Effects of Combined Dopamine and Serotonin Transporter Inhibitors on Cocaine Self-Administration in Rhesus Monkeys

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Received May 23, 2006; accepted November 10, 2006

ABSTRACT

Dopamine transporter (DAT) inhibitors may represent a promising class of drugs in the development of cocaine pharmacotherapies. In the present study, the effects of pretreatments with the selective DAT inhibitor 3β-(4-chlorophenyl)tropane-2β-[3-[4-α-methylphenyl]isoxazol-5-yl] hydrochloride (RTI-336) (0.3–1.7 mg/kg) were characterized in rhesus monkeys trained to self-administer cocaine (0.1 and 0.3 mg/kg/injection) under a multiple second-order schedule of i.v. drug or food delivery. In addition, RTI-336 (0.01–1.0 mg/kg/injection) was substituted for cocaine to characterize its reinforcing effects. Last, the dose of RTI-336 that reduced cocaine-maintained behavior by 50% (ED50) was coadministered with the selective serotonin transporter (SERT) inhibitors fluoxetine (3.0 mg/kg) and citalopram (3.0 mg/kg) to characterize their combined effects on cocaine self-administration. PET neuroimaging with the selective DAT ligand [(18)F]8-(2-[18F]fluoroethyl)-2β-carbomethoxy-3β-(4-chlorophenyl)nortropane quantified DAT occupancy at behaviorally relevant doses of RTI-336. Pretreatments of RTI-336 produced dose-related reductions in cocaine self-administration, and the ED50 dose resulted in approximately 90% DAT occupancy. RTI-336 was reliably self-administered, but responding maintained by RTI-336 was lower than responding maintained by cocaine. Doses of RTI-336 that maintained peak rates of responding resulted in approximately 62% DAT occupancy. Co-administration of the ED50 dose of RTI-336 in combination with either SERT inhibitor completely suppressed cocaine self-administration without affecting DAT occupancy. Hence, at comparable levels of DAT occupancy, coadministration of SERT inhibitors with RTI-336 produced more robust reductions in cocaine self-administration compared with RTI-336 alone. Collectively, the results indicate that combined inhibition of DAT and SERT warrants consideration as a viable approach in the development of cocaine medications.

The neurochemical mechanisms that mediate the behavioral effects of cocaine are complex and probably involve multiple neurotransmitter systems. In vitro studies have demonstrated that cocaine blocks the uptake of the monoamines dopamine, serotonin, and norepinephrine (Heikkila and Manzino, 1984; Reith et al., 1986). However, the behavioral effects of cocaine have been linked more closely to inhibition of dopamine uptake (Ritz et al., 1987; Woolverton and Kleven, 1988). Evidence to support this conclusion is derived from a variety of behavioral studies characterizing the acute effects of dopamine transporter (DAT) inhibitors administered alone or in combination with cocaine. Preclinical studies have demonstrated a relationship between the potency of cocaine analogs to bind to the DAT in vivo and their potency in producing locomotor-stimulant effects in rodents (Cline et al., 1992; Kuhar, 1993). Likewise, a high correlation was found between the potency of cocaine analogs to displace [3H]cocaine in the caudate and the potency of these compounds to produce cocaine-like behavioral effects in squirrel monkeys (Bergman et al., 1989; Madras et al., 1989; Spealman et al., 1989). Cocaine and selective DAT inhibitors exert similar effects on schedule-controlled behavior and are reli-

ABBREVIATIONS: DAT, dopamine transporter; GBR12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine; SERT, serotonin transporter; RTI-336, 3β-(4-chlorophenyl)tropane-2β-[3-[4-α-methylphenyl]isoxazol-5-yl] hydrochloride; FR, fixed ratio; PET, positron emission tomography; FECNT, 8-(2-[18F]fluoroethyl)-2β-carbomethoxy-3β-(4-chlorophenyl)nortropane; RTI-112, 3β-(3-methyl-4-chlorophenyl)-2β-carboxylic acid methyl ester hydrochloride; RTI-113, 3β-(4-chlorophenyl)tropane-2β-carboxylic acid phenyl ester hydrochloride; RTI-177, 3β-(4-chlorophenyl)tropane-2β-[3-(phenylisoxazol-5-yl)] hydrochloride.
ably self-administered in squirrel monkeys (Bergman et al., 1989; Howell and Byrd, 1991; Howell et al., 2000) and rhesus monkeys (Nader et al., 1997; Lile et al., 2003; Lindsey et al., 2004; Wilcox et al., 2005).

The relevance of the DAT in the reinforcing effects of cocaine is supported further by human and nonhuman primate neuroimaging studies. In human cocaine users, a significant correlation was observed between the level of DAT occupancy and the magnitude of the subjective high reported following administration of cocaine (Volkow et al., 1997) or the behavioral stimulant methylphenidate (Volkow et al., 1999). In rhesus monkeys, doses of cocaine that maintained peak responding in drug self-administration studies resulted in DAT occupancy greater than 65% (Wilcox et al., 2002; Lindsey et al., 2004). Doses of GBR12909 that decreased cocaine self-administration in rhesus monkeys resulted in DAT occupancy greater than 50% in baboons (Villemaigne et al., 1999) and rhesus monkeys (Lindsey et al., 2004). Likewise, doses of cocaine analogs with selectivity for DAT decreased cocaine self-administration in rhesus monkeys at DAT occupancies greater than 70% (Wilcox et al., 2002; Lindsey et al., 2004). Collectively, these results indicate that DAT occupancy is closely associated with the reinforcing effects of cocaine and with the effectiveness of DAT inhibitors to reduce cocaine self-administration.

