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Conformationally restricted analogs of BD1008 and an antisense oligodeoxynucleotide targeting σ_1 receptors produce anti-cocaine effects in mice

Rae R. Matsumoto [a,*], Kari A. McCracken [a], Michele J. Friedman [a], Buddy Pouw [a], Brian R. De Costa [b], Wayne D. Bowen [b]

[a] *Department of Pharmaceutical Sciences, College of Pharmacy, University of Oklahoma Health Sciences Center, PO Box 26901, CPB 337, Oklahoma City, OK 73190, USA*

[b] *Laboratory of Medicinal Chemistry, NIDDK / NIH, Bethesda, MD 20892, USA*

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Abstract

Cocaine's ability to interact with σ receptors suggests that these proteins mediate some of its behavioral effects. Therefore, three novel σ receptor ligands with antagonist activity were evaluated in Swiss Webster mice: BD1018 (3*S*-1-[2-(3,4-dichlorophenyl)ethyl]-1,4-diazabicyclo[4.3.0]nonane), BD1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine), and LR132 (1*R*,2*S*-(+)-*cis*-*N*-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamine). Competition binding assays demonstrated that all three compounds have high affinities for σ_1 receptors. The three compounds vary in their affinities for σ_2 receptors and exhibit negligible affinities for dopamine, opioid, $GABA_A$ and NMDA receptors. In behavioral studies, pre-treatment of mice with BD1018, BD1063, or LR132 significantly attenuated cocaine-induced convulsions and lethality. Moreover, post-treatment with LR132 prevented cocaine-induced lethality in a significant proportion of animals. In contrast to the protection provided by the putative antagonists, the well-characterized σ receptor agonist di-*o*-tolylguanidine (DTG) and the novel σ receptor agonist BD1031 (3*R*-1-[2-(3,4-dichlorophenyl)ethyl]-1,4-diazabicyclo[4.3.0]nonane) each worsened the behavioral toxicity of cocaine. At doses where alone, they produced no significant effects on locomotion, BD1018, BD1063 and LR132 significantly attenuated the locomotor stimulatory effects of cocaine. To further validate the hypothesis that the anti-cocaine effects of the novel ligands involved antagonism of σ receptors, an antisense oligodeoxynucleotide against σ_1 receptors was also shown to significantly attenuate the convulsive and locomotor stimulatory effects of cocaine. Together, the data suggests that functional antagonism of σ receptors is capable of attenuating a number of cocaine-induced behaviors. © 2001 Published by Elsevier Science B.V.

Keywords: Antisense; Cocaine; Convulsion; Locomotor activity; Psychomotor activity; σ Receptor; Toxicity

1. Introduction

Cocaine acts as a dopamine uptake blocker (Kuhar et al., 1988). It can additionally inhibit the reuptake of serotonin and norepinephrine, and bind to a number of neurotransmitter receptors (Kuhar et al., 1988). Of the myriad of sites with which cocaine interacts, the monoamine transporters, muscarinic receptors, and σ receptors are thought to mediate the psychological and physiological properties of cocaine (Kuhar et al., 1988). At these sites, the affinity of cocaine corresponds to concentrations that are achievable in vivo (Kuhar et al., 1988). Therefore, efforts to develop pharmacotherapies to treat cocaine abuse and overdose have logically focused on these sites. Over the years, numerous promising compounds have been developed that target the monoamine transporters and their corresponding neurotransmitter systems (Carroll et al., 1999; Newman, 2000). However, drug development efforts directed toward these strategies have yet to result in an effective treatment for cocaine abuse in humans (Appel, 2000). Previous investigations that targeted muscarinic systems were also limited in success (Heidbreder and Shippenberg, 1996; Ritz and George, 1997a,b; Witkin et al., 1989), and while a recent study suggests that compounds that act as partial agonists at muscarinic receptors

* Corresponding author. Tel.: +1-405-271-6593 ext. 47250; fax: +1-405-271-7505.

E-mail address: rae-matsumoto@ouhsc.edu (R.R. Matsumoto).

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may have promise (Rasmussen et al., 2000), concerns about common side effects associated with this class of compounds are difficult to circumvent. Early attempts to block the actions of cocaine by manipulating σ receptors were met also with mixed results (Menkel et al., 1991; Ritz and George, 1997a,b; Witkin et al., 1993). However, in recent years, significant advances have been made in our understanding of σ receptors, including the development of highly selective ligands. Indeed, a number of these selective ligands and antisense oligodeoxynucleotides have been shown to attenuate the convulsive, lethal, locomotor stimulatory, and rewarding properties of cocaine (Matsumoto et al., 2001; Matsumoto and McCracken, 1999; McCracken et al., 1999a,b; Romieu et al., 2000). These recent studies thus suggest that σ receptors may be viable targets for the development of medications to treat cocaine abuse.

σ Receptors are currently recognized as unique binding sites with a drug selectivity pattern and anatomical distribution that are distinct from any known receptor (Itzhak, 1994; Walker et al., 1990). The biological relevance of σ receptors is supported by evidence for endogenous ligands for these sites (Connor and Chavkin, 1991; Patterson et al., 1994; Su et al., 1986). In addition, correlations have been demonstrated between the σ receptor binding affinities of drugs and their potencies in numerous functional assays (Campbell et al., 1989; Kinney et al., 1995; Matsumoto et al., 1990; Walker et al., 1993; Wu et al., 1991). Biochemical and pharmacological studies indicate the existence of multiple σ receptor subtypes, with σ_1 and σ_2 sites being the best characterized (Quirion et al., 1992; Walker et al., 1990). Furthermore, σ receptors have been linked to the modulation or production of intracellular second messengers such as G-proteins, cGMP, inositol phosphates, and calcium (Bowen, 1994; Hayashi et al., 2000; Hong and Werling, 1998; Joseph and Bowen, 1998; Morin-Surun et al., 1999; Rao et al., 1991; Vilner and Bowen, 2000). Recently, several laboratories have sequenced and cloned possible receptor proteins (Pan et al., 1998; Prasad et al., 1998; Seth et al., 1997, 1998), and antisense oligodeoxynucleotides for these sequences have been shown to attenuate functional effects (King et al., 1997; Kitaichi et al., 1997; Maurice et al., 1997).

We have recently reported that the novel σ receptor ligand, BD1008 (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)ethylamine) and several of its analogs possess anti-cocaine actions (Matsumoto et al., 2001; McCracken et al., 1999a,b). Structure–activity evaluations of ligands for other receptors have revealed that conformational restriction can alter the interaction of drugs with the receptor protein, sometimes resulting in antagonistic properties. This feature may also apply to σ receptors because BD1063 (1-[2-(3,4-dichlorophenyl)-ethyl]-4-methylpiperazine), an analog of BD1008 in which the positions of the two nitrogens are restricted, possesses antagonist actions through σ receptors (Joseph and Bowen, 1998; Matsumoto et al., 1995; Monnet et al., 1996; Tran et al., 1998; Vilner and Bowen, 2000). In addition, a preliminary report on the effects of BD1063 on cocaine-induced behaviors showed it capable of attenuating the locomotor stimulatory actions of cocaine (McCracken et al., 1999b).

