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Characterization of two novel σ receptor ligands: antidystonic effects in rats suggest σ receptor antagonism

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Abstract

The novel σ receptor ligands, *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine (BD1047) and 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine (BD1063), were characterized in rats using binding assays and behavioral studies. In radioligand binding studies, the novel ligands showed marked selectivity for σ binding sites, generally having a 100-fold or better affinity for σ sites compared to nine other tested receptors (opiate, phencyclidine, muscarinic, dopamine, α_1-, α_2-, β-adrenoceptor, 5-HT_1, 5-HT_2); the only exception was the affinity of BD1047 for β-adrenoceptors. Competition assays further revealed that the drugs interacted with both σ_1 and σ_2 binding sites. Although both drugs had preferential affinities for σ_1 sites, BD1047 exhibited a higher affinity for σ_2 sites than BD1063. In behavioral studies, BD1047 and BD1063 had no effects on their own when unilaterally microinjected into the red nucleus of rats, but both compounds attenuated the dystonia produced by the high affinity σ ligands, di-*o*-tolylguanidine (DTG) and haloperidol. BD1047 and BD1063 dose-dependently attenuated the dystonia produced by DTG, suggesting a receptor-mediated mechanism, and the dose curve for DTG was shifted to the right in the presence of the novel ligands. BD1047 and BD1063 appear to act as antagonists at σ sites and may represent promising new tools for probing other functional effects associated with σ binding sites.

Keywords: Dystonia; Haloperidol; σ Receptor; Red nucleus; DTG (di-*o*-tolylguanidine)

1. Introduction

σ Binding sites are non-opiate, non-phencyclidine, non-dopamine receptors with a unique drug selectivity pattern and anatomical distribution in the brain (Gundlach et al., 1986; Quirion et al., 1992; Tam and Cook, 1984; Walker et al., 1990). Proteins with the characteristics of σ binding sites have been solubilized and purified (Kavanaugh et al., 1989; McCann and Su, 1991; Schuster et al., 1994). The physiological relevance of these sites is supported by brain extracts with σ activity, suggesting the existence of endogenous σ receptor ligands (Connor and Chavkin, 1991, 1992; Contreras et al., 1987; Su and Vaupel, 1988; Su et al., 1986). Further support for the biological relevance of σ binding sites comes from correlations between σ binding affinities and the efficacies or potencies of ligands in a number of functional systems: inhibition of agonist- and electrically stimulated contractions in the guinea pig ileum/myenteric plexus (Campbell et al., 1987), inhibition of a tonic K^+ current in whole-cell patch recordings from NCB-20 cells (Wu et al., 1991), potentiation of muscle contractions in the guinea pig vas deferens (Vaupel and Su, 1987; Wu et al., 1991), inhibition of agonist-stimulated phosphoinositide turnover (Bowen et al., 1992a), and the ability to produce dystonic postures and circling behavior after microinjection (Matsumoto et al., 1990; Walker et al., 1993).

Nevertheless, there is still some controversy regarding the physiological significance of σ binding sites, contributed in part, by the lack of pharmacological tools, such as selective antagonists, to facilitate func-

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tional studies. Therefore, we began screening novel σ ligands in an effort to identify functional or competitive antagonists. We recently identified two potential ligands, *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine (BD1047) and 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine (BD1063), using the in vitro turtle brain preparation (Matsumoto et al., 1992). In the studies herein, we tested the ability of these novel compounds to attenuate the dystonic postures produced by the prototypic σ ligands, di-*o*-tolylguanidine and haloperidol, following unilateral microinjection into the red nucleus of rats. This behavior is one of the few in vivo effects reported to date where a correlation has been found between σ binding and the ED_{50} for producing the behavior (Matsumoto et al., 1990). Furthermore, because the red nucleus is rich in σ binding sites, but virtually devoid of other receptors with which non-selective σ ligands interact (e.g. the red nucleus contains almost no dopamine, adrenergic, phencyclidine, 5-HT, or opiate receptors), it provides a permissive environment for studying the functional effects of σ ligands with minimal interference from other neurochemical systems (Boyson et al., 1986; Gundlach et al., 1986; Jones and Palacios, 1991; Mansour et al., 1987; McLean and Weber, 1988; Pazos and Palacios, 1985; Quirion et al., 1981; Sircar and Zukin, 1988).

To evaluate the selectivities of the novel ligands, the preferential affinities of the compounds for σ binding sites as compared to nine other receptors (opiate, phencyclidine, muscarinic, dopamine, α_1-, α_2-, β-adrenoceptor, 5-HT_1, 5-HT_2) were determined in the rat brain. To further evaluate the affinities of BD1047 and BD1063 for σ subtypes, radioligand binding assays were performed in guinea pig brain and rat liver. These tissues are rich in σ_1 and σ_2 binding sites respectively, and the assays were performed under conditions which selectively label these subtypes (Bowen et al., 1993; Hellewell et al., 1994; Leitner et al., 1994).

