Biochemical and Behavioral Characterization of Novel Methylphenidate Analogs

M. M. SCHWERI, H. M. DEUTSCH, A.T. MASSEY, AND S. G. HOLTZMAN

Division of Basic Medical Sciences, Mercer University School of Medicine, Macon, Georgia (M.M.S., A.T.M.); School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia (H.M.D.); and Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia (S.G.H.)

Received October 29, 2001; accepted January 10, 2002

This article is available online at http://jpet.aspetjournals.org

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 3H-(-)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane 1,5-naphthalenedisulfonate ([3H]WIN 35,428) ([3H]WIN binding to the dopamine transporter, compared with TMP. In general, parallel results were obtained for inhibition of [3H]dopamine ([3H]DA) 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 [3H]WIN binding than [3H]DA uptake did not attenuate inhibition by cocaine of synaposomal ([3H]DA transport. The compounds were significantly less potent in displacing [3H]citalopram 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

Supported by a grant from the National Institute on Drug Abuse (DA06305) to H.M.D., M.M.S., and S.G.H., and by a Senior Scientist Award (DA00008) to S.G.H.

Current address: Instructor, Division of Natural Sciences and Mathematics, Macon State College, Macon, GA.

ABBREVIATIONS: DAT, dopamine transporter; DR, discrimination ratio; [3H]CIT, [3H]citalopram; [3H]DA, [3H]dopamine; [3H]WIN, [3H]WIN 35,428, [3H]-(-)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane 1,5-naphthalenedisulfonate; SERT, serotonin transporter; 3CTMP, (±)-threo-3-chloromethylphenidate; 3CTMPNMe, (±)-threo-N-methyl-3-chloromethylphenidate; 4ATMP, (±)-threo-4-amonomethylphenidate; 4TMP, (±)-threo-4-fluoromethylphenidate; 4MeTMP, (±)-threo-4-methylphenidate; 3,4CTMP, (±)-threo-3,4-dichloromethylphenidate; THBNb, (±)-threo-N-benzyl-

The Journal of Pharmacology and Experimental Therapeutics
Vol. 301, No. 2
Copyright © 2002 by The American Society for Pharmacology and Experimental Therapeutics
0022-3565/02/3012-527–535$7.00
Printed in U.S.A.
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 [3H]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 effects of N-substitution, and/or re-
tion of [3H]DA was determined using a crude synaptosomal prep- 
K
from 25 to 800 nM.
the same conditions, except that the [3H]DA concentrations ranged 
GraphPad Prism, as described above.
Safe cocktail (Beckman Coulter, Fullerton, CA), and counted on a 
filters were transferred to scintillation vials, shaken vigorously for 30 min in the presence of 8 ml of Ready-
safe cocktail (Beckman Coulter, Fullerton, CA), and counted on a 
Benzyl-substituted com-

[3H]DA Uptake. The effect of test compounds on the accumula-
tion of [3H]DA was determined using a crude synaptosomal prepa-
ration 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 
25 to 800 nM. K_m and V_max 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 Institu-
tional 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 be-
tween 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 appropri-
ate for what was injected beforehand the 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 com-
pleted 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 re-
spect: after a response on the observing lever, a response on either 
choice lever that 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 methylpheni-
date; 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 
TMPNMe 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 pre-
seemed 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., ED_{50}) 
was calculated for individual rats. This was done by linear regression 
of the ascending limb of the stimulus-generalization curve, using 
log_{10} dose and at least three points. In those instances when only two 
points defined the ascending portion of the curve, ED_{50} values were 
derived by simple interpolation. The individual ED_{50} 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 ED_{50} values and 
on slopes were made by analysis of variance, followed, where 
appropriate, by the Student-Newman-Keuls test for multiple com-
parisons 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 IC_{50} values. The compounds show a hundred-fold variation in potency against [3H]WIN binding, ranging from IC_{50} values of about 5 nM for both 3CTMP and 3,4CTMP to about 500 nM for TMPNMe. A 200-fold range of potency was observed against [3H]DA uptake, from an IC_{50} of 7.0 ± 0.6 nM for 3,4CTMP to 1435 ± 5 nM for TMPNMe. The most potent compounds at the SERT are N-benzyl-substituted 
compounds in which the ester function has been converted to an
TABLE 1  
Effect of compounds on [3H]WIN and [3H]CIT binding and [3H]IDA uptake

