A Comprehensive In Vitro Screening of d-, l-, and dl-threo-Methylphenidate: An Exploratory Study

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ABSTRACT

dl-Methylphenidate (MPH) has been widely used to treat attention-deficit/hyperactivity disorder (ADHD) for the last half century. It had been exclusively available in the racemic form, i.e., a 50:50 mixture of d- and l-isomers. However, a single enantiomer formulation, d-MPH (dexamethylphenidate), became available for general clinical use in 2002. For this reason, the intrinsic pharmacological differences in the effects of d- and l-MPH have recently come under intense investigation. The primary therapeutic effects of MPH are generally recognized to reside in the d-isomer. The present investigation provides quantitative values for a broad range of receptor-level interactions of the individual MPH isomers to better characterize better the distinction between dl-MPH versus d-MPH versus l-MPH as it relates to binding affinity at sites associated with relevant central nervous system (CNS) pharmacology, as well as peripheral physiology. Overall, there were few differences in binding affinities between d-MPH and the racemate whereas there were more apparent differences between d-MPH and l-MPH. d-MPH exhibited prominent affinity at the norepinephrine transporter (NET) site, even exceeding such affinity at the dopamine transporter (DAT). These results further demonstrate that affinity for catecholaminergic sites largely resides in the d-MPH isomer. Although binding affinity was not demonstrable at the serotonin (5-HT) transporter site (SERT), novel findings of the study included affinity for the 5-HT_{1A} and 5-HT_{2B} receptor sites for both d- and l-MPH, with d-MPH exerting by far the most predominant effects at these sites. Thus, the emerging data of favorable therapeutic effects of ADHD treatment with d-MPH (and dl-MPH) may be underpinned by affinity and potential pharmacologic effects at NET and DAT sites, as well as sites relevant to serotonergic neurotransmission that may modulate mood, cognition, and motor behavior. However, the present exploratory studies reflect receptor binding affinities only. The specific pharmacological activities (i.e., agonism vs. antagonism) of these compounds await further exploration.

INTRODUCTION

ATTENTION-DEFICIT/HYPERACTIVITY DISORDER (ADHD) is a complex neurobehavioral disorder characterized by varying degrees of inattention, hyperactivity, and impulsivity (Holmer et al. 2000; Biederman 2005). It is also one of the most common chronic health prob-
lems afflicting school-age children, with an estimated worldwide prevalence of 8–12% (Faraone 2003). Up to 50% of diagnosed children demonstrate clinically significant symptoms and impairment as young adults (Mannuzza 1998; Barkley 2002), although its persistence is not universally associated with impaired outcomes (Biederman 2005).

The most widely used pharmacological treatment of ADHD is the racemic (50:50) mixture of d-threo-(R,R)-methylphenidate (MPH) and l-threo-(S,S)-MPH isomers (Fig. 1), a racemic mixture in general clinical use for over 50 years. Depicted in bold within these structures is the phenethylamine pharmacophore of d-MPH shared by the neuronal substrates dopamine (DA) and norepinephrine (NE). Both pharmacodynamic and pharmacokinetic differences between these isomers are well recognized. Although dl-MPH is available as either an immediate-release (IR) or extended-release (ER) formulations (Markowitz et al. 2003), an enantiopure d-MPH product [d-threo-(R,R)-MPH, dexmethylphenidate] was recently introduced into clinical practice (Keating and Figgit 2002).

The clinical effectiveness of racemic MPH appears to reside primarily in the d-isomer (see Patrick et al. 2005). Srinivas et al. (1992) demonstrated similar improvement on sustained attention testing after ADHD subjects were treated with d-MPH or twice the dose of dl-MPH, but not after l-MPH. This study also demonstrated similar pharmacokinetics of d-MPH after the administration of d-MPH as either the pure enantiomeric formulation or as twice the dose of the racemic mixture. The l-MPH isomer of racemic MPH formulations has long been regarded as an inactive or passive component, contributing little to the therapeutic or adverse event profile.

