Biochemical and Behavioral Characterization of Novel Methylphenidate Analogs

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ABSTRACT

As part of a project to develop treatment agents for cocaine abuse, (±)-threo-methylphenidate (TMP) and 11 analogs were characterized biochemically and behaviorally to assess their potential as anti-cocaine medications. The compounds contained aryl and/or nitrogen substitutions, and/or replacement of the ester function by an alcohol or ether. All of the analogs, except for the N-methyl-substituted compounds, showed increased inhibitory potency against \(^{3}\)H-(±)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane 1,5-naphthalenedisulfonate (\(^{3}\)HWIN 35,428) (\(^{3}\)HWIN) binding to the dopamine transporter, compared with TMP. In general, parallel results were obtained for inhibition of \(^{3}\)H-dopamine (\(^{3}\)HDA) uptake. Although compounds with N-substitutions were proportionally less potent at blocking DA uptake than WIN binding (compared with the unsubstituted compounds), one such compound that was 6-fold more potent against \(^{3}\)HWIN binding than \(^{3}\)HDA uptake did not attenuate inhibition by cocaine of synaptosomal \(^{3}\)HDA transport. The compounds were significantly less potent in displacing \(^{3}\)Hcitalopram binding from the serotonin transporter. In cocaine discrimination studies in rats, all but two of the analogs (both N-substituted) completely generalized with the cocaine stimulus. Robust positive correlations were observed between potency in the drug discrimination assay and activity at the dopamine transporter, but not the serotonin transporter. When tested for their ability to alter cocaine discrimination, four of the analogs (three of which had N-substitutions and shallow dose-response curves as cocaine substitutes) actually enhanced cocaine discrimination, often at combined doses of cocaine and test compound that were inactive when given separately. Taken together, the results suggest that TMP analogs may have potential as substitution therapies for the treatment of cocaine abuse.

I illicit use of cocaine is a major public health problem worldwide. There is an urgent need for treatment agents that could be used to block the pleasurable effects of cocaine and/or reduce craving for the drug, without causing deleterious side effects. To this end, our research efforts have been directed toward the synthesis of compounds that will interact with the DAT, the site in the brain thought to subserve the reinforcing effects of cocaine (Ritz et al., 1987; Howell and Wilcox, 2001). Although cocaine is known to block norepinephrine and serotonin transport, as well as conductance through sodium channels (Reith, 1988), its abuse potential is generally attributed to its inhibition of the reuptake of the neurotransmitter DA at nerve terminals of the mesolimbic system. To increase the probability that a proposed compound will target the DAT, our approach has been to use psychomotor stimulant agents (in this case, TMP) as the starting point for structural modification. TMP was selected for several reasons: 1) it has somewhat more inhibitory potency at the DAT than at the norepinephrine transporter or the SERT (Ritz et al., 1987); 2) it has several important chemical and structural properties in common with cocaine; and 3) based both on widespread clinical experience with the drug as an oral treatment agent for attention deficit disorder, and on several recent studies on its use as replacement therapy for cocaine addicts (Grabowski et al., 1997; Roache et al., 2000), TMP appears to have low abuse potential and few serious side effects. The

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As part of a project to develop treatment agents for cocaine abuse, (±)-threo-methylphenidate (TMP) and 11 analogs were characterized biochemically and behaviorally to assess their potential as anti-cocaine medications. The compounds contained aryl and/or nitrogen substitutions, and/or replacement of the ester function by an alcohol or ether. All of the analogs, except for the N-methyl-substituted compounds, showed increased inhibitory potency against \(^{3}\)H-(±)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane 1,5-naphthalenedisulfonate (\(^{3}\)HWIN 35,428) (\(^{3}\)HWIN) binding to the dopamine transporter, compared with TMP. In general, parallel results were obtained for inhibition of \(^{3}\)H-dopamine (\(^{3}\)HDA) uptake. Although compounds with N-substitutions were proportionally less potent at blocking DA uptake than WIN binding (compared with the unsubstituted compounds), one such compound that was 6-fold more potent against \(^{3}\)HWIN binding than \(^{3}\)HDA uptake did not attenuate inhibition by cocaine of synaptosomal \(^{3}\)HDA transport. The compounds were significantly less potent in displacing \(^{3}\)Hcitalopram binding from the serotonin transporter. In cocaine discrimination studies in rats, all but two of the analogs (both N-substituted) completely generalized with the cocaine stimulus. Robust positive correlations were observed between potency in the drug discrimination assay and activity at the dopamine transporter, but not the serotonin transporter. When tested for their ability to alter cocaine discrimination, four of the analogs (three of which had N-substitutions and shallow dose-response curves as cocaine substitutes) actually enhanced cocaine discrimination, often at combined doses of cocaine and test compound that were inactive when given separately. Taken together, the results suggest that TMP analogs may have potential as substitution therapies for the treatment of cocaine abuse.

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goal of this research is to create novel TMP analogs that will retain their selectivity for the DAT, and either serve as replacement therapy for cocaine (full or partial cocaine agonists) or prevent its effects by directly blocking its binding to the DAT (cocaine antagonists). Ideally, potential antagonists would inhibit the binding of cocaine to its recognition site on the DAT but not interfere with the binding and subsequent uptake of DA. Although this was once thought to be an unattainable goal, a large body of evidence now supports its feasibility (see references in Deutsch and Schweri, 1994).

Although the reinforcing effects of cocaine are not attributable to its action on serotonin transport, serotonin can modulate its subjective effects (Walsh and Cunningham, 1997). Serotonergic systems affect stimulant-induced behaviors in a somewhat complex manner. For instance, specific 5HT uptake blockers enhance cocaine discrimination (Kleven and Koek, 1998, and references therein), but serotonergic agonists attenuate some cocaine-reinforced behaviors and self-administration of amphetamine (see references in Ritz et al., 1987). Therefore, because alteration of the SERT activity of the TMP analogs could influence their utility as cocaine treatment agents, their inhibitory potency against \(^{3}H\)CIT binding to the SERT was also monitored.