Therefore, in the present study, three conformationally restricted analogs of BD1008 were tested for their ability to attenuate the convulsive, lethal, and locomotor stimulatory effects of cocaine: BD1018 (3*S*-1-[2-(3,4-dichlorophenyl)ethyl]-1,4-diazabicyclo[4.3.0]nonane), BD1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine), and LR132 (1*R*,2*S*-(+)-*cis*-*N*-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamine). For comparison, the well-established σ receptor agonist di-*o*-tolylguanidine (DTG) was also tested to determine if it would worsen the toxicity of cocaine. The novel σ receptor ligand BD1031 (3*R*-1-[2-(3,4-dichlorophenyl)ethyl]-1,4-diazabicyclo [4.3.0]nonane), which is the *trans*-isomer of BD1018, was evaluated as an additional comparison because it has been reported to possess agonistic actions at σ receptors (Matsumoto et al., 1999). Finally, an antisense oligodeoxynucleotide against σ_1 receptors (King et al., 1997) was used to obtain complementary evidence that interfering with access to σ receptors reduces the convulsive and locomotor stimulatory effects of cocaine.

2. Materials and methods

2.1. Drugs

BD1018 (3*S*-1-[2-(3,4-dichlorophenyl)ethyl]-1,4-diazabicyclo[4.3.0]nonane), BD1031 (3*R*-1-[2-(3,4-dichlorophenyl)ethyl]-1,4-diazabicyclo[4.3.0]nonane), BD1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine), and LR132 (1*R*,2*S*-(+)-*cis*-*N*-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamine) were synthesized as described previously (De Costa et al., 1992a,b; Radesca et al., 1991). The structures of the novel ligands are shown in Fig. 1. Di-*o*-tolylguanidine (DTG) was purchased from Aldrich (Milwaukee, WI, USA). Cocaine hydrochloride was obtained from Sigma (St. Louis, MO, USA). The radioligands were obtained from Dupont/New England Nuclear (Boston, MA, USA).

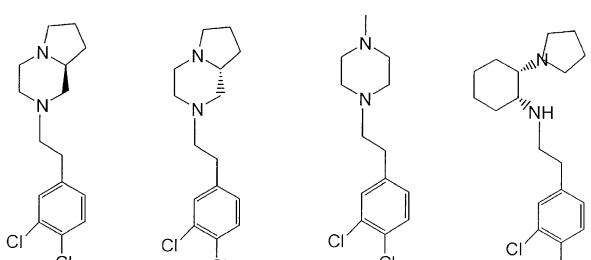

Fig. 1. Structures of the novel σ receptor ligands.

The 21-mer phosphorothioate-modified antisense oligodeoxynucleotide previously described by King et al. (1997) was used: 5′-GAGTGCCCAGCCACAACCAGG-3′. This antisense oligodeoxynucleotide was designed to target area −97 to −77 after the initiation codon of a cloned cDNA sequence for σ_1 receptors from mouse. As a control, three base pairs in the antisense sequence were reversed to obtain the following mismatch sequence: 5′-GAG**GT**CCC**GA**CCACA**CA**CAGG-3′ (King et al., 1997). The sense sequence was used as an additional control. The oligodeoxynucleotides were synthesized with an Applied Biosystems 394 DNA Sequencer and purified using HPLC (Molecular Biology Resource Facility, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA). No σ_2 antisense was tested because the sequence for σ_2 receptors is still unknown.

2.2. Competition binding assays

The affinities of the novel ligands for σ receptors were determined in tissues that are heavily concentrated with the respective subtypes. Methods previously published in detail were used (Bowen et al., 1993; Matsumoto et al., 1995). Briefly, σ_1 receptors were labeled in homogenates from guinea pig brain minus cerebellum using 5 nM [^{3}H](+)-pentazocine; σ_2 receptors were labeled in homogenates from rat liver with 3 nM [^{3}H]DTG in the presence of 1 μM dextrallorphan to mask σ_1 receptors. Non-specific binding was determined in the presence of 10 μM haloperidol. Twelve concentrations of test ligand (0.05–10,000 nM) were incubated for 120 min at 25°C to evaluate their ability to displace the binding of the radioligand.

Since many historic ligands for σ receptors are non-specific, exhibiting interactions with dopamine, opiate, or phencyclidine (PCP) binding sites in addition to σ receptors (cf. Itzhak, 1994; Walker et al., 1990), the relative selectivities of the novel ligands were determined. In addition, the affinities of the novel ligands for $GABA_A$ receptors were measured because many anticonvulsant drugs act through these receptors (Olsen et al., 1999). The affinities of the compounds for 5-HT_2 receptors were also examined because antagonists at these sites are capable of attenuating the behavioral toxicity of cocaine (Ritz and George, 1997a). The affinities of the novel ligands for dopamine, opiate, $GABA_A$, and 5-HT_2 receptors, as well as the PCP binding site on NMDA receptors were measured in homogenates from rat brain minus cerebellum using previously published methods (De Costa et al., 1992a,b; Matsumoto et al., 1995, 2001). Briefly, dopamine receptors were labeled with 5 nM [^{3}H](−)-sulpiride; non-specific binding was determined with 1 μM haloperidol. Opiate receptors were labeled with 2 nM [^{3}H]bremazocine; non-specific binding was determined with 10 μM levallorphan. PCP sites were labeled with 5 nM [^{3}H]TCP (1-[1-(2-thienyl)cyclohexyl]piperidine); non-specific binding was determined with 10 μM cyclazocine. $GABA_A$ receptors were labeled with 10 nM [^{3}H]muscimol; non-specific binding was determined with 1 mM GABA. 5-HT_2 receptors were labeled with 2 nM [^{3}H]ketanserin; non-specific binding was determined with 1 μM mianserin. The incubations were carried out for 60 min at 25°C for the dopamine and opiate receptor assays, 30 min at 37°C for the $GABA_A$ and 5-HT_2 receptor assays, and for 60 min at 4°C for the PCP assays.

All of the assays were terminated with the addition of ice-cold buffer and vacuum filtration through glass fiber filters. Counts were extracted from the filters using Ecoscint cocktail (National Diagnostics, Manville, NJ, USA) for at least 8 h prior to counting.