Profound differences between d- and l-MPH metabolism and disposition have consistently been demonstrated in enantiospecific investigations. The presystemic metabolism and metabolic clearance of dl-MPH is an enantioselective process resulting in markedly higher plasma concentrations of d-MPH relative to l-MPH (see Markowitz et al. 2003; Patrick et al. 2005). In an enantiospecific study of MPH administered intravenously, both isomers exhibited similar distribution characteristics, although the terminal elimination phase of the l-isomer was more rapid (Srinivas et al. 1993). In other studies using different oral formulations, the area under the plasma concentration–time curve (AUC_{inf}) value for the l-isomer has been reported to reach only approximately 1–15% of that of d-MPH (Ramos et al. 1999; Modi et al. 2000; Patrick et al. 2005). Although l-MPH exhibits lower oral bioavailability, l-

![Dopamine; R = H](image)

![Norepinephrine; R = OH](image)

**FIG. 1.** Stereoviews of methylphenidate enantiomers and the catecholamines d-methylphenidate mimics through its common phenethylamine (bold) pharmacophore.
METHYLPHENIDATE PHARMACOLOGY AND ADHD

MPH has also been reported to be more stable in human plasma than \( d \)-MPH (Srinivas et al. 1991). Furthermore, radiolabeled \( l \)-MPH, or a metabolite with the same chromatographic retention time as an active metabolite of \( l \)-MPH, \( p \)-hydroxymethylphenidate (Patrick et al. 1981), was recently reported to be taken up into the brain to a greater extent than \( d \)-MPH following oral administration of each isomer to rats or baboons (Ding et al. 2004). Interestingly, a recent report describes a normal volunteer study subject identified as a “poor metabolizer” of MPH, exhibiting an extended overall drug half-life and an unprecedented enrichment of \( l \)-MPH in plasma relative to \( d \)-MPH (Patrick et al. in press 2006). This abnormal profile is postulated to be due to a genetic deficiency in carboxylesterase activity (CES1A1). However, its frequency of occurrence in the general population cannot be estimated at present. These findings are currently under further investigation by this group. Finally, the recent availability of the \( dl \)-MPH transdermal patch reveals much higher plasma concentrations of \( l \)-MPH (i.e., values approaching those of \( d \)-MPH concentrations in some instances) throughout the day, a profile far different from any existing oral formulation of racemic MPH (Pierce et al. 2005). Thus, the determination of the pharmacologic profile or contribution of \( l \)-MPH compared to \( d \)-MPH has now gained greater relevance.

A number of preclinical studies have suggested a \( d \)-MPH--\( l \)-MPH pharmacodynamic interaction associated with the neuropharmacologic effects of racemic MPH. In a recent rodent study, the \( l \)-MPH isomer inhibited, in a dose-dependent fashion, the locomotor stimulation by \( d \)-MPH, cocaine, or apomorphine in rats (Baldessarini and Cambell 2001). These results differ from those observed in mice, where \( l \)-MPH has been reported to enhance the locomotor stimulatory activity following cocaine (Ding et al. 2002; Ding et al. 2004). Davids and co-workers (2002a) assessed the activity of the separate enantiomers in a rat model of ADHD following the neonatal lesioning of cerebral dopaminergic neurons with 6-hydroxydopamine to induce hyperlocomotion. Challenges with \( d \)-MPH, \( l \)-MPH, racemic MPH, or saline in these rats demonstrated that \( d \)-MPH was over three times more active in reducing motor activity than the racemate. A two-fold reduction would be expected if \( l \)-MPH were inert. Furthermore, pretreatment of the lesioned rats with \( l \)-MPH attenuated the motor activity response \( d \)-MPH. Although not yet tested in a human clinical study, these findings suggest that clinical efficacy might be obtained with \( d \)-MPH administration in doses lower than presumed equipotent doses (i.e., 50% of \( dl \)-MPH dose). In other recent rodent studies, the incidence of dilated pupils and vocalization was greater for the racemate than pure \( d \)-MPH administered at one-half the racemate dose (Teo et al. 2003). When comparing \( dl \)-MPH to \( d \)-MPH, the inclusion of the \( l \)-isomer in dosing regimens enhanced the degree of repetitive pawing, dilated pupils, and aggressive behavior observed in rats and head-bobbing and hyperpnea in rabbits (Teo et al. 2003).