We now report on the testing of TMP and 11 of its derivatives, which contain amino, methyl, or halide substituents on the aryl ring, methyl or benzyl substitutions on the piperidine nitrogen, and/or modifications of the methyl ester function. This is the first time, to our knowledge, that the effect of more radical structural changes (i.e., a disubstituted aryl ring, N-substitutions, and/or replacement of the ester function by an ether or alcohol) have been examined. Previous reports correlating the biochemical and behavioral effects of MP analogs have been limited to derivatives containing only monosubstituted aryl rings or modification of the ester function (e.g., Patrick et al., 1981; Schweri et al., 1985; Gatley et al., 1996a; Thai et al., 1998). Preliminary characterization of these novel compounds was conducted, using both in vitro and in vivo techniques. The inhibitory potencies of the compounds against \(^{3}H\)WIN binding to the cocaine recognition site on the DAT, \(^{3}H\)CIT binding to the SERT, and \(^{3}H\)DA uptake were determined, if not assessed previously. Based on the results, (\(\pm\)-threo-N-methyl-4-methyl-methylphenidate (4MeTMPNMe)) was selected as a representative compound for further in vitro tests designed to examine the nature of its interaction with the DAT, as well as its ability to attenuate the inhibition of DA uptake by cocaine. All of the analogs were tested for cocaine-like discriminative stimulus effects in rats trained to discriminate between saline and 10 mg/kg cocaine; selected compounds were further tested for their ability to block the discriminative effects of cocaine. The drug discrimination assay is a proven animal model of the subjective effects of drugs in humans, with predictive value for abuse potential (Holtzman, 1990). Possible candidates for further study were identified, and the combined data were evaluated with the aim of determining useful characteristics and possible correlations between the in vitro and in vivo results that might guide the design of future pharmacotherapies for cocaine abuse.

### Materials and Methods

#### Chemicals

**Synthetic Compounds.** The syntheses of (\(\pm\)-threo-methylphenidate (TMP), (\(\pm\)-threo-3,4-dichloromethylphenidate (3,4CTMP), (\(\pm\)-threo-4-fluoromethylphenidate (4FTMP), (\(\pm\)-threo-3-chloromethylphenidate (3CTMP), (\(\pm\)-threo-4-aminomethylphenidate (4ATMP), (\(\pm\)-threo-4-methylphenidate (4MeTMP), (\(\pm\)-threo-N-methyl-3-chloromethylphenidate (3CTMPNMe), (\(\pm\)-threo-N-methyl-4-methylphenidate (4MeTMPNMe), (\(\pm\)-threo-N-methyl-methylphenidate (TMPNMe), (\(\pm\)-threo-N-benzyl-methylphenidate (TMPNb), (\(\pm\)-threo-N-benzyliritalinol methyl ether (TROMeNbn), and (\(\pm\)-threo-N-benzyliritalinol (TROHNb) have been described previously (Deutsch et al., 1996; Friomowitz et al., 1997; Deutsch, 1998). 3CTMPNMe and TMPNMe were prepared as free bases; all other synthesized compounds were HCl salts. NMR analysis suggested that 3CTMPNMe contained approximately 10% of the (\(\pm\)-erythro enantiomers; this was likely due to epimerization that occurred at the N-methylation step.

**Other Chemicals.** The TMP used in the in vitro assays was a gift of Ciba-Geigy (Basel, Switzerland). (\(\pm\)-Cocaine HCl used in the drug discrimination studies was obtained from the National Institute on Drug Abuse (Bethesda, MD); that used in the in vitro assays was purchased from Sigma Chemical Co. (St. Louis, MO). Citalopram was a gift of H. Lundbeck A/S (Copenhagen, Denmark). All radioisotopes were purchased from PerkinElmer Life Sciences (Boston, MA); they are: [\(^{3}H\)-2,5,6\(^{3}H\)]dopamine; [\(^{3}H\)-N-methyl-[\(^{3}H\)]citalopram, and [\(^{3}H\)-N-methyl-[\(^{3}H\)]WIN 35,428. All other drugs were purchased from standard commercial sources.

#### In Vitro Studies

**Animals.** Male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 150 to 300 g, were anesthetized using CO\(_2\) gas and sacrificed by decapitation. Their brains were quickly removed and placed in ice-cold 0.32 M sucrose. Tissue preparations were prepared from those areas of the brain as required for the individual assays described below. This protocol was approved by the Institutional Animal Care and Use Committee of Mercer University and is in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**\(^{3}H\)WIN Binding to the DAT.** These assays were conducted using rat striatal tissue as previously described (Deutsch et al., 1999). IC\(_{50}\) values (that concentration of drug which inhibits 50% of specific binding of the radioligand) and Hill coefficients were determined by least-squares nonlinear regression analysis of sigmoideal dose-response curves using the GraphPad Prism (v.2) program (GraphPad Software, Inc., San Diego, CA).

