Novel C-1 Substituted Cocaine Analogs Unlike Cocaine or Benztropine^S

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ABSTRACT

Despite a wealth of information on cocaine-like compounds, there is no information on cocaine analogs with substitutions at C-1. Here, we report on (*R*)-(–)-cocaine analogs with various C-1 substituents: methyl (**2**), ethyl (**3**), *n*-propyl (**4**), *n*-pentyl (**5**), and phenyl (**6**). Analog **2** was equipotent to cocaine as an inhibitor of the dopamine transporter (DAT), whereas **3** and **6** were 3- and 10-fold more potent, respectively. None of the analogs, however, stimulated mouse locomotor activity, in contrast to cocaine. Pharmacokinetic assays showed compound **2** occupied mouse brain rapidly, as cocaine itself; moreover, **2** and **6** were behaviorally active in mice in the forced-swim test model of depression and the conditioned place preference test. Analog **2** was a weaker inhibitor of voltage-dependent Na⁺ channels than cocaine, although **6** was more potent than cocaine, highlighting the need to assay future C-1 analogs for this

activity. Receptorome screening indicated few significant binding targets other than the monoamine transporters. Benztropine-like “atypical” DAT inhibitors are known to display reduced cocaine-like locomotor stimulation, presumably by their propensity to interact with an inward-facing transporter conformation. However, **2** and **6**, like cocaine, but unlike benztropine, exhibited preferential interaction with an outward-facing conformation upon docking in our DAT homology model. In summary, C-1 cocaine analogs are not cocaine-like in that they are not stimulatory *in vivo*. However, they are not benztropine-like in binding mechanism and seem to interact with the DAT similarly to cocaine. The present data warrant further consideration of these novel cocaine analogs for antidepressant or cocaine substitution potential.

Introduction

Cocaine contains four asymmetric carbons (Fig. 1), implying 16 hypothetically possible stereoisomers. However, only eight of these isomers can exist, because of the geometric

constraints imparted by the tropane bridgehead amine. The natural plant alkaloid (*R*)-(–)-cocaine (**1**) is the only isomer that possesses psychostimulant activity (Carroll et al., 1992). Different substituents at nearly all of the positions of the cocaine molecule, except C-1 of the tropane skeleton (Fig. 1), have been introduced, providing a wealth of knowledge on structure-activity relationships (SARs) at monoamine transporters (Carroll et al., 1992). The major difficulty in the asymmetric synthesis of optically pure (*R*)-(–)-cocaine and its analogs is the problem of introducing tropane substituents stereoselectively, with the required *cis* relationship between the C-2 and C-3 tropane substituents, and the C-2 carbomethoxy moiety occupying the thermodynamically unfavorable β (*exo*) configuration (Fig. 1). No cocaine analogs

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ABBREVIATIONS: SAR, structure-activity relationship; ANOVA, analysis of variance; β -CFT, 2 β -carbomethoxy-3 β -(4-fluorophenyl)tropane; CPP, conditioned place preference; DA, dopamine; DAT, DA transporter; hDAT, human DAT; DMSO, dimethyl sulfoxide; HEK, human embryonic kidney; LeuT, leucine transporter; MOE, Molecular Operating Environment; NET, norepinephrine transporter; NSS, neurotransmitter/sodium symporter; PDSP, Psychoactive Drug Screening Program; RTI-15, 3 β -benzoyloxy-8-methyl-8-azabicyclo (3.2.1) octane-2-carboxylic acid phenyl ester; SBFI, sodium-binding benzofuran isophthalate; SERT, serotonin transporter; TM, transmembrane; TUI, triple uptake inhibitor.

Fig. 1. Chemical structures of (*R*)-(-)-cocaine, novel C-1-substituted cocaine analogs, and the atypical DAT inhibitors benztropine and modafinil. Tropane ring carbon atoms of cocaine and its analogs are numbered, and the absolute configuration of the stereoactive carbons in (-)-cocaine is indicated [formally, (-)-cocaine has a 1*R*,2*R*,3*S*,5*S* absolute configuration].

possessing substituents at the C-1 bridge position existed until recently, when we targeted such analogs with our novel sulfinimine (*N*-sulfinyl-imine) chemistry (Zhou et al., 2004; Senanayake et al., 2005; Davis, 2006; Morton and Stockman, 2006). Our asymmetric synthesis (Davis et al., 2010, 2012) generated chiral (*R*)-(-)-cocaine analogs with various C-1 substituents, including: methyl (**2**), ethyl (**3**), *n*-propyl (**4**), *n*-pentyl (**5**), and phenyl (**6**) moieties (structures shown in Fig. 1).

Cocaine exhibits nonselective binding to the three monoaminergic neurotransmitter-sodium symporter (NSS) proteins, the dopamine transporter (DAT), serotonin transporter (SERT), and norepinephrine transporter (NET), where it acts as a nontranslocated inhibitor. However, the strong locomotor stimulant and addictive effects of cocaine are mediated almost exclusively by its interaction with the DAT (Chen et al., 2006; Thomsen et al., 2009). Preliminary data obtained for (-)-1-methyl-cocaine (**2**) indicated broad-spectrum inhibition of monoamine uptake with potency similar to cocaine; but, unexpectedly, little or no locomotor stimulatory activity in behavioral tests. This curious finding raised the possibility that C-1 modification produces psychoactive compounds that inhibit monoamine uptake, but lack cocaine-like psychostimulant activity. In this context, it is of interest that certain “atypical” DAT inhibitors exhibit limited stimulatory and reinforcing properties compared with cocaine (Rothman et al., 2008; Newman and Katz, 2009). Examples are the tropane compound benztropine (**7**) and various benztropine derivatives that are devoid of benztropine’s antimuscarinic and antihistaminergic side effects (Katz et al., 2004; Campbell et al., 2005; Desai et al., 2005; Li et al., 2011). We thus sought to investigate whether C-1 substitution yields novel DAT ligands with a cocaine-like structure but an atypical-like pharmacological profile.

Our studies addressed the following properties of the analogs, with particular focus on two of the compounds: the series prototype (-)-1-methyl-cocaine (**2**) and the most potent DAT inhibitor of the series, (-)-1-phenyl-cocaine (**6**). First,

pharmacokinetic experiments were carried out to assess the entry of **2** into the brain. Second, the ability of all five analogs to inhibit radiolabeled substrate uptake at DAT, SERT, and NET was determined. Third, because cocaine has local anesthetic properties, analogs **2** and **6** were tested for their ability to interact with voltage-dependent sodium channels. Fourth, potential locomotor-stimulating properties of all analogs were monitored and compared with those engendered by cocaine, and compounds **2** and **6** were tested in a conditioned place preference (CPP) assay. Fifth, because the nonstimulatory analog **2** displayed a monoamine transporter binding profile indicative of a triple uptake inhibitor (TUI), compounds that have shown promising antidepressant action in preclinical studies (Dutta et al., 2008 and references therein), it was tested for potential antidepressant activity in an animal model of depression-like symptoms (Gopishetty et al., 2011), as was **6**. Finally, we performed *in silico* molecular modeling to assess whether **2** and **6** preferentially interact with an open-to-out DAT conformation, like cocaine and the phenyltropane stimulant β-CFT (Reith et al., 2001; Beuming et al., 2008), or instead interact with a closed-to-out (inward-facing or occluded) state, like benztropine (**7**), various benztropine analogs, and other atypical DAT inhibitors such as modafinil (**8**) (Loland et al., 2008; Schmitt et al., 2008; Schmitt and Reith, 2011).

