N-Substituted Benztropine Analogs: Selective Dopamine Transporter Ligands with a Fast Onset of Action and Minimal Cocaine-Like Behavioral Effects

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ABSTRACT
Previous studies suggested that differences between the behavioral effects of cocaine and analogs of benztropine were related to the relatively slow onset of action of the latter compounds. Several N-substituted benztropine analogs with a relatively fast onset of effects were studied to assess whether a fast onset of effects would render the effects more similar to those of cocaine. Only one of the compounds increased locomotor activity, and the increases were modest compared with those of 10 to 20 mg/kg cocaine. In rats trained to discriminate 10 mg/kg cocaine from saline none of the compounds produced more than 40% cocaine-like responses up to 2 h after injection. None of the compounds produced place-conditioning when examined up to 90 min after injection, indicating minimal abuse liability. The compounds had 5.6 to 30 nM affinities at the dopamine transporter (DAT), with uniformly lower affinities at norepinephrine and serotonin transporters (from 490-4600 and 1420–7350 nM, respectively). Affinities at muscarinic M1 receptors were from 100- to 300-fold lower than DAT affinities, suggesting minimal contribution of those sites to the behavioral effects of the compounds. Affinities at histaminic H1 sites were from 11- to 43-fold lower than those for the DAT. The compounds also had affinity for sigma, 5-hydroxytryptamine, (5-HT1), and 5-HT2 receptors that may have contributed to their behavioral effects. Together, the results indicate that a slow onset of action is not a necessary condition for reduced cocaine-like effects of atypical DAT ligands and suggest several mechanisms that may contribute to the reduced cocaine-like efficacy of these compounds.

Introductions
Previous studies indicated that dopamine (DA) transport inhibitors with varying potency produce behavioral effects that differ little from those of cocaine (e.g., Kuhar et al., 1991). More recently, several “atypical” DA uptake inhibitors were identified that do not completely reproduce cocaine’s behavioral effects, despite inhibiting DA uptake with high DA transporter (DAT) affinity (Tanda et al., 2009). For example, benztropine (BZT) analogs have been reported that did not stimulate locomotor activity as effectively as cocaine, did not fully substitute for cocaine in rats trained to discriminate cocaine from saline injections (Newman et al., 1995; Katz et al., 1999, 2004), were not self-administered to the same degree as cocaine or other DA uptake inhibitors (Woolerton et al., 2000; Ferragud et al., 2009; Hirani et al., 2009b), did not produce place conditioning comparable with cocaine (Li et al., 2005; Velázquez-Sánchez et al., 2009), and were less effective than cocaine in stimulating nucleus accumbens shell DA levels (Tanda et al., 2005, 2009). Although BZT analogs may be the most thoroughly studied atypical...
DAT inhibitors, examples from other structural classes exist, including analogs of the σ receptor ligand, rimcazole (Katz et al., 2003), and some analogs of cocaine (Navarro et al., 2009).

Structure-activity studies have indicated that BZT analogs bind to the DAT in a manner that differs from that for standard DAT inhibitors (Newman et al., 1995). Beuming et al. (2008) used a DAT model based on the crystallized structure of the bacterial leucine transporter (Yamashita et al., 2005) to model DAT binding. BZT analogs, as opposed to cocaine analogs, preserved a distance between Tyr156 and Asp79 that allowed hydrogen bonding between the two residues. That bond was suggested to close the binding pocket, shielding the binding site from extracellular space (Beuming et al., 2008). Furthermore, studies of the binding of the radiolabeled BZT analog, \( \text{N-(n-butyl)-3a-[bis(4'-fluorophenyl)methoxy]-tropane (JHW 007)} \), indicate that, as opposed to other DAT inhibitors, its binding is insensitive to sodium (Kopajtic et al., 2010), suggesting again that DAT binding of BZT analogs differs from that for other DAT inhibitors.

Pharmacological studies have suggested conformational changes in the DAT induced by uptake inhibitors, consistent with the modeling studies. Loland et al. (2008) found that cocaine analogs were less potent as DA uptake inhibitors in cells transfected with a DAT mutant (Y335A) that assumes an inward-facing conformation than in cells with WT DAT. In contrast, BZT analogs had more similar potencies for the two forms of DAT. Furthermore, there was a relationship between the behavioral effects of the compounds and the change in DA-uptake inhibition potency in Y335A- and WT-transfected cells. The behavioral effects of compounds for which there was a large ratio were comparable with those of cocaine. In contrast, a small ratio predicted atypical behavioral effects.

One BZT analog, JHW 007, is of interest because it blocks some of the effects of cocaine. When administered before cocaine, JHW 007 antagonized the stimulation of locomotor activity normally produced by cocaine (Desai et al., 2005a). Furthermore, combinations of cocaine with JHW 007, in contrast to typical DA uptake inhibitors, are less than additive, and at some concentrations JHW 007 antagonized the effects of cocaine on extracellular DA, consistent with the antagomism of cocaine’s behavioral effects (Tanda et al., 2009). Finally, JHW 007 antagonizes the self-administration of cocaine (Hiranita et al., 2009b) as well as the place conditioning induced by cocaine (Velázquez-Sánchez et al., 2010).

One feature of JHW 007 that may contribute to its atypical actions, as well as its cocaine-antagonist effects, is its slow onset of action. Desai et al. (2005a) reported that the rate at which JHW 007 displaced \( ^{[125]}\text{I}-\beta\text{-carboisopropoxy-3}-\text{cyclopropylmethyl)-3a-}[\text{4',4'-difluoro-diphenylmethoxy}]-\text{tropane HCl (JHW 013)} \) was substantially faster than that for JHW 007. Thus, to examine the hypothesis that a slow onset was critical for atypical effects of DAT inhibitors, we studied the acute behavioral effects of this group of N-substituted BZT analogs.

### Materials and Methods

**Subjects.** The subjects were Swiss-Webster mice and Sprague-Dawley rats that, respectively, weighed 20 to 25 and 180 to 200 g when received (both from Taconic Farms, Germantown, NY). They were housed in a temperature- and humidity-controlled animal facility with a 12-h light/dark cycle (lights on at 7:00 AM), with all experiments conducted during the light phase. Subjects were allowed to habituate to the animal facility for at least 1 week before experiments. Food and water were available at all times except during experimental sessions. Experiments were conducted in accordance with National Institutes of Health Guidelines under Institutional Animal Care and Use Committee-approved protocols.

**Dopamine Transporter Binding Assay.** Brains from male Sprague-Dawley rats weighing 200 to 225 g (Taconic Farms) were removed, and the striatum was dissected and quickly frozen. Membranes were prepared by homogenizing tissues in 20 volumes (w/v) of ice-cold modified sucrose-phosphate buffer (0.32 M NaCl, 7.74 mM NaHPO\(_4\), pH adjusted to 7.4) using a Brinkmann Instruments (Westbury, NY) Polytron (setting 6 for 20 s) and centrifuged at 50,000 g for 10 min at 4°C. The resulting pellet was resuspended in buffer, recentrifuged, and suspended in buffer again to a concentration of 10 mg/ml original wet weight (OWW).

