscopic properties will be reported elsewhere (Ucar et al., data not shown). Their chemical structures are presented in Fig. 1.

2.4. σ_1 and σ_2 receptor binding

Membranes were prepared from guinea-pig brain as described by Mach et al. (1995). Animals were decapitated, the brains rapidly removed and the crude P_2 membrane fraction was prepared. Tissue homogenisation was carried out at 4°C in 10 mM Tris–HCl/0.32 M sucrose (10 ml/g tissue weight), pH 7.4 using a Potter–Elvehjem tissue grinder. The homogenates were centrifuged at 1000 $\times g$ for 10 min and the supernatant saved on ice. The pellet was suspended in 2 ml/g tissue weight of ice-cold 10 mM Tris–HCl/0.32 M sucrose, pH 7.4. After centrifuging at 1000 $\times g$ for 10 min, the pellet was discarded and the supernatants were combined and centrifuged at 31 000 $\times g$ for 15 min. The pellet was then suspended in 3 ml/g 10 mM Tris–HCl (pH 7.4; 4°C) by vortexing and the suspension was allowed to incubate at 25°C for 15 min. Following centrifugation at 31 000 $\times g$ for 15 min, the



1, $R_1 = C_3H_7$, $R_2 = (CH)_2\text{-}C_5H_{10}N$, X = S
2, $R_1 = C_2H_5CO$, $R_2 = (CH)_3\text{-}C_5H_{10}N$, X = S
3, $R_1 = C_2H_5CO$, $R_2 = (CH)_3\text{-}C_5H_{10}N$, X = O
4, $R_1 = C_4H_9CO$, $R_2 = (CH)_2\text{-}C_5H_{10}N$, X = O
5, $R_1 = C_4H_9CO$, $R_2 = (CH)_3\text{-}C_5H_{10}N$, X = O
6, $R_1 = C_2H_5CO$, $R_2 = (CH)_2\text{-}C_5H_{10}N$, X = S
7, $R_1 = C_4H_9CO$, $R_2 = (CH)_2\text{-}C_5H_{10}N$, X = S
8, $R_1 = C_6H_5CO$, $R_2 = (CH)_2\text{-}C_4H_8N$, X = S
9, $R_1 = R_2 = C_2H_5CO$, X = S
10, $R_1 = C_2H_5CO$, $R_2 = (CH)_2\text{-}C_5H_{10}N$, X = O
11, $R_1 = C_7H_7$, $R_2 = (CH)_2\text{-}C_4H_8NO$, X = O
12, $R_1 = C_6H_5CO$, $R_2 = (CH)_2\text{-}C_5H_{10}N$, X = S
13, $R_1 = C_6H_5CO$, $R_2 = (CH)_2\text{-}C_2H_6N$, X = S
14, $R_1 = C_5H_{11}$, $R_2 = (CH)_2\text{-}C_4H_8NO$, X = O
15, $R_1 = C_6H_5CO$, $R_2 = (CH)_2\text{-}C_4H_8N$, X = O
16, $R_1 = C_6H_5CO$, $R_2 = (CH)_2\text{-}C_5H_{10}N$, X = O
17, $R_1 = C_4H_9CO$, $R_2 = (CH)_2\text{-}C_4H_8N$, X = O
18, $R_1 = C_6H_5CO$, $R_2 = (CH)_2\text{-}C_2H_6N$, X = O
19, $R_1 = C_2H_5CO$, $R_2 = (CH)_2\text{-}C_4H_8N$, X = S
20, $R_1 = C_2H_5CO$, $R_2 = (CH)_2\text{-}C_4H_8N$, X = O
21, $R_1 = C_4H_9CO$, $R_2 = (CH)_2\text{-}C_2H_6N$, X = S

Fig. 1. Chemical structure of 2(*3H*)-benzoxazolone and 2(*3H*)-benzothiazolone derivatives.

pellet was resuspended by homogenisation to 1.53 ml/g in 10 mM Tris–HCl (pH 7.4; 4°C) and aliquots were stored at −80°C until used. The protein concentration of the suspension was determined (Bradford, 1976).