2. Materials and methods

2.1. Drugs

[^{3}H]Di-*o*-tolylguanidine (DTG, 52.3 Ci/mmol), [^{3}H](−)-sulpiride (71.4 Ci/mmol), [^{3}H]TCP (49 Ci/mmol), [^{3}H]pirenzipine (70.4 Ci/mmol), [^{3}H]prazosin (79.2 Ci/mmol), [^{3}H]clonidine (57.83 Ci/mmol), [^{3}H]ketanserin (60 Ci/mmol), [^{3}H]dihydroalprenolol (DHA, 105.5 Ci/mmol), and [^{3}H]5-HT (26.4 Ci/mmol) were obtained from Dupont/New England Nuclear (Boston, MA, USA). [^{3}H]Etorphine (39 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, USA). [^{3}H](+)-Pentazocine (51.7 Ci/mmol) was synthesized as described elsewhere (Bowen et al., 1993; De Costa et al., 1989).

1,3-Di-*o*-tolylguanidine (DTG) was obtained from Aldrich (Milwaukee, WI, USA). BD1047 (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine) and BD1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine) were synthesized as described elsewhere (De Costa et al., 1992,1993). Haloperidol and 5-HT were purchased from Sigma (St. Louis, MO, USA). Prazosin, mianserin, (±)-quinuclidinyl benzilate, yohimbine, pargyline, and propranolol were obtained from Research Biochemicals International (Natick, MA, USA). Cyclazocine, (+)-pentazocine, dextrallorphan, and levallorphan were provided by the National Institute on Drug Abuse (Rockville, MD, USA) or synthesized in the laboratory of Dr. Kenner C. Rice (NIDDK, Bethesda, MD, USA).

2.2. Ligand binding assays

Membrane preparation

Rats were decapitated and the brains and livers rapidly removed. Crude P_2 membrane fractions were prepared from these tissues as well as from frozen guinea pig brain (Pel-Freeze, Rogers, AK, USA). Both rat and guinea pig brains were processed without cerebella as previously described (Bowen et al., 1993; Matsumoto et al., 1990). Tissues were homogenized in ice-cold 10 mM Tris-sucrose buffer (0.32 M sucrose in 10 mM Tris-HCl, pH 7.4) in a volume of 10 ml/g wet tissue weight using a Potter-Elvehjem homogenizer and 6–10 strokes of a motor-driven Teflon pestle. The homogenates were centrifuged at 4° C at $1000 \times g$ for 10 min and the supernatants saved. The pellets were resuspended in 2 ml/g Tris-sucrose buffer and centrifuged at 4° C at $1000 \times g$ for 10 min. The supernatants from both $1000 \times g$ spins were combined and centrifuged at 4° C at $31\,000 \times g$ for 15 min. The pellets were resuspended in 10 mM Tris-HCl, pH 7.4 in a volume of 3 ml/g and allowed to incubate for 30 min at 25° C. Following the incubation, the suspensions were centrifuged at 4° C at $31\,000 \times g$ for 15 min. The pellets were resuspended in 10 mM Tris-HCl, pH 7.4 in a final volume of 1.53 ml/g tissue. The suspensions were hand homogenized with five strokes in a Potter-Elvejhem homogenizer and the aliquots stored at −80° C until use. Protein content was determined using the method of Lowry et al. (1951).

Comparison of σ binding to other receptors in rat brain

To characterize the specificity of BD1047 and BD1063 for σ sites, binding studies were performed on the P_2 fraction of rat brain minus cerebellum. The preparation of the membranes and assay conditions were as previously described (Matsumoto et al., 1990). To measure affinities at σ binding sites, membranes

(500 μg membrane protein) were incubated with 3 nM [^{3}H]DTG and one of 12 concentrations (0.05–10 000 nM) of either BD1047 or BD1063. Incubations were carried out in a total volume of 0.5 ml of 50 mM Tris-HCl, pH 8.0 for 120 min. Non-specific binding was determined in the presence of 10 μM haloperidol. Assays were terminated with 5 ml ice-cold 10 mM Tris-HCl, pH 8.0 and vacuum filtration through glass fiber filters (Schleicher and Schuell, Keene, NH, USA) that were soaked in 0.5% polyethyleneimine for at least 30 min prior to use.