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substitution</th>
<th>[3H]WIN Binding</th>
<th>[3H]IDA Uptake</th>
<th>Discrimination Ratio</th>
<th>[3H]CIT Binding</th>
<th>Inhibition by 10 μM Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁</td>
<td>R₂</td>
<td>R₃</td>
<td>R₄</td>
<td>IC₅₀</td>
<td>nᵣ</td>
<td>IC₅₀</td>
</tr>
<tr>
<td>3CTMP</td>
<td>H</td>
<td>CO₂CH₃</td>
<td>Cl</td>
<td>H</td>
<td>5.1 ± 1.6</td>
<td>0.95 ± 0.12</td>
</tr>
<tr>
<td>3,4CTMP</td>
<td>H</td>
<td>CO₂CH₃</td>
<td>Cl</td>
<td>Cl</td>
<td>5.3 ± 0.7</td>
<td>2.08 ± 0.05</td>
</tr>
<tr>
<td>TROMeNBn</td>
<td>Bn</td>
<td>CH₂OCH₃</td>
<td>H</td>
<td>H</td>
<td>17.8 ± 1.1</td>
<td>1.12 ± 0.13</td>
</tr>
<tr>
<td>TROHNbN</td>
<td>Bn</td>
<td>CH₂OH</td>
<td>H</td>
<td>H</td>
<td>25.7 ± 3.3</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>4MeTMP</td>
<td>H</td>
<td>CO₂CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>33.0 ± 1.2</td>
<td>1.05 ± 0.02</td>
</tr>
<tr>
<td>4ATMP</td>
<td>H</td>
<td>CO₂CH₃</td>
<td>NH₂</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>CO₂CH₃</td>
<td>H</td>
<td>F</td>
<td>35.0 ± 3.0</td>
<td>0.97 ± 0.02</td>
</tr>
<tr>
<td>TMPNbN</td>
<td>Bn</td>
<td>CO₂CH₃</td>
<td>H</td>
<td>H</td>
<td>52.9 ± 2.3</td>
<td>1.08 ± 0.02</td>
</tr>
<tr>
<td>TMP</td>
<td>H</td>
<td>CO₂CH₃</td>
<td>H</td>
<td>H</td>
<td>83.0 ± 7.9</td>
<td>0.90 ± 0.09</td>
</tr>
<tr>
<td>4MeTMP</td>
<td>H</td>
<td>CO₂CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>140 ± 9</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>3CTMPMe</td>
<td>CH₃</td>
<td>CO₂CH₃</td>
<td>Cl</td>
<td>H</td>
<td>160 ± 18</td>
<td>0.96 ± 0.04</td>
</tr>
<tr>
<td>TMPNMe</td>
<td>CH₃</td>
<td>CO₂CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>499 ± 25</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td>(+)-Cocaine</td>
<td>160 ± 15</td>
<td>1.03 ± 0.01</td>
<td>404 ± 26</td>
<td>2.5</td>
<td>401 ± 27</td>
<td>1.27 ± 0.01</td>
</tr>
<tr>
<td>Citalopram</td>
<td>9.2 ± 2.3</td>
<td>1.00 ± 0.07</td>
<td>9.2 ± 2.3</td>
<td>1.00 ± 0.07</td>
<td>9.2 ± 2.3</td>
<td>1.00 ± 0.07</td>
</tr>
</tbody>
</table>

* Mean ± S.E.M. from two or more independent experiments in which triplicate samples were run at each of six different concentrations of test compound.
* 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 IC₅₀ for [3H]IDA uptake/IC₅₀ for [3H]WIN binding.
* Mean ± S.E.M. from two or more independent experiments in which triplicate samples were run at each of seven different concentrations of test compound.
* Mean ± S.E.M. from two or more independent experiments in which the inhibition due to 10 μM compound was determined in triplicate.

ether (TROMeNBn; IC₅₀, 166 ± 8 nM) or an alcohol (TROHNbN; IC₅₀, 204 ± 9 nM); the least potent is 4ATMP, with an IC₅₀ well over 10,000 nM.