In view of the reported differences between \( d \)- and \( l \)-MPH activities, the present in vitro study was carried out to assess further the individual affinity of MPH isomers, as well as the racemic mixture, in a broad array of receptor binding, ion channel, transporter, and cellular assays of interest in central nervous system (CNS) pharmacology inclusive of, and beyond the typical monoamine [i.e., NE, DA, and serotonin (5-HT)] neurotransmitters assays carried out to date. This study focuses on differences in MPH isomer affinity for pharmacologically relevant sites that may be pertinent to enantiospecific therapeutic and/or adverse effect profiles associated with each isomer or racemic MPH.

MATERIALS AND METHODS

Chemical compounds

\( l \)-threo-methylphenidate (\( l \)-MPH), \( d \)-threo-methylphenidate (\( l \)-MPH), and \( dl \)-threo-methylphenidate (\( dl \)-MPH) were purchased in their hydrochloride salt forms from Sigma-Aldrich (St. Louis, MO).

Receptor binding, ion channel, and transporter assays

All receptor binding assays, ion channel assays, transporter studies, and cellular uptake/
release assays were performed by CEREP (Celle l’Evescault, France). Unless otherwise specified in the results, all screening studies were initially carried out at concentrations of 10 μM in duplicate for all three MPH compounds. Concentrations of dl-MPH, d-MPH, and I-MPH were based upon weight, i.e., experiments using the racemate provided 50% less d-MPH than those using the pure enantiomer. If greater than 50% displacement of the receptor-specific ligand occurred at this screening concentration, and these values favored one isomer over another or the racemic mixture by ≥ two-fold, further experiments were undertaken assessing multiple concentrations of the compound of interest to generate specific IC₅₀ values. That is, the concentration of the drug of interest (e.g., MPH racemate or isomers) that competes for 50% of the receptors present (one-half maximal or IC₅₀). The binding equilibrium dissociation constant (Kᵢ) was then calculated from the determined IC₅₀ value by use of the Cheng–Pru-soff equation (Cheng and Prusoff 1973). Receptor binding affinity is generally quantified utilizing the Kᵢ value. In interpreting the Kᵢ, the subscript i represents the competitor inhibited radioligand binding. The Kᵢ is the concentration of the competing ligand that will bind to one-half the targeted binding sites at equilibrium, in the absence of a specific radioligand or other competitor. If the Kᵢ is low, the affinity of the receptor for the inhibitor is high.

Over 100 binding sites for the two individual MPH isomers as well as the racemate were assessed with regard to binding affinity that included approximately 90 CNS receptor binding sites for different biogenic amine and neuropeptide receptors, major ion channels, and major transporters. The individual receptors, ion channels, and transporters assessed as well as origin of cell type are listed in Table 1. Detailed descriptions of all assays performed may be accessed at the CEREP website (http://www.cerep.com).

SPECIFIC METHODS

DAT assay

Evaluation of the affinity of compounds for the human dopamine transporter (DAT) in transfected Chinese hamster ovary (CHO) cells was determined in a validated radioligand binding assay. Cell membrane homogenates (25 μg of protein) were incubated for 120 minutes at 4°C with 0.5 nM [³H]GBR 12935 in the absence or presence of each test compound in a buffer containing 50 mM of Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM EDTA, and 0.1% bovine serum albumin (BSA). Nonspecific binding was determined in the presence of 10 μM N-[1-(benzo[b]thien-2-yl-cyclohexyl)] piperidine (BTCP). Following incubation, the samples were filtered under vacuum through glass GF/B filters presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester. The filters were then dried and measured for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results were expressed as a percent inhibition of the control radioligand-specific binding. The standard reference compound was BTCP, which was tested at several concentrations to obtain a competition curve from which its IC₅₀ was calculated.