The results of this work indicate that C-1 modification of the cocaine molecule can generate compounds that are taken up into the brain as readily as cocaine, but are not cocaine-like in that they are not stimulatory *in vivo*, but yet are not benztropine-like because they do not preferentially interact with an inward-facing DAT conformation. This warrants further work on C-1 substituted phenyltropanes as novel leads in medication development as well as pharmacological probes to assist in mapping inhibitor-binding SARs in NSS proteins.

Materials and Methods

Animals, Cells, and Reagents. Details on animals (all male mice) are listed under each test. All radioligand uptake assays were performed with stably transfected human embryonic kidney (HEK) 293 cells. Cultured cells expressing the human DAT were as described previously (Chen et al., 2004; Dutta et al., 2008). The human SERT-expressing cells were those described previously by Eshleman et al. (1995), and the human NET-expressing cells used were those described previously by Reith et al. (2005). Voltage-gated sodium channel inhibition assays were performed with cultured neocortical neurons obtained from Swiss-Webster mice, as described briefly in the section below and in further detail elsewhere (Cao et al., 2008; Jabba et al., 2010). All of the C-1 cocaine analogs were synthesized by us (Davis et al., 2010, 2012). Other reagents were from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. All animal use protocols were approved by the Institutional Animal Care and Use Committee of Creighton University.

Brain Uptake of C-1 Cocaine Analog **2.** Pharmacokinetic analysis for brain uptake of compound **2** was performed by Jeffrey Crabtree, Dr. Sanford Mendonca, and Dr. Pat Noker of Southern Research Institute (Birmingham, AL). Six male CD-1 mice (Charles River Laboratories, Inc., Wilmington, MA), age 8 weeks, were injected intraperitoneally with 30 mg/kg of **2** and sacrificed at time points of 5 and 30 min (three animals per time point). Whole brain and plasma levels of **2** were determined by mass spectrometry.

Inhibition of Monoamine Uptake by DAT, SERT, and NET. The ability of test compounds to inhibit radiolabeled [³H]substrate

uptake via human monoamine transporters was monitored as described previously (Reith et al., 2005). In brief, DAT and SERT cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 5% bovine calf serum, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 2 μ g/ml puromycin at 37°C and 5% CO₂. NET cells were grown the same way, except that serum was only of the fetal bovine type (10%) and the selective antibiotic was G-418 (250 μ g/ml). All buffers and solutions were prepared with purified water (Nanopure Diamond, Barnstead; Thermo Fisher Scientific). Cell suspensions were prepared and incubated as described previously (Reith et al., 2005) in 122 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 10 mM glucose, 1 mM CaCl₂, 15 mM NaH₂PO₄, and 7.5 mM Na₂HPO₄, mixed to achieve pH 7.4 at room temperature (21°C) and containing 0.1 mM tropolone for the inhibition of catechol-*O*-methyltransferase. In 96-well plates, cells and test inhibitor compound were first preincubated at room temperature, tritiated substrate was added, incubation was continued, and the assay was terminated by rapid filtration through Whatman (Clifton, NJ) GF/C glass fiber filter mats with a 96-pin Brandel harvester (Brandel Inc., Gaithersburg, MD). After filter mat drying, tritium radioactivity was assessed by using a Wallac Microbeta Plus liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). The conditions for uptake assays with HEK human DAT, SERT, and NET cells, respectively, were as follows: the particular radiolabeled substrates used were [³H]dopamine (ring 2,5,6-³H)DA, 38.7 Ci/mmol; PerkinElmer Life and Analytical Sciences), [³H]serotonin (28.0 Ci/mmol; PerkinElmer Life and Analytical Sciences), and [³H]dopamine (ring 2,5,6-³H)DA, 38.7 Ci/mmol; Perkin-Elmer Life and Analytical Sciences). For each assay, test inhibitor ligands were preincubated with cells for 6 min, followed by addition of radiolabeled substrate and further incubation for 5, 8, or 7 min, respectively. Nonspecific uptake was defined in the presence of 100 μ M cocaine (for DAT), 100 μ M citalopram (for SERT), or 10 μ M desipramine (for NET). Dopamine has a high affinity for the NET (somewhat higher than norepinephrine itself) (Buck and Amara, 1994; Gu et al., 1994) and is readily translocated by the NET, with a $V_{\max}$ value in the range of that of norepinephrine (Buck and Amara, 1994; Gu et al., 1994). Hence, [³H]dopamine is well suited for use as a radiolabeled substrate in NET uptake assays. All compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted out in 10% (v/v) DMSO. Additions from the latter stocks resulted in final concentrations of DMSO of 1%, which by itself did not interfere with radioligand binding or uptake. The cocaine analogs did not need DMSO for dissolving, but were assayed as part of a larger series of compounds with varying solubility. At least five concentrations of the test compound were studied, spaced evenly around their IC₅₀ values.

Receptorome Radioligand Binding Screen. Broad-spectrum in vitro receptor binding data for analogs **2** and **6** were kindly provided by Dr. Bryan L. Roth (University of North Carolina, Chapel Hill, NC) as part of the National Institute of Mental Health's Psychoactive Drug Screening Program (PDSP). In brief, the PDSP assesses binding of novel compounds at a wide array of cloned human G protein-coupled receptors, membrane transporters, and other proteins known to be psychoactive drug targets (e.g., the σ_1 and σ_2 binding sites and the mitochondrial translocator protein TPO, originally known as the "peripheral" benzodiazepine receptor). For detailed description of the PDSP experimental protocols, refer to the PDSP web site at <http://pdsp.med.unc.edu/> and select the Binding Assay link.

Neocortical Neurons for Monitoring Voltage-Dependent Sodium Channels. Primary cultures of neocortical neurons were obtained from embryonic day 17 Swiss-Webster mice as described elsewhere (Cao et al., 2008; Jabba et al., 2010). In brief, pregnant mice were euthanized by CO₂ asphyxiation, and embryos were removed under sterile conditions. Neocortices were collected, stripped of meninges, minced by trituration with a Pasteur pipette, and treated with trypsin for 25 min at 37°C. The cells were then dissociated by two successive trituration and sedimentation steps in soy-

bean trypsin inhibitor and DNase containing isolation buffer, centrifuged, and resuspended in Eagle's minimal essential medium with Earle's salt and supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 IU/ml penicillin, and 0.10 mg/ml streptomycin, pH 7.4. Cells were plated onto poly-L-lysine-coated, 96-well (9 mm), clear-bottomed, black-well culture plates (MidSci, St. Louis, MO) at a density of 1.5×10^5 cells/well. Cells were then incubated at 37°C in a 5% CO₂ and 95% humidity atmosphere. Cytosine arabinoside (10 mM) was added to the culture medium on day 2 after plating to prevent proliferation of non-neuronal cells. The culture media were changed every other day, starting from day 5 in vitro by using a serum-free growth medium containing neurobasal medium supplemented with B-27, 100 IU/ml penicillin, 0.10 mg/ml streptomycin, and 0.2 mM L-glutamine. Neocortical cultures were used in experiments between 8 and 10 days in vitro. All animal use protocols were approved by the Institutional Animal Care and Use Committee of Creighton University.