Preclinical studies have indicated that serotonin can modulate the behavioral effects of psychomotor stimulants. For example, a negative relationship was observed between the toxicities of several cocaine- and amphetamine-like drugs in self-administration studies and their binding potencies to serotonin uptake sites (Ritz et al., 1987; Ritz and Kuhar, 1989). Monoamine-releasing agents had decreased reinforcing effectiveness in rhesus monkeys when serotonin-releasing potency was increased relative to dopamine (Wee et al., 2005). Moreover, a potent releaser of dopamine and serotonin lacked reinforcing effectiveness, yet it produced dose-dependent reductions in cocaine self-administration in rhesus monkeys (Rothman et al., 2005). The behavioral and neurochemical profile of DAT inhibitors also is influenced by actions at multiple monoamine transporters in squirrel monkeys (Ginsburg et al., 2005). Consistent with these results, administration of the SERT inhibitor fluoxetine decreased self-administration of cocaine in rodents (Carroll et al., 1990) and rhesus monkeys (Kleven and Woolverton, 1993). In squirrel monkeys, the SERT inhibitors citalopram, fluoxetine, and alaproclate attenuated the behavioral-stimulant effects of cocaine (Spealman, 1993; Howell and Byrd, 1995). Alaproclate also attenuated cocaine self-administration and cocaine-induced increases in extracellular dopamine in squirrel monkeys (Czoty et al., 2002) and cocaine-induced activation of prefrontal activity in rhesus monkeys (Howell et al., 2002). Collectively, there is strong evidence to suggest that SERT inhibition can attenuate the behavioral-stimulant and reinforcing effects of psychomotor stimulants.

The present study characterized the effects of the selective DAT inhibitor, RTI-336, on cocaine self-administration in rhesus monkeys. In addition, RTI-336 was substituted for cocaine to characterize its reinforcing effects. It was hypothesized that RTI-336 would reduce cocaine self-administration in a dose-related manner, and reliably maintain self-administration behavior when substituted for cocaine. Subsequent experiments coadministered RTI-336 with the selective SERT inhibitors, fluoxetine and citalopram, to characterize their combined effects on cocaine self-administration. It was hypothesized that coadministration of RTI-336 with the SERT inhibitors would be more effective in reducing cocaine self-administration compared with RTI-336 alone. PET neuroimaging with the selective DAT ligand, [18F]FECNT, quantified DAT occupancy by RTI-336 at doses that decreased cocaine self-administration and at doses that maintained self-administration when substituted for cocaine.

**Materials and Methods**

**General Methods**

**Subjects.** Four female (RRj-5, RNa-4, RMy-4, and REl-5) and two male (RvC-5 and ROk-5) adult rhesus monkeys (Macaca mulatta) weighing 8.0 to 13.0 kg were used as subjects. Each subject was housed individually and fed Purina monkey chow (Ralston Purina, St. Louis, MO), fruit, and vegetables. Water was continuously available, and food restriction protocols were not used. Food intake was monitored daily throughout the study by recording the number of chow delivered each feeding, and the number of chow remaining in the home cage each morning. Animal care procedures strictly followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Emory University.

**Surgery.** Each subject was prepared surgically with a chronic indwelling venous catheter under sterile surgical conditions using a technique described previously (Wilcox et al., 2002). Preoperative antibiotics (Rocephin (ceftriaxone, 25 mg/kg i.m.) or cefazolin (25 mg/kg i.m.)) were given on the day of the surgery to help prevent infection. A silicone catheter (0.65 mm i.d., 1.75 mm o.d.; Access Technologies, Skokie, IL) was implanted under a combination of Telazol (tiletamine hydrochloride and zolazepam hydrochloride, 4.0 mg/kg i.m.) and isoflurane anesthesia using aseptic techniques. The proximal end of the catheter terminated in the ven a cava above the right atrium, and the distal end was routed under the skin and attached to a subcutaneous vascular access port (Access Technologies) located in the center of the lower back. After surgery, the subject was returned to its home cage and received Banamine (flunixin meglumine, 1.0 mg/kg i.m.) every 6 h for 24 h postoperatively to reduce pain and discomfort associated with surgery. Catheters were flushed daily with 1.0 ml of heparinized (100 U/ml) saline to maintain patency.

**Drugs.** Cocaine HCl (National Institute on Drug Abuse, Bethesda, MD), RTI-336 HCl (Research Triangle Institute, Research Triangle Park, NC), fluoxetine HCl (Eli Lilly & Co., Indianapolis, IN), and citalopram HCl (H. Lundbeck AS, Copenhagen, Denmark) were dissolved in 0.9% saline, and all doses were determined as salts.