2.3. Animals

Male, Swiss Webster mice (21–30 g, Harlan, Indianapolis, IN, USA; Charles River, Portage, MI, USA) were used for the behavioral experiments. The mice were housed in groups of five to six with a 12:12-h light/dark cycle and ad libitum food and water. The animals were randomly assigned to their treatment groups. Mice from at least two different shipments were tested on different days to form the final data set for each dose/drug group. All procedures were performed as approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center.

2.4. Convulsions

The dose–response curve for cocaine-induced convulsions was determined by injecting mice with various doses of cocaine (10–90 mg/kg, i.p., $n = 52$). The animals were then placed in individual boxes and observed for the next 30 min for the occurrence of convulsions. Convulsions were operationally defined as clonic or tonic limb movements, which were accompanied by the loss of righting reflexes, wild running, and/or popcorn jumping.

To probe for anti-cocaine actions, mice were injected (i.p.), 15 min before administration of a convulsive dose of cocaine (60 mg/kg, i.p.), with one of the following pretreatments: BD1018 [0.1 ($n = 11$), 1 ($n = 13$), 5 ($n = 10$), 10 ($n = 10$), 20 ($n = 10$), 30 ($n = 7$) mg/kg], BD1031 [0.1 ($n = 8$), 30 ($n = 10$) mg/kg], BD1063 [0.1 ($n = 10$), 1 ($n = 10$), 5 ($n = 8$), 10 ($n = 9$), 20 ($n = 7$), 30 ($n = 10$), 40 ($n = 8$) mg/kg], LR132 [0.1 ($n = 10$), 1 ($n = 9$), 5 ($n = 10$), 15 ($n = 7$), 30 ($n = 10$) mg/kg], or saline ($n = 7$). The dose of cocaine used in this part of the study (60 mg/kg, i.p.) reliably produces convulsions in 100% of our animals without deaths (Brackett et al., 2000; Matsumoto et al., 2001; McCracken et al., 1999a). After the injection with cocaine, the animals were observed for the next 30 min for the occurrence of a convulsion.

To validate the assignment of agonist vs. antagonist actions of the various σ receptor ligands, the well-estab-

lished σ receptor agonist DTG was tested against various doses of cocaine to determine whether it could shift the ED_{50} value of the cocaine dose–response curve to the left. Similarly, the ability of the novel ligand BD1031 was tested in this portion of the study because it appears to act as an agonist at σ receptors (Matsumoto et al., 1999). For these studies, mice were injected (i.p.) with saline or a 30 mg/kg dose of DTG ($n = 49$) or BD1031 ($n = 22$), followed 15 min later with a dose of cocaine (5–60 mg/kg). The animals were then observed for the next 30 min for the occurrence of convulsions or lethality.

2.5. Lethality

The dose–response curve for cocaine-induced lethality was determined by injecting mice with various doses of cocaine (80–150 mg/kg, i.p., $n = 53$). The animals were then placed in individual boxes and observed for the next 30 min for death.

To evaluate the effects of the novel σ receptor ligands on cocaine-induced lethality, mice were injected (i.p.), 15 min before administration of a lethal dose of cocaine (125 mg/kg, i.p.), with one of the following pre-treatments: BD1018 [0.1 ($n = 10$), 1 ($n = 13$), 5 ($n = 10$), 15 ($n = 10$), 30 ($n = 7$) mg/kg], BD1031 [1 ($n = 7$), 30 ($n = 5$) mg/kg], BD1063 [0.1 ($n = 8$), 1 ($n = 8$), 10 ($n = 7$), 20 ($n = 10$), 30 ($n = 7$), 40 ($n = 5$) mg/kg], LR132 [0.1 ($n = 10$), 0.5 ($n = 5$), 1 ($n = 15$), 5 ($n = 7$), 30 ($n = 5$) mg/kg], or saline ($n = 9$). Similarly to the convulsion study, functional antagonism was tested against a single high dose of cocaine that produced lethality in 100% of our animals.

Although pre-treatment ensures that the receptors are occupied at the time of the overdose, to be of practical use, the drugs must be effective when administered after the overdose. Therefore, the compounds were tested in post-treatment studies in which mice were first administered a lethal dose of cocaine (125 mg/kg, i.p.). LR132 (0.1, 1 mg/kg, $n = 18$) or BD1063 (0.1, 1, 30 mg/kg, $n = 19$) was then administered after the onset of convulsions; post-treatment with saline served as the control ($n = 9$). BD1018 was not tested under the post-treatment condition because it was not as effective as the other two compounds when it was administered as a pre-treatment.

The mice were watched for 30 min following the cocaine injections and deaths were recorded. Those animals surviving the 30-min testing session were returned to their home cages where food and water were available, but they received no additional supportive therapies. Deaths after 24 h were also noted to assess the longer term effects of the protection.

2.6. Locomotor activity

To measure the locomotor stimulatory effects of cocaine, mice were first acclimated for 30 min to the plexiglass enclosures of an automated activity monitor (San Diego Instruments, San Diego, CA, USA). The mice were then administered cocaine (0–20 mg/kg, i.p., $n = 30$). Horizontal locomotor activity was quantified for the subsequent 30 min as disruptions in the 4×4 photobeam array that circumscribed each plexiglass enclosure. The dose of cocaine that produced the peak level of locomotor activity (10 mg/kg, i.p.) was selected for use in the subsequent antagonism portion of the study.

To select an appropriate antagonist dose to test against cocaine, the effects of BD1018, BD1063, or LR132 themselves on locomotor activity were first determined. After a 30-min acclimation period, mice were injected (i.p.) with saline ($n = 6$) or a 30 mg/kg dose of BD1018 ($n = 5$), BD1063 ($n = 6$), or LR132 ($n = 6$) to confirm that they produced effects no different from saline when administered alone. The 30 mg/kg dose was selected because it was a dose that elicited robust attenuation of the convulsive effects of cocaine.

For the antagonism experiments, mice were acclimated to the activity monitors for 15 min. The animals were then injected (i.p.) with saline ($n = 6$) or a 30 mg/kg dose of BD1018 ($n = 5$), BD1063 ($n = 6$), or LR132 ($n = 5$). After a 15-min pre-treatment period, cocaine (10 mg/kg, i.p.) was administered and horizontal locomotor activity was quantified for the subsequent 30 min in an automated activity monitoring system (San Diego Instruments) as the number of breaks in a 4×4 photobeam array.