**\(^{3}H\)CIT Binding to the SERT.** The method used here was modified from binding assays described elsewhere (D’Amato et al., 1987; Dutta et al., 1996). Cortical tissue from all brain areas except the olfactory tubercles and medial cortex extending to approximately 1 mm on either side of the interhemispheric fissure was combined and homogenized in 20 volumes (based on wet weight) of 0.32 M sucrose, using 10 up/down strokes of a motorized Potter-Elvehjem homogenizer. The homogenized tissue was centrifuged at 1,000g for 10 min at 0°C, and the resulting supernatant was further centrifuged at 20,000g for 20 min. The resulting pellet (P\(_{2}\) fraction) was suspended in 16 volumes of ice-cold assay buffer (25 mM sodium phosphate buffer, pH 7.7 at 0°C) using an Ultra-Turrax tissue homogenizer. Binding was initiated by addition of 150 \(\mu L\) of the P\(_{2}\) suspension to samples containing 750 \(\mu L\) of assay buffer, 50 \(\mu L\) of the test compound, 25 \(\mu L\) of water or clomipramine (to define nonspecific binding; final concentration, 1 \(\mu M\)) and 25 \(\mu L\) of \(^{3}H\)CIT (final concentration, 2 nM). Test compounds were dissolved in water, diluted dimethyl sulfoxide, or diluted methanol, with the concentration of organic solvent in any assay tube limited to 0.3% by volume. Usually, a range of seven drug concentrations was used to generate the dose-response curve; triplicate samples were run at each concent-
tion of [3H]DA was determined using a crude synaptosomal preparation of rat striatal tissue that was preincubated for 10 min in the presence of the test compound, then exposed to 30 nM [3H]DA over a 2-min period at 37°C, as described previously (Deutsch et al., 1999). In those experiments in which the effect of 4MeTMPNMe on the ability of cocaine to inhibit [3H]DA uptake was determined, both 4MeTMPNMe and cocaine were present for the entire preincubation period. Studies to characterize the effect of 4MeTMPNMe on the Michaelis-Menten kinetics of [3H]DA uptake were performed under the same conditions, except that the [3H]DA concentrations ranged from 25 to 800 nM. Km and Vmax were determined by nonlinear least-squares regression fit of specific [3H]DA uptake versus free [3H]DA to a rectangular hyperbola function using the GraphPad Prism program (see above). The resulting values were then used to generate the equations for the straight lines to allow plotting of the data in the Lineweaver-Burk format.

Drug Discrimination Studies

Subjects. Male rats of Sprague-Dawley descent (Charles River Laboratories, Raleigh, NC) were used in the study. They weighed 250 to 300 g when discrimination training began and were housed in pairs in a vivarium that had a 12-h light/dark cycle. Food and water always were available in the home cage. The vivarium was part of a facility that was accredited by the American Association for Accreditation of Laboratory Animal Care. The care and testing of the animals conformed to a protocol that was approved by the Institutional Animal Care and Use Committee of Emory University and were in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drug Discrimination. Rats were trained to discriminate between i.p. injections of 10 mg/kg cocaine and saline in a two-choice discrete-trial avoidance/escape procedure (Shannon and Holtzman, 1976). Injections of cocaine or saline were given 15 min before a session, which was conducted in a standard testing chamber. The beginning of a trial was signaled by the onset of white noise and illumination of the house light in the chamber. Five seconds after a trial began, a constant electrical current (1.0–1.5 mA) was applied to the grid floor of the chamber for 1.0 sec every 3.0 sec. To end a trial, the rat had to complete a two-response chain: press an “observing” lever in one wall of the chamber and then press one of two “choice” levers mounted in the opposite wall. The choice levers were 15 cm apart and were separated by a 5.0-cm-wide Plexiglas partition that extended from the floor to the ceiling. A response on the observing lever turned off the white noise and enabled a response on the correct choice lever to extinguish the house light and end the trial. A trial was recorded as correct if the rat pressed the choice lever appropriate for what was injected beforethe session (i.e., cocaine or saline) right after it pressed the observing lever. A trial was recorded as incorrect (and did not end) if the rat pressed the choice lever that was not appropriate for what was injected before the session between pressing the observing lever and pressing the appropriate choice lever. A trial also ended if the rat did not complete either the correct or incorrect sequence of responses within 30 sec and was recorded as an incomplete trial. Trials were separated by a 50-sec period, during which the chamber was illuminated by a red stimulus lamp. A session consisted of 20 trials and, with fully trained rats, usually lasted 19–21 min.

Approximately half the animals were trained to press the left choice lever in training sessions that followed an injection of 10 mg/kg cocaine and to press the right choice lever in training sessions that followed an injection of saline. The designation of choice levers was reversed for the rest of the animals. Rats were considered to be under the stimulus control of cocaine and saline when they completed the response sequence of observing lever-appropriate choice lever in at least 18 trials of 20 in four consecutive training sessions and two consecutive test sessions, half preceded by cocaine and half by saline. Test sessions differed from training sessions in one respect: after a response on the observing lever, a response on either choice lever ended a trial.

After rats met the criterion for acquisition of the discrimination, they all were tested first with graded doses of cocaine. Doses were injected i.p. 15 min before a session in a random sequence that also included saline. Other drugs were then tested in a nonsystematic order in groups of four to five rats. They were injected i.p. 30 min before a session based upon the time course of action of methylphenidate; each dose was administered once to each rat in the group. Drug test sessions in which both choice levers were activated usually were conducted twice weekly, 3 to 4 days apart. Training sessions in which only the pretreatment-appropriate choice lever was activated were conducted three times each week to maintain stable discrimination performance. Saline and cocaine were injected on alternate days before a training session. If an animal failed to complete at least 18 trials correctly in a training session, testing was suspended until it had done so again in four consecutive training sessions.

Drugs. In all cases, doses refer to the free base. Cocaine was dissolved in normal saline solution; 3CTMPNMe, 4MeTMPNMe, and TMMPMe were dissolved in one part dimethyl sulfoxide followed by three parts distilled water; all other compounds were dissolved in distilled water. Drugs usually were injected in a volume of 1.0 ml/kg of body weight; however, twice this volume was used for some of the higher doses.

Data Analysis. Data from stimulus-generalization tests are presented as the average number of trials completed on the cocaine-appropriate choice lever in a 20-trial session; the remaining trials of the session were completed on the choice lever appropriate for the drug vehicle. The dose of a drug that resulted in the selection of the cocaine-appropriate choice lever in 10 trials of a session (i.e., ED50) was calculated for individual rats. This was done by linear regression of the ascending limb of the stimulus-generalization curve, using log10 dose and at least three points. In those instances when only two points defined the ascending portion of the curve, ED50 values were derived by simple interpolation. The individual ED50 values were averaged to obtain a group mean and 95% confidence limits. Slopes of stimulus-generalization curves also were determined by linear regression of the log-dose data. Comparisons among ED50 values and among slopes were made by analysis of variance, followed, where appropriate, by the Student-Newman-Keuls test for multiple comparisons among all means. The alpha level was set at 0.05.