**Behavioral Methods**

**Apparatus.** During behavioral testing, each monkey was seated in a commercially available primate chair (Primate Products, Miami, FL). A response panel with one lever was mounted on the front of the chair. Located above the lever in the center of the response panel were red, green, and white stimulus lights. Once the monkey was seated in the chair, a Huber needle (Access Technologies) was inserted into the venous access port. The polyvinyl chloride tubing attached to the Huber needle was connected to a motor-driven syringe (Coulbourn Instruments, Allentown, PA) located outside of the chamber containing the drug solution. A volume of 2.0 ml infusion was delivered over 7 s. M&M chocolate candies were dispensed by an M&M dispenser (MED Associates, St. Albans, VT) located on the top of the response panel. Testing during daily 1-h sessions occurred in a ventilated, sound-attenuating chamber. IBM-compatible computers controlled experimental events and recorded data.
**Procedure.** Subjects responded for i.v. infusions of cocaine or M&Ms under a multiple, second-order schedule of reinforcement. When the daily session began, the red light on the response panel was illuminated and indicated availability of food reinforcement. Each fixed ratio (FR) of 20 responses completed during a 10-min fixed interval changed the stimulus light from red to white for 2 s. The first FR20 completed after the 10-min fixed interval had elapsed resulted in the simultaneous delivery of three M&Ms in a tray located in the lower center portion of the response panel, and it changed the stimulus light from red to white for 15 s. There was a 30-s limited hold for completion of the first FR20 after the 10-min fixed interval had elapsed, and food was not delivered if the limited hold expired. The purpose of the limited hold was to limit the component duration and to require a minimal rate of responding for the subject to receive a reinforcer. After the food delivery or the limited hold expired, there was a 1-min time-out during which all stimulus lights were extinguished and responding on the lever had no programmed consequences. When the second component of the daily session began, the green light was illuminated and indicated availability of cocaine reinforcement. Otherwise, all schedule parameters were identical to the food component. The food and cocaine components continued to alternate during a daily session until each component was presented three times.

The sequence of drug treatments and subject assignment for the entire study are shown in Table 1. The six subjects were assigned to overlapping protocols to ensure that \( n = 4 \) for each experimental condition. Unfortunately, subject REI-5 died due to health problems unrelated to the study and was unable to complete the SERT inhibitor coadministration experiments. The training conditions described above remained in effect until responding for 0.1 mg/kg/injection cocaine and food was stable (\(< 20\% \) variance in daily response rate over five consecutive sessions), after which saline was substituted for cocaine until responding in the cocaine component decreased to below 30\% of responding for the training dose of cocaine. Typically, response rates declined gradually over two to four sessions. After saline extinction, the maintenance dose of cocaine was reinstated and responding was allowed to stabilize (\(< 20\% \) variance in daily response rate over five consecutive sessions).

For cocaine substitution studies, each subject was allowed to self-administer several doses of cocaine in random order. Substitution for each drug dose continued for at least five consecutive sessions and until responding stabilized (\(< 20\% \) variance in daily response rate). Typically, stable performance required nine to 12 sessions for a given drug dose.

For pretreatment studies, a given dose of RTI-336 was administered i.v. 15 min presession for 5 consecutive days. No trend was present across treatment days for any drug dose, so daily response rates were averaged across the 5 days. Saline vehicle was administered on alternate weeks and served as the control condition for drug pretreatment effects. There was little variation in response rates over multiple blocks of saline pretreatments, indicating that cocaine self-administration was stable in the absence of RTI-336 pretreatments. Each pretreatment dose was administered on at least two separate occasions and the sequence of doses was quasi-random. Data for individual subjects were based on the mean response rate over multiple determinations at each dose. All doses of RTI-336 were studied in combination with 0.1 mg/kg/infusion cocaine first. Subsequently, the maintenance dose of cocaine was changed to 0.3 mg/kg infusion and responding was allowed to stabilize. Drug pretreatments were repeated as described above for the lower maintenance dose of cocaine.

For RTI-336 substitution studies, each subject was allowed to self-administer several doses of RTI-336 in random order. Substitution for each drug dose continued for at least five consecutive sessions and until responding stabilized (\(< 20\% \) variance in daily response rate). Typically, stable performance required 10 to 15 sessions for a given drug dose. RTI-336 was the only drug available during these substitution studies. Two subjects (RNa-4 and ROk-5) assigned to RTI-336 substitution studies first participated in the RTI-336 pretreatment studies as described above. The other two subjects (RMy-4 and RRj-5) only had a history of cocaine self-administration.

The dose of RTI-336 that reduced cocaine self-administration by 50\% of baseline (ED\(_{50}\)) was determined for individual subjects and subsequently administered in combination with the SERT inhibitors fluoxetine (3.0 mg/kg) or citalopram (3.0 mg/kg), while subjects self-administered the 0.1 mg/kg/injection maintenance dose of cocaine. The pretreatment doses of fluoxetine and citalopram were the highest doses that could be administered without producing overt behavioral effects when given alone (Spealman, 1993; Czoty et al., 2002). In one condition, the SERT inhibitors were administered i.m. chronically for four consecutive weeks, and RTI-336 was administered during the last week of treatment. The 4-week treatment period with the SERT inhibitors was selected on the basis of the time course of therapeutic effects observed in the treatment of depression. In the other condition, the SERT inhibitors were administered i.m. acutely for five consecutive days during coadministration of RTI-336. The 5-day treatment period with the SERT inhibitors was selected to detect potential changes in sensitivity associated with repeated dosing. The order of drug testing was chronic fluoxetine, acute fluoxetine, acute citalopram, and chronic citalopram. Last, the dose of RTI-336 that reduced cocaine self-administration by 10\% of baseline (ED\(_{10}\)) was determined for individual subjects and subsequently administered in combination with fluoxetine (3.0 mg/kg), while subjects self-administered the 0.1 mg/kg/injection maintenance dose of cocaine. The rationale for evaluating the lower, ED\(_{10}\), dose of RTI-336 was to determine whether robust effects of the DAT inhibitor are required to observe drug interactions during coadministration with fluoxetine. Fluoxetine was administered i.m. acutely for five consecutive days during coadministration of RTI-336. In all coadministration