Ligand binding experiments were conducted in assay tubes containing 0.5 ml of sucrose-phosphate buffer. Each tube contained 0.5 mM \( ^{3}\text{H}\text{-}(\text{2-β-carboisopropoxy-3-β,4-fluorophenyl)tropane-1,5-napthalenedisulfonate (WIN 35,428) (specific activity 84 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA) and 1.0 mg of striatal tissue (OWW). The reaction was started with the addition of tissue, and the tubes were incubated for 120 min on ice. Non-specific binding was determined using 0.1 mM cocaine HCl (Sigma-Aldrich, St. Louis, MO). The affinities of these compounds were reported previously (Agoston et al., 1997; Robarge et al., 2000) but were assayed again because previous results were obtained with a...
HEPES buffer, which typically renders 3-fold lower Kᵢ values than the presently used buffer (unpublished data).

**Norepinephrine Transporter Binding Assay.** Frontal cortex was dissected from brains of male Sprague-Dawley rats (Taconic Farms) and frozen for later use. Tissue was thawed and homogenized in 20 volumes (w/v) of 50 mM Tris containing 120 mM NaCl and 5 mM KCl (pH 7.4 at 25°C), using a Brinkmann Instruments Polytron (setting 6 for 20 s). The tissue was centrifuged at 50,000g for 10 min at 4°C. The resulting pellet was resuspended in buffer and centrifuged again. The final pellet was resuspended in cold buffer to a concentration of 80 mg/ml OWW.

Ligand binding experiments were conducted in assay tubes containing 0.5 ml of buffer, 0.5 mM [H]niosoxetine (PerkinElmer Life and Analytical Sciences), and 8 mg of frontal cortex tissue OWW. The reaction was started with the addition of the tissue, and the tubes were incubated for 60 min at 0 to 4°C. Nonspecific binding was determined using 1 μM desipramine (Sigma-Aldrich).

**Serotonin Transporter Binding Assay.** Midbrain was dissected from brains of male Sprague-Dawley rats (Taconic Farms) and frozen for later use. Tissue was thawed and homogenized in 20 volumes (w/v) of 50 mM Tris containing 120 mM NaCl and 5 mM KCl (pH 7.4 at 25°C), using a Brinkmann Instruments Polytron (setting 6 for 20 s). The tissue was centrifuged at 50,000g for 10 min at 4°C. The resulting pellet was resuspended in buffer and centrifuged again. The final pellet was resuspended in cold buffer to a concentration of 15 mg/ml (OWW).

Ligand binding experiments were conducted in assay tubes containing 0.5 ml of buffer, 1.4 nM [H]citalopram (PerkinElmer Life and Analytical Sciences), and 1.5 mg of midbrain tissue. The reaction was started with the addition of the tissue, and the tubes were incubated for 60 min at 25°C (room temperature). Nonspecific binding was determined using 10 μM fluoxetine (Sigma-Aldrich).

**α₁ Receptor Binding.** Frozen whole guinea pig brains (minus cerebellum) were thawed on ice, weighed, and homogenized (with a glass and Teflon homogenizer) in 10 mM Tris-HCl with 0.32 M sucrose, pH 7.4 (10 ml/g tissue). The homogenate was centrifuged at 1000g for 10 min at 4°C. The supernatant was collected into a clean centrifuge tube, and the remaining pellet was resuspended by vortexing in 10 ml of buffer (tissue) and centrifuged again at 50,000g for 15 min at 4°C. The resulting pellet was resuspended at 3 ml of (OWW) in 10 mM Tris-HCl with 0.32 M sucrose, pH 7.4, and mixed by vortexing. The pellet was gently resuspended in experimental buffer to 80 mg/ml (OWW).

Ligand binding experiments were conducted in polypropylene assay tubes containing 0.5 ml of 50 mM Tris-HCl buffer, pH 8.0. Each tube contained 3 nM [H]-1,3-di-ortho-tolylguanidine (DTG) (Perkin Elmer Life and Analytical Sciences), 200 nM (+)-pentazocine, and 8.0 mg of tissue (OWW). Nonspecific binding was determined using 100 μM haloperidol.

Incubations for all binding assays were terminated by rapid filtration through Whatman (Clifton, NJ) GF/B filters, presoaked in polyethyleneimine, using a Brandel R46 filtering manifold (Brandel Inc., Gaithersburg, MD). The filters were washed twice with 5 ml of ice-cold buffer and transferred to scintillation vials. Beckman Ready Safe (3.0 ml) was added, and the vials were counted the next day using a Beckman 6000 liquid scintillation counter (Beckman Coulter, Fullerton, CA) at 50% efficiency. Assays were typically conducted in at least three independent experiments, each performed in triplicate.

The ICₒ values from the displacement data were computed using a nonlinear, least-squares regression analysis (Piriss; GraphPad Software Inc., San Diego, CA). Inhibition constants (Kᵢ values) were calculated using the equation of Cheng and Prusoff (1973), the concentration of radioligand used in the assay, and the historical value for the Kᵢ value of the radioligand determined in this laboratory.

**Receptor Screen.** The compounds were also screened for their activity at various receptor sites by examining their competition with the appropriate radioligands (ProfilingScreen procured from MDS Panlabs Pharmacology Services, Bothell, WA). The screen consisted of assays designed to assess the activity of the compounds at various mammalian receptors listed in Table 1. Each compound was tested in duplicate in each assay at a concentration of 10 μM. Concurrent vehicle and reference standards were conducted with each assay. If displacement at 10 μM was more than 50% the result was considered positive, otherwise a Ki value was considered to be more than 100 μM. For JHW 013, all positive results were repeated at concentrations from 0.1 to 100 μM in log-unit increments, to obtain an estimate of the affinity for the site. For the other compounds, selected positive results were repeated to obtain affinity estimates. For sites at which activity was identified, ICₒ values were computed as described using the obtained ICₒ value, the concentration of radioligand used in the assay, and the MDS Panlabs historical value for the Kᵢ value of the radioligand. Because ICₒ values were determined from four concentrations of cold compound, the derived binding constants should be interpreted as estimates. Significant details of the assay procedures are also provided in Table 1.