In addition, the affinities of BD1047 and BD1063 for the following nine receptors were measured: opiate, phencyclidine, dopamine, muscarinic M_1, α_1-, α_2-, β-adrenoceptors, 5-HT_1, 5-HT_2. These receptors were chosen for the specificity experiments because the chemical structures of BD1047 and BD1063 are related to or derived from compounds that interact with the receptors in question. In initial experiments, competing concentrations of 10 000 and 100 000 nM of BD1047 or BD1063 were used. If there was greater than 30% displacement of radioligand binding at the 10 000 nM concentration, then competition curves (concentration range 1–10 000 nM) were constructed. A modification of previously described procedures was used (Cash et al., 1975; De Costa et al., 1992; Hoyer et al., 1985; Oshita et al., 1991). The data were analyzed using the iterative curve fitting program CDATA (EMF Software, Baltimore, MD, USA) or GraphPad InPlot (San Diego, CA, USA).

Binding to σ subtypes

The affinities of BD1047 and BD1063 for σ_1 and σ_2 binding sites were determined under conditions which selectively label each of the putative σ subtypes (Bowen et al., 1993; Hellewell et al., 1994; Quirion et al., 1992). Affinities of the ligands for σ_1 sites were determined in guinea pig brain using [^{3}H](+)-pentazocine. Briefly, membranes (500 μg membrane protein) were incubated with 3 nM [^{3}H](+)-pentazocine and one of 12 concentrations (0.05–10 000 nM) of either BD1047 or BD1063. Incubations were carried out in a total volume of 0.5 ml of 50 mM Tris-HCl, pH 8.0 for 120 min. Non-specific binding was determined in the presence of 10 μM haloperidol or (+)-pentazocine. Incubations were terminated with 5 ml ice-cold 10 mM Tris-HCl, pH 8.0 and vacuum filtration through glass fiber filters (Schleicher and Schuell, Keene, NH, USA) that were soaked in 0.5% polyethyleneimine for at least 30 min prior to use. After termination of the incubation, the filters were then washed twice with ice-cold buffer. Scintillation counting was performed after overnight extraction of the counts from the filters using Ecoscint cocktail (National Diagnostics, Manville, NJ, USA). The data were analyzed using the iterative curve fitting program CDATA (EMF Software, Baltimore, MD, USA) or GraphPad InPlot (San Diego, CA, USA).

Affinities of the ligands for σ_2 sites were determined in rat liver using [^{3}H]DTG in the presence of a saturating concentration of dextrallorphan to mask σ_1 sites, as previously described (Hellewell et al., 1994). Membranes (160–200 μg membrane protein) were incubated with 3 nM [^{3}H]DTG, 1 μM dextrallorphan, and one of 12 concentrations (0.05–10 000 nM) of either BD1047 or BD1063. Incubations were carried out in a total volume of 0.5 ml of 50 mM Tris-HCl, pH 8.0 for 120 min. Non-specific binding was determined in the presence of 10 μM haloperidol. Incubations were terminated and counts quantified as described for brain tissue.

2.3. Behavioral experiments

Surgical procedure

Male Sprague-Dawley rats, obtained from either Charles River Laboratories (San Diego, CA, USA) or Zivic-Miller (Zelienople, PA, USA), were used in these studies. The animals were group housed prior to surgery and individually thereafter. The rats had free access to food and water. All animal care procedures followed those approved by the University of California Irvine Institutional Animal Care and Use Committee.

Surgical procedures were as previously described (Matsumoto et al., 1989, 1990; Walker et al., 1988). Briefly, 3–7 days before behavioral testing, each animal (250–380 g) was anesthetized with 50 mg/kg sodium pentobarbital and mounted in a stereotaxic apparatus. A guide cannula, constructed from 24-gauge stainless steel tubing, was implanted with its tip 4.0 mm above the red nucleus of each animal (coordinates: 2.5 mm anterior, 0.8 mm lateral, 4.0 mm ventral from lambda and the skull surface; Paxinos and Watson, 1986). In initial experiments, the guide cannulae were implanted unilaterally because we did not know the extent to which the ligands might produce permanent damage. In later experiments, cannulae were implanted bilaterally so that unilateral microinjections could be made into each red nucleus, with wash-out times of at least 24 h between the injections. Cannulae were secured to stainless steel skull screws with dental acrylic.

Microinjection and testing procedures

Microinjection and testing procedures were as previously described (Matsumoto et al., 1989,1990; Walker et al., 1988). Three to seven days after surgery, each rat received a single microinjection of either BD1047 (10 nmol, $n = 6$) or BD1063 (10 nmol, $n = 7$). The drugs were dissolved in saline or water and prepared on the day of testing. The chosen doses consistently produced σ-mediated dystonia using other ligands in previous studies (Matsumoto et al., 1990; Walker et al., 1988). In addition, some rats were microinjected with normal

saline ($n = 12$) as a vehicle control. The solutions were administered in a volume of 0.5 μl over 60 s through a 31 gauge microneedle that was constructed to extend 4.0 mm beyond the tips of the guide cannulae.