### Table 1

**Sequence of drug treatments and subject assignment**

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Subject</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-admin. training (0.1 mg/kg/inj cocaine)</td>
<td>n = 6; REI, RMy, RNa, ROk, RRj, RVc</td>
</tr>
<tr>
<td>Extinction–saline subst. for cocaine</td>
<td>n = 6; REI, RMy, RNa, ROk, RRj, RVc</td>
</tr>
<tr>
<td>Re-establish self-admin. (0.1 mg/kg/inj cocaine)</td>
<td>n = 6; REI, RMy, RNa, ROk, RRj, RVc</td>
</tr>
<tr>
<td>Establish dose-effect curve for cocaine subst.</td>
<td>n = 6; REI, RMy, RNa, ROk, RRj, RVc</td>
</tr>
<tr>
<td>RTI-336 pretreatments + 0.1 mg/kg/inj cocaine</td>
<td>n = 4; REI, RMy, RNa, ROk, RRj, RVc</td>
</tr>
<tr>
<td>RTI-336 pretreatments + 0.3 mg/kg/inj cocaine</td>
<td>n = 4; REI, RMy, RNa, ROk, RRj, RVc</td>
</tr>
<tr>
<td>Establish dose-effect curve for RTI-336 subst.</td>
<td>n = 4; REI, RNa, ROk, RVc</td>
</tr>
<tr>
<td>Re-establish self-admin. (0.1 mg/kg/inj cocaine)</td>
<td>n = 4; REI, RNa, ROk, RVc</td>
</tr>
<tr>
<td>Chronic fluoxetine + RTI-336 (ED(_{50})) on week 4</td>
<td>n = 3; RNA, ROK, RVc</td>
</tr>
<tr>
<td>Acute fluoxetine + RTI-336 (ED(_{50})) over 5 days</td>
<td>n = 3; RNA, ROK, RVc</td>
</tr>
<tr>
<td>Acute citalopram + RTI-336 (ED(_{50})) over 5 days</td>
<td>n = 3; RNA, ROK, RVc</td>
</tr>
<tr>
<td>Chronic citalopram + RTI-336 (ED(_{50})) on week 4</td>
<td>n = 3; RNA, ROK, RVc</td>
</tr>
<tr>
<td>Acute fluoxetine + RTI-336 (ED(_{50})) over 5 days</td>
<td>n = 3; RNA, ROK, RVc</td>
</tr>
<tr>
<td>Blood level determinations of RTI-336</td>
<td>n = 3; RNA, ROK, RVc</td>
</tr>
</tbody>
</table>

* admin., administration; inj, injection; and subst., substitution.*
tion studies, the SERT inhibitor was administered i.m. 30 min pre-
session, and RTI-336 was administered i.v. 15 min presession.
Subjects self-administered the maintenance dose of cocaine alone for
at least two consecutive weeks between each experimental condition
involving coadministration of a SERT inhibitor to re-establish stable
self-administration behavior (<20% variance in daily response rate).

**Data Analysis.** Response rates for cocaine- and food-maintained
behavior were analyzed separately for individual subjects as a func-
tion of cocaine maintenance dose (0.1 or 0.3 mg/kg/injection) and RTI-336 pretreatment dose. Responses and time elapsed during the
time-out were used as presentations of the white stimulus lights were not
included in response rate determinations. Mean response rates for
the group were compared with values obtained after saline pretreat-
ments. RTI-336 doses predicted to reduce response rates by 50%
(ED50) or 10% (ED10) of baseline were derived from standard linear
regression analysis (GraphPad Prism; GraphPad Software Inc., San
Diego, CA) of data from individual subjects. The analysis did not
force the regression lines through zero. The goodness of fit value (r2)
was 0.84, 0.95, 0.72, and 0.97 for subjects REl-5, RNa-4, ROk-5, and
RVC-5, respectively.

**PET Neuroimaging Methods.**

**Apparatus.** PET neuroimaging was performed at the Emory Uni-
versity PET Center on a microPET scanner (Concorde MicroSys-
tems, Knoxville, TN). The PET ligand FECNT is an N-fluoroethyl
norpropamine derivative that is selective for the DAT (Goodman et al.,
2000). Moreover, displacement studies with cocaine determined that
FECNT and cocaine bind to the same site in the striatum and that
the dose of cocaine gave dose-dependent (Votaw et al., 2002). All images were reconstructed with mea-
sured attenuation correction, zoom factor 8, and Shepp-Logan recon-
struction filter cut-off at 1 cycle/cm. The axial slice thickness was
3.375 mm. All images were decay-corrected to the time of injection.
Regions of interest were manually drawn on the late images over the
putamen and cerebellum. The regions of interest were then overlaid
on all images to obtain time-activity curves.

**Procedure.** Measures of DAT occupancy for RTI-336 were ob-
tained after the completion of behavioral studies with the drug in
individual subjects. In drug pretreatment studies, the ED50 doses of
RTI-336 were administered as a single i.v. bolus. In drug substitu-
tion studies, the dose of RTI-336 that maintained peak response
rates was administered as an i.v. bolus of the total dose the animal
received during its self-administration sessions (dose in milligrams
per kilogram per infusion × average number of infusions per ses-
on). On days of the PET studies, subjects were immobilized in their
home cage with 4.0 mg/kg Telazol and transported to the Emory
University PET Center. Subsequently, they were intubated, and
anesthesia was maintained with 1.0 to 2.0% isoflurane. Subjects
were positioned in the scanner, and a 15-min transmission scan was
obtained for attenuation correction. Subsequently, a slow bolus of
approximately 5.0 mCi of [18F]FECNT (specific activity 1.5 Ci/µmol)
was injected over 5 to 6 min at a rate of 1.0 ml/min. Scanning began
coincident with the start of injection. The initial acquisition was a
28-frame dynamic sequence starting with 30-s scans and ending with
20-min scans for a total duration of 1.5 h. At 1.5 h, a single bolus dose
of RTI-336 was injected, and a second dynamic sequence was ac-
quired starting with 2-min frames and ending with 5-min frames for
a total duration of 3 h for both sequences.