Intracellular Sodium Concentration Measurement. Intracellular sodium concentration measurement and full in situ calibration of sodium-binding benzofuran isophthalate (SBFI) fluorescence ratio were performed as described previously (Jabba et al., 2010). Cells grown in 96-well plates were washed four times with Locke's buffer (8.6 mM HEPES, 5.6 mM KCl, 154 mM NaCl, 5.6 mM glucose, 1.0 mM MgCl₂, 2.3 mM CaCl₂, and 0.1 mM glycine, pH 7.4) by using an automated microplate washer (BioTek Instruments, Winooski, VT). After measuring the background fluorescence of each well, cells were incubated for 1 h at 37°C with dye-loading buffer (100 μ l/well) containing 10 μ M SBFI-AM (Invitrogen, Carlsbad, CA) and 0.02% Pluronic F-127 (Invitrogen). Cells were washed five times with Locke's buffer, leaving a final volume of 100 μ l in each well. The plate was then transferred back to the incubator for 15 min to allow the cells to equilibrate after washing and placed in a FlexStation II chamber (Molecular Devices, Sunnyvale, CA) to detect Na⁺-bound SBFI emission at 505 nm (cells were excited at 340 and 380 nm). Fluorescence readings were taken once every 5 s for 60 s to establish the baseline, and then 50 μ l of different concentrations of either the cocaine analogs (**2** and **6**), lidocaine, or cocaine was added to each well from the compound plate at a rate of 26 μ l/s. After 180 s, 50 μ l of veratridine was added in each well, yielding a final volume of 200 μ l/well. After correcting for background fluorescence, SBFI fluorescence ratios (340/380) and concentration-response graphs were generated by using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Locomotor Activity in Mice. Locomotor activity of male C57BL/6 mice, 8 to 9 weeks of age, was measured in activity boxes equipped with infrared beams as described previously (Sershen et al., 2002). In brief, an Opto-Varimex Auto-Track System (version 4.10, 8 Opto-Varimex-3 photocell activity monitors; Columbia Instruments, Columbia Station, OH) was used. Mice were housed singly in a standard mouse cage (7 $\times$ 12 $\times$ 5 inches) 1 day before activity monitoring. The cage was placed in the activity monitor, and activity was measured starting after intraperitoneal drug injection (1–30 mg/kg). Locomotor activity was calculated based on total ambulatory counts (consecutive beams broken during ambulation; single beams broken repeatedly were not counted). Data were expressed as ambulatory beam breaks over a 60- to 120-min period (in 5-min segments or total counts).

Forced-Swim Test. Antidepressant-like activity of analogs **2** and **6** was tested in the forced-swim test model as described previously (Porsolt et al., 1977; Castagne et al., 2011). In brief, adult male C57BL/6 mice ($n = 6$ –10/group) were placed into individual glass cylinders (45 cm high and 21 cm in diameter) containing clean 25°C ($\pm 1^\circ$ C) water, 15 cm deep for 15 min. Mice were removed, dried, and returned to their home cages. A second 6-min swim test was given 24 h after the first. Saline (3 ml/kg i.p.), **2** (30 mg/kg i.p.), **6** (3 mg/kg i.p.), or desipramine (10 mg/kg i.p.; a well known antidepressant, used as a positive control) were administered 23.5 h, 5 h, and 30 min before the second swim test (i.e., 30 min, 19 h, and 23.5 h after the

first swim). Test sessions were video recorded. Behavior during the 6-min swim test was scored from the videotapes. The cumulative amount of time that the mouse was immobile in the water during the last 4 min of the test was measured, as was the latency for the mouse to show the first bout of immobility behavior. Immobility was defined as the mouse performing no movements except those necessary to keep the head above the water (no swimming or climbing behaviors). The dose of compound **2** was set at 30 mg/kg i.p. with the consideration that 1) **2** and cocaine have comparable DAT affinity, 2) 30 mg/kg i.p. is a behaviorally active dose for cocaine, and 3) this dose of **2** did not produce locomotor stimulation (see *Results*).

Conditioned Place Preference Testing. For CPP the Optovarimex platform was divided into two sections (eight squares each) by a plastic partition (painted black to within 2 inches of the bottom) containing a 3 × 3 removable door. The two sections had different floor surfaces (smooth and rough textures). One side was covered by black paper and had a metal mesh floor. The other side was uncovered and had a smooth metal floor. The 9-inch high sides had a plastic cover, half of which was black colored. The room light was diminished, so that the light side had only a soft illumination. One side corresponded to the least preferred side of the animal during a preconditioning trial, when the mouse had access to both sides through a 3 × 3-inch opening in the center divider. Before the trial (preconditioning), the mice were put in the apparatus and allowed to explore freely for 1 h to decrease novelty during the trial. After conditioning, they were tested for preference again to see whether the time spent on the conditioned (nonpreferred) side had increased.

Animals were handled gently several times per day for 2 to 3 days before they were allowed to explore the test area. Between sessions in the test box, the box was dismantled and thoroughly cleaned with 70% ethanol. The first day of testing the mice had equal access to both sides and were monitored for time in the box for 30 min to determine preference for the light or dark side. On days 2 to 4, the mice were given an intraperitoneal injection of either cocaine, the designated C-1 analog test compound, or saline and confined to the nonpreferred side for 30 min during one session. They were given saline on the preferred side during another session. Time of the sessions was alternated, morning or afternoon. On day 5, they were again given access to both sides, and the difference in time spent on the nonpreferred side was calculated (postcondition minus precondition time in seconds in nonpreferred side; these two times were subjected to statistical analysis). The cocaine dose (10 mg/kg i.p.) was chosen based on the literature (for references see Bardo and Bevins, 2000). Compound **2** with comparable DAT affinity (see *Results*) was also tested at 10 mg/kg i.p.; the dose of compound **6**, which was approximately five times more potent at DAT, was set at 3 mg/kg i.p.

DAT Homology Modeling and Flexible Ligand Docking. Homology modeling of the hDAT protein and flexible ligand docking was performed according to procedures described previously (Schmitt et al., 2010; Schmitt and Reith, 2011 and references therein). In brief, the crystal structure of the bacterial leucine transporter (LeuT), a prokaryotic NSS protein from *Aquifex aeolicus*, bound to the ligands leucine and the tricyclic antidepressant desipramine (Zhou et al., 2007; Protein Data Bank ID Code 2QJU), was used as the structural template, using the NSS-family protein amino acid sequence alignment as described previously by Beuming et al. (2008). The sodium ions were initially placed in the DAT model based on their location in the LeuT crystal, but were allowed to move freely during energy minimization, docking, and optimization rounds. The chloride ion that is cotransported during the DAT translocation cycle was initially placed at the position corresponding to Glu290 in the LeuT structure (see Schmitt and Reith, 2011 for further discussion of NSS protein Cl⁻ ion binding and our molecular model).