Results

In Vitro Studies. The potencies of the compounds as inhibitors of [3H]WIN binding, [3H]CIT binding, and [3H]DA uptake in vitro are shown in Table 1, expressed as IC50 values. The compounds show a hundred-fold variation in potency against [3H]WIN binding, ranging from IC50 values of about 5 nM for both 3CTMP and 3,4CTMP to about 500 nM for TMMPMe. A 200-fold range of potency was observed against [3H]DA uptake, from an IC50 of 7.0 ± 0.6 nM for 3,4CTMP to 1435 ± 5 nM for TMMPMe. The most potent compounds at the SERT are N-benzyl-substituted compounds in which the ester function has been converted to an
ether (TROMeNBn; IC\textsubscript{50}, 166 ± 8 nM) or an alcohol (TROHNBn; IC\textsubscript{50}, 204 ± 9 nM); the least potent is 4ATMP, with an IC\textsubscript{50} well over 10,000 nM.

All of the analogs are selective for the \textsuperscript{3}H\textsubscript{1}WIN over the \textsuperscript{3}H\textsubscript{1}CIT binding site (Table 1). The parent compound, TMP, exhibits well over 120-fold selectivity for the DAT. For the most part, N-substituted derivatives show the least separation in binding affinities at the two transporters (e.g., 4MeTMPNMe, TROMeNBn, and TROHNBn are only 8- to 9-fold more potent against \textsuperscript{3}H\textsubscript{1}WIN binding than \textsuperscript{3}H\textsubscript{1}CIT binding), whereas derivatives with single substitutions on the aromatic ring exhibit the greatest degree of selectivity (e.g., 3CTMP bound with more than 2000-fold affinity to the DAT over the SERT).

Although the potency against \textsuperscript{3}H\textsubscript{1}DA uptake parallels that found for \textsuperscript{3}H\textsubscript{1}WIN binding, the correlation is not perfect (Pearson \(r = 0.9380, p < 0.0001\)). This modest deviation from perfect linearity results in discrimination ratios (DRs; the ratio of the IC\textsubscript{50} of a given compound against \textsuperscript{3}H\textsubscript{1}DA uptake to its IC\textsubscript{50} against \textsuperscript{3}H\textsubscript{1}WIN binding) ranging from 1.3 for 3,4CTMP to 7.7 for TROHNBn. The DR was calculated as a possible tool for use in the identification of compounds with potential as partial or full cocaine antagonists. In theory, structural modifications that increase the DR of a compound should result in greater antagonist properties, because they reflect the improved ability of the compound to block the binding of cocaine to its recognition site, while leaving DA uptake largely unaffected. Interestingly, when the analogs containing a substituent on the nitrogen were compared with unsubstituted analogs, it was found that the N-substituted compounds had significantly higher DRs (5.0 ± 0.8 versus 3.3 ± 0.5, one-tailed independent \(t\) test, \(p < 0.05\)).

As a compound with one of the higher DRs in this series (6.6), 4MeTMPNMe was selected for further study to determine the nature of its interaction with the DAT, as well as its ability to affect the inhibition by cocaine of DA uptake. In the first series of studies, the effects of 300 and 600 nM 4MeTMPNMe on the \(K_m\) and \(V_{max}\) of \textsuperscript{3}H\textsubscript{1}DA uptake were examined. The saturation curves obtained in the presence and absence of 4MeTMPNMe were analyzed according to Michaelis-Menten kinetics. Data from a representative experiment are shown as a Lineweaver-Burk plot in Fig. 1; results from three independent experiments are summarized in Table 2. Results characteristic of a competitive inhibitor were obtained; i.e., the apparent \(K_m\) increased with increasing concentrations of 4MeTMPNMe, whereas the apparent \(V_{max}\) values did not differ significantly from the corresponding control values.

The ability of 4MeTMPNMe to block the inhibition of \textsuperscript{3}H\textsubscript{1}DA uptake by cocaine was examined using the method described by Simoni et al. (1993). In these experiments, the effect of 200, 500, and 1000 nM 4MeTMPNMe on the IC\textsubscript{50} of cocaine was determined (Table 3). If cocaine and 4MeTMPNMe competed for the same domain on the DAT, the IC\textsubscript{50} for cocaine would be expected to rise by a predicted theoretical amount. On the other hand, if cocaine and 4MeTMPNMe acted at two distinct and unrelated sites, the IC\textsubscript{50} for cocaine in the presence of added 4MeTMPNMe would be expected to change very little from its value in the absence of 4MeTMPNMe, whereas if the compounds acted at two overlapping but

**TABLE 1**

Effect of compounds on \textsuperscript{3}H\textsubscript{1}WIN and \textsuperscript{3}H\textsubscript{1}CIT binding and \textsuperscript{3}H\textsubscript{1}DA uptake