σ_1 Binding sites were labelled with [^{3}H](+)-pentazocine (31.6 Ci/mmol); σ_2 binding sites were labelled with [^{3}H]DTG (35 Ci/mmol).

σ_1 Binding assay was performed as described (De-Haven-Hudkins et al., 1992). Briefly, each assay tube contained 500 μg of membrane proteins, 3 nM [^{3}H](+)-pentazocine, Tris–HCl 50 mM, pH 7.4 (37°C). Non-specific binding was determined by 10 μM haloperidol. The reaction was performed for 150 min at 37°C and terminated by rapid filtration over Whatman GF/B glass fiber filters (pre-soaked in a 0.1% polyethyleneimine solution). The value of the apparent dissociation constant (K_d) for [^{3}H](+)-pentazocine was 4.3 ± 0.8 nM ($n = 3$). Radioactivity of the filters was measured by liquid scintillation spectrometry using a Beckman LS 1701 counter after overnight incubation in scintillation cocktail.

σ_2 Binding assay was carried out using the method described by Mach et al. (1995). Membranes (360 μg of membrane proteins) were incubated with 3 nM [^{3}H]DTG ($K_d = 9.9 \pm 0.8$ nM; $n = 3$) in the presence of 100 nM (+)-NANM to mask σ_1 sites. Incubations were carried out in 50 mM Tris–HCl (pH 8.0; 25°C) for 120 min at room temperature and assays were terminated by the addition of ice-cold 10 mM Tris–HCl (pH 8.0) followed by rapid filtration through Whatman GF/B glass fibers presoaked in a 0.1% polyethyleneimine solution. Non-specific binding was determined in the presence of 5 μM DTG.

2.5. Binding assay to κ-, μ- and δ-opioid receptors

To evaluate the specific binding to κ sites, a crude membrane fraction from guinea-pig cerebella was prepared as described (Gillan et al., 1980). Aliquots (800 μg/ml of membrane proteins) were incubated at 25°C for 60 min with 5 nM [^{3}H]diprenorphine ($K_d = 0.55 \pm 0.7$ nM; $n = 5$) and various concentrations of test compounds. Binding studies were performed in the presence of DAMGO (300 nM) and DADLE (300 nM) to eliminate the interaction with μ and δ receptors, respectively. Non-specific binding was determined by the addition of U50488 (1 μM).

Binding to μ and δ sites was carried out on crude membrane fraction obtained from the whole rat brain minus cerebellum as previously reported (Gillan et al., 1980). To evaluate the specific binding to μ sites, displacement of the binding of [^{3}H]diprenorphine (1 nM; Amersham) was measured by the addition of U50488 (300 nM) and of DADLE (300 nM) added to saturate the κ and δ receptors, respectively (the K_d of the radioligand was 0.22 ± 0.03 nM; $n = 5$). Non-specific binding was determined by the addition of naloxone (10 μM). To evaluate the specific binding to δ sites, the displacement of the binding of [^{3}H]diprenorphine (the K_d of the radioligand was 0.44 ± 0.03 nM; $n = 5$) was measured in the presence of DAMGO (300 nM) and U50488 (300 nM) added to saturate the κ- and μ-opioid receptors, respectively. Binding assays were carried out as previously referred. Non-specific binding was determined in the presence of DADLE (10 μM).

2.6. Binding assays to dopamine D_2, muscarinic M_2 and 5-HT_2 receptors

Dopamine D_2 receptor binding assay was performed using 0.5 nM [^{3}H]spiroperidol (18.5 Ci/mmol; $K_d = 1.98 \pm 0.4$ nM; $n = 3$) and rat striatal membranes according to Briley and Langer (1978). Non-specific binding assay was measured in the presence of 10 μM haloperidol.

Muscarinic M_2 receptor binding assay was performed using [^{3}H]NMS (79.5 Ci/mmol; $K_d = 0.48 \pm 0.03$ nM; $n = 3$) and rat heart homogenates according to the procedure described by Waelbroeck et al. (1986). Non-specific binding was assessed in the presence of 10 μM atropine.

5-HT_2 receptor binding assay was performed using 1 nM [^{3}H]ketanserin (77.1 Ci/mmol; $K_d = 1.49 \pm 0.4$ nM; $n = 3$) and rat frontal cortex membranes according to the procedure described by Tam et al. (1992). Non-specific binding was measured in the presence of 1 μM methisergide.