Homology modeling was performed by using the MODELLER algorithm (<http://salilab.org/modeller/manual/>), and the resultant lowest energy structure was imported into the Molecular Operating Environment (MOE) program suite (version 2009.10; Chemical Computing Group, Montreal, CA). The Protonate3D function in MOE was

used in combination with the AMBER99 force field and generalized Born (GB/VI) implicit solvation model to prepare the hDAT protein and assign partial charges. After multiple minimization rounds, model stereochemical quality was analyzed with PROCHECK (<http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/>) and Ramachandran plots. Ligand binding sites in the DAT model were identified with the Site Finder tool implemented in MOE, two binding pockets (approximately overlapping with the central S1 and the putative vestibular S2 substrate sites of LeuT) were identified. For review of the structural characteristics of LeuT and discussion of the two-site substrate-translocation model, see Nyola et al. (2010). For the present analysis of cocaine, bupropion, and C-1 analog interaction with the DAT, we focused on binding at the central S1 substrate-binding site. This decision was made to facilitate comparison of our docking results with other reported DAT molecular modeling studies, which indicate that both phenyltropane and bupropion-based DAT inhibitors preferentially bind at the S1 site (Beuming et al., 2008; Bisgaard et al., 2011). Dummy atoms were placed at the centroids of alpha spheres defining the S1 site to assist in ligand docking. For docking, ligand structures were imported into MOE, protonated, assigned partial charges, and energy-minimized ($< 0.001 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-1}$) by using the MMFF94x force field with GB/VI implicit solvation. Energetically favorable poses were output to a database, and a representative pose was then refined by further minimization rounds; protein backbone atoms were weakly tethered ($1 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-1}$ force constant), and the side-chain and ligand atoms were completely unconstrained to allow for flexible “ligand adaptive” docking. For the last minimization round, the backbone tethering constant was increased to $10 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-1}$, and the convergence gradient was set at $0.01 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-1}$. Final ray-traced models depicted in the figures were rendered with PyMOL (Schrödinger LLC, New York, NY). All MOE simulations were performed on a standard quad-core x64 computer running Windows 7 (Microsoft, Redmond, WA).

Data Analysis and Statistics. The IC₅₀ for a compound to inhibit radiolabeled [³H]substrate uptake was estimated by logistic fitting of data by using the Origin 7.5 software suite (OriginLab, Northampton, MA), and IC₅₀ values were converted to K_i values with the Cheng-Prusoff equation as described previously (Schmitt et al., 2010). IC₅₀ values for the inhibition of voltage-gated sodium channel activity were obtained via nonlinear regression analysis by using GraphPad Prism. Transporter data and immobility scores on the forced-swim test were analyzed by one-way ANOVA, followed by the Dunnett multiple comparisons test or Bonferroni post hoc analysis, respectively. Locomotor data were compared between equal drug doses tested within the same experimental set by Student's *t* test. CPP postconditioning and preconditioning times were compared by paired Student's *t* test. Data were log-transformed where appropriate for comparing groups with equal variation. The accepted level of significance was $P < 0.05$.

Results

Brain Uptake of C-1 Analog **2.** Upon intraperitoneal administration to mice of 30 mg/kg of (–)-1-methyl-cocaine (**2**), the plasma and whole brain levels at 5 and 30 min (Fig. 2) were comparable with the cerebrocortical levels found after intraperitoneal administration of 25 mg/kg (–)-cocaine (Benuck et al., 1987). For brain, the levels of **2** and cocaine were 8.6 ± 0.7 and $6.7 \pm 1.2 \text{ }\mu\text{g/g}$, respectively, at 5 min, indicating rapid brain entry for the C-1 analog. The brain levels were 3.8 ± 0.2 and $2.3 \pm 0.3 \text{ }\mu\text{g/g}$ at 30 min; for plasma, the respective values were 3.3 ± 0.2 and $1.1 \pm 0.1 \text{ }\mu\text{g/ml}$ at 5 min and 1.7 ± 0.3 and $0.40 \pm 0.05 \text{ }\mu\text{g/ml}$ at 30 min [mean $\pm$ S.E.M., compare data in Fig. 2 with results of Benuck et al. (1987)]. The dose-independent parameter, half-life of disap-

Fig. 2. Pharmacokinetic data for compound (–)-2 administered to CD-1 male mice with three animals per time point. Data shown are mean $\pm$ S.E.M. (vertical bars).

pearance from the brain, was estimated at 27 min for compound **2** (Fig. 2) and 18 min for cocaine (Benuck et al., 1987; Reith et al., 1987); the brain/plasma ratios for the two compounds were 3 and 7. Thus, analog **2** seems to enter the brain quickly, accumulates in brain at levels above those in plasma (but somewhat less pronounced than cocaine itself), and is eliminated from brain at a slightly slower rate than cocaine.

The predicted octanol-water partition coefficients ($\log P$ values) computed for compounds **2** and **6** were 2.67 and 3.77, respectively, compared with 2.30 for (–)-cocaine and 2.72 for β -CFT (calculated by using the XLogP3 algorithm of Cheng et al., 2007). Predicted $\log P$ values for analogs **3**, **4**, and **5** are 3.20, 3.56, and 4.64, respectively, showing the expected increase in lipophilicity with increasing alkyl chain length. Because there is a general correlation between $\log P$ and blood-brain barrier permeability, increasing chain length could be expected to enhance permeability (Rapoport, 1992), but this correlation becomes less predictable for compounds with $\log P$ values exceeding 2, which is the case for our compounds, including cocaine. However, the above pharmacokinetic data for compound **2** show it to be rapidly taken up into brain, as are cocaine and other phenyltropane stimulants, such as β -CFT (Cline et al., 1992; Stathis et al., 1995).

Uptake Inhibitory Profile of all Analogs at DAT, SERT, and NET. In comparison with (–)-cocaine itself, the 1-methyl- and 1-ethyl-analogs (compounds **2** and **3**) had similar or somewhat higher potency (lower K_i) with respect to the DAT; these substitutions had little or no effect on inhibitory potency at the SERT or NET (Table 1). Extending the length of the C-1 alkyl substituent to *n*-propyl and *n*-pentyl (compounds **4** and **5**, respectively) reduced DAT potency. This also seemed to be the case for the SERT and NET. However, the C-1 phenyl analog (**6**), was highly interesting in displaying an order of magnitude increase in affinity for the DAT compared with cocaine, whereas its affinity for the SERT and NET was more comparable with that of **3** and **5**, respectively (Table 1). Further work focused on analogs with potent DAT activity. Thus, among compounds **2** to **5**, we chose analog **2**, which inhibited transmitter uptake mediated by DAT with a

TABLE 1

K_i values for inhibiting neurotransmitter uptake in HEK-293 cells heterologously expressing human monoamine transporters. Uptake of [3 H]dopamine by DAT- or NET-expressing cells and [3 H]serotonin by SERT-expressing cells was monitored as described under *Materials and Methods*. Average $\pm$ S.E.M. for three to eight independent experiments is shown.