**Locomotor Activity.** Experimentally naive mice were placed singly in clear acrylic chambers (40 cm³) containing within monitors (Arcusen Instruments, Inc., Columbus, OH) that were equipped with light-sensitive detectors. The detectors were spaced 2.5 cm apart along two perpendicular walls with infrared light sources mounted on the opposing walls and directed at the detectors. Activity counts were registered for each interruption of a light beam. Mice were injected (intraperitoneally in volumes of 1 ml/100 g), and then immediately placed in the apparatus for 60 min or for 8 h in other experiments. Activity counts were collected every 10 min. Mice were used only once, and each dose was studied in six to eight mice. The doses of the N-substituted BZT analogs tested in half log-unit steps were as follows: GA 1-69, 0.03 to 1.0 mg/kg; GA 2-50, 3.0 to 30.0 mg/kg; GA 2-99, 3.0 to 30.0 mg/kg; and JHW 013, 0.1 to 10.0 mg/kg. The doses of cocaine tested were 5.0 to 40.0 mg/kg in one-third log-unit steps.

**Cocaine Discrimination.** Details have been described previously (Katz et al., 2004). In brief, experimentally naive male rats were individually housed and maintained at 325 to 350 g by providing 10 to 15 g of food daily at least 1 h after testing. Experiments were conducted at the same time daily, with subjects placed in 29.2 × 24.2 × 21-cm operant-conditioning chambers (modified ENV-001; MED Associates, St. Albans, VT) containing two response keys (levers requiring a downward force of 0.4 N) with pairs of green and yellow light-emitting diodes above each. A dispenser delivered 45 mg food pellets (BioServ, Frenchtown, NJ) to a tray located between the response keys, and a light was mounted near the ceiling to provide...
<table>
<thead>
<tr>
<th>Assay Target</th>
<th>Ligand</th>
<th>Nonselective Binding</th>
<th>Tissue</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine A₁</td>
<td>1 nM [³H]DPAT</td>
<td>100 nM Rp-adenosine 3',5'-cyclic monophosphate (cAMP)</td>
<td>Human recombinant CHO cells</td>
<td>90 min at 25°C</td>
</tr>
<tr>
<td>Adenosine A₂A</td>
<td>0.05 nM [³H]CGS-21680</td>
<td>50 nM NECA</td>
<td>Human recombinant HEK-293 cells</td>
<td>90 min at 25°C</td>
</tr>
<tr>
<td>Adrenergic α₁, nonselective</td>
<td>0.25 nM [³H]prazosin</td>
<td>0.1 µM Prazosin</td>
<td>Rat brain</td>
<td>30 min at 25°C</td>
</tr>
<tr>
<td>Adrenergic α₁A</td>
<td>0.25 nM [³H]prazosin</td>
<td>10 nM Phenolamine</td>
<td>Rat submaxillary gland</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>Adrenergic α₂B</td>
<td>0.25 nM [³H]prazosin</td>
<td>10 nM Phenolamine</td>
<td>Rat liver</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>Adrenergic α₁A, nonselective</td>
<td>0.7 nM [³H]prazosin</td>
<td>1 µM Yohimbine</td>
<td>Rat cortex</td>
<td>30 min at 25°C</td>
</tr>
<tr>
<td>Adrenergic α₂A</td>
<td>1 nM [³H]MK-912</td>
<td>10 µM BB-94</td>
<td>Human recombinant insect S9 cells</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>Adrenergic β, nonselective</td>
<td>0.25 nM [³H]diiodo-β-estradiol</td>
<td>1 µM [±]-propranolol</td>
<td>Human recombinant Rar 16 cells</td>
<td>120 min at 25°C</td>
</tr>
<tr>
<td>Adrenergic β₁</td>
<td>0.03 nM [³H]ICI-118551</td>
<td>100 nM (S)-propranolol</td>
<td>Human recombinant CHO-NKB1 cells</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>Adrenergic β₂</td>
<td>0.2 nM [³H]CGP-20712</td>
<td>10 µM ICI-118551</td>
<td>Human recombinant CHO-NKB1 cells</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>Ca²⁺ channel-L, dihydropyridine</td>
<td>0.1 nM [³H]nitrendipine</td>
<td>1 µM Nifedipine</td>
<td>Rat cortex</td>
<td>90 min at 25°C</td>
</tr>
<tr>
<td>Dopamine D₁</td>
<td>1.4 nM [³H]Sch 23390</td>
<td>10 µM (S)-Butaclamol</td>
<td>Human recombinant CHO cells</td>
<td>120 min at 37°C</td>
</tr>
<tr>
<td>Dopamine D₂</td>
<td>0.16 nM [³H]spiperone</td>
<td>10 µM Haloperidol</td>
<td>Human recombinant CHO cells</td>
<td>120 min at 25°C</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;, agonist site (muscle)</td>
<td>1 nM [³H]muscimol</td>
<td>100 nM Muscimol</td>
<td>Rat brain (minus cerebellum)</td>
<td>10 min at 4°C</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;, benzodiazepine central</td>
<td>1 nM [³H]flunitrazepam</td>
<td>10 µM Dizepam</td>
<td>Rat brain (minus cerebellum)</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;, chloride channel</td>
<td>3 nM [³H]PCPA</td>
<td>200 nM Picrotoxin</td>
<td>Rat cortex</td>
<td>20 min at 25°C</td>
</tr>
<tr>
<td>Glutamate, NMDA, phencyclidine</td>
<td>4 nM [³H]TCP</td>
<td>1 µM MK-801</td>
<td>Rat cortex</td>
<td>45 min at 25°C</td>
</tr>
<tr>
<td>Glycine, strychnine-sensitive</td>
<td>10 nM [³H]strychnine</td>
<td>1 nM Glycine</td>
<td>Rat cortex</td>
<td>30 min at 37°C</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.03 nM [¹²⁵I]insulin</td>
<td>1 µM Insulin</td>
<td>Rat liver</td>
<td>16 h at 4°C</td>
</tr>
<tr>
<td>Muscarinic M₂</td>
<td>0.29 nM [³H]N-n-propylcarbamol (0.8 nM for JHW 013)</td>
<td>1 µM Atropine</td>
<td>Human recombinant insect s9 cells (human recombinant CHO cells)</td>
<td>60 min at 25°C (120 min at 25°C)</td>
</tr>
<tr>
<td>Muscarinic M₃</td>
<td>0.29 nM [³H]N-n-propylcarbamol (0.8 nM for JHW 013)</td>
<td>1 µM Atropine</td>
<td>Human recombinant insect s9 cells (human recombinant CHO cells)</td>
<td>60 min at 25°C (min at 25°C)</td>
</tr>
<tr>
<td>Nicotinic acetylcholine</td>
<td>0.1 nM [¹²⁵I]epibatidine</td>
<td>300 nM (±)-nicotine</td>
<td>Human IMR-32 cells</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>Opiate, nonselective</td>
<td>1.0 nM [³H]naloxone</td>
<td>1 µM Naloxone</td>
<td>Rat brain</td>
<td>40 min at 25°C</td>
</tr>
<tr>
<td>Opiate-δ</td>
<td>0.9 nM [³H]naltrindole</td>
<td>10 µM Naloxone</td>
<td>Human recombinant CHO cells</td>
<td>120 min at 25°C</td>
</tr>
<tr>
<td>Opiate-κ</td>
<td>0.6 nM [³H]diprenorphine</td>
<td>1 µM Naltrexone</td>
<td>Human recombinant HEK-293 cells</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>Opiate-μ</td>
<td>0.6 nM [³H]diprenorphine</td>
<td>10 µM Naloxone</td>
<td>Human recombinant CHO cells</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>Phorbol ester</td>
<td>3 nM [³H]PDBu</td>
<td>1 µM Phorbol 12,13-dibutyrate</td>
<td>Mouse brain</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>K⁺ channel [K&lt;sub&gt;ATP&lt;/sub&gt;]</td>
<td>5 nM [³H]glibenclamide</td>
<td>1 µM Glyburide</td>
<td>Hamster pancreatic β cells HIT-T15</td>
<td>120 min at 25°C</td>
</tr>
<tr>
<td>K⁺ channel [K&lt;sub&gt;ATP&lt;/sub&gt;]</td>
<td>1.5 nM [³H]astemizole</td>
<td>10 µM Astemizole</td>
<td>Human recombinant HEK-293 cells</td>
<td>60 min at 25°C</td>
</tr>
<tr>
<td>Progestrone</td>
<td>2 nM [³H]progesterone</td>
<td>0.41 µM Progesterone</td>
<td>Bovine uterus</td>
<td>16 h at 4°C</td>
</tr>
<tr>
<td>5-HT₁, nonselective</td>
<td>2 nM [³H]nor-5-HT</td>
<td>10 µM 5-HT</td>
<td>Rat cortex</td>
<td>10 min at 37°C</td>
</tr>
<tr>
<td>5-HT₃, nonselective</td>
<td>0.5 nM [³H]ketanserin</td>
<td>1 µM Ketanserin</td>
<td>Rat brain</td>
<td>40 min at 37°C</td>
</tr>
<tr>
<td>Na⁺ Channel, site 1</td>
<td>50 nM [³H]haraftocortatin A 20-α-azabenzene</td>
<td>100 nM Verapamil</td>
<td>Rat brain</td>
<td>60 min at 37°C</td>
</tr>
</tbody>
</table>