2.7. Antisense

Mice were surgically implanted with chronic indwelling guide cannulae through which the antisense could be administered because previous studies have shown that oligodeoxynucleotides do not cross the blood–brain barrier very effectively (Davidkova and Weiss, 1998). For the surgeries, mice were deeply anesthetized with sodium pentobarbital (55 mg/kg, i.p.), immediately preceded by a pre-anesthetic dose of chlorpromazine (10 mg/kg, s.c.). Guide cannulae, constructed from 24 gauge stainless steel tubing, were implanted with their tips in the left lateral ventricles: 0.3 mm anterior, 0.7 mm lateral, and 2.5 mm ventral from bregma and the skull surface. Cannulae were secured to the skull surface with U-shaped wire and dental acrylic. Stainless steel stylets kept the cannulae sealed except during drug infusion. The dosing schedule to knock down σ receptors was as previously reported by King et al. (1997). A total of three intracerebroventricular infusions (each 10 μg/5 μl) of the antisense oligodeoxynucleotide ($n = 10$) were administered on Days 1, 2 and 4. As controls, a mismatch ($n = 5$) or sense ($n = 8$) sequence or saline ($n = 11$) was administered using the same regimen. On Day 5, each mouse was evaluated behaviorally after being challenged with a convulsive (60 mg/kg, i.p.) or locomotor stimulatory (10 mg/kg, i.p.) dose of cocaine, as described above.

All of the cannulae placements were histologically confirmed. Following the behavioral assessments, the mice were sacrificed and cresyl violet dye (5 μl) was infused into the cannulae. The brains were removed and coronal knife cuts were made through the site of penetration of the cannulae tips and at the level of the cerebellum; the lateral and fourth ventricles were examined for the presence of cresyl violet dye. Only those animals with histologically confirmed injections into the ventricles were used in the data analyses.

Immediately following confirmation of each injection site, the brain was frozen and stored at −80°C. The elapsed time from the death of each mouse to freezing of the brain was approximately 1 min. Tissues of mice receiving the same treatment were later pooled and saturation assays were performed using [^{3}H](+)-pentazocine as the radioligand (Bowen et al., 1993) to quantify knock down of σ_1 receptors.

2.8. Statistics

The data from the binding assays were analyzed using GraphPad Prism (San Diego, CA, USA). Apparent K_i values were calculated using the Cheng–Prusoff equation and K_d values previously determined (Bowen et al., 1993; Hellewell et al., 1994).

The data from the behavioral toxicity studies were analyzed with Fisher's exact tests (GraphPad InStat San Diego, CA, USA). The data from the locomotor studies were evaluated with repeated measures analyses of variance, followed by post-hoc Dunnett's tests (comparisons against the vehicle control) for the pharmacological antagonism study or Student–Newman–Keuls tests (pairwise comparisons) for the antisense study. $P < 0.05$ was considered statistically significant. ED_{50} values for cocaine-induced convulsions were calculated in the absence and presence of DTG or BD1031 (InStat San Diego, CA, USA).

3. Results

3.1. Binding affinities

The K_i values of the novel ligands for σ_1, σ_2, dopamine, opiate, NMDA, $GABA_A$ and 5-HT_2 receptors are summarized in Table 1. All of the ligands had high affinities for σ_1 receptors. They also interacted with σ_2 receptors, although they exhibited a range of affinities at this subtype. In contrast to their significant affinities at σ receptors, the compounds were inactive at dopamine, opiate, $GABA_A$ and NMDA receptors. Some of the ligands had low micromolar affinity for 5-HT_2 receptors, while others had negligible affinity for these sites.

Table 1
Binding affinities of novel ligands for σ receptors and other binding sites. Affinities (K_i in nM) were determined in competition assays, as described in the Materials and methods. The values in the table represent the means ± S.E.M. from two to three experiments, each performed in duplicate or triplicate. Values of > 10,000 nM in the table signify that there was less than 30% displacement of radioligand at these concentrations.

Receptor	BD1018	BD1031	BD1063	LR132
σ Receptors				
σ_1	5 ± 0.7	1 ± 0.2	9 ± 1[a]	2 ± 0.1
σ_2	49 ± 4	80 ± 9	449 ± 11[a]	701 ± 375
Non-σ receptors				
Dopamine	> 10,000	> 10,000	> 10,000[a]	> 10,000
Opiate	> 10,000	> 10,000	> 10,000[a]	> 10,000
NMDA	> 10,000	> 10,000	> 10,000[a]	> 10,000
5-HT_2	1246 ± 14	2670 ± 769	2552 ± 2417[a]	> 10,000
$GABA_A$	> 10,000	> 10,000	> 10,000	> 10,000

[a] Data from Matsumoto et al., 1995.

3.2. Convulsions

Similar to previous studies, the dose–response curve for cocaine-induced convulsions was very steep (Brackett et al., 2000; McCracken et al., 1999a). None of the mice convulsed after receiving 50 mg/kg of cocaine, while all of the mice convulsed after receiving 60 mg/kg of cocaine. The calculated ED_{50} value for cocaine-induced convulsions was 57 mg/kg, i.p.

Similar to results previously reported, pre-treatment of mice with saline failed to protect them from cocaine-induced convulsions (Matsumoto et al., 2001; McCracken et al., 1999a). In contrast, BD1018, BD1063, and LR132 provided significant protection from cocaine-induced convulsions ($P < 0.005$ at the best dose; Fig. 2).

In contrast, BD1031, the *trans*-isomer of BD1018, which has known agonist actions (Matsumoto et al., 1999), worsened the convulsive effects of cocaine, lowering the ED_{50} for cocaine-induced convulsions from 57 mg/kg in the absence of BD1031, to 47 mg/kg in its presence (Fig. 3). The σ receptor agonist DTG likewise exacerbated the toxicity of cocaine, lowering the ED_{50} for cocaine-induced convulsions to 31 mg/kg in its presence (Fig. 3). The presence of DTG further precipitated death in some mice in response to doses of cocaine that are otherwise not lethal.

3.3. Lethality

Similar to previous studies, the dose–response curve for cocaine-induced lethality was quite steep following acute i.p. administration (Brackett et al., 2000; McCracken et al., 1999a). The calculated LD_{50} for cocaine-induced lethality was 109 mg/kg, i.p.