2.7. In vivo tests

Maximal electroshock test, subcutaneous pentylenetetrazol test and rotarod neurotoxicity test were carried out by the Anticonvulsant Drug Development Program, Epilepsy Branch, National Institutes of Health (Bethesda, MD, USA) (Krall et al., 1978; Porter et al., 1984). All compounds were tested for anticonvulsant activity with male Carworth Farms #1 mice in 18–25 g weight range. Each compound was administered i.p. to mice at three or sometimes four dose levels (10–300 mg/kg). The compounds were suspended in 0.5% methylcellulose.

Maximal electroshock seizures were induced 30 min after drug treatment by application of a 60 Hz current of 50 mA for 0.2 s via corneal electrodes into the eyes. The protection was defined as the abolition of hind-leg tonic maximal extension component of the seizure. The subcutaneous pentylenetetrazol (Metrazol) seizure threshold test was carried out by an i.p. administration of 85 mg/kg of penthylenetetrazol. Mice were observed over 30 min. Failure to observe the generalized clonic seizures was defined as protection. Locomotor incapacitation was measured by the rotarod test. Mice were placed on a 1 inch diameter knurled plastic rod rotating at 6 rpm after the administration of the drug and their ability to maintain the equilibrium was evaluated. Locomotor deficit was indicated by the inability of the animal to maintain its equilibrium for 1 min on the rotating rod in each of three trials. All the compounds were administered 30 min or 4 h prior to perform the tests.

2.8. Data analysis

Scatchard parameters and inhibition constants (K_i values) were calculated using the EBDA (McPherson, 1985) and LIGAND (Munson and Rodbard, 1980) programs. The results are expressed as mean ± S.E.M. All assays were done in duplicate and for each compound at least three independent experiments were done.

3. Results

The affinity of a new series of 2(*3H*)-benzoxazolone and 2(*3H*)-benzothiazolone derivatives for σ_1 and σ_2 binding sites was determined in competition binding experiments by using [^{3}H](+)-pentazocine and [^{3}H]DTG, respectively. Binding constants (K_i values) are presented in Table 1. Several of these derivatives were found to have preferential affinities for σ_1 sites. Compound **1** was the most potent σ_1 receptor ligand ($K_i = 0.6$ nM) with a selectivity (K_i for σ_2/K_i for σ_1) of 29. The affinity of compound **1** at σ_1 binding sites is almost 3-fold greater than that of the reference σ receptor ligands haloperidol, (+)-pentazocine, *N,N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride (NE-100) and 1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine dihydrochloride (SA4503) (Table 1). Compounds **2** and **3** interacted with σ_1 displaying K_i values of 2.3 and 8.5 nM, respectively. Compound **2** had a 87-fold greater affinity for σ_1 sites than for σ_2 binding sites. Compound **3** had a 58-fold greater affinity for σ_1 sites as compared to σ_2 binding sites. Compound **12** showed moderate affinity ($K_i = 83.4$ nM) for σ_1 binding sites with a rather good selectivity (Table 1). Compounds **13** and **18** did not bind significantly to σ_2 receptor subtype (Table 1). Competition binding curves of haloperidol and of compounds **1**, **2** and **3** to σ_1 and σ_2 receptor subtypes are represented in Fig. 2.

Table 1
Affinities of 2(*3H*)-benzoxazolone and 2(*3H*)-benzothiazolone derivatives for σ receptor subtypes