**Data Analysis.** A generalized reference tissue method was used to
analyze the data (Votaw et al., 2002). Data were collected in two
sections with the assumption that drug infusion changed only the k3
(Bmax, βmax) rate constant (i.e., dopamine compete with the FECNT for
binding the DAT and decrease the apparent Bmax from k3 (predrug
infusion) to k3 (postdrug infusion). Five rate constants (R, k3, k4, k5,
and k6) and the time-activity curve from the reference region
(cerebellum) were used to model the putamen time-activity curve. In
the modeling, it was assumed that the competing ligand did not affect
the blood-brain barrier parameters (flow or extraction; R or k6) or the
transporter-FECNT kinetic properties (kmax, kα = kβ). Thus, these
parameters were kept constant over the entire experiment. After
optimization, the covariance matrix was numerically determined and
used to estimate the uncertainty in the fit values. From this, it
was found that the variance in the kβ/kα ratio is much less than the
variance in either parameter alone due to their nonvanishing covari-
ance. Accordingly, the kβ/kα ratio was taken as a measure of DAT
density. The fraction of transporters occupied by the competing lig-
and was then estimated as 1 − (kβ/kα)/kα.

**Drug Plasma Concentration Methods.**

A temporary catheter was implanted into the saphenous vein for
repeated blood samples while subjects sat quietly in a primate chair.
The ED50 dose of RTI-336 was administered as an i.v. bolus injection
through a separate vascular access port and indwelling venous cath-
eter. Blood samples (2.0 ml) were collected from the temporary
venous catheter immediately before drug administration and then at
0.5, 1.0, and 2.0 h following drug administration. Blood samples
were collected into tubes containing sodium heparin as the anticoagulant
and stored on wet ice until centrifuged. All blood samples were
centrifuged within 1 h of collection. After centrifugation, plasma was
separated and stored in polypropylene tubes and kept frozen at
approximately −70°C. The plasma storage tubes were labeled with
animal identification, date, and time of sample collection. All sam-
ple collected were shipped frozen on sufficient dry ice to the bioana-
litical facility for analysis.

RTI-336 concentrations in plasma were determined using a vali-
dated method of liquid chromatography with mass spectrometric
detection at the Analytical Division-Drug Studies Unit (Department
of Biopharmaceutical Sciences, University of California, San Fran-
cisco, CA). Plasma samples (0.2 ml each) were extracted with 2 ml of
methyl-ter-butyl ether and then separated by liquid chromatography
and detected by mass spectrometry. The liquid chromatography with
mass spectrometric detection system consisted of a PE-Sciex API
3000 system equipped with a BDS Hypersil C18 column (4.6 × 150
mm; 5-μm particle size), a mobile phase of acetonitrile/water/trifu-
oroacetic acid (75:25:0.04, v/v/v) with 5 mM ammonium acetate
and mass spectrometric detection with sample inlet by heated nebulizer,
positive ionization by atmospheric pressure chemical ionization and
mass scanning by multiple reaction monitoring analysis. The linear
range of assay was from 0.2 to 80 ng/ml. The lower limit of quanti-
tation of the assay was 0.2 ng/ml using 0.2 ml of plasma for the
analysis.

**Results.**

**RTI-336 Pretreatment Studies.** Unit doses of 0.1 and 0.3
mg/kg/infusion cocaine were selected for drug pretreatment
experiments because they were positioned on the peak and
the initial portion of the descending limb of the dose-effect
curve for the group (Fig. 1, inset). Both unit doses maintained
high rates of responding in all subjects. Pretreatments with
RTI-336 produced dose-dependent reductions in cocaine-
maintained responding in all subjects tested (Fig. 1). No
adverse behavioral effects (e.g., agitation, hyperventilation,
and salivation) were observed at any pretreatment dose of
RTI-336, and daily food intake was unaffected. RTI-336 was
equally effective in reducing cocaine self-administration at
both maintenance doses of cocaine as determined by linear
regression analyses of the RTI-336 dose-effect curves shown in
Fig. 1. Therefore, data were pooled for each maintenance
dose of cocaine to determine the ED50 doses for drug pretreat-
ments in individual subjects (Table 2). The ED50 dose of
RTI-336 for the group was 1.07 ± 0.17 mg/kg. Percentage of
DAT occupancy at the ED50 dose of RTI-336 for the group
was 90 ± 5.3.
Food-maintained responding was stable in all subjects, although baseline rates of responding for food were lower than those maintained by either unit dose of cocaine (Fig. 1). Moreover, rates of food-maintained responding were lower when the food component alternated with the higher unit dose of cocaine. Pretreatments with RTI-336 produced dose-dependent reductions in food-maintained responding in all subjects (Fig. 1). When the food component alternated with the lower unit dose of cocaine, the ED₅₀ dose of RTI-336 for the group was 0.98 ± 0.55 mg/kg. This value corresponded closely with the ED₅₀ dose of RTI-336 for reducing cocaine-maintained responding. When the food component alternated with the higher unit dose of cocaine, food-reinforced responding was not reliably maintained during RTI-336 pretreatments. Accordingly, an accurate ED₅₀ value could not be determined.