All of the analogs are selective for the [3H]WIN over the [3H]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 [3H]WIN binding than [3H]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 [3H]IDA uptake parallels that found for [3H]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 IC₅₀ of a given compound against [3H]IDA uptake to its IC₅₀ against [3H]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 Kₘ and Vₘₐₓ of [3H]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 Kₘ increased with increasing concentrations of 4MeTMPNMe, whereas the apparent Vₘₐₓ values did not differ significantly from the corresponding control values.

The ability of 4MeTMPNMe to block the inhibition of [3H]IDA 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 IC₅₀ of cocaine was determined (Table 3). If cocaine and 4MeTMPNMe competed for the same domain on the DAT, the IC₅₀ 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 IC₅₀ 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...
nonidentical regions of the transporter, the IC₅₀ 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 IC₅₀ for cocaine increased significantly as the concentration of 4MeTMPNMe rose from 200 to 1000 nM, it never differed significantly from the theoretical value that would be predicted if cocaine and 4MeTMPNMe were directly competing for the same site.

**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>
<th>[³H]DA Uptake Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td>Apparent Kₘ</td>
<td>Vₘₐₓ</td>
</tr>
<tr>
<td>0</td>
<td>157 ± 11</td>
<td>153 ± 21</td>
</tr>
<tr>
<td>300</td>
<td>235 ± 23*</td>
<td>159 ± 29</td>
</tr>
<tr>
<td>600</td>
<td>326 ± 6**</td>
<td>175 ± 27</td>
</tr>
</tbody>
</table>

* Significant effect of [4MeTMPNMe] on apparent Kₘ by one-way ANOVA with repeated measures (F₄,₄ = 41.04, p = 0.002).

** Significant effect of [4MeTMPNMe] on Vₘₐₓ by one-way ANOVA with repeated measures (F₄,₄ = 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).

** Significant different from control by t test with Bonferroni correction (p < 0.01).

**Fig. 1.** Lineweaver-Burk analysis of the effect of 4MeTMPNMe on [³H]DA uptake by rat striatal synaptosomes. Accumulation of [³H]DA (25–800 nM) was measured over a 2-min period in an S₁ fraction of rat striatal tissue that had been mixed with 25 mM phosphate buffer, then preincubated for 10 min at 37°C with water (controls), 300 nM 4MeTMPNMe, or 600 nM 4MeTMPNMe before the addition of the [³H]DA. In this representative experiment, the Vₘₐₓ values were 134.8 (12.53–14.42), 134.2 (11.52–15.31, and 152.5 (14.67–15.84) pmol/mg protein × 2 min) and the apparent IC₅₀ values were 180 (146–214), 239 (157–322), and 333 (304–361) nM for the water, 300 nM 4MeTMPNMe, and 600 nM 4MeTMPNMe treatment groups, respectively (95% confidence intervals are shown in parentheses). The results from three such experiments are presented in Table 2.

**Table 3.**

<table>
<thead>
<tr>
<th>4MeTMPNMe</th>
<th>Observed</th>
<th>Theoretical (IC₅₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>454 ± 38</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>632 ± 73</td>
<td>593 ± 53</td>
</tr>
<tr>
<td>500</td>
<td>881 ± 7</td>
<td>801 ± 75</td>
</tr>
<tr>
<td>1000</td>
<td>1208 ± 58</td>
<td>1149 ± 113</td>
</tr>
</tbody>
</table>

* The observed IC₅₀ values for cocaine differed in the presence of varying concentrations of 4MeTMPNMe (one-way ANOVA with repeated measures; F₄,₄ = 7.41, p < 0.0001). The observed IC₅₀ for cocaine increased in the presence of increasing 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.