Following the injection, each rat was placed in a clear, plastic chamber (23 × 36 cm). Torticollis was quantified by measuring the torsional deviation of the head from the horizontal plane, using the eyes of the animals as a reference. Measurements were taken 1, 5, 10, 15, 20, and 30 min after the injection.

A series of experiments were then conducted to test the ability of BD1047 and BD1063 to block the dystonia produced by the prototypic σ ligands, DTG and haloperidol. For the antagonism studies, the testing procedures were identical to those described above except that each rat was injected twice: once in the presence and once in the absence of the putative antagonists. For the coadministrations, the total drug volume remained 0.5 μl and at least 24 h intervened between the injections. Twenty-four hours was deemed a sufficient washout period because almost all rats resumed a normal posture within 90 min of an intrarubral microinjection of DTG or haloperidol (Walker et al., 1988).

In one set of studies, BD1047 and BD1063 were tested for their ability to attenuate the dystonia produced by the high affinity, non-selective σ ligand haloperidol. Unilateral microinjections of a single high dose of haloperidol (10 nmol) were made into the rat red nucleus alone, and in the presence of either BD1047 (10 nmol, $n = 6$) or BD1063 (10 nmol, $n = 6$). In this portion of the study, haloperidol alone had to produce a criterion head angle of at least 10° for antagonism to be tested. This criterion, which has previously been used for the calculation of ED_{50} values with this paradigm (Matsumoto et al., 1990), was implemented to reliably assess statistically significant attenuation of torticollis by the putative antagonists. Although the study was designed so that the order of the injections was counterbalanced when testing for antagonism, as an additional control, two injections of haloperidol (10 nmol, $n = 6$) were made in some animals to eliminate the possibility that the first injection was producing permanent, mechanical damage to the tissue. This additional control was necessary because only animals that were later shown to have histolologically confirmed injection sites in the red nucleus were included in the data analyses and as such, we could not ensure that the final data set would reflect counterbalancing during the testing.

Due to the non-selective nature of haloperidol for σ binding sites, further characterization of the antagonist actions of BD1047 and BD1063 were conducted under conditions where DTG was used to induce dystonia. DTG was chosen for this characterization because it is one of the most potent and selective σ ligands available (Walker et al., 1990; Weber et al., 1986). The dose dependency of the antagonism was tested by administering various doses of BD1047 or BD1063 (0–20 nmol) in the red nucleus in the presence and absence of a fixed dose of DTG (10 nmol). The extent to which various doses of BD1047 could block the DTG-mediated dystonia was evaluated (0 nmol $n = 6$, 1 nmol $n = 7$, 2.5 nmol $n = 5$, 5 nmol $n = 5$, 10 nmol $n = 8$, 20 nmol $n = 6$). A similar comparison was made for various doses of BD1063 (0 nmol $n = 6$, 5 nmol $n = 8$, 10 nmol $n = 7$, 20 nmol $n = 8$). Again, efforts were made to counterbalance the order of the injections, but as an additional control, two injections of DTG (10 nmol, $n = 6$) were made in some animals to eliminate the possibility that the order of the injections affected the results.

A second set of studies was conducted in an effort to evaluate whether BD1047 and BD1063 were acting as competitive or non-competitive antagonists. In these experiments, various doses of DTG (5, 10, 15, 20 nmol) were tested in the absence and presence of a fixed concentration (10 nmol) of BD1047 ($n = 22$) or BD1063 ($n = 27$). As in the other antagonism experiments described above, efforts were made to counterbalance the order of the injections, with at least 24 h intervening between the injections.

Histology

At the end of the experiments, each animal was injected (i.p.) with a fatal dose of sodium pentobarbital and perfused intracardially with saline, followed by 10% formalin. The brains were fixed further by immersion in a 30% sucrose-formalin solution. Coronal sections (40 μm) were taken throughout the extent of the injection site. The sections were stained in cresyl violet and examined under a microscope to localize injection sites. Only those animals with histologically confirmed injection sites in the red nucleus were used in the data analyses.

Statistics

Unless otherwise specified, the peak angle of head deviation during the 30 min testing session was used in the data analyses. Student's t-tests were used to compare the effects of the novel ligands to control injections of vehicle (0.9% saline). Analysis of variance was used to compare the drug effects of DTG in the presence and absence of BD1047 or BD1063. However, non-parametric statistics were used to analyze the antagonism data when haloperidol was used to induce the dystonia because rats had to meet a 10° criterion to be included in the study; thus, Wilcoxon signed rank tests were used for this comparison.