Compound	K_i (nM)		$K_i(\sigma_2)/K_i(\sigma_1)$
	σ_1	σ_2	
1	0.6 ± 0.3	18.1 ± 6.2	29
2	2.3 ± 0.3	202 ± 18	87
3	8.5 ± 0.5	496 ± 50	58
4	23 ± 3	84 ± 9	4
5	40 ± 3	155 ± 13	4
6	47 ± 5	481 ± 21	10
7	49 ± 4	232 ± 35	5
8	54 ± 3	225 ± 16	4
9	63 ± 4	670 ± 40	11
10	72 ± 6	900 ± 42	13
11	72 ± 6	515 ± 25	7
12	83 ± 7	1906 ± 257	23
13	105 ± 6	> 10000	> 95
14	177 ± 21	966 ± 93	5
15	185 ± 10	125 ± 11	0.7
16	236 ± 25	780 ± 69	3
17	323 ± 20	685 ± 50	2
18	365 ± 15	> 10000	> 27
19	571 ± 42	919 ± 87	1.6
20	617 ± 32	494 ± 33	0.8
21	628 ± 40	4612 ± 525	7
Haloperidol	1.6 ± 0.3	13 ± 2	8
(+)-pentazocine [a]	2.1	1348	642
NE-100 [b]	1.5	85	57
SA4503 [c]	17.4	1784	103

Affinity constants (K_i) values are the mean ± S.E.M. of three separate experiments, each carried out in duplicate. A one-site model was the best fit to all curves. All Hill coefficients were not significantly different from unity ($P > 0.05$). σ_1 Binding assays were performed in guinea-pig brain using [^{3}H](+)-pentazocine. σ_2 Binding assays were determined in guinea-pig brain using [^{3}H]DTG in the presence of an excess of (+)-NANM to mask σ_1 binding sites.
[a] Taken from Hudkins et al. (1994).
[b] Taken from Chaki et al. (1994).
[c] Taken from Matsuno et al. (1996).

The selectivity of the first three compounds for the σ binding sites was examined and these substances were found to have no apparent affinity at 5-HT$_2$, dopamine D$_2$, muscarinic M$_2$, and μ-, δ- and κ-opioid receptors (all K_i values were over 10 μM).

Anticonvulsant and locomotor screening data in mice

were provided by the Antiepileptic Drug Development (ADD) Program, Epilepsy Branch, Neurological and Developmental Disorders. The data of the most effective compounds (administered i.p. to mice) are shown in Table 2. These new σ_1 receptor ligands were effective, injected 30 min before performing the test, against maximal electroshock-induced seizures while they were ineffective against subcutaneous pentylenetetrazol-induced seizures. In this regard, their pharmacological profile is similar to phenytoin and carbamazepine (Krall et al., 1978). Compound **1** was the most potent anti-maximal electroshock compound of this series; it protected 50% of treated mice against maximal electroshock-induced seizures when administered at the dose of 10 mg/kg whereas it induced locomotor incapacitation (as determined by the ability of mice to maintain their position in the rotarod test) in 50% of tested mice when injected at the dose of 30 mg/kg. In the maximal electroshock test, compound **6** displayed significant protection against convulsions (100% of mice were protected at the dose of 30 mg/kg) while it induced locomotor incapacitation in 50% of treated mice when injected at the dose of 300 mg/kg. Compound **9** was found to be active against maximal electroshock-induced seizures (25% of treated mice were protected against seizures at the dose of 10 mg/kg) and induced locomotor incapacitation only at a much higher dose (50% of mice displayed locomotor deficit at the dose of 300 mg/kg). Compounds **7**, **8**, **14**, **19** and **21** protected against maximal electroshock-induced seizures at the dose of 30 mg/kg whereas motor incapacitation was observed at the dose of 100 mg/kg (data not shown). Compounds **3**, **4**, **10**, **11**, **15**, **17** and **18** displayed anticonvulsant activity in the maximal electroshock test, concomitantly with significant rotarod ataxia, only after the i.p. injection of higher doses (100 and 300 mg/kg; data not shown). Compounds **2**, **12**, **13**, **16**, and **20** were not tested.

Table 2
Anticonvulsant and locomotor screening data in mice [a]

Compd.	Dose (mg/kg)	MES [b]		scMet [c]		Rotarod test [d]	
		30 min	4 h	30 min	4 h	30 min	4 h
1	10	2/4	nd	nd	nd	0/4	0/4
	30	1/1	1/1	0/1	0/1	2/4	0/4
	100	1/1	0/1	0/1	0/1	8/8	0/1
6	30	1/1	0/1	0/1	0/1	0/4	0/2
	100	3/3	0/3	0/1	0/1	0/8	0/4
	300	1/1	1/1	0/1	0/1	2/4	0/2
9	10	1/4	nd	nd	nd	0/4	nd
	30	1/1	0/1	0/1	0/1	0/4	0/2
	100	3/3	0/3	0/1	0/1	0/8	0/4
	300	1/1	0/1	0/1	0/1	2/4	0/2

nd, not determined.
[a] Test compounds were administered intraperitoneally.
[b] Maximal electroshock test (number of animals protected/number of animals tested).
[c] Subcutaneous pentylenetetrazol test.
[d] Rotarod test (number of animals fallen off the rotarod/number of animals tested).