<table>
<thead>
<tr>
<th>Substitution</th>
<th>\textsuperscript{3}H\textsubscript{1}WIN Binding</th>
<th>\textsuperscript{3}H\textsubscript{1}DA Uptake</th>
<th>Discrimination Ratio</th>
<th>\textsuperscript{3}H\textsubscript{1}CIT Binding</th>
<th>Inhibition by 10 (\mu)M Compound</th>
<th>IC\textsubscript{50} \textsuperscript{3}H\textsubscript{1}CIT/IC\textsubscript{50} \textsuperscript{3}H\textsubscript{1}WIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>(R_1)</td>
<td>(R_2)</td>
<td>(R_3)</td>
<td>(R_4)</td>
<td>IC\textsubscript{50}</td>
<td>(nM)</td>
</tr>
<tr>
<td>3CTMP</td>
<td>H</td>
<td>CO\textsubscript{2}CH\textsubscript{3}</td>
<td>Cl</td>
<td>H</td>
<td>5.1 ± 1.6</td>
<td>0.95 ± 0.12</td>
</tr>
<tr>
<td>4MeTMP</td>
<td>H</td>
<td>CO\textsubscript{2}CH\textsubscript{3}</td>
<td>CH\textsubscript{3}</td>
<td>H</td>
<td>33.0 ± 3.2</td>
<td>1.05 ± 0.02</td>
</tr>
<tr>
<td>4ATMP</td>
<td>H</td>
<td>CO\textsubscript{2}CH\textsubscript{3}</td>
<td>NH\textsubscript{2}</td>
<td>H</td>
<td>34.5 ± 4.0</td>
<td>0.96 ± 0.09</td>
</tr>
<tr>
<td>4TMP</td>
<td>H</td>
<td>CO\textsubscript{2}CH\textsubscript{3}</td>
<td>H</td>
<td>F</td>
<td>35.0 ± 3.0</td>
<td>0.97 ± 0.02</td>
</tr>
<tr>
<td>TMFPNMe</td>
<td>H</td>
<td>CO\textsubscript{2}CH\textsubscript{3}</td>
<td>H</td>
<td>H</td>
<td>85.0 ± 7.9</td>
<td>0.90 ± 0.09</td>
</tr>
<tr>
<td>4MeTMPCNMe</td>
<td>CH\textsubscript{3}</td>
<td>CO\textsubscript{2}CH\textsubscript{3}</td>
<td>CH\textsubscript{3}</td>
<td>H</td>
<td>140 ± 9</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>4MeTMPCNMe</td>
<td>CH\textsubscript{3}</td>
<td>CO\textsubscript{2}CH\textsubscript{3}</td>
<td>Cl</td>
<td>H</td>
<td>160 ± 18</td>
<td>0.96 ± 0.04</td>
</tr>
<tr>
<td>TMPCNMe</td>
<td>CH\textsubscript{3}</td>
<td>CO\textsubscript{2}CH\textsubscript{3}</td>
<td>CH\textsubscript{3}</td>
<td>H</td>
<td>499 ± 25</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td>(−)-Cocaine</td>
<td>H</td>
<td>CO\textsubscript{2}CH\textsubscript{3}</td>
<td>H</td>
<td>H</td>
<td>160 ± 15</td>
<td>1.03 ± 0.01</td>
</tr>
<tr>
<td>Citalopram</td>
<td>H</td>
<td>CO\textsubscript{2}CH\textsubscript{3}</td>
<td>H</td>
<td>H</td>
<td>9.2 ± 2.3</td>
<td>1.00 ± 0.07</td>
</tr>
</tbody>
</table>

\(\text{IC}_{50}\) means ± S.E.M. from two or more independent experiments in which triplicate samples were run at each of seven different concentrations of test compound.

\(\text{IC}_{50}\) mean ± S.E.M. from two or more independent experiments in which duplicate samples were run at each of five different concentrations of test compound.

Discrimination ratio is IC\textsubscript{50} for \textsuperscript{3}H\textsubscript{1}DA uptake/IC\textsubscript{50} for \textsuperscript{3}H\textsubscript{1}WIN.

Discrimination ratio is IC\textsubscript{50} for \textsuperscript{3}H\textsubscript{1}WIN binding.

Discrimination ratio is IC\textsubscript{50} for \textsuperscript{3}H\textsubscript{1}CIT binding.

Discrimination ratio is IC\textsubscript{50} for \textsuperscript{3}H\textsubscript{1}WIN binding.
nondentical regions of the transporter, the IC$_{50}$ for cocaine in the presence of a fixed concentration of 4MeTMPNMe would be expected to exceed the theoretical value that was calculated, assuming a straightforward competition for the same binding site. The results show that, although the IC$_{50}$ for cocaine increased significantly as the concentration of 4MeTMPNMe increased, the IC$_{50}$ for cocaine never differed significantly from the theoretical value that was calculated.

**Drug Discrimination Studies.** A total of 23 rats were trained to discriminate between 10 mg/kg cocaine and saline in a median of 32 sessions (range: 13–90). Doses of cocaine from 1.0 to 10 mg/kg occasioned orderly increases in the number of trials completed on the cocaine-appropriate choice lever (Fig. 2). The group averaged more than 19 trials on the cocaine-appropriate choice lever at the training dose of cocaine and at the highest dose tested, 17.5 mg/kg. TMP also occasioned dose-dependent responding on the cocaine-appropriate choice lever and was three times more potent than cocaine in this regard (Fig. 2; Table 4).

With the exceptions of 3CTMPNMe and TMPNBn, all of the analogs of TMP occasioned dose-dependent increases in the number of trials completed on the cocaine-appropriate choice lever; at the highest dose they generalized completely (i.e., ≥18 trials to the cocaine-appropriate choice lever) or almost completely with the training dose of cocaine (Fig. 3).

**Table 2**

<table>
<thead>
<tr>
<th>[4MeTMPNMe]</th>
<th>[3H]DA Uptake Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td>Apparent $K_m$</td>
</tr>
<tr>
<td>0</td>
<td>157 ± 11</td>
</tr>
<tr>
<td>300</td>
<td>235 ± 23*</td>
</tr>
<tr>
<td>600</td>
<td>326 ± 6**</td>
</tr>
</tbody>
</table>

Significant effect of [4MeTMPNMe] on apparent $K_m$ by one-way ANOVA with repeated measures ($F_{2,4} = 41.04, p = 0.002$).

Significant effect of [4MeTMPNMe] on $V_{max}$ by one-way ANOVA with repeated measures ($F_{2,4} = 7.41, p = 0.045$), but no significant difference between samples with and without 4MeTMPNMe by t test with Bonferroni correction.

Significantly different from control by t test with Bonferroni correction ($p < 0.05$).

Significantly different from control by t test with Bonferroni correction ($p < 0.01$).

**Table 3**

<table>
<thead>
<tr>
<th>[4MeTMPNMe]</th>
<th>Observed$^a$</th>
<th>Theoretical (IC$_{50}$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>454 ± 38</td>
<td>593 ± 53</td>
</tr>
<tr>
<td>200</td>
<td>632 ± 73</td>
<td>801 ± 75</td>
</tr>
<tr>
<td>500</td>
<td>881 ± 7</td>
<td>1149 ± 113</td>
</tr>
<tr>
<td>1000</td>
<td>1208 ± 58</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The observed IC$_{50}$ values for cocaine differed in the presence of varying concentrations of 4MeTMPNMe, with the following rank order as a function of [4MeTMPNMe]: 1000 nM > 500 nM > 200 nM = 0 nM, by Scheffe multiple comparisons.