NET assay

Evaluation of the affinity of compounds for the human norepinephrine transporter (NET) in transfected CHO cells was determined in a radioligand binding assay similar to the procedures described for the DAT assay. Cell membrane homogenates (25 μg of protein) were incubated for 90 minutes at 4°C with 1 nM [³H]nisoxetine in the absence or presence of each test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, and 5 mM KCl. Nonspecific binding was determined in the presence of 1 μM of desipramine. Following incubation, the samples were filtered rapidly under vacuum through glass fiber filters (GF/B, Packard), presoaked with 0.3% PEI, and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters were then dried and counted for radioactivity. The results were expressed as a percent inhibition of the control radioligand-specific binding. The standard reference compound was the tricyclic antidepres-
Receptor binding profiles

Adenosine A<sub>1</sub>, A<sub>2A</sub>, and A<sub>3</sub> receptors; rat cerebral cortex adrenergic receptor (α<sub>1</sub>); rat cerebral cortex (central) and (peripheral) benzodiazepine receptors (BZD); rat cerebral cortex bombesin (BB); bradykinin receptors 1 and 2 (B<sub>1</sub>, B<sub>2</sub>); calcitonin gene-related peptides (CGRP); cannabinoid (CB<sub>1</sub>); cholecystokinin receptors 1 and 2 (CCK<sub>1</sub>, CCK<sub>2</sub>); complement 5 alpha (C5a); pituitary gland corticotropin-releasing factor (CRF); DA<sub>D<sub>1</sub></sub>, DA<sub>D<sub>2</sub></sub>, D<sub>D<sub>3</sub></sub>, and D<sub>D<sub>4</sub></sub> receptors; rat cerebral cortex γ-aminobutyric acid (GABA) receptors GABA<sub>A</sub> and GABA<sub>B</sub>; galanin (GAL) receptors GAL1 and GAL2; rat cerebral cortex glutamate (AMPA); kainate, NMDA; rat spinal cord glycine (strychnine-sensitive) and rat cerebral cortex glycine (strychnine nonsensitive); vascular endothelial growth factor (VEGF); histamine receptors H<sub>1</sub>, H<sub>2</sub>, and H<sub>3</sub>; rat imidazoline type 2 (I<sub>2</sub>) receptors (central and peripheral); rat cerebral cortex opiate (nonselective); neuropeptide Y (nonselective); neurotensin (NT<sub>1</sub>); neurexin U2 (NmuU2); rat cerebral cortex nicotinic (N); rat cerebral cortex orbitope (nonselective); orphanin (ORL1); rat cerebral cortex phenylcyclidine (PCP); prostanoid (TXA<sub>2</sub>/PGH<sub>2</sub>); rat urinary bladder purinergic (P2X); mouse brain rolipram; serotonin (5-HT) receptor types 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>3</sub>, 5-HT<sub>4C</sub>, 5-HT<sub>4D</sub>, 5-HT<sub>4E</sub>, 5-HT<sub>5A</sub>, 5-HT<sub>5B</sub>, 5-HT<sub>5C</sub>, 5-HT<sub>5D</sub>, 5-HT<sub>5E</sub>, 5-HT<sub>5F</sub>; rat cerebral cortex sigma (σ, nonspecific); somatostatin (SST); and vasopressin (V<sub>1a</sub>). All assays were run using recombinant human receptors, except where noted.

Ion channel assays

Rat cerebral cortex calcium (Ca<sup>2+</sup>) channel (L, DHP site and L, diltiazem site); rat cerebral cortex potassium (K<sup>+</sup>) channel (K<sup>+</sup>-MPH, and K<sup>+</sup>-MPH); rat cerebral cortex sodium (Na<sup>+</sup>) channel (site 2); rat cerebral cortex chloride (Cl<sup>-</sup>) channel.