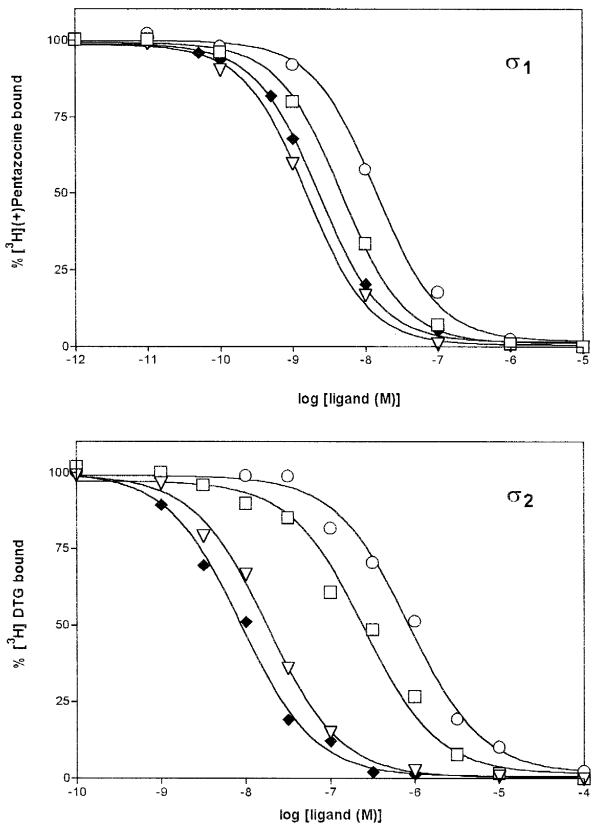


Fig. 2. Competition binding curves of selected ligands against [^{3}H](+)-pentazocine (σ_1) and [^{3}H]DTG (σ_2) in guinea-pig brain membranes. Binding affinities to σ_1 and σ_2 binding sites were assessed with 3 nM [^{3}H](+)-pentazocine or 3 nM [^{3}H]DTG with 100 nM (+)NANM, respectively. Each data point represents the mean of two determinations. The curves are from a representative experiment which has been repeated at least three times. Key: (◆) haloperidol; (▽) compound 1; (□) compound 2; (○) compound 3.

4. Discussion

Despite the purification and molecular cloning of σ_1 binding sites (Hanner et al., 1996), the functional role of σ receptors remains still unknown and this is due, in part, to the lack of selective σ receptor ligands. Radioligand binding assays carried out on this novel class of compounds revealed that compounds **1**, **2**, and **3** appear to be highly potent and selective for σ receptors. Indeed, they did not bind to 5-HT$_2$, dopamine D$_2$, muscarinic M$_2$, and μ-, δ- and κ-opioid receptors. Compound **1** is among the most potent σ receptor ligands known up to date, with a good selectivity for the σ_1 receptor subtype. Indeed, this compound is more potent than haloperidol, (+)-pentazocine, SA4503 (Matsuno et al., 1996) and NE-100 (Chaki et al., 1994) for the binding to the σ_1 receptor subtype and

more selective of the potent σ_1 ligand [2-(4-phenylpiperidinyl)ethyl 1-(4-nitrophenyl)cyclopentanecarboxylate HCl] (RLH-033; $K_i = 0.05$ nM) which also binds to dopamine D_2 and muscarinic M_2 receptors (Hudkins et al., 1994). Compounds **2** and **3** are interesting regarding their affinity and selectivity for σ_1 binding sites. Compound **2** has a high affinity for σ_1 receptor subtype with almost the same potency as haloperidol and NE-100. However, its selectivity for σ_1 binding sites is greater than that of the above prototype σ_1 ligands. In fact, haloperidol displays high affinity for dopamine D_2 receptors while NE-100 has a moderate affinity for σ_2 binding sites ($K_i = 85$ nM; Chaki et al., 1994). Therefore, compound **2** could be used as a σ receptor probe to investigate the structure and possible functions of σ receptors.