**RTI-336 Substitution Studies.** RTI-336 was substituted for cocaine to compare their reinforcing effects. Both cocaine and RTI-336 reliably maintained drug self-administration at levels greater than saline in all subjects and the shape of the dose-effect curves resembled an inverted-U shape function (Fig. 2). However, rates of responding were lower for RTI-336 compared with those maintained by cocaine across a range of doses. Food-reinforced behavior was reliably maintained during RTI-336 substitution at response rates that approximated those shown in Fig. 1. No adverse behavioral effects were observed at any substitution dose of RTI-336. The unit dose of RTI-336 that maintained peak responding for the group was 0.10 ± 0.06 mg/kg/injection (Table 3). When the dose of RTI-336 that maintained peak responding was administered as an i.v. bolus of the total dose each animal received during self-administration sessions, percentage of DAT occupancy was 62 ± 12 (Table 3).

**RTI-336 and SERT Inhibitor Coadministration Studies.** Chronic administration of fluoxetine (3.0 mg/kg/day) for three consecutive weeks had only modest effects on cocaine self-administration (Fig. 3A). Mean response rate maintained by cocaine for the group over the 3-week period decreased by 23 ± 6%. However, coadministration of the ED₅₀ dose of RTI-336 during week 4 of chronic fluoxetine treatment completely suppressed responding in all subjects. Likewise, chronic administration of citalopram (3.0 mg/kg/day) for three consecutive weeks had only modest effects on cocaine self-administration (Fig. 3B). Mean response rate maintained by cocaine for the group over the 3-week period decreased by 18 ± 5%. However, coadministration of the ED₅₀ dose of RTI-336 during week 4 of chronic citalopram treatment completely suppressed responding in all subjects. The drug interactions observed did not require chronic administration of the SERT inhibitors. Subchronic administration of fluoxetine (Fig. 4A) or citalopram (Fig. 4B) for five consecutive days during coadministration of the ED₅₀ dose of RTI-336 also completely suppressed cocaine-maintained responding in all subjects. Note that food-maintained behavior was also completely suppressed during coadministration of the ED₅₀ dose of RTI-336 and the SERT inhibitors. Similar to the results obtained with the ED₅₀ dose of RTI-336, when the ED₁₀ dose of RTI-336 (0.21 ± 0.12 mg/kg) was coadministered with fluoxetine (Fig. 4C), the rate-decreasing effects were more pronounced than the effects of RTI-336 alone. Mean response rate maintained by cocaine for the group over the 5-day period decreased by 37 ± 6%. In all experiments involving SERT inhibitor pretreatments, there was recovery of cocaine- and food-maintained responding within 1 to 2 days following the termination of drug pretreatments. When DAT occupancy of the ED₅₀ dose of RTI-336 was redetermined during coadministration of fluoxetine (3.0 mg/kg), percentage of DAT occupancy for the group was 90 ± 7.5 (Table 2).

**Drug Plasma Concentration Studies.** RTI-336 concentrations in plasma over an interval of 2 h after an i.v. bolus of the ED₅₀ dose were determined to be in the range of 56 to 147 ng/ml (mean, 82 to 111 ng/ml). The dose administered and drug plasma concentrations are shown for individual subjects in Table 4. Peak plasma concentrations were observed 0.5 to 1.0 h postinjection. At 2 h postinjection, plasma concentrations were approximately 70% of peak values for the group.
RTI-336 is a highly selective DAT inhibitor with 1000- and 400-fold selectivity relative to SERT and norepinephrine transporter binding, respectively (Kuhar et al., 1999). Pretreatments with RTI-336 produced dose-dependent reductions in cocaine self-administration behavior, and RTI-336 maintained its effectiveness when the unit dose of cocaine was increased from 0.1 to 0.3 mg/kg/injection. Direct, within-subject comparisons were made between drug effects on behavior and in vivo DAT occupancy measured with PET neuroimaging. The ED$_{50}$ dose of RTI-336 for reducing cocaine self-administration resulted in approximately 90% DAT occupancy for the group. Subsequently, the ED$_{50}$ dose of RTI-336 was coadministered with the selective SERT inhibitors fluoxetine and citalopram. Administration of the SERT inhibitors alone had little effect on cocaine self-administration behavior. However, coadministration of the ED$_{50}$ dose of RTI-336 completely suppressed cocaine self-administration behavior in all subjects, and the drug interactions observed did not require chronic administration of the SERT inhibitors. Subchronic administration of either SERT inhibitor for five consecutive days during coadministration of the ED$_{50}$ dose of RTI-336 also completely suppressed responding in all subjects. Likewise, coadministration of fluoxetine with the ED$_{10}$ dose of RTI-336 had more pronounced effects compared with RTI-336 alone. When DAT occupancy for the ED$_{50}$ dose of RTI-336 was redetermined during coadministration of fluoxetine, approximately 90% DAT occupancy was observed for the group. Hence, at comparable levels of DAT occupancy, coadministration of fluoxetine produced more robust reductions in cocaine self-administration compared with RTI-336 alone.