NMDA, N-methyl-D-aspartate; CGP-12177, 4-[3(1,1-dimethylethylamino)-2-hydroxypropyl]-1,3-dihydro-2H-benzo[d]imidazole-2-one; CGP-54626, [S(+)-R,R]-[3-[3-(4,4-dichlophenethyl)ethyl]-amino]-2-hydroxypropyl]-1,3-dihydro-2H-benzene. MEK901, 5,10,15,20-tetrahydro-5H-dibenzo[a,d]cycloheptene-5,10-imine hydrochloride; NMDA, 5-N-methylcarboxamidoadenosine; PDBu, phorbol-12,13-dibutyrate; PLA, N,N′-phenylpropylenediamine; R(-)-isopropylveratridine, 5-HT<sub>3</sub>-agonist site (muscle); SCH 23390, R(-)-7-chloro-8-hydroxy-5-(1,3-dimethylsalamethoxyphenylethyl)aminoethyl)-1,4-benzoxazolone.
conducted exactly as in the preconditioning phase. Subjects were

Each of the N-substituted BZT analogs was first studied when in-

were: saline, cocaine (10 mg/kg), GA 1-69 (3–17 mg/kg), GA 2-50 (3–17 mg/kg), GA 2-99, and JHW 013 (1–10 mg/kg). The treatments that

alternated with vehicle injections in different groups of subjects

compartments. The door between compartments was in place, re-

sessions) injections of saline or the test compound were administered

from the study. Approximately 25% of the subjects were removed

subject spent more than 67% of its time on one side, it was removed

Testing sessions were initiated after subjects met the criteria on

four consecutive sessions of at least 85% cocaine- or saline-appropri-

The postconditioning phase was one 15-min session that was con-

reinforced.

Receptor Binding. The affinities ($K_i$ values) of the BZT

analogs at the various primary sites studied are shown in

Table 2. Affinity at the dopamine transporter varied from

overall illumination. The chamber was contained within a ventilated

enclosure that provided sound attenuation and was supplied with

white noise to further mask extraneous noise.

Rats were initially trained with food reinforcement to press both

levers, and they were subsequently trained to press one after cocaine

(10 mg/kg i.p.) and the other after saline (intraperitoneal) injection. Each response produced an audible click. The ratio of responses to

food pellets (fixed ratio (FR)) was gradually increased until, under

the final conditions, the completion of 20 consecutive responses on the cocaine- or saline-appropriate lever produced food. Incorrect responses reset the FR response requirement. The right versus left

assignments of cocaine and saline keys were counterbalanced among subjects. Subjects were injected and placed in chambers. Sessions started after a 5-min timeout period during which lights were off and responses had no scheduled consequences, other than producing a
click. After the timeout, the house light was turned on until the completion of the FR 20-response requirement and the presentation of food. Sessions ended after 20 food presentations or 15 min, whichever occurred first, and they were conducted 5 days per week, with cocaine or saline sessions scheduled in a double-alter-

nation sequence.

Place Conditioning. Details have been described previously (Li

et al., 2002). In brief, subjects were first habituated to handling

followed by the experiment proper, which was conducted with sub-

jects placed daily in acrylic test chambers (Accuscan Instruments,

Inc.). The chambers were divided into two 20 × 40-cm compartments

separated by a clear acrylic 10 × 6-cm guillotine door. One compart-

ment was black with a floor constructed of stainless-steel mesh,

under which there was β-chip bedding. The other compartment was

black with a floor constructed of stainless-steel rods, under which

there was no bedding. Infrared light sources directed at detectors on

the opposing walls were spaced 2.5 cm apart and allowed recording of

time spent in each compartment and locomotor activity (total number of beams interrupted).

During the first preconditioning phase (four consecutive 15-min
daily sessions) of the experiments, subjects were placed in the cham-

ber close to the middle, with both compartments accessible. The time

spent in each compartment was recorded on the fourth day. If a

subject spent more than 67% of its time on one side, it was removed

from the study. Approximately 25% of the subjects were removed

based on this criterion.

During the conditioning phase (eight consecutive daily 30-min
sessions) injections of saline or the test compound were administered

before alternate sessions, with subjects placed in one of the two

compartments. The door between compartments was in place, re-

stricting the subject to the one compartment. The treatments that

alternated with vehicle injections in different groups of subjects

were: saline, cocaine (10 mg/kg), GA 1-69 (1–17 mg/kg), GA 2-50

(3–17 mg/kg), GA 2-99 (3–17 mg/kg), and JHW 013 (1–10 mg/kg).

Each of the N-substituted BZT analogs was first studied when in-

jected immediately before subjects were placed in the chamber. In

separate studies, subjects were injected and placed back in their

home cages until 45 or 90 min later when they were placed into the
test chamber. The effect on locomotor activity of the first admin-

istration of each treatment was assessed by tabulating the number of

photocell beams broken during the 30-min exposure period.