$^b$ IC$_{50}$ was calculated as described in Simoni et al. (1993) and expressed as mean ± S.E.M. from three separate experiments. For two inhibitors acting at the same site, IC$_{50}$ = $K_C + [DA]/K_{DA} + [4MeTMPNMe]/K_{4MeTMPNMe}$, where $K_C$ and $K_{4MeTMPNMe}$ are the equilibrium dissociation constants for cocaine and 4MeTMPNMe, respectively, obtained in each individual experiment. $K_D$ is the Michaelis constant for DA uptake determined under the same conditions (157 nM; see Table 2).

$^c$ No significant difference was found between the observed and theoretical IC$_{50}$ values for cocaine at any concentration of 4MeTMPNMe.
of 10 trials per session to the cocaine-appropriate choice lever (discriminative effects). Potency of methylphenidate and analogs for producing cocaine-like discriminative stimuli correlated significantly with their order of potency for displacing the binding of \(^{3}H\)WIN and for inhibiting \(^{3}H\)DA uptake in striatal preparations (Fig. 4). Correlation coefficients of 0.61 (p = 0.048) and 0.75 (p = 0.008) were obtained between the log ED\(_{50}\) for cocaine discrimination and the log of the \(^{3}H\)WIN binding IC\(_{50}\) or \(^{3}H\)DA uptake IC\(_{50}\) respectively. The correlation between the log ED\(_{50}\) for cocaine discrimination and the log of the DR approached significance (Pearson r = 0.60; p = 0.0534). No correlation was observed between the log of the ED\(_{50}\) values for cocaine discrimination and the log of inhibitory potencies against \(^{3}H\)CIT binding to the SERT, based on the four analogs for which \(^{3}H\)CIT binding IC\(_{50}\) values could be calculated (Pearson r = 0.534, p < 0.46).

On the basis of stimulus-generalization curves, DR values, and chemical structures, four of the TMP analogs were selected for testing in combination with cocaine: 4ATMP, 4MeTMPNMe, TROHN Bn, and TMPN Bn. 4ATMP is a more potent compound than cocaine that required a 100-fold range of doses to define the ascending limb of the stimulus-generalization curve. 4MeTMPNMe is a less potent compound than cocaine that occasioned a low level of drug-appropriate responding over a 30-fold dose range. The last three compounds had the highest DR values of all of the analogs studied in this series, suggesting that a dose might be found at which they could inhibit cocaine binding to the transporter but not block transport of the substrate. The last two com-

\*\* Dose resulting in selection of the cocaine-appropriate choice lever in an average of 10 trials per session (n = 5 for all drugs except TROHN Bn (n = 4) and cocaine (n = 23)).

\*\* Potency relative to methylphenidate, based upon ED\(_{50}\) values (\(\mu\)mol/kg).

\*\*\* ED\(_{50}\) values that do not have a superscript in common are significantly different from each other, p < 0.05.

\*\*\*\* Estimated from group means and not included in the ANOVA of ED\(_{50}\) values.

to the cocaine-appropriate choice lever (F\(_{11,259}\) = 1.92, p = 0.037). However, post hoc tests failed to identify differences between specific compounds.

Comparison of the slopes of the stimulus-generalization curves obtained for the N-substituted compounds (8.5 ± 1.4 trials/log dose) with the slopes obtained for the compounds containing no N-substitution (13.2 ± 1.5 trials/log dose) revealed that the average slope for the unsubstituted compounds was about 55% higher than the average slope for the N-substituted compounds, although the difference did not quite reach statistical significance (t\(_{9}\) = 2.196; p = 0.056). The slope value for TMPN Bn was not included in these calculations because a reliable slope could not be calculated from the available data.

TABLE 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED(_{50}) (95% Confidence Limits)*</th>
<th>Relative Potency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4CTMP</td>
<td>0.38 (0.2–0.73) 0.43 (0.13–1.36)*</td>
<td>7.98</td>
</tr>
<tr>
<td>4FTMP</td>
<td>0.26 (0.15–0.36) 1.03 (0.72–1.43)*</td>
<td>3.33</td>
</tr>
<tr>
<td>3CTMP</td>
<td>0.38 (0.2–0.73) 1.42 (0.75–2.73)*</td>
<td>2.42</td>
</tr>
<tr>
<td>4ATMP</td>
<td>0.39 (0.09–1.79) 1.57 (0.36–7.22)*</td>
<td>2.18</td>
</tr>
<tr>
<td>TMP</td>
<td>0.80 (0.61–1.05) 3.43 (2.62–4.51)*</td>
<td>1.00</td>
</tr>
<tr>
<td>4MeTMP</td>
<td>1.15 (0.25–3.33) 4.65 (1.01–21.6)*</td>
<td>0.74</td>
</tr>
<tr>
<td>3CTMPNMe</td>
<td>2.00* 7.10*</td>
<td>0.48</td>
</tr>
<tr>
<td>Cocaine</td>
<td>2.58 (2.03–3.28) 8.45 (6.66–10.8)</td>
<td>0.41</td>
</tr>
<tr>
<td>TROHN Bn</td>
<td>9.40* 31.6*</td>
<td>0.11</td>
</tr>
<tr>
<td>4MeTMPNMe</td>
<td>9.06 (3.24–25.4) 34.7 (12.4–97.3)</td>
<td>0.10</td>
</tr>
<tr>
<td>TMPNMe</td>
<td>11.4 (3.47–37.2) 46.1 (14.0–151)*</td>
<td>0.07</td>
</tr>
<tr>
<td>TMPN Bn</td>
<td>&gt;30 &gt;92</td>
<td>&lt;0.04</td>
</tr>
</tbody>
</table>

- ED\(_{50}\) values for cocaine discrimination and the log of inhibitory potencies against \(^{3}H\)CIT binding IC\(_{50}\) or \(^{3}H\)DA uptake IC\(_{50}\) respectively. The correlation between the log ED\(_{50}\) for cocaine discrimination and the log of the DR approached significance (Pearson r = 0.60; p = 0.0534). No correlation was observed between the log of the ED\(_{50}\) values for cocaine discrimination and the log of inhibitory potencies against \(^{3}H\)CIT binding to the SERT, based on the four analogs for which \(^{3}H\)CIT binding IC\(_{50}\) values could be calculated (Pearson r = 0.534, p < 0.46).