Compound (C-1 Moiety)	K_i at DAT	K_i at SERT	K_i at NET
(–)-1 Cocaine (H)	326 $\pm$ 106	513 $\pm$ 143	358 $\pm$ 69
(–)-2 (Me)	163 $\pm$ 23	435 $\pm$ 77	488 $\pm$ 101
(–)-3 (Et)	95.1 $\pm$ 17.0*	1,106 $\pm$ 112	598 $\pm$ 179
(–)-4 (<i>n</i> -Pr)	871 $\pm$ 205*	2,949 $\pm$ 462**	796 $\pm$ 195
(–)-5 (<i>n</i> -C ₅ H ₁₁)	1,272 $\pm$ 199**	1,866 $\pm$ 400*	1,596 $\pm$ 21**
(–)-6 (Ph)	32.3 $\pm$ 5.7**	974 $\pm$ 308	1,980 $\pm$ 99**

*, $P < 0.05$ and **, $P < 0.01$ compared with (–)-1 (cocaine) (one-way ANOVA followed by Dunnett's multiple comparisons test).

K_i of 163 $\pm$ 23 nM, a value not statistically different from the 326 $\pm$ 106 nM observed for cocaine. In addition, compound **2** had the interesting feature of nonselectively inhibiting transmitter uptake by all three biogenic amine transporters with K_i values within a 3-fold window (Table 1). Finally, compound **6** was selected for more extensive characterization because of its selectivity for DAT (K_i of 32.3 nM) versus SERT (974 nM) and NET (1980 nM).

Broad-Spectrum Receptorome Binding Screen of Analogs 2 and 6. To assess whether the lack of locomotor stimulatory effect of the C-1 analogs is mediated by activity at targets other than monoamine transporters, compounds **2** and **6** were submitted to the National Institute of Mental Health's PDSP (data not shown). Other than the expected activity at the DAT, SERT, and NET, the only notable binding hits ($K_i < 10 \mu\text{M}$) revealed by the receptorome screen were the two σ -receptor subtypes. Compound **2** lacked appreciable affinity for the σ_1 receptor, but bound to the σ_2 receptor with low micromolar affinity ($K_i = 1.13 \mu\text{M}$). Compound **6**, on the other hand, showed nonselective sigmaergic activity, binding to the σ_1 receptor with an affinity of 524 nM and the σ_2 receptor with an affinity of 198 nM. The results of the PDSP screen indicate that, except for σ receptors, activity at sites other than the DAT is unlikely to underlie the unique properties of the C-1 analogs **2** and **6**. It should be noted here that a recent study by Hiranita et al. (2011) shows that compounds with dual DAT affinity and σ antagonist activity are capable of attenuating cocaine self-administration. In fact, compound **6** (more than **2**) has a relatively significant affinity for σ receptors (0.2–0.5 μM), similar to the σ affinity of rimzacole (0.2–0.9 μM), and both compound **6** and rimzacole display appreciable DAT affinity (for rimzacole activity see Hiranita et al., 2011).

Cocaine Analog Inhibition of Veratridine-Stimulated Na⁺ Influx in Neocortical Neurons. The ability of cocaine, lidocaine, and cocaine analogs to block voltage-gated sodium channels was assessed in neocortical neurons loaded with SBFI. We specifically determined the ability of these compounds to antagonize veratridine-stimulated Na⁺ influx in these neurons. As depicted in Fig. 3, both cocaine analogs (**2** and **6**), lidocaine, and cocaine produced concentration-dependent antagonism of the increase in neuronal intracellular sodium concentration produced by veratridine. The concentration-response curves for the cocaine analogs **2** and **6**, lidocaine and cocaine, were best-fit by a three-parameter logistic equation yielding IC₅₀ values of 16.01 $\pm$ 1.90, 0.29 $\pm$ 0.07, 39.6 $\pm$ 2.4, and 6.99 $\pm$ 2.43 μM , respectively. These

Fig. 3. Nonlinear regression analysis of the response of the Na^+ -sensitive fluorescent indicator SBFI (ratiometric measurement of fluorescence intensity at 505 nm with sequential excitation at 340/380 nm) to veratridine (VRT), in the absence and presence of the C-1 cocaine analogs (–)-2 and (–)-6 (A and B), lidocaine (C), and cocaine (D). Data shown denote the mean $\pm$ S.E.M. of two representative experiments performed with two to four replicates. IC_{50} values represent the mean $\pm$ S.E.M. of four to six experiments, each performed with two to four replicates.

results indicate that 1-phenyl-cocaine (**6**) is approximately 55-fold more potent than the 1-methyl analog (**2**). Furthermore, in comparison with the IC_{50} value of 6.99 μM for (–)-cocaine itself, analog **2** is 2.3-fold less and analog **6** is 24-fold more active as an inhibitor of voltage-dependent Na^+ channels than the parent compound (Fig. 3). Previously, cocaine had been demonstrated to have an IC_{50} of 6 μM and lidocaine an IC_{50} of $73.6 \pm 10 \mu\text{M}$ as inhibitors of veratridine-induced Na^+ flux (Matthews and Collins, 1983; Deffois et al., 1996). This potency of cocaine and lidocaine is therefore in good agreement with the data presented here.

Effect of All C-1-Substituted Cocaine Analogs on Locomotor Activity. The first compounds tested, the 1-methyl- and 1-propyl-cocaine analogs (**2** and **4**), had little, if any, effect on locomotor activity in adult mice, at doses up to 30 mg/kg i.p. (Fig. 4; compare with saline and cocaine). Subsequently tested compounds **3**, **5**, and **6** (the 1-ethyl, 1-pentyl, and 1-phenyl derivatives, respectively) were also nonstimulatory, showing no significant effects on locomotion at doses of 10 or 30 mg/kg i.p. (Fig. 5). In contrast, (–)-cocaine dose-dependently increased locomotion, with intraperitoneal administration of 10 mg/kg producing a slight elevation above saline and 30 mg/kg producing pronounced increases (Figs. 4 and 5). Overall, each of the novel C-1 analogs, even at a dose of 30 mg/kg, did not stimulate locomotion to levels above those seen with 10 mg/kg of cocaine. This finding is remarkable for compounds **3** and **6**, which exhibit greater potency than cocaine in blocking the DAT (Table 1). This is especially true for **6**, which is 10-fold more potent than cocaine at blocking DAT-mediated substrate uptake in HEK-hDAT cells (the K_i values for the inhibition of [^3H]DA uptake for compound **6** and cocaine were 32.3 and 326 nM, respectively). Thus, compared with cocaine, in the context of DAT activity, doses of 10 and 30 mg/kg of analog **6** are relatively high, and

we therefore additionally tested lower doses of 1 and 3 mg/kg (Fig. 5B). Administration of 1 and 3 mg/kg of compound **6** produced locomotor counts comparable with the counts observed with 10 mg/kg. This indicates it is unlikely we somehow missed an inverted U-shaped dose-response curve for locomotion with higher doses becoming inhibitory or inducing motor stereotypy that might mask increases in locomotor behavior. In this context, doses of compounds **4** and **5** higher than 30 mg/kg would be expected to be needed for behavioral activity involving DA based on their lower DAT affinity compared with cocaine (~3- to 4-fold); such higher doses, however, were not tested to avoid complications from potentially deleterious off-target effects.