The results obtained with RTI-336 are consistent with previous reports documenting the effectiveness of DAT inhibitors to suppress cocaine self-administration in nonhuman primates. The phenyltropanes RTI-113 and RTI-177 effectively decreased cocaine self-administration in nonhuman primates under a second-order schedule (Howell et al., 2000; Wilcox et al., 2002; Lindsey et al., 2004). Similar results have been observed with the phenyltropane 8-methyl-2-propanoyl-3-[(1-methylphenyl)-8-azabicyclo[3.2.1]octane in rhesus monkeys trained under a fixed-interval schedule (Nader et al., 1997). The effectiveness of DAT inhibitors to

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**TABLE 3**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Peak Reinforcing Dose</th>
<th>Unit Dose</th>
<th>Total Dose$^a$</th>
<th>DAT Occupancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRj-5</td>
<td>0.3 mg/kg/injection</td>
<td>0.1 mg/kg/session</td>
<td>55</td>
<td>79</td>
</tr>
<tr>
<td>ROk-5</td>
<td>0.1 mg/kg/injection</td>
<td>0.3 mg/kg/session</td>
<td>36</td>
<td>79</td>
</tr>
<tr>
<td>RNa-4</td>
<td>0.1 mg/kg/injection</td>
<td>0.3 mg/kg/session</td>
<td>36</td>
<td>79</td>
</tr>
<tr>
<td>RMy-4</td>
<td>0.03 mg/kg/injection</td>
<td>0.09 mg/kg/session</td>
<td>79</td>
<td>79</td>
</tr>
</tbody>
</table>

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$^a$ Note that the DAT occupancy values correspond to the total dose the animal received during self-administration sessions, administered as an i.v. bolus during PET studies.
reduce cocaine self-administration in rhesus monkeys also extends to the phenylpiperazine derivative GBR12909 (Glowa et al., 1995; Lindsey et al., 2004). However, it is important to note that high levels of DAT occupancy were observed for doses of selective DAT inhibitors that produced robust decreases in cocaine self-administration. At ED₅₀ doses, DAT occupancy was approximately 70 to 80% (Wilcox et al., 2002; Lindsey et al., 2004). Interestingly, RTI-336 has

Table 4

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Dose (mg/kg)</th>
<th>Sample Time</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Predose</td>
<td>0.5 h</td>
<td>1.0 h</td>
<td>2.0 h</td>
</tr>
<tr>
<td>RNa-4</td>
<td>1.34</td>
<td>0</td>
<td>109.0</td>
<td>114.0</td>
<td>83.6</td>
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<tr>
<td>ROK-5</td>
<td>0.98</td>
<td>0</td>
<td>139.0</td>
<td>147.0</td>
<td>108.0</td>
</tr>
<tr>
<td>RVc-5</td>
<td>0.63</td>
<td>0</td>
<td>85.5</td>
<td>66.9</td>
<td>55.5</td>
</tr>
</tbody>
</table>

Fig. 3. Effects of coadministration of 3.0 mg/kg/day fluoxetine (A) or 3.0 mg/kg citalopram (B) in combination with the ED₅₀ dose of RTI-336 in a group of three rhesus monkeys. Fluoxetine or citalopram was administered i.m. chronically for four consecutive weeks and RTI-336 was coadministered during the last week of treatment. Error bars indicate S.E.M. for group data. Abscissa, consecutive days of treatment. Ordinate, mean response rate (responses per second).

Fig. 4. Effects of coadministration of 3.0 mg/kg/day fluoxetine (A) or 3.0 mg/kg citalopram (B) in combination with the ED₅₀ dose of RTI-336 or 3.0 mg/kg/day fluoxetine (C) in combination with the ED₅₀ dose of RTI-336 in a group of three rhesus monkeys. Fluoxetine or citalopram was administered i.m. subchronically for five consecutive days during coadministration of RTI-336. Error bars indicate S.E.M. for group data. Abscissa, consecutive days of treatment. Ordinate, mean response rate (responses per second).
even greater selectivity for DAT relative to SERT, and the ED$_{50}$ dose of RTI-336 resulted in approximately 90% DAT occupancy. In contrast, the ED$_{50}$ dose of the mixed action DAT/SERT inhibitor RTI-112 did not exhibit levels of DAT occupancy above the threshold of detection (Lindsey et al., 2004). Hence, it seems that the greater the selectivity for DAT, the higher the level of DAT occupancy required to suppress cocaine self-administration.

Doses of RTI-336 that effectively suppressed cocaine self-administration also suppressed food-maintained behavior under the multiple second-order schedule. Hence, the present study did not show selectivity of drug pretreatment effects on cocaine-maintained behavior. Published studies with monoamine transporter inhibitors have yielded equivocal results concerning selectivity of drug pretreatment effects. GBR12909 selectively attenuated cocaine-maintained behavior compared with food-maintained behavior in food-deprived rhesus monkeys at a low unit dose of cocaine but not at a high unit dose (Glowa et al., 1995). Likewise, administration of cocaine as a continuous infusion had selective effects on cocaine-maintained behavior compared with food-maintained behavior only at low unit doses of self-administered cocaine (Glowa and Fantegrossi, 1997). However, other studies with monoamine transporter inhibitors have demonstrated nonselective reductions in cocaine-maintained behavior and behavior maintained by alternate reinforcers (Kleven and Woolverton, 1993; Howell et al., 2000). It should be emphasized that the subjects in the present study were not food-deprived. Moreover, the food and cocaine components alternated during daily 1-h sessions. Responding maintained by food presentation was suppressed in the absence of drug pretreatments when the unit dose of cocaine was increased to 0.3 mg/kg/injection. Hence, the conditions were not optimal to maintain robust food-maintained behavior during drug pretreatment studies. Selective reductions in cocaine-maintained behavior would have been a preferred outcome from the standpoint of medication development. However, no adverse behavioral effects were observed at any pretreatment dose of RTI-336 administered alone or in combination with the SERT inhibitors, and food intake and body weight were unaffected over the course of the study.