On the basis of stimulus-generalization curves, DR values, and chemical structures, four of the TMP analogs were selected for testing in combination with cocaine: 4ATMP, 4MeTMPNMe, TROHN Bn, and TMPN Bn. 4ATMP is a more potent compound than cocaine that required a 100-fold range of doses to define the ascending limb of the stimulus-generalization curve. 4MeTMPNMe is a less potent compound than cocaine that occasioned a low level of drug-appropriate responding over a 30-fold dose range. The last three compounds had the highest DR values of all of the analogs studied in this series, suggesting that a dose might be found at which they could inhibit cocaine binding to the transporter but not block transport of the substrate. The last two com-

Fig. 2. Stimulus-generalization curves for methylphenidate and cocaine in rats trained to discriminate between 10 mg/kg cocaine and saline in a two-choice discrete-trial avoidance/escape procedure. Drugs were administered i.p. either 30 (methylphenidate) or 15 min (cocaine) before a session. Points are means based upon one observation in each of 5 (methylphenidate) or 23 (cocaine) rats. They indicate the number of trials completed on the cocaine-appropriate choice lever in a 20-trial session; the remaining trials of the session were completed on the choice lever appropriate for saline (Veh). The upper and lower dashed horizontal lines indicate the minimum level of performance maintained in training sessions that followed an injection of 10 mg/kg cocaine or saline, respectively.

Fig. 3. Stimulus generalization curves for methylphenidate and 11 analogs in rats trained to discriminate between 10 mg/kg cocaine and saline. Drugs were administered i.p. 30 min before a session. Points are means based upon one observation in each of five or four (TROHN Bn) rats. Solid lines indicate compounds that have a substitution on the ring nitrogen, and dashed lines indicate compounds that do not. The curve for methylphenidate (TMP) is reproduced from Fig. 2. Other details are the same as in Fig. 2.

3,4CTMP was the most potent of the analogs, 8 times more potent than TMP; TMPNMe was the least potent of the compounds for which an ED\(_{50}\) could be calculated, 1/14 as potent as TMP (Table 4). Thus, relative to the parent compound, the analogs spanned a potency range of 107-fold. The inclusion of TMPN Bn increases the potency range to more than 200-fold. The relative potencies of TMP and the 10 analogs for which ED\(_{50}\) values could be determined as cocaine-like discriminative stimuli correlated significantly with their order of potency for displacing the binding of \(^{3}H\)WIN and for inhibiting \(^{3}H\)DA uptake in striatal preparations (Fig. 4): Correlation coefficients of 0.61 (p = 0.048) and 0.75 (p = 0.008) were obtained between the log ED\(_{50}\) for cocaine discrimination and the log of the \(^{3}H\)WIN binding IC\(_{50}\) or \(^{3}H\)DA uptake IC\(_{50}\) respectively. The correlation between the log ED\(_{50}\) for cocaine discrimination and the log of the DR approached significance (Pearson r = 0.60; p = 0.0534). No correlation was observed between the log of the ED\(_{50}\) values for cocaine discrimination and the log of inhibitory potencies against \(^{3}H\)CIT binding to the SERT, based on the four analogs for which \(^{3}H\)CIT binding IC\(_{50}\) values could be calculated (Pearson r = 0.534, p < 0.46).

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pounds have N-benzyl substitutions, and one, TMPNBn, occasioned little cocaine-appropriate responding up to a dose of 30 mg/kg. Administered 15 min before cocaine, each of the TMP analogs tended to shift the cocaine stimulus-generalization curve upward and to the left (Fig. 5). This effect is particularly notable with 3.0 mg/kg TROHNBn (Fig. 5, upper right) and 10 mg/kg TMPNBn (Fig. 5, lower right), doses that occasioned little or no cocaine-appropriate responding when tested alone.

The ED_{50} of cocaine was lowered from 3.27 (2.10–5.08) mg/kg to 1.38 (0.37–5.14) and 1.43 (0.36–5.70) mg/kg after pretreatment with 0.03 or 0.1 mg/kg 4ATMP, respectively. It was lowered from 2.60 (1.82–3.72) mg/kg to 2.25 (0.99–5.10) and 1.58 (0.43–5.79) mg/kg, after pretreatment with 1.0 or 3.0 mg/kg 4MeTMPNMe, respectively. However, none of these changes was statistically reliable, according to ANOVA. Pretreatment with 3.0 mg/kg TMPNBn lowered the cocaine ED_{50} nonsignificantly from 2.72 (1.69–4.36) mg/kg to a group mean of 1.87 mg/kg.

ED_{50} values could not be calculated for cocaine after pretreatment with 10 mg/kg 4MeTMPNMe, 3.0 mg/kg TROHNBn, or 10 mg/kg TMPNBn because the threshold for detection of cocaine was reduced so markedly. However, a reduction in ED_{50} can be inferred from the fact that pretreatment with each of the four compounds resulted in a flattening of the stimulus-generalization curve for cocaine. ANOVA confirmed significant differences among the three curves in the 4ATMP (F_{[2,15]} = 5.69, p = 0.015) and TMPNBn (F_{[2,15]} = 14.32; p < 0.001) series as well as among the four curves in the 4MeTMPNMe series (F_{[3,20]} =

Fig. 4. The order of potency of methylphenidate and 10 analogs to produce cocaine-like discriminative effects in rats discriminating between 10 mg/kg cocaine and saline correlates significantly with their order of potency to inhibit [3H]WIN binding to the DAT (left) and [3H]DA uptake (right) in rat striatal preparations. ○, the N-substituted analogs; •, the analogs with no N-substitution. TMPNBn (open square with arrow) was not included in the regression analysis, because it did not generalize sufficiently to the cocaine stimulus to allow a valid calculation of its ED_{50} (see Table 4). It is shown here to underscore its unique activity and allow comparison with the other N-substituted compounds.