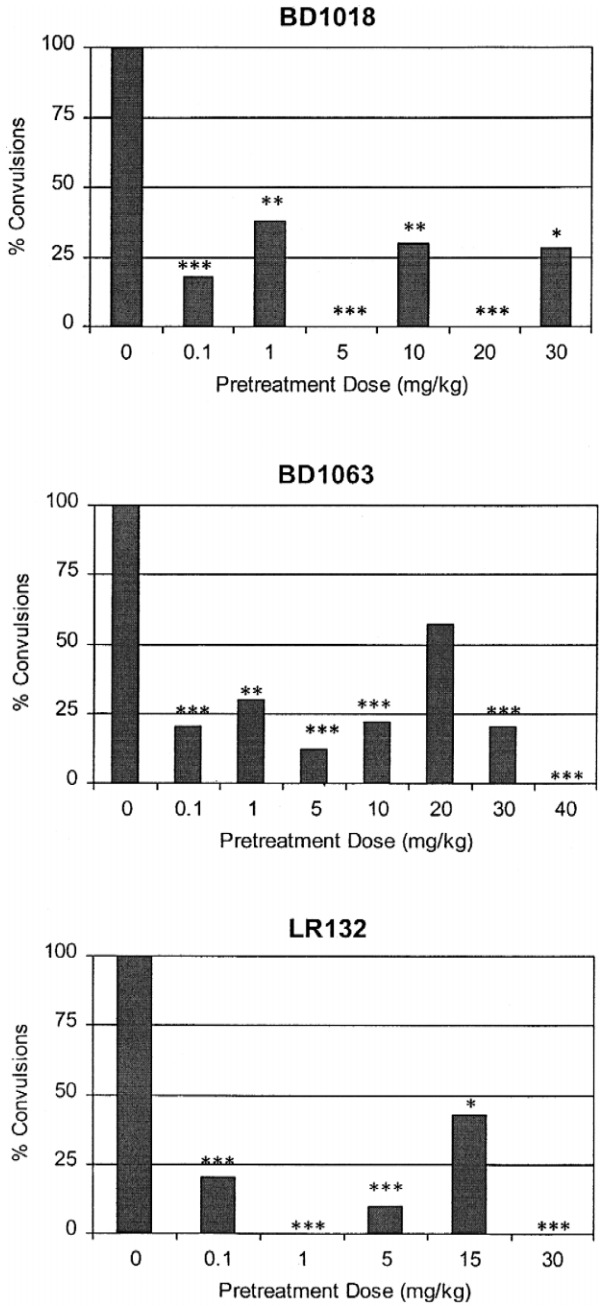

Fig. 2. Pre-treatment with BD1018, BD1063, or LR132 attenuates cocaine-induced convulsions. Mice were pre-treated with one of the novel compounds, followed 15 min later with a convulsive dose of cocaine (60 mg/kg, i.p.). The data are represented as the number of mice convulsing during the 30 min testing period/the total number of mice tested × 100%. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

Pre-treatment of mice with BD1018, BD1063, or LR132 significantly attenuated cocaine-induced lethality ($P < 0.05$ for at least one dose; Fig. 4). The dose curves for some of the compounds were U-shaped, with the loss of protection at higher doses. There was no significant difference between the survival rates of the animals 30 min vs. 24 h after the overdose (Fisher's exact tests, n.s.). Similar to the pattern observed when convulsions were used as the behavioral endpoint, DTG exacerbated the lethal effects of cocaine, shifting the LD_{50} for cocaine to 29 mg/kg, i.p. BD1031, another putative σ receptor agonist failed to attenuate the lethal effects of cocaine. However, due to the limited quantities of BD1031, it was not tested against multiple doses of cocaine.

Post-treatment with LR132 prevented death in a significant proportion of animals even when it was administered after the onset of convulsions. The 0.1 mg/kg dose of LR132 prevented death in 7/10 mice (70% protection, $P < 0.05$), while the 1 mg/kg dose of LR132 rescued 4/8 mice (50% protection, $P < 0.05$). In contrast, Fisher's exact test revealed that post-treatment with BD1063 failed to prevent death in a significant number of animals when administered as a post-treatment.

3.4. Locomotor activity

A repeated measures analysis of variance revealed a significant effect of cocaine dose on horizontal locomotor activity ($F[4,20] = 35.05$, $P < 0.0001$). Post-hoc Dunnett's tests further revealed that there was a significant difference between the saline control vs. each of the cocaine doses (5 mg/kg: $q = 3.14$, $P < 0.05$; 10 mg/kg: $q = 9.36$, $P < 0.01$; 15 mg/kg: $q = 9.09$, $P < 0.01$; 20 mg/kg: $q = 8.26$, $P < 0.01$). Similar to previous studies, the dose–response curve for the locomotor stimulatory effect of cocaine peaked at 10 mg/kg, i.p. (McCracken et al., 1999b). The

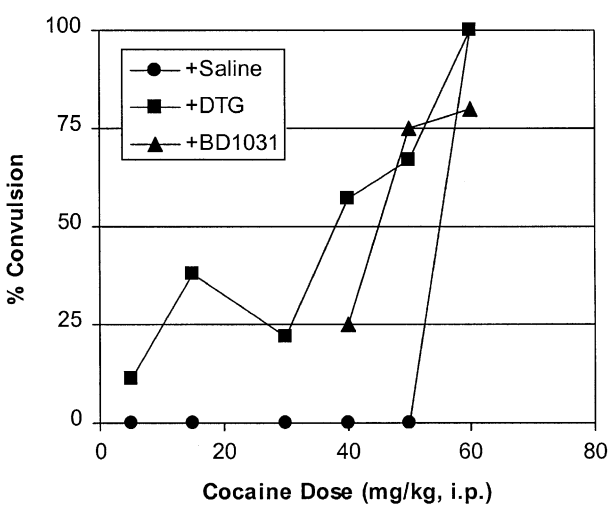

Fig. 3. Pre-treatment with σ receptor agonists worsens the convulsive effects of cocaine. Mice were pre-treated (i.p.) with either saline, DTG (30 mg/kg) or BD1031 (30 mg/kg), followed 15 min later with a dose of cocaine (5–60 mg/kg, i.p.). There was a shift to the left in the dose curve for cocaine-induced convulsions in the presence of DTG or BD1031. The data are represented as the number of mice convulsing during the 30-min testing period/the total number of mice tested × 100%.