Table 1
Affinities (IC_{50} in nM) of BD1047 and BD1063 for different receptors in rat brain

Receptor Radioligand	σ DTG	Opiate Etorphine	PCP TCP	Dopamine (−)Sulpiride	Muscarinic Pirenzipine
BD1047	36 ± 14	> 10000	> 100000	> 10000	8000 ± 1000
BD1063	45 ± 31	> 10000	> 100000	> 10000	9460 ± 750
Receptor Radioligand	α_1 Prazosin	α_2 Clonidine	β DHA	5-HT_1 5-HT	5-HT_2 Ketanserin
BD1047	> 10000	> 10000	145 ± 100	> 10000	4667 ± 2626
BD1063	> 10000	> 10000	> 10000	> 10000	2552 ± 2417

Competition assays were run as previously described in the Materials and methods section to determine the affinity and selectivity of BD1047 and BD1063 for σ binding sites. The values in the table represent the mean ± S.E.M. from at least two experiments, each carried out in duplicate. IC_{50} values were derived using the iterative curve fitting program CDATA (EMF Software, Baltimore, MD, USA) or GraphPad InPlot (San Diego, CA, USA). DTG = di-*o*-tolylguanidine, DHA = dihydroalprenolol, 5-HT = 5-hydroxytryptamine, PCP = phencyclidine, TCP = 1-[(2-thienyl)cyclohexyl]piperidine.

Table 2
Affinities of BD1047 and BD1063 for putative σ subtypes

	BD1047	BD1063
σ_1	0.93 ± 0.14	9.15 ± 1.28
σ_2	47 ± 0.60	449 ± 11
σ_1/σ_2	0.019	0.020

Affinities (K_i in nM) were determined in competition assays. σ_1 binding was determined in guinea pig brain using [^{3}H](+)-pentazocine. σ_2 binding was determined in rat liver using [^{3}H]DTG in the presence of a saturating concentration of dextrallorphan. The values in the table represent the mean ± S.E.M. from 2–3 experiments, each carried out in duplicate.

3. Results

3.1. Binding assays

In the rat brain, BD1047 and BD1063 showed marked selectivity for σ sites, generally having a 100-fold or better affinity for σ sites compared to the other tested receptors (Table 1). The only notable exception was the significant affinity of BD1047 for β-adrenoceptors.

BD1047 and BD1063 interacted with both σ_1 and σ_2 binding sites under conditions where the subtypes were selectively labelled (i.e. in guinea pig brain and rat liver). Both compounds had preferential affinities for σ_1 sites, with K_i values below 10 nM (Table 2). BD1047 had a 51-fold greater affinity for σ_1 binding sites as compared to σ_2 sites. BD1063, likewise, had a 49-fold greater affinity for σ_1 sites than σ_2 sites. However, while BD1047 showed high affinity for σ_2 sites, BD1063 exhibited only moderate affinity.

3.2. Behavioral studies

Control injections

Control injections of saline produced negligible changes in head angle after unilateral microinjection into the red nucleus (mean ± S.E.M. for saline was 0.67° ± 1.68°). Alone, neither BD1047 nor BD1063 had a significant effect on head posture when microinjected into the red nucleus of rats (BD1047: $t = 0.20$, n.s.; BD1063: $t = 0.57$, n.s.). The mean ± S.E.M. head angle produced by microinjection of BD1047 into the

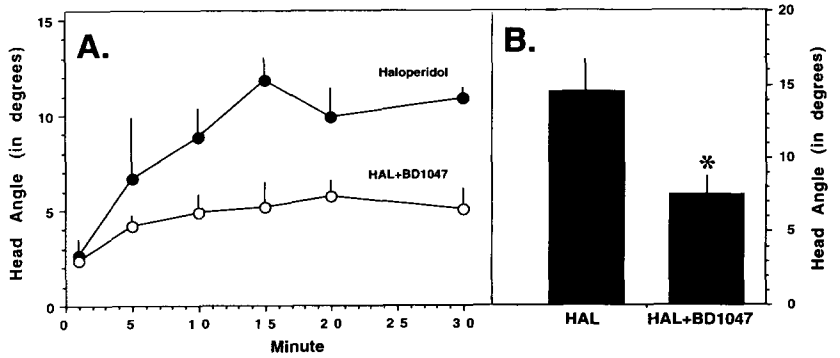

Fig. 1. Effects of BD1047 on haloperidol-induced dystonia. Panel A: Time course of the extent of dystonia (head angle in degrees) produced by unilateral microinjection of haloperidol (10 nmol) alone, or in the presence of BD1047 (10 nmol). Panel B: Summary of the peak head angles produced by haloperidol alone (HAL) or haloperidol when coadministered with BD1047 (HAL + BD1047). Paired injections were made in the same animals ($n = 6$) with at least 24 h intervening between applications. The attenuation of haloperidol-induced dystonia by BD1047 was statistically significant ($P < 0.02$).