Transporter assays

Guinea-pig cerebral cortex adenosine (ADO) transporter, NET, DAT; rat cerebral cortex GABA transporter, rat striatum choline transporter, and SERT. The assays were run at 10 μM of each isomer (i.e., d- and l-MPH) as well as racemic (dl-MPH), and the percentage of inhibition is given as the mean of three determinations. All transporter assays were carried out using human transporters in transfected Chinese hamster ovary (CHO) cells.

Cell-based assays

Cellular assays were conducted to provide a functional measure of activity of each isomer and racemate with regard to DA, NE, and 5-HT uptake and release. These assays utilized rat brain synaptosomal preparations.

<table>
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<th>TABLE 1. ASSAYS PERFORMED</th>
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Method for determinations of IC<sub>50</sub> values for the human 5-HT<sub>2B</sub> and 5-HT<sub>1A</sub> receptors

Evaluation of the affinity of d- and l-MPH and the racemate for the agonist site of the human 5-HT<sub>2B</sub> receptor was carried out in transfected CHO cells determined in a radioligand binding assay. Cell membrane homogenates (5–10 μg of protein) were incubated for 15 minutes at 37°C with 0.2 nM [<sup>125</sup>I]DOI (1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane) in the absence or presence of each test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 10 μM pargyline, and 0.1% ascorbic acid. Nonspecific binding was determined in the presence of 1 μM DOI. Following incubation, the samples were filtered under vacuum through glass fiber filters (GF/B, Packard), presoaked with 0.3% PEI, and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters were dried and then counted for radioactivity. The results were expressed as a percent inhibition of the respective control radioligands for specific binding. The standard reference compound was DOI, which was tested in each experiment to generate a competition curve from which its IC<sub>50</sub> was calculated.
Evaluation of the affinity of all MPH compounds for the human 5-HT$_{1A}$ receptor was accomplished in a similar manner, but used transfected HEK-293 cells determined in a radioligand binding assay. Cell membrane homogenates (75–100 μg of protein) were incubated for 60 min at 22°C with 0.5 nM $[^3]$H8-OH-DPAT ([±]-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene) in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM MgSO$_4$, 0.5 mM EDTA, and 0.1% ascorbic acid. Nonspecific binding was determined in the presence of 10 μM 8-OH-DPAT. Following incubation, the samples were filtered rapidly under vacuum as described above, dried, and then counted for radioactivity. The results were expressed as a percent inhibition of the control radioligand-specific binding. The standard reference compound is 8-OH-DPAT, which was tested in each experiment to calculate an IC$_{50}$, as described above for 5-HT$_{2B}$ receptors.

**Cell-based assays**

**NE uptake:** The evaluation of the effects of the isomers and racemate on NE uptake employed synaptosomes prepared from the rat hypothalamus. The synaptosomes (100 μg) were incubated for 20 minutes at 37°C with 0.1 μCi $[^3]$HNE in the absence (control) or presence of the test compound or the reference compound in a buffer containing 118 mM NaCl, 5 mM KCl, 2.5 mM MgSO$_4$, 1.2 mM NaH$_2$PO$_4$, 25 mM NaHCO$_3$, 11 mM glucose, 10 μM EGTA, and 50 μM ascorbic acid (pH 7.4). Basal control activity was determined by incubating the same mixture for 20 minutes at 0°C in the presence of 1 μM protriptyline to block the uptake. After incubation, the samples were filtered and counted, and the results expressed as a percent inhibition of the control uptake of $[^3]$HNE. The standard inhibitory reference compound was GBR12909, which was tested in each experiment at several concentrations to obtain an inhibition curve from which its IC$_{50}$ value was calculated.

**RESULTS**

For the vast majority of receptor systems, transporters, and assays assessed (see Table 1), no significant binding affinity was apparent for either of the individual MPH isomers or the racemate at the utilized concentration of 10 μM, suggesting a remarkably “clean” binding profile for MPH in general. The concentration of MPH employed in this initial screening study (i.e., 10 μM) is widely used in general high-throughput pharmacological screening procedures and is accepted to be sufficiently high in pharmacological screening of this nature so that if any significant binding affinity would be a characteristic of a given compound, it would be apparent under the assay conditions employed.