Effect of Analogs **2 and **6** in Forced-Swim Test Sensitive to Depression-Like Symptoms.** Saline and analog **2** (30 mg/kg i.p.), analog **6** (3 mg/kg i.p.), and the classic tricyclic antidepressant desipramine (10 mg/kg i.p.) were administered 23.5, 5, and 1 h before the 6-min test swim, respectively (Fig. 6). Compound **2** was as effective as the antidepressant desipramine in reducing immobility time (Fig. 6A; $F_{2,22} = 21.26$; $P = 0.0001$). In addition, the latency to the first episode of immobility was significantly increased over saline, similar to desipramine (Fig. 6B; $F_{2,22} = 20.52$; $P < 0.0001$). In a separate experiment, a significant immobility reduction and latency increase was also observed for compound **6** (Fig. 6, C and D, with, respectively, $F_{2,20} = 5.336$, $P = 0.013$ and $F_{2,20} = 6.191$, $P < 0.0085$). Together, these results suggest that (–)-1-methyl-cocaine (**2**) and (–)-1-phenyl-cocaine (**6**) have antidepressant-like action.

Effect of Analogs **2 and **6** in Conditioned Place Preference Test.** In the CPP test, analog **2** (10 mg/kg), like cocaine (**1**) (10 mg/kg), increased the postconditioning time (over preconditioning time) in the nonpreferred side (Fig. 7A). That is, analog **2** produced CPP, without reducing the CPP

Fig. 4. The effect of acute cocaine (Coc), saline, or the C-1 analogs (–)-2 (left) and (–)-4 (right) on ambulatory counts (measure of consecutive IR beams interrupted by C57BL/6 male mice during ambulatory activity) over a 120-min period after injection (in 5-min intervals). Doses administered (either 10 mg/kg or 30 mg/kg i.p.) are indicated. Data are mean $\pm$ S.E.M. (vertical bars). *, $P < 0.01$ ($n = 5-6$), compared with C-1 compound at the same dose (Student's t test).

engendered by cocaine itself (Fig. 7A); saline was ineffective in increasing the postconditioning time in the nonpreferred side as expected. In a separate experiment in a different batch of mice, analog **6** (3 mg/kg) did not produce CPP, with postconditioning time in the nonpreferred side being equal to the preconditioning time (Fig. 7B). Because only one dose of **6** was tested here, caution is needed in concluding the compound completely lacks CPP properties. Intriguingly, pretreatment with compound **6** (3 mg/kg) before cocaine (10 mg/kg) (Fig. 7B) caused the difference between postconditioning and preconditioning time to only approach statistical significance ($P = 0.053$; paired Student's t test); in contrast, the difference observed with cocaine alone in a separate experiment (Fig. 7A) indicated a statistically significant CPP at $P = 0.013$. The results taken together suggest that although some C-1 analogs may possess rewarding qualities, other C-1 substitutions can give compounds that 1) may not produce CPP, 2) may interfere with CPP engendered by cocaine, and 3) are not locomotor stimulants. To find a compound that engenders CPP but does not stimulate locomotion (both DA-associated behaviors) is not in itself surprising. It is

noteworthy that dissociation among genetically different mouse strains for cocaine to produce CPP and locomotor stimulation has been reported previously (Eisener-Dorman et al., 2011) with not entirely overlapping brain circuits for reward and locomotion. Likewise, a dissociation between the locomotor effect and the discriminative stimulus effect of cocaine-like compounds has been reported previously for the C2-phenyl ester-substituted cocaine analog 3 β -benzoyloxy-8-methyl-8-azabicyclo (3.2.1) octane-2-carboxylic acid phenyl ester (RTI-15) (Cook et al., 1998).

Docking of Cocaine, Analog 2, and Analog 6 into hDAT Homology Model. It was originally assumed that all ligands acting as nontranslocated DAT inhibitors possessed reinforcing effects identical to those of cocaine and differed solely in affinity for the transporter (hence, in the dose required to elicit cocaine-like behavioral reactions). However, various tropane compounds with a benztropine-type structure can exhibit remarkably high affinity for the DAT; but, rather than being powerful central stimulants like cocaine, these atypical DAT inhibitors show mild (if any) locomotor stimulant effects, as well as attenuate the effects of concom-

Fig. 5. The effect of acute cocaine and analogs (–)-3, (–)-5 (A) or analog (–)-6 (B) on ambulatory counts. Details are as in Fig. 4; however, a wider dose range (1–30 mg/kg) was tested for compound (–)-6, to account for its increased potency as a DAT inhibitor compared with cocaine. *, $P < 0.05$ ($n = 6–12$), compared with C-1 compound at the same dose (Student's t test). Statistics were applied to C-1 groups compared with cocaine groups (at the same dose) examined within one and the same experimental set; cocaine values shown are the average of all experiments.

Fig. 6. The effect of analog (–)-2 and (–)-6 in the forced-swim test model of antidepressant-like activity in C57BL/6 male mice. A and B, similar to the clinically useful tricyclic antidepressant desipramine (DMI; 10 mg/kg i.p.), (–)-2 (30 mg/kg i.p.) decreased immobility time (A) and increased latency to first episode of immobility (B) compared with saline-injected controls. C and D, the same was true for (–)-6 (3 mg/kg i.p.) and immobility time (C) and latency to first episode of immobility (D). Data are mean $\pm$ S.E.M. *, $P < 0.05$; ***, $P < 0.001$ ($n = 7–10$; one-way ANOVA and Bonferroni post hoc tests).

itantly administered cocaine (Agoston et al., 1997; Li et al., 2011). There is ample evidence that different classes of DAT ligands preferentially bind to (or induce upon binding) distinct transporter conformational states (Reith et al., 2001; Chen et al., 2004; Loland et al., 2008). The lack of cocaine-like locomotor stimulation seen with atypical inhibitors (like bupropion) is thought to result from their propensity to stabilize the DAT in a closed-to-out (inward-facing or occluded) conformational state, as opposed to cocaine-like ligands, which stabilize an open-to-out (outward-facing) transporter conformation (Loland et al., 2008; Schmitt et al., 2008; Schmitt and Reith, 2011). We considered the possibility that the C-1 cocaine analogs fall in the bupropion-like category with respect to their conformational effects on the DAT. Thus, we sought to investigate the interaction of certain C-1 analog compounds with the DAT *in silico*, using our homology model of the human DAT and flexible ligand-adaptive docking procedures (Schmitt et al., 2010; Schmitt and Reith, 2011). We performed docking simulations with two of the novel C-1 analogs, the prototypical series member 1-methylcocaine (2) and the most potent DAT inhibitor of the series, 1-phenylcocaine (6), as well as (–)-cocaine itself and bupropion to serve as a basis for comparison. The hDAT model was based on the structure of LeuT, with ligands docked at the primary (S1) substrate-binding site. For each ligand, an energetically favorable docking pose was selected for further geometric optimization of the transporter/ligand complex (see *Materials and Methods*).