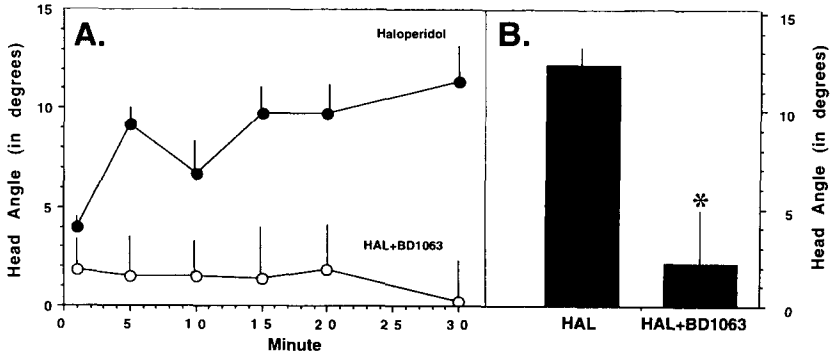

Fig. 2. Effects of BD1063 on haloperidol-induced dystonia. Panel A: Time course of the extent of dystonia produced by unilateral microinjection of haloperiol (10 nmol) alone, or haloperidol (10 nmol) in the presence of BD1063 (10 nmol). Panel B: Summary of the peak head angles produced by haloperidol alone (HAL) or haloperidol when coadministered with BD1063 (HAL + BD1063). Paired injections were made into the same animals ($n = 6$) with at least 24 h intervening between administration. The ability of BD1063 to antagonize the effects of haloperidol was statistically significant ($P < 0.02$).

red nucleus was 1.33° ± 3.44°, whereas the corresponding measurement for BD1063 alone was 0.43° ± 2.58°.

For the antagonism portions of the study, there was no significant difference between the extent of dystonia produced by the first and second injections when rats were microinjected twice with either haloperidol (Wilcoxon stat = 9.50, n.s.) or DTG (Wilcoxon stat = 9.00, n.s.), suggesting that the order of injections did not affect the pattern of results. For the haloperidol microinjections, the mean ± S.E.M. head angle for the first injection was 11.83° ± 1.05°, which did not differ significantly from the 12.33° ± 1.52° measurement obtained from the second injection. A similar pattern of results was observed for DTG with a mean ± S.E.M. head angle of 14.00° ± 1.07° for the first injection and a mean head angle of 14.00° ± 2.13° for the second injection.

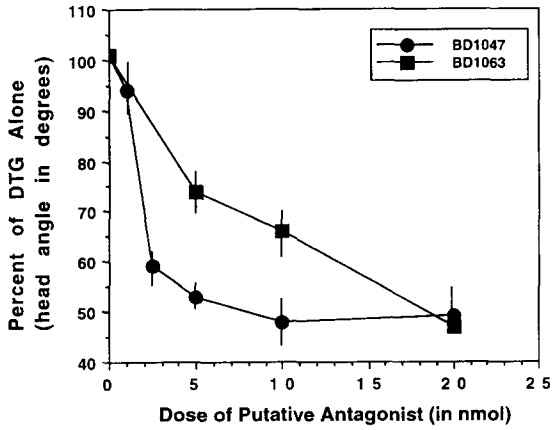

Fig. 3. Dose-dependent antagonism of DTG-induced dystonia by BD1047 and BD1063. A fixed concentration of DTG (10 nmol) was administered alone, or in the presence of another dose of BD1047 or BD1063 (0–20 nmol). Paired microinjections were made into the red nucleus of rats ($n = 60$) with at least 24 h intervening between the injections. The head angles produced in the presence of BD1047 or BD1063 are represented as a percentage of the head angle produced by a microinjection of DTG alone. 100% signifies no change from the extent of dystonia produced by DTG alone. The attenuation of the dystonia produced in the presence of BD1047 and BD1063 was statistically significant ($P < 0.01$).

Attenuation of the effects of haloperidol

The extent of dystonia produced by unilateral microinjection of the high affinity, non-selective σ ligand, haloperidol, into the rat red nucleus was attenuated by coadministration of either BD1047 or BD1063 (Figs. 1 and 2). The antagonism of the dystonia produced by haloperidol was statistically significant (BD1047: Wilcoxon stat = 0.00, $P < 0.02$; BD1063: Wilcoxon stat = 0.00, $P < 0.02$).