The first preconditioning phase was one 15-min session that was con-
ducted 1 day after the last conditioning session. This session was

conducted exactly as in the preconditioning phase. Subjects were

placed in the chamber close to the middle with both compartments

accessible. The time spent in each compartment was recorded.

Compounds. The compounds studied were cocaine hydrochloride

(Sigma-Aldrich), and several N-substituted analogs of BZT (Fig. 1):

GA 1-69, GA 2-50, GA 2-99, and JHW 013. The synthesis of these

analogos was conducted in the Medicinal Chemistry Section of

the National Institute on Drug Abuse Intramural Research Program

and has been described previously (Agoston et al., 1997). All compounds

were dissolved in distilled water with heat and sonication, as neces-

sary except cocaine, which was dissolved in saline. All solutions were

administered at 1 ml/kg i.p. except GA 2-50, which was given at 2

and 3.4 ml/kg at the higher doses because of solubility limitations.

Statistical Analysis. Radioligand binding data were analyzed by

using Prism software (GraphPad Software Inc.). The $K_i$ values from individual experiments were determined and averaged to provide a

single value with S.E.M. To obtain a more accurate resolution of

potential multiple-site binding, the data from the $\alpha$-receptor binding

assays were globally fit to one- or two-site binding models. The two

models were compared by $F$ test to determine which model better

fit the data, and those $K_i$ values and their 95% confidence limits are

reported. Locomotor activity in mice was assessed with counts col-

lected during each successive 10-min epoch for 8 h after injection.

Because effects were most prominent in the first hour after injection,

these data were further analyzed as a function of dose using stan-

dard analysis of variance (ANOVA) and linear regression techniques.

For cocaine discrimination, overall rate of responding on both keys

and the percentage of responses emitted on the cocaine-appropriate

key were calculated. The mean values for the group of subjects were

calculated for each measure at each drug dose tested. Because the

percentage of responses emitted on the cocaine-appropriate key is a

relative measure, it is largely independent of the rate of response.

However, when response rates are extremely low, the small sample

size may render the measure an unreliable indication of the discrim-

inative effect of the drug. Therefore, a data exclusion criterion was

implemented by which the percentage of cocaine-appropriate re-

sponding was not calculated if fewer than half of the subjects re-

sponded at a particular dose. This data exclusion criterion was met

once in the course of these studies (for GA 2-99 at 17.0 mg/kg

administered 60 min before testing).

The data were analyzed using standard ANOVA and linear re-

gression techniques to calculate the dose producing a half-maximal

effect (50% cocaine-appropriate responding). For these analyses,

points on the linear part of the ascending portions of the dose-effect

curves were used. Pairs of $ED_{50}$ values were considered to be signif-

icantly different if their 95% confidence limits did not overlap. Dif-

ferences in the effectiveness of selected pairs of compounds were

analyzed by examining maximal effects with Student's $t$ test.

For place conditioning, the time spent in the compound-paired

compartment during the postconditioning session was expressed as a

difference from that during the last preconditioning session (condi-

tioned place preference (CPP) score). Because the time spent in the

compound-paired compartment is an allocation of total time, it is

largely independent of the subjects' activity level. The exception to

this is when activity is virtually eliminated, which did not occur in

the present study. The locomotor activity was expressed as counts/minute during the first compound conditioning sessions. The differ-

ences in group means were analyzed by one-way ANOVA followed by

Dunnett's multiple comparisons of treatments versus saline controls
detecting change from control in either direction. The effects of single

doses of cocaine were compared with vehicle controls by unpaired $t$

tests for independent samples. Effects with a calculated $p$ value < 0.05 were considered statistically significant.

Results

Receptor Binding. The affinities ($K_i$ values) of the BZT

analogos at the various primary sites studied are shown in

Table 2. Affinity at the dopamine transporter varied from
TABLE 2
Affinities of N-substituted BZT analogs in binding to the transporters for dopamine, norepinephrine, and serotonin, and M₁ muscarinic, histamine H₁, σ₁, and σ₂ receptors.

<table>
<thead>
<tr>
<th>Target</th>
<th>GA 1-69</th>
<th>GA 2-50</th>
<th>GA 2-99</th>
<th>JHW 013</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAT</td>
<td>29.2 ± 3.24</td>
<td>13.2 ± 1.50</td>
<td>5.59 ± 0.619</td>
<td>24.6 ± 1.70</td>
</tr>
<tr>
<td>SERT</td>
<td>490 ± 56.4</td>
<td>3870 ± 135</td>
<td>4600 ± 680</td>
<td>1420 ± 116</td>
</tr>
<tr>
<td>NET</td>
<td>7350 ± 934</td>
<td>2130 ± 160</td>
<td>1429 ± 125</td>
<td>1640 ± 153</td>
</tr>
<tr>
<td>M₁</td>
<td>3280 ± 221²</td>
<td>4020 ± 592³</td>
<td>1250 ± 138⁴</td>
<td>257 ± 28.9⁵</td>
</tr>
<tr>
<td>H₁</td>
<td>353 ± 22.6⁶</td>
<td>218 ± 15.5⁷</td>
<td>129 (110–150)</td>
<td>6.90 (6.31–7.57)</td>
</tr>
<tr>
<td>σ₁</td>
<td>430 (385–479)</td>
<td>12.1 (10.9–13.5)</td>
<td>156 (111–219)⁸</td>
<td>25.9 (20.0–33.8)⁹</td>
</tr>
<tr>
<td>σ₂</td>
<td>65.8 (50.8–85.1)¹⁰</td>
<td>18.1 (15.0–21.8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Data were published in Kulkarni et al., 2004.

Data were published in Robarge et al., 2000.

SERT: serotonin transporter.

Data were published in Campbell et al., 2005.

The Kᵢ values reported are from a [³H]DTG binding assay in which the data uniformly modeled better for two than one binding site. The Kᵢ value for the higher affinity site is displayed. The obtained high-affinity site is the site recognized as the σ₂ receptor, whereas the low-affinity site is currently not identified. Values for the low-affinity DTG site and their 95% confidence limits were as follows: GA 1-69, 14,600 (3860–55,000); GA 2-50, 6500 (2830–14,900); GA 2-99, 8490 (424–171,900); JHW 013, 12,100 (4280–34,000).

5.59 nM for GA 2-99 to 29.2 nM for GA 1-69. Affinities at monoamine transporters were uniformly lower, varying from 490 to 4600 and 1420 to 7350 nM, respectively, for the serotonin and norepinephrine transporters (Table 2). In general, the N-substituted compounds retained selectivity for the dopamine transporter over M₁ muscarinic and H₁ histaminic sites. GA 1-69, GA 2-50, and GA 2-99 had from 100- to 300-fold lower affinity for M₁ and 11- to 43-fold lower affinity for H₁ sites. JHW 013 was less selective than the other compounds (Table 2). The affinities of GA 2-50 and JHW 013 for σ₁ receptors were similar to or greater than the affinities of the compounds for the DAT. In contrast, selectivity for the DAT over σ receptors varied more substantially across the compounds studied. Affinities of GA 1-69 and GA 2-99 for σ₁ receptors were approximately 15- to 20-fold lower than their affinities for the DAT, whereas those for GA 2-50 and JHW 013 were, respectively, similar to or greater than their DAT affinities. Only GA 2-99 had an affinity for σ₂ receptors that was appreciably lower than its affinity for the DAT.