In preliminary studies, we have observed that several compounds which display high affinity for σ_1 receptors possess in vivo anticonvulsant activity whereas rotarod ataxia is observed when these compounds are administered at higher doses. Compound **1** which is the most potent σ_1 receptor ligand of this novel series, is also the most active anticonvulsant compound in the maximal electroshock test. In addition, compounds **6** and **9** which bind to σ_1 receptor subtype in the nanomolar range, exhibit a high degree of protection against seizures induced by maximal electroshock. It has been reported that σ receptor ligands induce stereotyped behavior and locomotor hyperactivity, which are often used as an indication of 'psychotic behavior' (Contreras et al., 1986; Marquis et al., 1989); however, they also cause impairment of motor coordination which must be taken into account when interpreting behavioral effects (Jerram et al., 1966; Walker et al., 1993; Carter, 1994). Therefore, motor incapacitation observed after the administration of these novel compounds could be related to their interaction with σ receptor. Interestingly, the most active anticonvulsant compounds induce rotarod ataxia only at higher doses. Taken together, present data add more evidence to the hypothesis that σ_1 receptor ligands may represent a novel class of anticonvulsant compounds (Tortella et al., 1988).

In conclusion, this study reports the synthesis of a new class of potent and selective σ receptor ligands which bind preferentially at σ_1 receptor subtype. Some in vivo observations revealed that several of these potent σ_1 receptor ligands have significant anticonvulsant activity against maximal electroshock-induced seizures in mice.

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References

Bowen, W.D., Bertha, C.M., Vilner, B.J., Rice, K.C., 1995. CB-64D and CB-184: Ligands with high σ_2 receptor affinity and subtype selectivity. Eur. J. Pharmacol. 278, 257–260.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72, 248–254.

Briley, M., Langer, S.Z., 1978. Two binding sites for [^{3}H]-spiroperidol on rat striatal membranes. Eur. J. Pharmacol. 50, 283–284.

Carter, A.J., 1994. Many agents that antagonize the NMDA receptor-channel complex in vivo also cause disturbances of motor coordination. J. Pharmacol. Exp. Ther. 269, 573–580.

Chaki, S., Tanaka, M., Muramatsu, M., Otomo, S., 1994. NE-100, a novel potent σ_1 binding sites in guinea pig brain. Eur. J. Pharmacol. 251, R1–R2.

Contreras, P.C., Rice, K.C., Jacobson, A., O'Donohue, T.L., 1986. Stereotyped behavior correlates better than ataxia with phencyclidine-receptor interactions. Eur. J. Pharmacol. 121, 9–18.

Contreras, P.C., Ragan, D.M., Bremer, M.E., Lanthorn, T.H., Gray, N.M., Iyengar, S., Jacobson, A.E., Rice, K.C., De Costa, B.R., 1991. Evaluation of U50,488H analogs for neuroprotective activity in the gerbil. Brain Res. 546, 79–82.

Dalkara, S., Calis, U., Sunal, R., 1988. Synthesis and anticonvulsant activity of some new 2(*3H*)-benzoxazolone derivatives. J. Pharm. Belg. 43, 372–378.

DeHaven-Hudkins, D.L., Fleissner, C., Ford-Rice, F.Y., 1992. Characterization of the binding of [^{3}H] (+)-pentazocine to σ recognition sites in guinea pig brain. Eur. J. Pharmacol. 227, 371–378.

DeHaven-Hudkins, D.L., Ford-Rice, F.Y., Allen, J.T., Hudkins, R.L., 1993. Allosteric modulation of ligand binding to [^{3}H](+)-pentazocine-defined sigma recognition sites by phenytoin. Life Sci. 53, 41–48.

Deutsch, S.I., Weizman, A., Goldman, M.E., Morihasa, J.M., 1988. The sigma receptor: A novel site implicated in psychosis and antipsychotic drug efficacy. Clin. Neuropharmacol. 11, 105–119.