Attenuation of the effects of DTG

The ability of BD1047 and BD1063 to dose-dependently attenuate the dystonia produced by DTG is summarized in Fig. 3. Although the animals received different concentrations of putative antagonists, they all received the same dose of DTG (10 nmol) to induce the dystonia. Analyses of variance showed that there was no significant difference in the extent of dystonia produced by DTG alone among the different groups of animals for BD1047 ($F(5,30) = 0.16$, n.s.) or BD1063 ($F(3,24) = 0.89$, n.s.). Therefore, the reductions in dystonia produced in the presence of the putative antagonists were analyzed as a percentage of the degree of head angle produced by DTG alone. Analysis of variance showed that BD1047 attenuated the DTG-induced dystonia in a dose-dependent manner ($F(5,26) = 3.68$, $P < 0.01$). A similar pattern of results was observed for BD1063 ($F(3,23) = 5.49$; $P < 0.006$), although as compared to BD1047, higher doses of BD1063 were required to achieve maximum reductions in the extent of dystonia produced by DTG.

Although the nature of these microinjection experiments precluded the construction of comprehensive dose curves, similar to the pattern of results previously

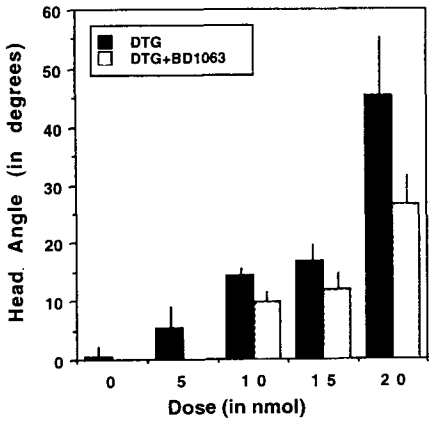

Fig. 4. Decreased effectiveness of various concentrations of DTG in the presence of a fixed concentration of BD1063. Unilateral microinjection of DTG (0–20 nmol) into the rat red nucleus produced dystonia in a dose-dependent manner. Coadministration of BD1063 (10 nmol) with DTG shifted the dose curve to the right ($P < 0.02$). The injections of DTG in the presence and absence of BD1063 were made into the same animals ($n = 27$) with at least 24 h intervening between administrations.

reported (Walker et al., 1988), DTG elicited dose-dependent dystonia after unilateral administration into the red nucleus (in the absence of BD1047: $F(4,29) = 10.43$, $P < 0.001$; in the absence of BD1063: $F(4,34) = 12.22$, $P < 0.001$). However, in the presence of a fixed concentration of BD1063, the dose-related responses elicited by DTG were shifted to the right (Fig. 4) and an analysis of variance revealed a significant difference between the effects of DTG in the presence and absence of BD1063 ($F(1,17) = 7.67$; $P < 0.02$). A similar pattern of results was observed for BD1047 ($F(1,16) = 30.39$; $P < 0.001$), suggesting that the ligands were acting as competitive antagonists. Higher doses of DTG were not tested in these studies because neurotoxic effects of DTG, as expressed as extensive lesions or persistent dystonia lasting for more than 24 h became increasingly apparent at the highest dose (20 nmol) used in this study; animals expressing these symptoms were not included in the data analyses.

4. Discussion

Radioligand binding studies revealed that the novel ligands, BD1047 and BD1063, were among the most selective σ ligands developed to date. Both ligands possessed marked selectivity for σ binding sites, generally having a 100-fold or better affinity for σ sites compared to nine other tested receptors (opiate, phencyclidine, muscarinic, dopamine, α_1-, α_2-, β-adrenoceptor, 5-HT_1, 5-HT_2). The only exception was the significant affinity of BD1047 for β-adrenoceptors. Both compounds were also shown to interact with σ_1 and σ_2 binding sites, with preferential affinities for σ_1 sites. However, BD1047 had a higher affinity for σ_2 sites as compared to BD1063.

In functional studies, BD1047 and BD1063 appeared to act as σ antagonists. They had no effects on their own in the present studies, but were capable of blocking the dystonic effects produced by unilateral intrarubral microinjection of DTG or haloperidol, two prototypic σ ligands. The ability of BD1047 and BD1063 to attenuate the dystonia produced by both DTG and haloperidol supports the involvement of σ binding sites in these effects, rather than a mechanism specific to a particular drug. Furthermore, although BD1047 has some affinity for β-adrenoceptors and the effects of this ligand on β-adrenergic function are currently unknown, it is unlikely that a β-adrenergic mechanism alone accounts for the antidystonic effects produced by the novel ligands because (1) BD1063, which also has antidystonic effects, is inactive at β-adrenoceptors, (2) isoproterenol and propranolol, a β agonist and antagonist respectively, failed to produce a significant amount of dystonia after mircoinjection into the rat red nucleus, and (3) significant concentrations of β-adrenoceptors have not been reported in the red nucleus, the part of the brain into which the injections were made (Jones and Palacios, 1991; Nelson et al., 1991). The involvement of a σ-mediated mechanism in the effects observed herein is further supported by the ability of BD1047 and BD1063 to dose-dependently attenuate the dystonia produced by DTG. Taken together with the high σ-selectivity of BD1047 and BD1063 and the fact that the antagonism occurred upon co-injection into an area of the brain where there is minimal interference from other neurochemical systems, the results suggest that the effects of BD1047 and BD1063 were mediated through σ binding sites.