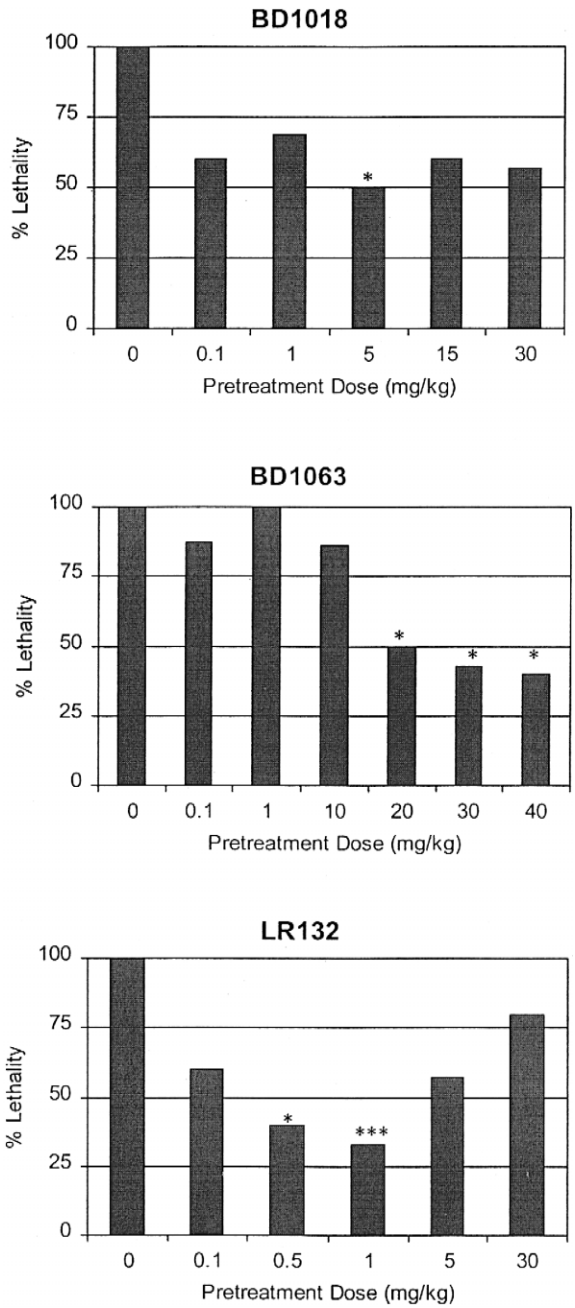

Fig. 4. Pre-treatment with BD1018, BD1063, or LR132 attenuates cocaine-induced lethality. Mice were pre-treated with one of the novel compounds, followed 15 min later with a lethal dose of cocaine (125 mg/kg, i.p.). The data are represented as the number of mice dying during the 30-min testing period/the total number of mice tested × 100%. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

behaviorally inactive doses of BD1018, BD1063, and LR132 that were used in the antagonism portions of the study are shown in Fig. 5; there was no significant difference between these doses and the saline vehicle ($F[3,15] = 0.28$, n.s.). Although alone, they produced no significant effects on locomotor activity, BD1018, BD1063, and LR132 dramatically attenuated the locomotor stimulatory effects of cocaine (Fig. 5). A repeated measures analysis of variance revealed a significant effect of treatment ($F[3,15] = 35.49$, $P < 0.0001$), and post-hoc Dunnett's tested showed a significant difference between mice pre-treated with saline vs. BD1018 ($q = 9.33$, $P < 0.01$), BD1063 ($q = 8.22$, $P < 0.01$), or LR132 ($q = 7.17$, $P < 0.01$).

3.5. Antisense

There was a 45% and 38% reduction in the B_{max} of σ_1 receptors in pooled brain membranes from mice treated

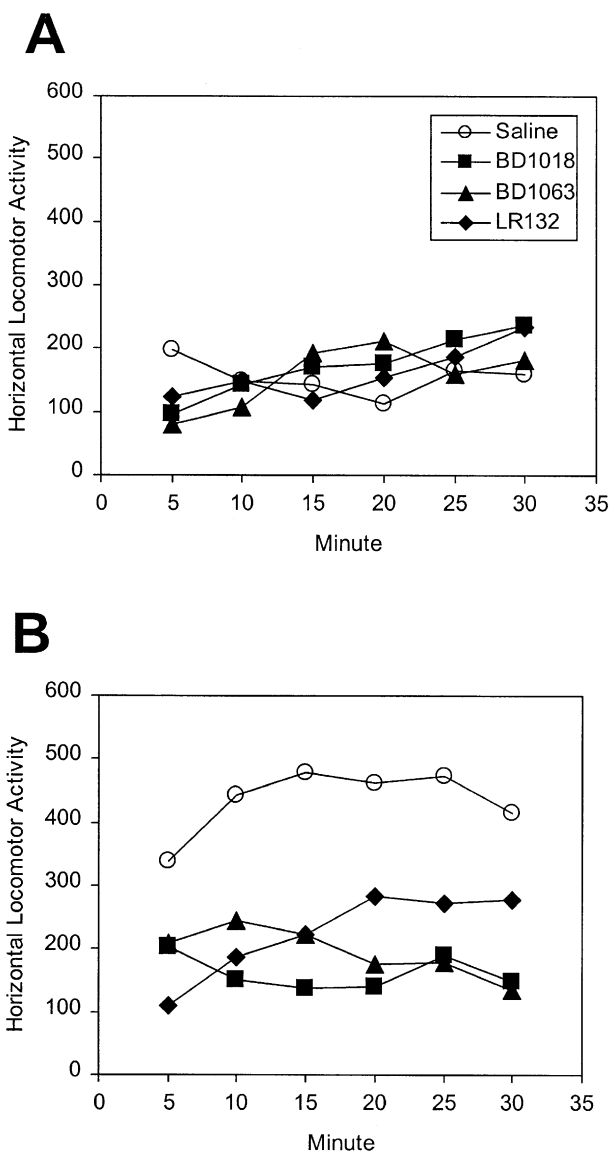

Fig. 5. Effects of the conformationally restricted ligands on spontaneous (A) and cocaine-induced (B) locomotor activity. Panel A: Mice were injected (i.p.) with BD1018 (30 mg/kg), BD1063 (30 mg/kg), or LR132 (30 mg/kg). Horizontal locomotor activity was measured for the next 30 min. Alone, BD1018, BD1063, and LR132 produced effects that did not differ significantly from saline. Panel B: Mice were injected (i.p.) with saline, or 30 mg/kg of BD1018, BD1063, or LR132, followed 15 min later with cocaine (10 mg/kg). Horizontal locomotor activity was monitored for the next 30 min. Pre-treatment with BD1018, BD1063, or LR132 significantly attenuates the locomotor stimulatory effects of cocaine ($P < 0.0001$).

with antisense (189 fmol/mg protein) as compared to mismatch (344 fmol/mg protein) or saline (305 fmol/mg protein), respectively. This reduction in the number of σ_1 receptors appears to have functional significance because prior administration of an antisense oligodeoxynucleotide against σ_1 receptors provided significant protection against the convulsive effects of cocaine ($P < 0.05$; Fig. 6A). Prior to receiving cocaine, animals treated intracerebroventricularly with saline, antisense, or sense oligodeoxynucleotides exhibited a level of baseline locomotor activity that was comparable to mice injected intraperitoneally with saline (Fig. 6B). However, following challenge with a locomotor stimulatory dose of cocaine, the antisense-treated animals exhibited an attenuated response ($F[3,15] = 4.16$, $P < 0.05$; Fig. 6C). Student–Newman–Keuls pairwise comparisons revealed that there was a significant difference between the antisense vs. sense ($q = 3.37$, $P < 0.05$) or saline ($q = 4.53$, $P < 0.05$) treatment groups.