Final energy-minimized poses of the model hDAT/ligand complexes are shown in Fig. 8. After flexible docking, cocaine was oriented such that the protonated tropane amine faced the S1 site residues of TM1 and exhibited strong molecular interactions with residues Asp79 (via a hydrogen bond) and Phe76 (via a cation- π interaction with the aromatic side chain of Phe76). The 3 β -benzyloxy and 2 β -carbomethoxy moieties were positioned toward TMs 3 and 8, enveloped by the side chains of residues Val152, Gly153, Tyr156, and Asn157, as well as Ser422 and Ala423 (Fig. 8A). The docking orientation of cocaine and the binding-pocket residues found

Fig. 7. CPP test on C57BL/6 male mice treated with saline, cocaine, (-)-2, or (-)-2 preceding cocaine by 20 min (A) or treated with (-)-6, or (-)-6 preceding cocaine by 20 min (B) [all drugs at 10 mg/kg i.p. except (-)-6, which was at 3 mg/kg i.p.]. Data are mean $\pm$ S.E.M. *, $P < 0.05$ ($n = 8$), compared with preconditioning time in nonpreferred side (paired Student's t test).

were to be consistent with those reported by Beuming et al. (2008) in their docking model of cocaine at the DAT S1 site. The docking model of **2** suggested that the analog binds in a similar manner as cocaine, with the extra methyl group oriented toward Asp79 and surrounding residues of TM1 (Phe76, Ala77, and Val78), but still readily accommodated within the binding pocket (Fig. 8B). In contrast, the additional steric heft of the C-1 phenyl substituent of **6** was not as readily accepted, and the compound adopted an entirely different binding orientation than cocaine and **2** (Fig. 8C). The extra C-1 phenyl group was positioned downward (toward the cytosolic vestibule), adjacent to the aromatic side chains of residues Phe76 and Phe326. In addition, the β -benzoyloxy moiety was oriented upward, slightly above the extracellular vestibule gating network residues Arg85, Phe320, and Asp476, enabling formation of a cation- π interaction between the benzoyloxy aromatic ring and Arg85 (Fig. 8C). In the benztropine docking model, the diphenylmethoxy ring system faced Val152, Gly153, and Tyr156 of TM3 and the tropane *N*-methyl amine group was surrounded by Phe76 (which formed a cation- π interaction with the amine nitrogen), Val78, and Asp79 of TM1 (Fig. 8D). Similar to the docking results for benztropine reported by Bisgaard et al.

(2011), we found that one of the diphenylmethoxy system rings was positioned relatively high up in the S1 binding site, close to residues Ala479 and Ala480 of TM10 (Fig. 8D).

Despite their benztropine-like lack of locomotor stimulatory activity in vivo (Figs. 4 and 5), our in silico docking results do not support the theory that the C-1 analogs interact with the DAT in an atypical manner like benztropine. In their study combining DAT modeling and site-directed mutagenesis, Beuming et al. (2008) showed that the presence or absence of a hydrogen bond between Asp79 and Tyr156 in a given DAT/ligand complex offers an indication of the DAT conformation bound by the ligand. Tyr156 (which is highly conserved across NSS protein family members) interacts with substrates upon binding at the S1 site and also participates in the vestibular gating network consisting of Asp79, Arg85, Tyr156, Phe320, and Asp476 that partitions the S1 and S2 sites (Shan et al., 2011). When DAT substrates (such as dopamine, dextroamphetamine, and 3,4-methylenedioxymethylamphetamine) bind at the S1 site, a hydrogen bond formed between the side chain oxygen atoms of Asp79 and the hydroxyl moiety of Tyr156 helps to close the vestibular gate, protecting the substrate and ions from permeation of extracellular water into the S1 site (Beuming et al., 2008).

Fig. 8. Final energy-minimized poses of DAT/ligand complex after flexible docking of cocaine, the novel C-1-substituted cocaine analogs (–)-**2** and (–)-**6**, and benztropine at the DAT primary (S1) substrate-binding site. Selected binding pocket residues are labeled and rendered as sticks; bound ligand molecules (also shown as sticks) are highlighted as gray-colored carbon atoms. Cotransported sodium and chloride ions are labeled and rendered as purple and green spheres, respectively. A to C, the distance between the carboxylate oxygen atom of residue Asp79 and the ring hydroxyl moiety of residue Tyr156 (accentuated by green dashed lines connecting the two oxygens) is indicated in the lower right in green text. Binding of (–)-cocaine (A) and the C-1 analogs (–)-**2** (B) and (–)-**6** (C) disrupts the hydrogen bond between the side chains of Asp79 and Tyr156 (interatomic distance >3.5 Å), indicating that these compounds promote an open-to-out DAT conformational state. D, in contrast, in the binding model of benztropine, the D79Y156 interatomic distance is 3.29 Å, suggesting that benztropine interacts with a more inward-facing (closed-to-out) DAT conformation.

Hence, an interatomic distance of less than 3.5 Å (indicative of an intact hydrogen bond) between the oxygen atoms of Asp79 and Tyr156 during ligand binding in the S1 site suggests the ligand interacts with a closed-to-out transporter state, whereas D79-Y156 distances larger than the 3.5-Å maximum for hydrogen bonding signify interaction with an open-to-out (outward-facing) state. In our case, cocaine binding resulted in a terminal D79-Y156 distance of 6.03 Å, consonant with many other literature findings of cocaine's selective interaction with an outward-facing DAT conformation, and benztropine binding resulted in a D79-Y156 distance of 3.29 Å, indicating preferential interaction with an inward-facing conformation (Fig. 8, A and D). Unexpectedly, docking of the C-1 analogs **2** and **6** yielded respective terminal D79-Y156 distances of 4.40 and 4.89 Å (Fig. 8, B and C), suggesting that these compounds, like cocaine, exhibit preferential interaction with an outward-facing DAT conformation.

Binding of C-1 Analogs to Different DAT Conformations. Binding affinity for conformationally biased DAT mutants can be indicative of preferential interaction with either an outward or inward facing conformation of the transporter (Loland et al., 2008; Schmitt et al., 2008; Schmitt and Reith, 2011). Thus, a compound that prefers an inward-facing form of the DAT displays a loss of binding affinity for a mutant biased toward an outward-facing conformation, whereas an outward preferring compound gains affinity at such a mutant. The latter is what we observed for compounds **2**, **3**, and **6** comparing their affinities for displacement of [3 H]CFT binding at W84L, a DAT mutant with an outward-facing bias, and wild-type transporters (Supplemental Table S1). This is consonant with the docking data presented above for compounds **2** and **6** (see Supplemental Results and Discussion).

Discussion

C-1 Modification of the Cocaine Molecule and Brain Uptake.

The lack of appreciable locomotor effect of the C-1 compounds could be explained if they have difficulty crossing the blood-brain barrier. This, however, would be unexpected for cocaine-like structures, with rapid brain entry observed for phenyltropane cocaine analogs (Cline et al., 1992; Stathis et al., 1995). Another hypothesis for the lack of cocaine-like locomotor stimulant effects of **2** is that the compound is either metabolized to inactive products more rapidly than cocaine or is actively expelled from the brain by the ATP-coupled multidrug efflux transporter P-glycoprotein, which is expressed in the endothelial cells forming the blood-brain barrier. However, the fact that **2** was detected in the brain at 30 min postadministration, in concentrations similar to those achieved after administration of cocaine, makes this explanation implausible. Furthermore, central effects observed for **2** in the forced-swim and CPP tests strongly indicate brain penetration of this analog.