<table>
<thead>
<tr>
<th>4MeTMPNMe</th>
<th>Observed</th>
<th>Theoretical (IC₅₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>454 ± 38</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>632 ± 73</td>
<td>593 ± 53</td>
</tr>
<tr>
<td>500</td>
<td>881 ± 7</td>
<td>801 ± 75</td>
</tr>
<tr>
<td>1000</td>
<td>1208 ± 58</td>
<td>1149 ± 113</td>
</tr>
</tbody>
</table>

* The observed IC₅₀ values for cocaine differed in the presence of varying concentrations of 4MeTMPNMe (one-way ANOVA with repeated measures; F₄,₄ = 7.41, p < 0.0001). The observed IC₅₀ for cocaine increased in the presence of increasing 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.

* The observed IC₅₀ values for cocaine differed in the presence of varying concentrations of 4MeTMPNMe (one-way ANOVA with repeated measures; F₄,₄ = 7.41, p < 0.0001). The observed IC₅₀ for cocaine increased in the presence of increasing 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.

* No significant difference was found between the observed and theoretical IC₅₀ values for cocaine at any concentration of 4MeTMPNMe.
of 10 trials per session. Points are means based upon one observation in each of five (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.

TABLE 4

Potency of methylphenidate and analogs for producing cocaine-like discriminative effects

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED$_{50}$ (95% Confidence Limits)$^a$</th>
<th>Relative Potency$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4CTMP</td>
<td>0.38 (0.2–0.73) 0.43 (0.13–1.36)$^c$</td>
<td>7.98</td>
</tr>
<tr>
<td>4TMP</td>
<td>0.26 (0.15–0.36) 1.03 (0.72–1.43)$^d$</td>
<td>3.33</td>
</tr>
<tr>
<td>3CTMP</td>
<td>0.38 (0.2–0.73) 1.42 (0.75–2.73)$^d$</td>
<td>2.42</td>
</tr>
<tr>
<td>4ATMP</td>
<td>0.39 (0.09–1.79) 1.57 (0.36–7.22)$^d$</td>
<td>2.18</td>
</tr>
<tr>
<td>TMP</td>
<td>0.80 (0.61–1.05) 3.43 (2.62–4.51)$^d$</td>
<td>1.00</td>
</tr>
<tr>
<td>4MeTMP</td>
<td>1.15 (0.25–3.33) 4.65 (1.01–21.6)$^d$</td>
<td>0.74</td>
</tr>
<tr>
<td>3CTMPNMe</td>
<td>2.00$^a$ 7.10$^a$</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>TROHNb</td>
<td>9.40$^a$ 31.6$^a$</td>
<td>0.11</td>
</tr>
<tr>
<td>4MeTMPNMe</td>
<td>9.06 (3.24–25.4) 34.7 (12.4–97.3)$^a$</td>
<td>0.10</td>
</tr>
<tr>
<td>TMPNMe</td>
<td>11.4 (3.47–37.2) 48.1 (14.0–151)$^a$</td>
<td>0.07</td>
</tr>
<tr>
<td>TMPNbn</td>
<td>&gt;80                     &gt;80                     &lt;0.04</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Dose resulting in selection of the cocaine-appropriate choice lever in an average of 10 trials per session [n = 5 for all drugs except TROHNb (n = 4) and cocaine (n = 23)].

$^b$ Potency relative to methylphenidate, based upon ED$_{50}$ values (µmol/kg).

$^c$ ED$_{50}$ values that do not have a superscript in common are significantly different from each other, p < 0.05.

$^d$ 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 TROHNb was not included in these calculations because a reliable slope could not be calculated from the available data.

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 TMPNbn 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).

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, TROHNb, and TMPNbn. 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 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-
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 ED50 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 ED50 nonsignificantly from 2.72 (1.69–4.36) mg/kg to a group mean of 1.87 mg/kg. ED50 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 ED50 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 ED50 (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.
Discussion

The methylphenidate analogs described here exhibit many cocaine-like properties, both in vitro and in vivo, suggesting 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 TMPNBN, all of the compounds fully substituted for 10 mg/kg cocaine in dopamine 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, despite the various structural modifications made to the methylphenidate molecule. Substitutions on the aryl ring, or introduction of an N-benzyl group (with or without simultaneous modification of the ester function) increased the affinities of the resulting compounds for the WIN binding site, compared with the parent compound. On the other hand, introduction of an N-methyl substitution (with or without simultaneous substitution on the aryl ring) caused up to a 6-fold loss in affinity. Hill coefficients of approximately 1 were obtained for all of the compounds in the WIN binding assay, with the notable exception of 3,4CTMP, which had a $n_H$ of 2, as reported previously (Deutsch et al., 1996). For the most part, parallel results were obtained for the inhibition of [3H]DA uptake by the various derivatives.