Gillan, M.G.C., Kosterlitz, H.W., Paterson, S.J., 1980. Comparison of the binding characteristics of tritiated opiates and opioid peptides. Br. J. Pharmacol. 70, 481–490.

Gilligan, P.J., Kergaye, A.A., Lewis, B.M., McElroy, J.F., 1994. Piperidinyltetralin sigma ligands. J. Med. Chem. 37, 364–370.

Gundlach, A.L., Largent, B.L., Snyder, S.H., 1985. Phencyclidine and sigma opiate receptors in brain: Biochemical and autoradiographic differentiation. Eur. J. Pharmacol. 113, 465–466.

Hanner, M., Moebius, F.F., Flandorfer, A., Knaus, H.G., Striessnig, J., Kempner, E., Glossmann, H., 1996. Purification, molecular cloning, and expression of the mammalian sigma$_1$-binding site. Proc. Natl. Acad. Sci. USA 93, 8072–8077.

Hellewell, S.B., Bowen, W.D., 1990. A sigma-like binding site in rat pheochromocytoma (PC12) cells: Decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain. Brain Res. 527, 244–253.

Hudkins, R.L., Mailman, R.B., DeHaven-Hudkins, D.L., 1994. RLH-033, a novel, potent and selective ligand for the σ_1 recognition sites. Eur. J. Pharmacol. 271, 235–236.

Itzhak, Y., Stein, I., 1991. Regulation of σ receptors and responsiveness to guanine nucleotides following repeated exposure of rats to haloperidol: Further evidence for multiple σ binding sites. Brain Res. 566, 166–172.

Jerram, A.H., Smith, P.F., Darlington, C.L., 1966. The effects of (+)-SKF10047 and ketamine hydrochloride on stereotyped behavior, locomotor activity and ataxia in guinea pig. Eur. J. Pharmacol. 307, 269–273.

Karbon, E.W., Naper, K., Pontecorvo, M.J., 1991. [^{3}H]DTG and [^{3}H]-3-PPP label pharmacologically distinct σ binding sites in guinea pig brain membranes. Eur. J. Pharmacol. 193, 21–27.

Krall, R.L., Penry, J.K., White, B.G., Kupferberg, H.J., Swinyard, E.A., 1978. Antiepileptic drug development: II. Anticonvulsant drug screening. Epilepsia 19, 400–428.

Largent, B.L., Gundlach, A.L., Snyder, S.H., 1984. Psychomimetic opiate receptors labeled and visualized with (+)-[^{3}H]3-3(3-hydroxyphenyl)-*N*-(1-propyl) piperidine. Proc. Natl. Acad. Sci. USA 81, 4983–4987.

Mach, R.H., Smith, C.R., Childers, S.R., 1995. Ibogaine possesses a selective affinity for σ_2 receptors. Life Sci. 57, PL57–PL62.

Marquis, K.L., Paquette, N.C., Gussio, R.P., Moreton, J.E., 1989. Comparative electroencephalographic and behavioral effects of phencyclidine, (+)-SKF-10,047 and MK-801 in rats. J. Pharmacol. Exp. Ther. 251, 1104–1112.

Martin, W.R., Eades, C.E., Thompson, J.A., Huppler, R.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., Hemstreet, M.K., Lai, N., Thurkauf, A., De Costa, B.R., Rice, K.R., Hellewell, S.B., Bowen, W.D., Walker, J.M., 1990. Drug specificity of pharmacological dystonia. Pharmacol. Biochem. Behav. 36, 151–155.

Matsuno, K., Nakazawa, M., Okamoto, K., Kawashima, Y., Mita, S., 1996. Binding properties of SA4503, a novel and selective σ_1 receptor agonist. Eur. J. Pharmacol. 306, 271–279.

McLean, S., Weber, E., 1988. Autoradiographic visualization of haloperidol sensitive sigma receptors in guinea-pig-brain. Neuroscience 25, 259–269.

McPherson, G.A., 1985. Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. J. Pharmacol. Methods 14, 213–228.

Mendelsohn, L.G., Kalra, V., Johnson, B.G., Kerchner, G.A., 1985. Sigma opioid receptor: Characterization and co-identity with the phencyclidine receptor. J. Pharmacol. Exp. Ther. 233, 597–602.