The results of the binding screen show activities at sites that varied across the different compounds (Table 3). Sites at which all of the compounds tested uniformly had activity that was less than 10 μM, or within 1000-fold of the compound’s affinity at the DAT, included: α-adrenergic receptors, DA D₂ receptors, muscarinic M₂ receptors, muscarinic M₃ receptors, μ-opioid receptors, κ-opioid receptors, and 5-HT₂ receptors.

**Locomotor Activity.** Cocaine produced dose-related increases in locomotor activity during the first 30 min after injection (Fig. 2, left). Levels of activity were increased at all of the doses examined, and the dose-effect curve had a characteristic bell shape. The ANOVA indicated a significant overall effect of dose (F₄,₁₂₀ = 4.26; p = 0.012), and post hoc tests indicated that doses of 10 and 20 mg/kg significantly increased activity. In contrast to the effects of cocaine, none of the N-substituted BZT analogs significantly increased activity (Fig. 2, open symbols). At the higher doses of GA 1-69, GA 2-50, and GA 2-99, post hoc tests indicated significant decreases in locomotor activity. With JHW 013 there was a nonsignificant ANOVA, with small nonsignificant increases in activity at the highest two doses. Because of this small effect, and because in previous studies several BZT analogs had a long duration of action, we examined the effects of the N-substituted BZT analogs over an 8-h period.

GA 1-69 (0.01–1.0 mg/kg) produced dose- and time-related decreases in locomotor activity (Fig. 3, top left). Decreases in activity were significant between 20 and 40 min after injection depending on dose and returned to approximately control levels at approximately 150 min after injection. Two-way ANOVA of the effects of GA 1-69 indicated significant effects of time (F₁₀,₄₁₄₀ = 85.3; p < 0.001), dose (F₅,₁₄₁₀ = 9.43; p < 0.001), and their interaction (F₅,₁₄₁₀ = 2.05; p < 0.001). Post hoc comparisons with control indicated that all of the significant effects were decreases in locomotor activity (p < 0.05), and that those decreases were obtained predominantly within the first 2½ h after injection. Likewise, both GA 2-50 and GA 2-99 produced dose- and time-related decreases in locomotor activity (Fig. 3, top right and bottom left, respectively). Effects of GA 2-50 at 30 mg/kg, and GA 2-99 at 10 and 30 mg/kg were significant at the first time point examined (10 min after injection). The two-way ANOVAs indicated significant effects of time (F values = 61.9 and 37.0, respectively; p < 0.001), dose (F values = 18.2 and 28.6, respectively; p < 0.001), and their interactions (F values = 1.55 and 2.26; p < 0.001). Post hoc comparisons with control indicated that all of the significant effects were decreases in locomotor activity (p < 0.05), and that those decreases were obtained predominantly within the first hour after injection for each compound.

In contrast to the effects of the other compounds, JHW 013 (at doses from 0.1 to 10.0 mg/kg) significantly increased locomotor activity at some time points after injection (Fig. 3, bottom right). Two-way ANOVA of the effects of JHW 013 indicated significant effects of time (F₁₀,₁₆₅₆₆ = 74.4; p < 0.001), dose (F₅,₁₆₅₆₆ = 2.95; p = 0.026), and the interaction of the two (F₂₃₅,₁₆₅₆₆ = 1.32; p = 0.002). Post hoc comparisons indicated that the significant increases in locomotor activity (p < 0.05) were obtained predominantly at 70 to 200 min after injection, although at 3.0 mg/kg increases in activity were obtained immediately after injection.

**Cocaine Discrimination.** As has been shown previously, cocaine produced a dose-related increase in the percentage of drug-appropriate responses in subjects trained to discriminate cocaine (10 mg/kg) from saline (Fig. 4, filled symbols). ANOVA indicated a significant effect of dose (F₁₆,₁₆₅₆₆ = 55.6; p < 0.001) and an ED₅₀ value of 3.73 mg/kg (95% confidence limits 3.06 to 4.63). In contrast to the effects of cocaine, none of the N-substituted BZT analogs fully substituted for cocaine at any of the doses studied (Fig. 4, top) across the range...
of doses from those that had no effects to those that virtually eliminated responses (Fig. 4, bottom). Each of the BZT analogs was studied up to 120 min after injection, and JHW 013 was studied 180 min after injection. If increasing the time between injection and testing appreciably increased the maximum substitution, a still longer time was studied until there were no substantial increases in maximum substitution or there was an apparent decrease in potency. The maximum substitution that was obtained was no more than 40% with any of the studied doses and times after injection. The ANOVAs of the effects indicated that only GA 2-99 had effects that were significantly related to dose ($F_{2,41} = 3.55; \ p = 0.038$, with $F$ values for the effect of dose for the other compounds < 2.74 and all $p > 0.05$). None of the compounds had effects that depended on time ($F$ values < 0.938; $p > 0.475$). In contrast to the negative outcomes in substituting for cocaine, all of the compounds had effects of dose on response rates (Fig. 4, bottom) that were significant ($F$ values > 9.82; $p < 0.015$), with only the effects of JHW 013 significant with respect to time ($F_{3,5} = 6.46; \ p = 0.036$).

**Place Conditioning and Locomotor Activity.** The increase from preconditioning to postconditioning in time spent in the drug-paired compartment (CPP score) with cocaine (10 mg/kg) was significantly greater ($t_{14} = 2.32; \ p < 0.05$) than that produced by saline (Fig. 5, compare filled bars). In addition, the first exposure to this dose of cocaine significantly increased locomotor activity over that obtained with saline (Fig. 5, bottom, compare filled and open circles; $t_{14} = 4.54; \ p = 0.0005$).

Doses of GA 1-69 from 1.0 to 17.0 mg/kg failed to produce a significant place conditioning when administered immediately before conditioning sessions (Fig. 5A; $F_{4,43} = 0.925; \ p = 0.458$). Because increased time between injection and conditioning sessions can increase effectiveness in place conditioning (e.g., De Beun et al., 1992), conditioning sessions were also conducted with injections administered at 90 min and 90 min before conditioning sessions. GA 1-69 was ineffective in conditioning when administered 45 min before (Fig. 5B; $F_{3,36} = 0.0988; \ p = 0.960$) or 90 min before (Fig. 5C; $F_{3,36} = 0.297; \ p = 0.827$) conditioning sessions. There were no significant effects of GA 1-69 on locomotor activity at any of the times tested (Fig. 5D; 0 min: $F_{4,43} = 0.775, \ p = 0.548$; 45 min: $F_{3,36} = 0.755, \ p = 0.527$; 90 min: $F_{3,36} = 2.28, \ p = 0.0955$).