The specific results of the in vitro screening for receptor binding for d-, l-, and dl-MPH in which significant binding occurred (i.e., ≥50% displacement of the specific receptor ligand) are presented in Table 2. There was measurable, albeit relatively weak, binding affinity of both MPH isomers and the racemate to a number of muscarinic receptors including M$_1$, M$_2$, M$_3$, M$_4$, and M$_5$. Generally, binding affinity was similar between the two isomers and racemate, although binding of d-MPH appeared to be significantly weaker than that of l-MPH for the M$_2$ muscarinic receptor (Table 2; 34 vs. 64%
inhibition of control specific binding). The potential clinical consequences of this modest binding, if any, are unknown and would require further exploration.

An unexpected finding was the measurable stereoselective binding observed for the 5-HT1A and 5-HT2B receptor sites (Table 2). For each of these sites, the binding was largely attributable to d-MPH rather than l-MPH. Although almost all previous investigations, including the present study, have found little interaction of MPH with the 5-HT transporter (SERT), the possibility of direct serotonergic agonist or antagonist activity at these specific 5-HT receptors was of particular interest. Accordingly, further studies were undertaken to generate IC50 and Ki values for the 5-HT1A and 5-HT2B (agonist site) receptor subtypes. These results are presented in Table 3. The data revealed IC50s and Ki values of below 10 μM for d-MPH and above 100 μM for l-MPH for both serotonergic sites.

Binding to the cellular monoamine transporters of the most prominent neurotransmitters [NE, DA, 5-HT, γ-aminobutyric acid (GABA), and choline] revealed that overall activity was the greatest for the NET and DAT while being negligible for GABA, choline, and 5-HT transporters. Furthermore, MPH isomers exhibited stereoselectivity in transporter binding with greater affinity noted for d-MPH relative to l-MPH at both the NET and DAT sites (Table 4; GABA and choline data not shown).

The results of cellular assays are presented in Table 5 and reveal that cellular uptake inhibition occurred and was more prominent with d-MPH than l-MPH for both NE and DA. However, 5-HT uptake did not differ significantly between the three test compounds and was not a prominent effect relative to the aforementioned effects upon NE or DA.

**DISCUSSION**

This study presents the results of a comprehensive in vitro pharmacological assessment of affinity with receptors, transporters, and ion channels pertinent to CNS activity. Racemic MPH was compared against the separate d- and l-MPH isomers in all assays. Contemporary high-throughput pharmacological screen-
ing techniques, generally used to assess novel drug entities or lead therapeutic compounds during the preclinical development phase (Entzeroth et al. 2000) were applied to racemic MPH and its respective isomers. Additionally, beyond the standard binding affinity assessments at the major monoamine transporters (e.g., DAT, NET) where MPH and other psychostimulants are widely believed to exert their primary pharmacological activity, this investigation was extended to include the assessment of drug binding affinity to a wide array of CNS receptors, ion channels, and transporters (Table 1) in an effort to characterize these compounds better. Such an enantiospecific study can provide preliminary evidence of pharmacologic activity heretofore unrecognized as a component of MPH therapeutic action and/or side effects. Additionally, cellular uptake and release studies were carried out assessing MPH effects on DA, NE, and 5-HT. It must be emphasized that these data are preliminary in terms of representing binding affinities only and do not discriminate between agonist or antagonist activity. Thus, the ability to speculate on the potential clinical implications of these findings is highly limited.