Fig. 5. Interactions of four analogs of methylphenidate with cocaine in rats discriminating between 10 mg/kg cocaine and saline. Cocaine (n = 7–9) was administered i.p. 15 min before a session, and each analog (n = 5) was administered i.p. 30 min before a session. Points above Veh represent the effect of an injection of the indicated dose of the methylphenidate analog followed 15 min later by an injection of saline, the vehicle for cocaine. Other details are the same as in Fig. 2.
were obtained for all of the compounds in the WIN binding introduction of an
tilities of the resulting compounds for the WIN binding site, despite the various structural modifications made to the
compounds fully substituted for 10 mg/kg cocaine in cocaine discrimination studies. The analogs with and without N-substitutions exhibit some unique differences that may influence their usefulness as drug therapy for cocaine abuse.
All of the derivatives retained activity at the DAT in vitro, the cocaine discrimination assay. In the absence of cocaine,
that they may ultimately prove useful as substitution therapies for the treatment of cocaine addiction. Like cocaine, they potently inhibit both the binding of [3H]WIN to the DAT, as well as the uptake of [3H]DA by striatal synaptosomes. With the exception of 3CTMPNMe and TMPBNb, all of the compounds without substitutions at the piperidinyl nitrogen. The shallower dose-response curves of this group are attributable to differences in onset of action caused by pharmacokinetic factors, such as absorption, binding to plasma proteins, and subsequent transfer across the blood-brain barrier.
There was no correlation between the potencies of compounds to substitute for cocaine and their inhibitory potentials against [3H]CIT binding to the SERT.
As a group, the N-substituted analogs also have DR values that are approximately 50% higher than those obtained for compounds without substitutions at the piperidinyl nitrogen. The DR is an empirical measure utilized in our laboratory as a preliminary screen to identify potential cocaine antagonists. Because it represents the ratio of the IC_{50} of a compound against [3H]WIN binding to its corresponding IC_{50} against [3H]DA uptake, high values would be expected to be associated with compounds that are more effective at blocking cocaine binding than DA uptake. For example, a compound that inhibited 80% of [3H]WIN binding sites while blocking only 10% of [3H]DA uptake would theoretically have a DR value of 36 (Deutsch et al., 1999). The DR is utilized only as one means of comparison of compounds assayed under rigorously maintained experimental conditions: even small variations in the assay conditions can drastically alter the DR value (Rothman et al., 1993; Xu et al., 1995). Although the DR values of the tertiary amines were significantly higher than those for the unsubstituted analogs, their mean absolute value (5.0 ± 0.8) nevertheless was quite low relative to the value theoretically required for a compound to exhibit predominantly antagonist-like characteristics against cocaine; this suggests that these compounds will have greatest utility as substitution therapy for cocaine.
In an effort to learn more about the interaction of these compounds with cocaine, both in vitro and in vivo studies were conducted. In vitro, the effect of 4MeTMPNMe on cocaine inhibition of [3H]DA uptake into synaptosomes was examined. 4MeTMPNMe was selected because of its relatively high DR (6.6) and its shallow dose-response curve in the cocaine discrimination assay. In the absence of cocaine,
4MeTMPNMe acted as a classic competitive antagonist of \(^{3}H\)DA uptake. When tested in combination with cocaine, 4MeTMPNMe appeared to act additively with cocaine at the same or similar site(s) to block the transport of \(^{3}H\)DA; it did not prevent access of cocaine to its binding site on the transporter in such a fashion as to raise the IC\(_{50}\) of cocaine over and above that predicted for the combination of two inhibitors acting at the same site.

When examined in vivo in combination with cocaine, most of the analogs tested appeared to potentiate cocaine discrimination, even at doses that by themselves had little or no cocaine-like discriminative stimulus activity. It is unlikely that these compounds potentiate effects of cocaine in vivo by interfering with its metabolism, because even the nonesterified analogs (which would not be expected to compete for metabolism) caused this effect. It is conceivable that the potency of these compounds against serotonin transporter underlies their potentiating effect of the discriminative stimulus effects of cocaine. Although it is generally acknowledged that inhibition of DA transport is the internal cue responsible for the discrimination of cocaine by rats (Cunningham and Callahan, 1991), serotonin transporter inhibitors increase the sensitivity for detection of this cue, even though they cannot by themselves substitute for cocaine (Kleven and Koek, 1998, and references therein). Indeed, TROHBNb, which potentiated cocaine discrimination by 8-fold when rats were pretreated with approximately 0.3 ED\(_{50}\) of it, was one of the most potent analogs against \(^{3}H\)CIT binding, whereas approximately the same relative dose of 4ATMP, which has virtually no activity at the SERT, caused only a 3-fold increase in cocaine discrimination. On the other hand, synergism with cocaine has also been reported for 1-[2-[bis(4-fluorophenyl)ethoxy]ethyl]-4-(3-phenylpropyl)piperazine (GRB-12909) and 4-chlorobenzotropine, structurally diverse dopamine uptake inhibitors with little activity at the SERT (Tolliver et al., 1999; Holtzman, 2001).

In summary, structural modification of TMP yields compounds with high to moderate potency as inhibitors of the DAT, and varying degrees of activity at the SERT, that may prove useful as substitution therapy for cocaine abuse. Further systematic studies, several of which are under way in our laboratories, are needed to investigate and exploit the unique (although sometimes conflicting) properties identified in this study for the N-substituted analogs. Their shallow dose-response curves may augment their utility as substitution therapy by providing a broader therapeutic dosing range before the threshold for full cocaine-like character is reached. If further chemical modification can generate compounds with even higher DR values, clinically useful partial agonists/antagonists may result. On the other hand, if the left-shift of the cocaine discrimination curve seen with these agents reflects their ability to potentiate the rewarding effects of cocaine, their appeal would diminish.

Acknowledgments

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References

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