GA 2-50 from 3.0 to 17.0 mg/kg immediately before sessions was ineffective in producing a significant change in time spent in the compound-paired compartment (Fig. 5E; $F_{3,28} = 0.551; \ p = 0.651$). The absence of significant place conditioning with GA 2-50 was obtained across this range of doses despite dose-dependent and significant decreases in locomotor activity (Fig. 5H, diamonds; $F_{3,28} = 33.4; \ p < 0.0001$), with 17 mg/kg producing the maximum decrease (Dunnett’s test $q = 8.90, \ q < 0.01$). Neither the 45-min (Fig. 5F; $F_{3,28} = 1.53; \ p = 0.228$) nor the 90-min (Fig. 5G; $F_{3,28} =
1.11, \( p = 0.362 \) pretreatment time significantly increased CPP scores across the range of doses examined. At 45 min after the first administration of GA 2-50 locomotor activity was significantly altered (\( F_{3,28} = 19.3; p < 0.0001 \), with 3.0 mg/kg producing a significant increase (Dunnett’s test \( q = 3.79; p < 0.01 \)) and 17 mg/kg producing a significant decrease (Dunnett’s test \( q = 3.64; p < 0.01 \)). At 90 min after injection, one-way ANOVA showed an overall significant difference in locomotor activity counts (\( F_{3,28} = 7.76; p < 0.001 \), although the effects were noticeably less than those obtained at earlier time points.

As with the other compounds, neither GA 2-99 (3–17 mg/kg) nor JHW 013 (1–10 mg/kg) produced significant place conditioning when given immediately before conditioning sessions (respectively, Fig. 5I, \( F_{3,28} = 0.829, p = 0.489 \) and Fig. 5M, \( F_{3,36} = 0.517, p = 0.673 \)). Neither a 45- nor a 90-min increase in the time between injection and placement in the chambers for conditioning increased the efficacy of either compound (45 min GA 2-99: Fig. 5J, \( F_{3,28} = 1.53, p = 0.229 \); 45 min JHW 013: Fig. 5N, \( F_{3,36} = 1.32, p = 0.284 \); 90 min GA 2-99: Fig. 5K, \( F_{3,28} = 0.0560, p = 0.982 \); 90 min JHW 013: Fig. 5O, \( F_{3,36} = 0.269, p = 0.847 \)). GA 2-99 also significantly decreased locomotor activity in a dose-dependent manner with maximal decreases in locomotor activity obtained immediately after injection and lesser decreases at 45 and 90 min after injection (Fig. 5L). The effects were significant at each pretreatment time (0 min: \( F_{3,28} = 14.1, p < 0.0001 \); 45 min: \( F_{3,28} = 8.83, p < 0.001 \); 90 min: \( F_{3,28} = 8.56, p < 0.001 \); Fig. 2D). When tested immediately and 45 min after injection, JHW 013 also produced significant decreases in locomotor activity (0 min: \( F_{3,36} = 10.4, p < 0.0001 \); 45 min: \( F_{3,36} = 8.58, p = 0.0002 \)). However, no significant effects on locomotor activity were observed when JHW 013 was injected 90 min before conditioning sessions (\( F_{3,36} = 1.45, p = 0.244 \)) (Fig. 5P).
Fig. 4. Effects of various doses of cocaine and N-substituted BZT analogs in rats trained to discriminate injections of cocaine (10 mg/kg) from saline at various times after injection. Ordinates at the top indicate percentage of responses on the cocaine-appropriate key, and ordinates at the bottom indicate the rates at which responses were emitted (as a percentage of response rates after saline administration). Abscissae: drug dose in milligram per kilogram (log scale). Each point represents the effect in six rats. *, the effects of cocaine, reproduced in each for reference. ○, the effects obtained with the drug injected 5 min before testing. □, the effects obtained with the drug injected 60 min before testing. △, the effects obtained with the drug injected 90 min before testing. ▽, the effects obtained with the drug injected 2 h before testing. ⌂, the effects obtained with the drug injected 3 h before testing.

Discussion

The presently studied N-substituted BZT analogs were less effective than cocaine in producing stimulant-like behavioral effects that indicate abuse liability. These compounds showed affinity for the DAT that ranged from approximately 5 to 30 nM and were previously shown to inhibit the uptake of DA (Agoston et al., 1997). Actions at the DAT suggest cocaine-like in vivo effects (Kuhar et al., 1991) that were not obtained in the present study, results similar to those obtained with several other BZT analogs (Newman et al., 2009; Tanda et al., 2009).

The reduced cocaine-like effects of the present BZT analogs could be caused by their pharmacokinetics. Syed et al. (2008) reported brain-to-plasma partition coefficients for GA 1-69 (1.32) and JHW 013 (1.51) that were lower than several previously reported BZT analogs that ranged from 2.0 to 5.6 (Raje et al., 2003). In contrast, the brain-to-plasma ratio for GA 2-50 for was reported to be approximately 10, depending on the dose administered (Othman et al., 2008). Comparable studies have not been conducted with GA 2-99. Because the brain-to-plasma ratios were calculated over large time periods, probably more important are concentrations in brain shortly after injection, which were 900, 800, and 610 ng/g for GA 1-69, GA 2-50, and JHW 013, respectively, each at 5.0 mg/kg i.v. (Othman et al., 2008; Syed et al., 2008). These values reflect estimated molar concentrations from 65 to 150 times their $K_i$ values. Because the pharmacokinetic studies were designed to examine blood-brain barrier transport, values are based on total brain concentrations, including those that presumably bound nonspecifically. Thus, although definitive information is not presently available, it seems that unless more than 98% of available drug is otherwise occupied more than adequate concentrations of the drugs are available in brain for DAT binding.

In addition to their activity at the DAT, the compounds were relatively selective for the DAT among the monoamine transporters, having affinities at the norepinephrine transporter and serotonin transporter, respectively, that ranged from 67- to 250-fold and 17- to 800-fold lower than their DAT affinities. Moreover, the compounds had affinities at the DAT that were from 10- to 300-fold higher than at M$_1$ receptors and 2- to 43-fold higher than at H$_1$ receptors. Previous studies have suggested that actions at M$_1$ and H$_1$ sites are unlikely contributors to the reduced effectiveness of the BZT analogs compared with cocaine (see Tanda et al., 2009 for a review).