Although the in vivo nature of the study strengthens the physiological relevance of the observed effects, one limitation of this approach is that it precludes the generation of comprehensive dose curves such that a change in maximal effect can be unequivocably evaluated. As increased concentrations of drug are microinjected into the brain, confounding factors such as limits to drug solubility, nonspecific drug actions, and competing physiological effects such as neurotoxicity become a problem. It has been shown that high doses of σ ligands produce σ receptor-mediated cytotoxic effects in vitro when exposed to cells in culture (Bowen and Vilner, 1994; Vilner et al., 1995). Furthermore, some σ ligands, such as reduced haloperidol and BD614, have been shown to have neurotoxic effects when microinjected into the red nucleus (Bowen et al., 1990, 1992b). Although in the present study, histological examination of brain tissue confirmed that the reported behavioral changes could not be attributed to neurotoxic effects, it is worth noting that some animals that were tested with the highest dose (20 nmol) had to

be excluded from the data set because of lesions in the red nucleus and unusually long-lasting alterations in posture. Therefore, the data suggest that as higher doses of σ ligands are used, the cytotoxic properties of these compounds must be considered. Nevertheless, within the dose range tested and excluding those changes that may have been associated with cytotoxic effects, the dose curves for DTG were shifted to the right in the presence of BD1047 or BD1063, suggesting that the ligands act as competitive antagonists. The data, taken together, provide strong evidence that BD1047 and BD1063 have antagonist actions in vivo. However, in vitro studies which allow the testing of a wider dose range and the reliable determination of a maximal effect are needed to confirm whether these ligands are competitive (vs. non-competitive) antagonists.

It has been conclusively determined that both σ_1 and σ_2 binding sites are present in the rat brain (Bowen et al., 1993; Leitner et al., 1994). Therefore, in terms of the subtype selectivity of the actions of BD1047 and BD1063, it appears that in the present study, the ligands acted through σ_2 sites. Previous studies have shown that: (1) there is a significant correlation between the behavioral potencies of ligands for producing the dystonic effects and their σ binding affinities for [^{3}H]DTG, but not [^{3}H](+)-pentazocine, and (2) non-benzomorphan σ ligands such as DTG and haloperidol have a higher potency for the behavioral effects than benzomorphan σ ligands (Matsumoto et al., 1990). Both of these patterns are consistent with a σ_2-mediated mechanism (Quirion et al., 1992), suggesting that the dystonic effects are mediated through the σ_2 subtype. In this respect, it is noteworthy that BD1047 which has a 47 nM K_i for σ_2 sites, as compared to BD1063 with a 449 nM K_i, was also able to block the head angles produced by DTG with greater potency.

However, since binding studies showed that BD1047 and BD1063 exhibit preferential affinities for σ_1 sites, we have also tested the effects of the novel compounds in a functional system that is thought to involve σ_1 sites. (+)-Pentazocine has previously been shown to modulate agonist-stimulated phosphoinositide turnover via a σ_1-mediated mechanism (Bowen et al., 1992a), and in preliminary studies, BD1047 and BD1063 were able to attenuate this effect (W.D. Bowen, unpublished observation; Cutts et al., 1994; Hsu et al., 1993). The ability of both ligands to inhibit the effect of (+)-pentazocine in the phosphoinositide assays, suggests that BD1047 and BD1063 possess antagonist properties through σ_1 sites as well as through σ_2 sites.

In addition to the dystonic effects and modulation of phosphoinositide turnover described above, previous studies have reported agonist-antagonist interactions of σ ligands in other functional systems: the modulation of NMDA-induced responses in hippocampal neurons, locomotor stimulatory effects of cocaine, activation of neurons in the mesocorticolimbic dopamine system, and drug discrimination paradigms (Bergeron et al., 1993; Ceci et al., 1988; Glennon et al., 1993; Monnet et al., 1992; Witkin et al., 1993). It is likely that at least some of these effects involve direct σ receptor blockade, but the non-selective nature of the ligands available at the time of the studies and the systemic administration routes used made it difficult to determine whether the effects represented functional antagonism or actions mediated directly through σ binding sites. The tentative identification of BD1047 and BD1063 as selective σ antagonists provides promising new tools for probing the functional effects associated with σ binding sites and could lead to important insights about σ receptor structure and function.

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