In contrast to their activity against [3H]WIN binding to the DAT, the compounds were much less potent as inhibitors of [3H]CIT binding to the SERT. Comparison of the IC$_{50}$ values of the analogs in the two assays showed that the derivatives ranged from 8- to more than 2000-fold less potent against [3H]CIT binding than against [3H]WIN binding (Table 1). Cocaine exhibited much less selectivity than the methylphenidate derivatives for the DAT: its IC$_{50}$ against [3H]WIN binding was only 2.5-fold less than its IC$_{50}$ against [3H]CIT. In agreement with previous reports (Ritz et al., 1987; Gatley et al., 1996), TMP had little inhibitory activity at the SERT. N-Benzyl modification increased affinity for the [3H]CIT binding site, and simultaneous conversion of the ester function to an alcohol or methyl ether increased it even more (10-fold). In contrast to the effect of ring halogenation on [3H]WIN binding, where 3-Cl and 3,4-CI modifications produced equivalent increases in potency, 3,4CTMP is much more potent than 3CTMP against [3H]CIT binding. Although dichloro substitution increased potency at the SERT compared with unmodified TMP, it did not raise the $n_H$ to 2, as it did against [3H]WIN binding. Because the [3H]WIN and [3H]CIT binding assay conditions are so similar, this finding suggests that the $n_H$ obtained for this compound in the [3H]WIN assay is valid and not an artifact caused by its high lipophilicity.

In general, the in vitro interactions of these agents with the DAT are predictive of their activity in the in vivo cocaine discrimination studies (Fig. 4), consistent with the generally accepted notion that inhibition of DA uptake is the chief internal cue for identification of cocaine-like drugs (Kleven and Koek, 1998, and references therein; Howell and Wilcox, 2001). In accord with this are the positive correlations observed between the logs of the [3H]WIN binding IC$_{50}$, [3H]DA uptake IC$_{50}$, or DR, and the log ED$_{50}$ for cocaine discrimination. However, results from in vitro assays were not perfect predictors of potency in vivo. Although the low in vivo potency of the N-Me-substituted compounds reflects their low potency in vitro, this was not the case with the N-Bn-substituted compounds. TROMeBn, for instance, was approximately 5 times more potent than TMP against [3H]WIN binding and two and a half times more potent against [3H]DA uptake than TMP; yet it was almost 8-fold less potent than TMP as a substitute for cocaine. TMPNBN, the low potency of which in the cocaine discrimination studies precluded a calculation of its ED$_{50}$, was comparable with TMP in the [3H]WIN binding and [3H]DA uptake assays. Five of the six N-substituted compounds lie above the regression line, whereas all of the compounds without an N-substitution fall on or below the line (Fig. 4). As a group, the N-substituted compounds had shallower stimulus-generalization curves, leading to higher ED$_{50}$ values than would have resulted had their slopes resembled those of the unsubstituted compounds. N-substituted analogs are more hydrophobic than their secondary amine counterparts. It is possible, therefore, that 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 potencies 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]DA uptake to its corresponding IC$_{50}$ against [3H]WIN binding, 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 nonsterified 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 potentiation 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 Calhahan, 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, TROHNBn, which potentiates 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-(4-fluorophenyl)-methoxy]-ethyl-4-(3-phenylpropyl)piperazine (GBR-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

We thank Monica Stafford and Adam Eason for assistance in the in vitro assays, and Christine Engels for assistance in the drug discrimination assays.

References


Address correspondence to: Dr. Margaret M. Schweri, Division of Basic Medical Sciences, Mercer University School of Medicine, 1550 College Street, Macon, GA 31207. E-mail: schweri_mm@mercer.edu