From the IC$_{50}$ data of NE and DA uptake (Table 5), it was revealed that the most potent in vitro pharmacologic effect of MPH, considered as either single enantiomers or as the racemate, was on inhibition of the NET. This differential binding affinity of d-MPH versus l-MPH at the major amine transporters appears to replicate the findings of several others that have been reported previously (Patrick et al. 1987; Pan et al. 1994; Wall et al. 1995). In microdialysis studies in rats, both MPH and the NE reuptake inhibitor atomoxetine have been found to increase significantly the rate of NE release in the prefrontal cortex in a dose-dependent fashion (Bymaster et al. 2002). The d-MPH isomer was found to be 13.8 times more potent at the NET relative to the l-MPH isomer in the present study (0.47/0.034 = 13.8). Nu-

### Table 4. Results of In Vitro Transporter Binding Affinities for MPH Isomers of the Major Monoamine Transporters

<table>
<thead>
<tr>
<th>Transporter (h)</th>
<th>Norepinephrine (NET)</th>
<th>Dopamine (DAT)</th>
<th>Serotonin (SERT)</th>
<th>Methylphenidate compound (% inhibition of control specific binding)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[3H]Nisoxetine</td>
<td>[3H]GBR-12935</td>
<td>[3H]Imipramine</td>
<td>d-MPH</td>
</tr>
<tr>
<td>Norepinephrine (NET) (h)</td>
<td>99</td>
<td>44</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Dopamine (DAT) (h)</td>
<td>61</td>
<td>37</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Serotonin (SERT) (h)</td>
<td>98</td>
<td>45</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

MPH = methylphenidate; NET = norepinephrine transporter; DAT = dopamine transporter; SERT = serotonin transporter; h = human; BTCP = N-[1-(Benzo[b]thien-2-yl-cyclohexyl)]piperidine; [3H]GBR-12935 = 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine.

Concentrations of dl-MPH, d-MPH, and l-MPH were weight-based, i.e., experiments using the racemate provided 50% less d-MPH than those using the pure isomer.

### Table 5. Results of In Vitro Cellular Uptake Studies of d-MPH, l-MPH, and dl-MPH

<table>
<thead>
<tr>
<th>Assay</th>
<th>Substrate</th>
<th>Reaction product</th>
<th>Methylphenidate (IC$_{50}$ expressed in μM)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>d-MPH</td>
</tr>
<tr>
<td>NE uptake</td>
<td>[3H]NE</td>
<td>[3H]NE incorporation into synaptosomes</td>
<td>0.034</td>
</tr>
<tr>
<td>DA uptake</td>
<td>[3H]DA</td>
<td>[3H]DA incorporation into synaptosomes</td>
<td>0.19</td>
</tr>
<tr>
<td>5-HT uptake</td>
<td>[3H]5-HT</td>
<td>[3H]5-HT incorporation into synaptosomes</td>
<td>17</td>
</tr>
</tbody>
</table>

MPH = methylphenidate; NE = norepinephrine; DA = dopamine; 5-HT = serotonin.
merous previous reports assessing transporter binding of MPH to the major monoamine transporters have shown a general interstudy consistency of relative binding affinities for NE, DA, and 5-HT transporters. Recently, Arnsten and Dudley (2005), using a rat model, reported that some of the beneficial effects of racemic MPH on cognition were attributed to a facilitation of NE- and DA-mediated stimulation of $\alpha_2$ adrenoreceptors and $D_1$ receptors, respectively, in the prefrontal cortex.

Although the present study did not find any direct binding affinity at $\alpha_2$ or $D_1$ receptors by any MPH isomer, the work of Arnsten and Dudley highlights the fact that indirect effects of MPH are clearly possible at these and other receptors via modulation of endogenous monoamines. ADHD has been described as a disorder with a significant noradrenergic component (Biederman et al. 1999; Bymaster et al. 2002; Pliszka 2005), and, interestingly, Yang and associates (2004) recently reported an association between response to $d$-MPH and a genetic polymorphism associated with the NET.