Whether actions at a site other than the DAT are responsible for the reduced cocaine-like effects of these and other BZT analogs has been of considerable interest. The present results of the receptor screen suggest other sites that may be involved, and parsimony suggests that the sites first considered are those at which all of the BZT analogs had affinity that approached that for the DAT. For example, whereas GA 2-50 had $\sim$18 nM affinity for adrenergic $\beta_1$ receptors, the other compounds studied had undetectable affinity at that site up to concentrations of 10 $\mu$M, suggesting that the diminished cocaine-like effects, which were exhibited by all of the compounds, were not caused by actions at adrenergic $\beta_1$ receptors.

The sites at which all four compounds had discernable affinity included $\alpha$-adrenergic, dopaminergic, opioid, serotonin, and $\sigma$ receptors. A previous study of other BZT analogs (Katz et al., 2004) reported low affinity of those compounds at adrenergic $\alpha_1$ and $\alpha_2$ receptors, opioid-$\delta$, and opioid-$\kappa$ receptors, suggesting that high affinity at those sites is not necessary for the diminished cocaine-like effects. In unpublished studies (R. I. Desai, A. H. Newman, D. K. Grandy, and J. L. Katz), JHW 007 antagonized the locomotor stimulant effects of cocaine in DA D2R knockout and wild-type mice. In that study, the locomotor-stimulant effects of cocaine were diminished in the DA D2R knockout compared with WT mice as in previous studies (Kelly et al., 1998; Chausmer et al., 2002). However, the cocaine antagonist effects of JHW 007 were evident, suggesting that at least the antagonism of cocaine’s locomotor-stimulant effects by BZT analogs, and probably their diminished cocaine-like effects as well, do not depend on actions at DA D2Rs. Some studies suggest that actions at 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors can block the effects of cocaine (Bubar and Cunningham, 2006). The actions of the BZT analogs at those sites, beyond the nonselective 5-HT$_2$ assay conducted in the present study, will be further addressed in future studies.

Several previous studies have indicated that $\sigma$-receptor antagonists can block several of the effects of cocaine, including locomotor stimulation, sensitization, and place conditioning (see review by Matsumoto 2009). It is noteworthy that the $\sigma$ antagonists that block some of the above-mentioned effects of cocaine were not effective in blocking the self-administration of cocaine (Hiranita et al., 2010). However, we have found that combined treatment with $\sigma$-receptor antagonists

![Image of a diagram with graphs and data points related to cocaine and BZT analogs' effects on cocaine discrimination and locomotor responses.](diagram.png)
and dopamine uptake inhibitors blocks the self-administration of cocaine, whereas the drugs administered alone do not (Hiranita et al., 2009a). Those findings suggest the actions of BZT analogs at σ receptors may be interacting with their activity at the dopamine transporter to limit their cocaine-like efficacy and possibly contribute to their cocaine-antagonist effects. GA 2-50 and JHW 013 had affinities at σ₁ or σ₂ receptors that were similar to their affinities at the DAT. Ongoing studies are directed at the hypothesis that actions at σ receptors combined with their affinities at the DAT are critical for the decreased cocaine-like behavioral effects of the present compounds.

Previous studies with several N-substituted BZT analogs showed that cocaine-like effects became more prominent with time after injection. For example, the N-methyl analog of the present compounds [3α-[(4-fluorophenyl)methoxy]tropane (AHN 1-055)] (Fig. 1) produced complete substitution for the discriminative-stimulus effects of cocaine at 90 min after injection, but not at 5 min after injection. Other BZT analogs became more effective, although they did not fully substitute for cocaine (Katz et al., 1999). Compared with cocaine, several N-substituted BZT analogs had a slow time course for DAT binding in vivo (Desai et al., 2005a, b) and had a relatively slow onset of increases in extracellular DA levels detected by in vivo microdialysis (Tanda et al., 2005, 2009). Pharmacokinetic studies indicated that these compounds reached sufficient concentrations in the brain to bind to the dopamine transporter shortly after systemic injection (Raje et al., 2003), suggesting a relatively slow association with the DAT, which was confirmed in vitro for the N-butyl BZT analog, JHW 007 (Kopajtic et al., 2010). These observations suggest that some aspect of the slow onset of action was important for the diminished cocaine-like effects of these DAT ligands (Tanda et al., 2009).

In contrast with those results, the present compounds were less effective than cocaine and those effects did not become greater with time. Only JHW 013 increased locomotor activity, although still less than cocaine. The other compounds only decreased locomotor activity, and those decreases diminished within 2 h after injection. As described above, pharmacokinetic studies indicated that brain-to-plasma partition coefficients varied for the present compounds, but likely are in brain at sufficient concentrations to bind at the DAT. Thus the present N-substituted compounds provide a distinctive set of pharmacodynamic and pharmacokinetic effects that suggest that a slow in vivo association may not be necessary for the diminished cocaine-like effects of BZT analogs.

A previous study suggested that the Tyr335 residue in the DAT is critical for regulating the equilibrium between open and closed states of the DAT (Loland et al., 2002). In addition, the potencies of cocaine and its analogs for inhibiting DA transport in cells transfected with a Y335A DAT mutant were decreased by approximately 100-fold compared with potencies with WT DAT. In contrast, potencies of BZT analogs were only 7- to 58-fold less in cells with the Y335A mutant (Loland et al., 2008). Furthermore, there was a significant relationship between the decrease in potencies of the BZT analogs caused by this DAT mutation and their behavioral effectiveness. The only one of the present BZT analogs studied, GA 2-99, like other BZT analogs had a ratio of potencies in the Y335A mutant that was lower than those obtained with cocaine or WIN 35,428 (Loland et al., 2008). Together, these results suggest that the BZT structure promotes conformational changes of the DAT that may lead to effects unlike those of cocaine, and that those conformational changes in vivo may occur faster than previously anticipated.

In summary, the present compounds, like other BZT analogs, have high affinity for the DAT, but are less effective than cocaine in producing various effects related to drug abuse. Subsequent studies will examine the potential of these compounds to antagonize the effects of cocaine. The present compounds depart from those previously reported in
that their effectiveness is slowly low, and their onsets of action are relatively fast. Sites other than the DAT have been identified, and an alternate mode of action at the DAT has been suggested as mechanisms that may be responsible for the reduced cocaine-like efficacy of these compounds. Subsequent studies will examine those mechanisms and the interaction of these drugs with cocaine to better assess their potential as treatments for cocaine abuse and further investigate mechanistic hypotheses.

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Authorship Contributions

Participated in research design: Li, Kopajtic, O’Callaghan, and Katz.
Conducted experiments: Li, Kopajtic, and O’Callaghan.
Contributed new reagents or analytic tools: Agoston, Cao, and Newman.
Performed data analysis: Li, Kopajtic, O’Callaghan, and Katz.
Wrote or contributed to the writing of the manuscript: Li, Kopajtic, Cao, Newman, and Katz.

References


Action of these drugs with cocaine to better assess their reduced cocaine-like efficacy of these compounds. Subsequent studies will examine those mechanisms and the interaction of these drugs with cocaine to better assess their potential as treatments for cocaine abuse and further investigate mechanistic hypotheses.