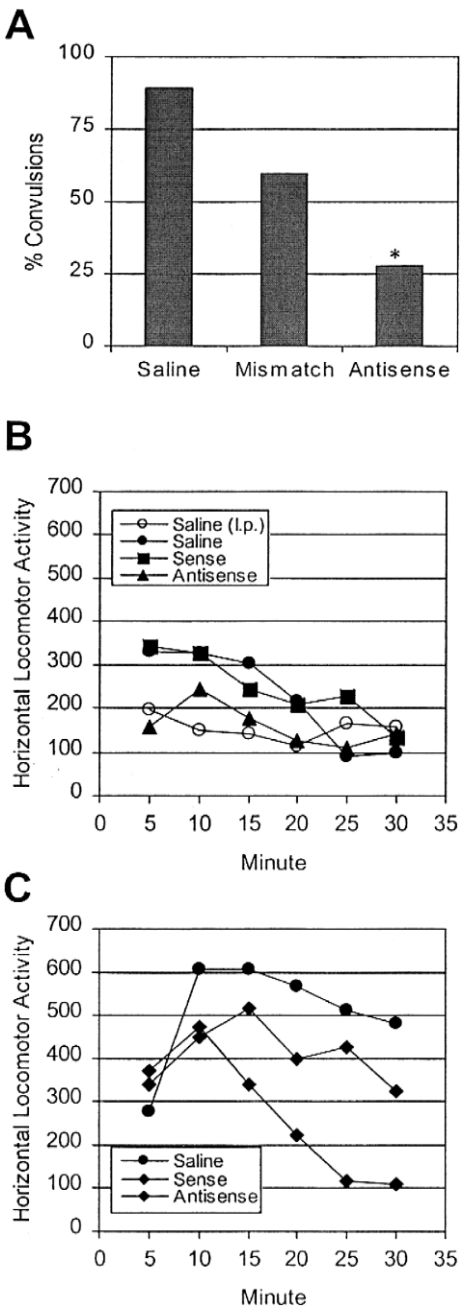

Fig. 6. Effects of antisense treatment on cocaine-induced convulsions (A), baseline locomotor activity (B), and cocaine-induced locomotor activity (C). Mice were injected i.c.v. with saline or 10 μg/5 μl of an antisense against σ_1 receptors or its mismatch or sense sequences on Days 1, 2, and 4. On Day 5, the mice were challenged with a convulsive (60 mg/kg, i.p.) or locomotor stimulatory (10 mg/kg, i.p.) dose of cocaine. There was a significant attenuation of cocaine-induced convulsions (A) and locomotor activity (C) in mice that first received antisense oligodeoxynucleotides. There was no significant effect of antisense treatment on baseline locomotor activity (B).

4. Discussion

BD1018, BD1063, and LR132, three conformationally restricted analogs of the σ receptor ligand BD1008, have high affinities for σ_1 receptors. They vary in their affinities for σ_2 receptors and exhibit micromolar to negligible affinities for 5-HT_2 receptors. The ability of the conformationally restricted σ receptor ligands to bind to σ_1, σ_2, and 5-HT_2 receptors suggests that these interactions may contribute to the anti-cocaine effects of the compounds. Moreover, the ability of a σ_1 receptor antisense oligodeoxynucleotide to significantly attenuate the behavioral actions of cocaine demonstrates that antagonism of σ_1 receptors alone is sufficient to elicit an anti-cocaine effect. Together, the data suggest that antagonism of σ_1 receptors may be a significant mechanism underlying the anti-cocaine actions observed herein, with additional interactions with σ_2 and 5-HT_2 receptors also contributing to the overall effectiveness of a given compound.

Some of the compounds tested herein interact with 5-HT_2 receptors. These interactions may influence the data because earlier studies reported that 5-HT_2 receptor antagonists such as ketanserin, cinaserin, and pirenperone attenuate the convulsive effects of cocaine (Ritz and George, 1997a). However, the pattern of the data in the present study suggests that while 5-HT_2 interactions could have a contributory role, serotonergic mechanisms alone are not sufficient to explain the ability of the compounds to protect against cocaine-induced convulsions. First, some of the compounds tested in the present study, such as LR132, do not interact with 5-HT_2 receptors and still exhibit anti-cocaine actions. If antagonism of 5-HT_2 receptors was the primary mechanism involved in the protective actions observed herein, LR132 should have been ineffective. Second, the protective actions of the compounds against cocaine-induced convulsions could be produced at low doses in the present study, a pattern that is consistent with the high affinities of the compounds for σ_1 receptors, but not with their almost 1000-fold weaker interactions with 5-HT_2 receptors. Third, in addition to the results reported

herein, structurally related BD1008 analogs with *N*-alkyl substitutions (Matsumoto et al., 2001), pyrrolidinyl alterations (McCracken et al., 1999a), or aryl monosubstitutions (McCracken et al., 2000) also block the convulsive effects of cocaine, but have negligible affinities for 5-HT_2 receptors. Although they are effective against the convulsive effects of cocaine, 5-HT_2 receptor antagonists are unable to attenuate the lethal effects of cocaine (Ritz and George, 1997b). This inability of 5-HT_2 receptor antagonists to attenuate the lethal effects of cocaine would be consistent with the observation that BD1018, the compound with the highest affinity for 5-HT_2 receptors, provided the least protection against the lethal endpoint. Thus, while interactions 5-HT_2 receptors cannot alone account for protective actions against cocaine in the present study, they may contribute to the overall pattern of activity of some of the compounds.

Several lines of evidence suggest that functional antagonism of σ receptors is the most likely mechanism explaining the anti-cocaine effects of the novel compounds in the present study. First, the compounds exhibit high and preferential affinity for σ receptors, particularly the σ_1 subtype, as compared to other binding sites. Not withstanding the ability of some of the compounds to interact with 5-HT_2 receptors at micromolar concentrations, receptor binding studies confirmed that the compounds lacked significant affinities for dopamine, NMDA, $GABA_A$ and opiate receptors. In addition, BD1018, BD1031, and BD1063 have micromolar or lower affinities for muscarinic, α_1, α_2, β-adrenergic, and 5-HT_1 receptors (Matsumoto et al., 1995; unpublished data). Second, BD1063 which produced anti-cocaine actions herein, acts as a functional antagonist at σ receptors in several other in vivo and in vitro assays (Joseph and Bowen, 1998; Matsumoto et al., 1995; Monnet et al., 1996; Tran et al., 1998; Vilner and Bowen, 2000), making it likely that it also acts as an antagonist in the present studies. Third, in contrast to the protective effects provided by the putative antagonists, σ receptor agonists exacerbated the toxic effects of cocaine. The well-characterized σ receptor agonist DTG worsened both the convulsive and lethal effects of cocaine. In addition, BD1031 (the *trans*-isomer of BD1018) was unable to prevent cocaine-induced convulsions and lethality, demonstrating a stereospecificity for this response. BD1031 has previously been shown to possess agonistic activity in other σ-mediated functional effects, such as the ability to produce dystonic postures after microinjection into the rat red nucleus (Matsumoto et al., 1999), and it shifted the dose–response for cocaine-induced convulsions to the left in the present study. Finally, the directionality of the effect of the putative antagonists was comparable to that produced by an antisense oligodeoxynucleotide against σ_1 receptors. Since antisense oligodeoxynucleotides are thought to act by interfering with the synthesis of new receptor proteins (Davidkova and Weiss, 1998), they are expected to act as functional antagonists with a high degree of selectivity when used with the proper controls. In this particular case, the antisense, but not the sense or mismatch, sequences attenuated cocaine-induced convulsions and locomotor activity. Therefore, when considered together, the results suggest that compounds with antagonistic actions at σ receptors attenuate the convulsive and lethal effects of cocaine, while compounds with agonistic actions at the receptor exacerbate the behavioral toxicity of cocaine.