A possible limitation to the use of selective DAT inhibitors as medications for treatment of cocaine addiction is their potential for abuse, given their documented reinforcing effects (Howell and Wilcox, 2001). In the present study, RTI-336 reliably maintained self-administration behavior in all subjects, consistent with results reported for phenyltropanes (Howell et al., 2000; Wilcox et al., 2002; Lindsey et al., 2004) and the phenylpipерazinc GBR12909 (Bergman et al., 1989; Howell and Byrd, 1991; Lindsey et al., 2004) in nonhuman primates. Moreover, DAT occupancy at the dose of RTI-336 that maintained peak responding was actually less than DAT occupancy at the ED$_{50}$ dose for suppressing cocaine self-administration. The bolus dosing protocol used for estimating occupancy associated with self-administration doses of RTI-336 may have overestimated actual occupancy obtained after repeated injections of the unit dose. The finding that lower DAT occupancy was associated with the reinforcing effects of RTI-336 compared with its effects in reducing cocaine self-administration may be problematic from the standpoint of medication development. However, RTI-336 maintained lower rates of responding compared with cocaine across a broad range of doses, even though DAT occupancy was equal to or greater than that observed for cocaine (Wilcox et al., 2002). In behavioral studies in rodents and nonhuman primates, these compounds had a slower onset and a longer duration of action compared with cocaine (Howell et al., 2000; Kimmel et al., 2001). The pharmacokinetic data reported in the present study also documented a longer duration of action for RTI-336 compared with cocaine. Hence, the reinforcing effects and pattern of drug self-administration may be influenced by pharmacokinetics in addition to steady-state levels of DAT occupancy.

It is interesting to note that DAT occupancy at the doses of RTI-336 that maintained peak rates of responding was equivalent for two subjects even though the doses differed by 1.0 log units. Moreover, there was no obvious difference in their drug histories that may account for this finding. In general, there did not seem to be a close relationship between absolute drug dose in milligrams per kilogram and DAT occupancy when comparing data across subjects. However, note that the doses selected for individual subjects in DAT occupancy determinations were functionally equivalent based on RTI-336 self-administration or RTI-336-induced reductions in cocaine self-administration. Hence, when subjects received functionally equivalent doses of RTI-336, the corresponding values for DAT occupancy were fairly consistent.

The reinforcing effects of DAT inhibitors also may be limited by dual actions at the SERT. The mixed action DAT/SERT inhibitor RTI-112 was not reliably self-administered when substituted for cocaine, yet it suppressed cocaine self-administration at low levels of DAT occupancy in rhesus monkeys (Lindsey et al., 2004). Likewise, monoamine-releasing agents had decreasing reinforcing effectiveness in rhesus monkeys when serotonin-releasing potency was increased relative to dopamine (Wee et al., 2005). Accordingly, combined inhibition of DAT and SERT may be effective in reducing cocaine use and limit the reinforcing effectiveness and abuse liability of the potential medications. Paradoxically, cocaine is a nonselective inhibitor of DAT and SERT, and it is a robust reinforcer with high abuse liability. However, cocaine exhibits a different pharmacokinetic profile and has lower affinity for DAT and SERT compared with the compounds being considered as pharmacotherapies.

Compelling data have emerged from clinical research supporting indirect agonist-like pharmacotherapy for stimulant abuse and dependence (Grabowski et al., 2004). The concept of indirect agonist pharmacotherapy implies that the medication will exhibit some cocaine-like properties at a neurochemical and behavioral level. In this regard, selective SERT inhibitors do not exhibit cocaine-like behavioral effects and do not seem appropriate as indirect agonist pharmacotherapies (Grabowski et al., 1995). In contrast, selective DAT inhibitors clearly exhibit cocaine-like behavioral effects and seem promising as cocaine medications based on preclinical evaluations. However, their reinforcing effects in animal models still indicate that they may exhibit high abuse liability that could limit their clinical utility. In addition, the effects of high levels of DAT occupancy that are required to reduce cocaine use are unknown. RTI-336 alone may prove to be a suitable pharmacotherapy, but an even better approach may be to use RTI-336 in combination with a SERT inhibitor. Alternatively, development of a pharmacotherapy involving dual actions at DAT and SERT could lead to compounds with
caine-like properties appropriate in indirect agonist pharmacotherapy but with limited abuse liability.

In summary, RTI-336 produced dose-related reductions in cocaine self-administration behavior but required high levels of DAT occupancy. In addition, RTI-336 was reliably self-administered and functioned as a reinforcer but was less effective than cocaine in maintaining high rates of responding. Coadministration of RTI-336 with two selective SERT inhibitors produced more robust reductions in cocaine self-administration compared with RTI-336 alone without affecting DAT occupancy. The present results obtained with combined administration of DAT and SERT inhibitors are consistent with those reported previously with mixed action DAT/SERT inhibitors. It seems that combined inhibition of DAT and SERT may be effective in reducing cocaine use and potentially limit the reinforcing effectiveness and abuse liability of DAT inhibitors.

Acknowledgments

We gratefully acknowledge the technical assistance of Stephanie Dyson, Amy Maguire, and Peggy Plant.

References


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