Monnet, F.P., Debonnel, G., de Montigny, C., 1992. In vivo electrophysiological evidence for a selective modulation of *N*-methyl-D-aspartate-induced neuronal activation in rat CA_3 dorsal hippocampus by sigma ligands. J. Pharmacol. Exp. Ther. 261, 123–130.

Munson, P.J., Rodbard, D., 1980. LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107, 220–239.

Porter, R.J., Cereghino, J.J., Gladding, G.D., Hessie, B.J., Kupferberg, H.J., Scoville, B., White, B.G., 1984. Antiepileptic drug development program. Clevel. Clin. Q. 51, 293–305.

Quirion, R., Bowen, W.D., Itzhak, Y., Junien, J.L., Musacchio, J.M., Rothman, R.B., Su, T.P., Tam, S.W., Taylor, D.P., 1992. A proposal for the classification of sigma binding sites. Trends Pharmacol. Sci. 13, 85–86.

Sircar, R., Nichtenhauser, R., Ieni, J.R., Zukin, S.R., 1986. Characterization and autoradiographic visualisation of (+)-[^{3}H]SKF 10,047 binding in rat and mouse brain: Further evidence for phencyclidine/'sigma opiate' receptor commonality. J. Pharmacol. Exp. Ther. 237, 681–688.

Snyder, S.H., Largent, B.L., 1989. Receptor mechanisms in antipsychotic drug action: Focus on sigma receptors. J. Neuropsychiatry 1, 7–15.

Su, T.P., 1982. Evidence for sigma opioid receptor: Binding of [^{3}H]-SKF-10047 to etorphine-inaccessible sites in guinea pig brain. J. Pharmacol. Exp. Ther. 223, 284–290.

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

Tam, S.W., 1983. Naloxone-inaccessible sigma receptor in rat central nervous system. Proc. Natl. Acad. Sci USA 80, 6703–6707.

Tam, S.W., Steinfels, G.F., Gillan, P.J., Schmidt, W.K., Cook, L., 1992. Dup 734 [1-(cyclopropyl-methyl)-4-(2′(4″-fluorophenyl)2′-oxoethyl)-piperidine HBr]. A sigma and 5-Hydroxytriptamine$_2$ receptor antagonist: Receptor binding, electrophysiological and neuropharmacological profiles. J. Pharmacol. Exp. Ther. 263, 1167–1174.

Tortella, F.C., Witkin, J.M., Musacchio, J.M., 1988. Caramiphen: A non-opioid antitussive with potent anticonvulsant properties in rats. Eur. J. Pharmacol. 155, 69–75.

Waelbroeck, M., Gillard, M., Robberecht, P., Christophe, J., 1986. Kinetic studies of [^{3}H]-N-Methylscopolamine binding to muscarinic receptors in the rat central nervous system: Evidence for the existence of three classes of binding sites. Mol. Pharmacol. 30, 305–314.

Walker, J.M., Matsumoto, R.R., Bowen, W.D., Gans, D.L., Jones, K.D., Walker, F.O., 1988. Evidence for a role of haloperidol-sensitive sigma opiate receptors in the motor effects of antipsychotic drugs. Neurology 38, 961–965.

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

Walker, J.M., Bowen, W.D., Patrick, S.L., Williams, W.L., Mascarella, S.W., Bai, X., Carroll, F.I., 1993. A comparison of (−)-deoxybenzomorphans devoid of opiate activity with their dextrorotatory phenolic counterparts suggests a role of σ_2 receptors in motor function. Eur. J. Pharmacol. 231, 61–68.

Yamamoto, H., Yamamoto, T., Sagi, N., Klenerova, V., Goji, K., Kawai, N., Baba, A., Takamori, E., Moroji, T., 1995. Sigma ligands indirectly modulate the NMDA receptor-ion channel complex on intact neuronal cells via σ_1 site. J. Neurosci. 15, 731–736.

Zukin, S.R., Brady, K.T., Slifer, B.L., Balster, R.L., 1984. Behavioral and biochemical stereo-selectivity of sigma opiate/PCP receptors. Brain Res. 294, 174–177.