The specific σ receptor subtype(s) involved in the protective effects has yet to be conclusively determined. The ability of the antisense oligodeoxynucleotide to attenuate the convulsive and locomotor stimulatory effects of cocaine provides strong evidence for an involvement of σ_1 receptors, since it was designed to selective target the mRNA sequence for σ_1 receptors. Moreover, all of the compounds have high affinities for σ_1 receptors and produce robust anti-cocaine actions, further supporting the importance of the σ_1 subtype in the protective effects. Lacking a σ_2 antisense, the importance of this subtype is difficult to ascertain at this time. However, it is noteworthy that LR132, which has relatively weak affinity for σ_2 receptors, retained marked protective ability. Therefore, together with the antisense data, the results with LR132 suggest that antagonism of σ_1 receptors alone is sufficient to elicit anti-cocaine actions. However, additional studies are needed to fully evaluate the contribution of the σ_2 subtype.

The ability of the conformationally restricted compounds and an antisense oligodeoxynucleotide to attenuate both the behavioral toxicity and locomotor stimulatory actions of cocaine may stem from their ability to directly interfere with cocaine's access to one of its target proteins (i.e. σ receptors), and to additionally modulate downstream neurotransmitter systems that are involved in the behavioral toxicity and locomotor stimulatory effects of cocaine. Dopamine systems have a very important role in the psychomotor stimulatory actions of cocaine and σ receptor agonists are known to stimulate dopamine release and synthesis (Booth and Baldessarini, 1991; Iyengar et al., 1990; Patrick et al., 1993; Weiser et al., 1995; Weatherspoon et al., 1996). Although the actions of σ receptor antagonists on dopamine systems have yet to be studied, it is plausible that this class of compounds would negatively modulate dopamine systems that mediate the actions of cocaine. Similarly, NMDA receptors appear to be involved in the end stage events of a cocaine overdose, such as convulsions, respiratory distress, and cardiovascular problems (Brackett et al., 2000). Again, σ receptor agonists have been reported to enhance NMDA-mediated responses, which can then be attenuated with antagonists (Iyengar et al., 1990; Monnet et al., 1992; Yamamoto et al., 1995). Therefore, σ receptor antagonism has the potential to negatively modulate downstream neurochemical systems that have an important role in the toxic and psychomotor actions of cocaine, in addition to directly

interfering with cocaine's access to a subset of its target proteins.

In addition to the overall trends discussed above, two peculiarities involving deviations from traditional, expected dose–response curves require comment. First, the dose curves for the protection provided by the conformationally restricted σ ligands against cocaine are quite steep, exhibiting more of a threshold effect going from ineffective to effective doses than a traditional gradual dose–response. Second, the protective effects of the antagonists against cocaine-induced lethality are sometimes U-shaped. Both of these features may result from mechanistic properties associated with cocaine itself. For example, the dose–response curve for cocaine itself is very steep, going from no effect to a maximal response within a narrow dose range, when convulsions, lethality, and locomotor activity are used as behavioral endpoints in mice (McCracken et al., 1999a,b; Brackett et al., 2000). Even in humans, imaging studies show that cocaine must occupy about 50% of its receptors before its reinforcing effects are perceptible, and that at doses of cocaine that are typically abused by addicts, receptor occupancy is in the 80–90% range (Gatley et al., 1997), suggesting that a large proportion of cocaine's receptors must be occupied for it to produce its physiological actions. Given the characteristics of the dose–response curve for cocaine itself, it would not be surprising for a true cocaine antagonist to also have a relatively steep dose curve, with perhaps a breakpoint dose at which its therapeutic effects become apparent.

Although it is unclear why the protective effects of LR132, and possibly BD1018, against cocaine-induced lethality are U-shaped, two explanations are plausible. First, the compounds may act as partial agonists rather than full antagonists. At higher doses, a partial agonist would produce weak agonist actions, which in this case would be expected to worsen the behavioral toxicity of cocaine. Since the parent compound BD1008 is best described as a partial agonist, further studies involving these conformationally restricted analogs may reveal them to also exhibit similar properties. Second, it is possible that the compounds have significant affinity for another binding site that was not examined in this project, which might influence the response to cocaine. If this were true, we would expect that the affinity of the compounds is weaker at this other receptor than it is for σ receptors, and that the unidentified interaction would serve to worsen or mimic the actions of cocaine. If these mechanisms underlie the U-shaped dose–response curves observed herein, then high selectivity and unequivocal antagonism at σ receptors would be favorable features to target because they would enable a compound to produce anti-cocaine effects over a wide range of doses.

In any event, it is noteworthy that LR132 prevented death when administered after an overdose of cocaine. Such effectiveness under post-treatment conditions would be an important requirement of a clinically useful compound. BD1063, although unable to prevent death under the post-treatment condition, exhibited improved protective ability the earlier it was administered. As compared to the pre-treatment (before cocaine) and post-treatment (after cocaine and the onset of convulsions), BD1063 produced an intermediate level protection when administered after a lethal dose of cocaine but before the onset of convulsions. Since under the post-treatment condition (i.p. injections after the onset of convulsions), the compounds only had 2–4 min in which to elicit therapeutic effects, differences in pharmacokinetic factors could have a large influence on the outcome. The pharmacokinetic properties of these novel σ receptor ligands have yet to be characterized, but the existing data suggest that administration routes that facilitate their absorption will be needed if these compounds are to have potential clinical relevance in overdose situations.

Together, the data demonstrate that interfering with cocaine's access to σ receptors, using either pharmacological antagonists or antisense oligodeoxynucleotides, attenuates the behavioral toxicity and locomotor stimulatory effects of cocaine. Further modulatory actions may be mediated through σ_2 and 5-HT_2 receptors. Additional studies to define the pharmacodynamic and pharmacokinetic properties that enable a compound to protect against the behavioral actions of cocaine are needed in order to optimize a rational approach to the development of medications for cocaine abuse.

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