Properties of C-1 Analogs are Unlike Either Cocaine or Benztropine. All C-1 cocaine analogs synthesized thus far (Fig. 1) were devoid of the locomotor stimulant activity associated with cocaine (Figs. 4 and 5). At the same time, analogs **3** and **6** were more potent inhibitors of the DAT than cocaine, and analog **2** was at least as potent. This combination of nonstimulatory character with potent DAT interaction equally applies to benztropine analogs (Li et al., 2011), some of which attenuate behavioral effects of cocaine (Desai et al., 2005). However, we do not think that the C-1 cocaine analogs investigated here are in the same pharmacological category as benztropine (and derivatives), because our *in silico* modeling data indicate that, unlike benztropine, the C-1 analogs do not seem to interact with an inward-facing DAT conformation (Fig. 8). In contrast, the C-1 analogs seem to bind, like cocaine, to an open-to-out state: compare the terminal distance between oxygen atoms of DAT residues D79-Y156 for cocaine, **2** and **6** (Fig. 8, A–C) with that of benztropine (Fig. 8D). Only in the benztropine docking model is the interatomic distance lower than the 3.5-Å maximum required for formation of a hydrogen bond. Hence, of the four ligands tested in our docking models, only benztropine allows the DAT extracellular gating network to operate, giving rise to a

closed-to-out conformation. Results from interaction experiments involving W84L, a DAT mutant conformationally biased toward the outward-facing transporter state, point to the same conclusion for compounds **2**, **3**, and **6** (see Supplemental Data). In this context it is of interest that modafinil (**8**), which possesses a benzhydrylsulfinyl moiety that is similar to the diphenylmethoxy group of benztropine (albeit with a sulfinyl moiety in lieu of oxygen), has shown promise as a therapeutic for cocaine addiction and interacts with a closed-to-out, inward-facing DAT conformation (Schmitt and Reith, 2011 and references therein). The novel C-1 cocaine analogs described here are interesting probes for mapping inhibitor-binding properties in monoamine transporters.

Novel Pharmacological Tools in Medication Development for Psychiatric Disorders. Some of the C-1 cocaine analogs described here display a novel pharmacology: cocaine-like brain uptake and potent DAT interaction combined with a benztropine-like lack of locomotor stimulation (although molecular interaction with the DAT seems to differ from that of benztropine). The combination of monoamine transporter interaction with a lack of stimulatory effect warrants further exploration of this type of compounds for central effects other than stimulation. Analogs **2** and **6** provide proof of principle for C-1 cocaine analogs to be able to exert central effects, but not locomotor stimulatory activity. Thus, **2** and **6** displayed antidepressant-like activity in the forced-swim test as effectively as the classic antidepressant desipramine (Fig. 6). It will therefore be important to further explore SARs for strengthening the antidepressant effect while maintaining the lack of locomotor stimulation. One obvious possibility is to remove the benzoyl ester linkage from the source compounds, generating more metabolically stable phenyltropanes, as in 2 β -carbomethoxy-3 β -phenyltropane (troparil), the prototypical compound of the WIN series of phenyltropane stimulants (Clarke et al., 1973). In addition, it can be expected that the phenyltropane versions will be more potent as monoamine transporter inhibitors than their respective cocaine (benzoyloxytropane) counterparts (Reith et al., 1986; Carroll et al., 1992).

It is also likely that removal of the benzoyl ester moiety will attenuate cocaine-like inhibitory activity at voltage-gated Na⁺ channels, reducing the possible risk of cardiotoxicity (Matthews and Collins, 1983). In this respect, the present data emphasize the importance of monitoring all C-1 cocaine analogs for activity at voltage-gated Na⁺ channels. Analog **2** was less potent (16.01 μ M) than cocaine itself (6.99 μ M) in inhibiting Na⁺ flux, reducing the risk of cardiotoxicity. In contrast, **6** was more potent than cocaine (by an order of magnitude, 0.29 versus 6.99 μ M).

Based on the realization that dopamine dysfunction plays a large role in anhedonia and depression (Nestler and Carlezon, 2006), there has been revival of the idea that DAT blockade can contribute to antidepressant activity, which has given rise to the development of TUI compounds as potential antidepressants (Dutta et al., 2008 and references therein). Current antidepressants are either selective for SERT (selective serotonin reuptake inhibitors) or target both SERT and NET (serotonin-norepinephrine reuptake inhibitors). These medications all suffer from delayed onset of antidepressant effect, risking patients to abandon treatment within the required 2-week period. In addition, many patients are not

responsive to currently available antidepressants, and the majority of patients do not experience complete remission of depressive symptoms when treated with selective serotonin reuptake inhibitors alone (Stewart et al., 2009). Therefore the search needs to be continued for novel treatments and lead TUI compounds such as the present C-1 analogs not available until now.

Another obvious area where cocaine-like compounds could be useful is the addiction field. There is no accepted pharmacotherapy currently available for psychostimulant users. Off-label prescription of dopaminergic agents, particularly DAT substrates like dextroamphetamine or atypical DAT blockers like modafinil, has beneficial effects in attenuating drug craving (Shearer, 2008; Herin et al., 2010), but a novel, widely applicable, treatment is in high demand. In this context, it is of interest that analog **6** does not produce CPP, attenuates CPP engendered by cocaine, and is not a locomotor stimulant (Figs. 5 and 7B). Manipulation of dopaminergic and serotonergic neurotransmission can affect self-administration of psychostimulant drugs (Spealman, 1993; Rothman and Baumann, 2006; Howell et al., 2007); for example, SERT activity has been shown to attenuate the locomotor stimulator effects of cocaine and amphetamine (Spealman, 1993; Howell et al., 2007). Hence, nonstimulatory C-1 analogs combining DAT and SERT inhibition could be useful. The nonstimulatory analog **2** displayed this combination. Unlike analog **6**, however, compound **2** did not reduce cocaine CPP and itself produced CPP (Fig. 7A). It should be noted that CPP tests can be susceptible to effects other than reward reduction or enhancement (Bardo and Bevins, 2000), and more addiction-related tests are needed. In this context, it should also be recalled that compound **6** displays σ activity (see Results), and σ antagonists have been reported to block CPP development (see Katz et al., 2011).

In the context of antiaddiction drug development, one issue to consider for C-1 cocaine analogs is the distinction between substitution and antagonist therapies. The goal of antagonist therapy is pharmacologically enforced abstinence via use of a selective, high-affinity antagonist of the addictive drug's binding target (e.g., naltrexone, a potent μ -receptor antagonist used in opioid addiction). In the case of treatment of cocaine addiction, a long-held view has been that we may be able to antagonize cocaine action at the DAT with a compound that does not interfere with DA recognition (a "DA-sparing" compound) (Carroll et al., 1992). Although this is still a theoretical possibility and may have some limited practical use in treating cocaine overdose, attention has shifted to being able to substitute the psychostimulant drug of addiction with a milder, longer-acting "agonist-like" agent that itself has lower abuse liability (Shearer, 2008; Herin et al., 2010). The potential value of our novel cocaine analogs, we feel, lies in the latter category. The prototypical compound **2** exhibits an antidepressant effect (as indicated by the forced-swim test data) and is not a locomotor stimulant; if it retained some cocaine-like, mildly rewarding effect it would be of interest to test it preclinically as a cocaine replacement therapeutic. However, its activity in the CPP test necessitates future behavioral work to ensure that this compound and other nonstimulatory C-1 analogs do not have the potential of addictive liability.

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Authorship Contributions

Participated in research design: Reith, Schmitt, Murray, Ser-shen, Unterwald, and Davis.

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Wrote or contributed to the writing of the manuscript: Reith, Schmitt, Murray, Ser-shen, Unterwald, and Davis.

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