The 5-HT$_{1A}$ receptors are localized dendritically as inhibitory autoreceptors on serotonergic cell bodies of the median raphe nucleus, which predominantly innervates the dorsal hippocampus, septum, and hypothalamus, as well as the dorsal raphe nucleus providing input into the frontal cortex, ventral hippocampus, and striatum (Barnes and Sharpe 1999). Furthermore, postsynaptic 5-HT$_{1A}$ sites are abundant in the frontal cortex, hippocampus, and other corticolimbic structures. Accordingly, both pre- and postsynaptic 5-HT$_{1A}$ receptors are likely to contribute to the modulation of mood, cognition, and motor behavior (Barnes and Sharpe 1999). In addition, the 5-HT$_{1A}$ receptors are known to modulate dopaminergic activity directly and the 5-HT$_{1A}$ agonist 8-OH-DPAT has been shown to reverse certain MPH-induced stereotyped gnawing (Kleven et al. 1996). At present, relative to 5-HT$_{1A}$, much less is known about the 5-HT$_{2B}$ receptor, its brain distribution, or general neuropharmacology (Barnes and Sharpe 1999).

The finding of modest, yet stereoselective, binding of $d$-MPH to the 5-HT$_{1A}$ and 5-HT$_{2B}$ receptor sites was not anticipated at the outset of this study. This finding is of interest given the recognized role of serotonergic neurotransmission in many psychiatric disorders (Naughton et al. 2000; Huang et al. 2004) as well as associations between 5-HT and ADHD (Faraone 2005). Additionally, there is some data indicating that psychostimulant medications may directly or indirectly influence 5-HT (Gainetdinov et al. 1999; Quist 2001). However, it must be emphasized that the nature of the current binding affinity studies preclude distinguishing between agonist, antagonist, partial agonist, or mixed agonist-antagonist activity at either 5-HT receptor site, and further functional assays and/or animal experiments are required for this determination. Furthermore, the pharmacological consequences of the observed 5-HT binding in vitro, if any, may be entirely regiospecific within the CNS. It is intriguing that, although the majority of published research on ADHD, its pathogenesis, and its pharmacotherapy with MPH has focused on DA and/or NE transport or receptor binding, there is an existing literature supporting at least a partial role for 5-HT in ADHD and the pharmacological activity of medications used to treat ADHD successfully, including MPH (Gainetdinov et al. 1999; Quist and Kennedy 2001; Davids et al. 2002b; Quist et al. 2003). Additionally, molecular genetic studies have identified several candidate 5-HT-related genes that may be implicated in the pathogenesis of ADHD (see Faraone 2005).

In conclusion, this exploratory investigation found in vitro pharmacological evidence for a potential multimodal effect of MPH on neurotransmitter sites likely to be involved in producing therapeutic effects in the treatment of ADHD. The most prominent effect, as measured by NET binding and cellular uptake studies, appeared to implicate NE as a key neurotransmitter in MPH effects with somewhat lesser effects on DA. In both instances, $d$-MPH accounted for essentially all of the activity. Binding affinity was also apparent at two of sixteen 5-HT subreceptor binding sites assessed; 5-HT$_{1A}$ and 5-HT$_{2B}$. Further studies are necessary to determine the nature of receptor binding at these sites (i.e., agonist vs antagonist), and to relate binding affinity to any potential behavioral effects of administered
d-MPH in humans. Finally, all pharmacologic effects were more potently mediated by the d-MPH isomer in comparison to l-MPH or d,l-MPH, further establishing d-MPH as the active component of racemic MPH pertaining to the treatment of ADHD.

**DISCLOSURES**

Dr. Markowitz has affiliations with Eli Lilly & Co. and Novartis Pharmaceuticals. Dr. Devane has affiliations with Janssen Pharmaceuticals and Eli Lilly & Co and has been a consultant to Novadel Pharmaceuticals, GlaxoSmithKline, Janssen Pharmaceuticals, Eli Lilly & Co, and Bristol Myers Squibb. Ms. Pestreich in an employee of Novartis Pharmaceuticals. Dr. Patrick has affiliations with Celgene, Johnson and Johnson, and Clariant. Dr. Muniz is an employee of Novartis Pharmaceuticals.

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