Synthesis and Pharmacology of Ethylphenidate Enantiomers: The Human Transesterification Metabolite of Methylphenidate and Ethanol

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Received November 9, 2004

Ethanol elevates methylphenidate (**1**) plasma concentrations and yields the metabolite ethylphenidate (**2**). The therapeutic implications are under investigation. The IC_{50} for dopamine reuptake inhibition by (+)-**2** was 27 nM compared to 367 nM for cocaine and 1730 nM for (−)-**2**. Binding selectivity for dopamine versus norepinephrine transporters was greater for (+)-**2** than for cocaine. Intraperitoneal (+)-**2** was approximately half as active as (+)-**1** in stimulating mouse motor activity at 5 mg/kg, but (+)-**2** was as active as (+)-**1** at 10 mg/kg.

Introduction

Attention-deficit hyperactivity/disorder (ADHD) is the most frequently diagnosed childhood neurobehavioral health problem, and (±)-*threo*-methylphenidate [(±)-**1**, Figure 1] is the drug most widely prescribed for its treatment.[1] Since the 1980s, the persistence of ADHD into adolescence and adulthood has been increasingly recognized.[2] Appropriate drug therapy for this older ADHD population requires a special consideration of lifestyle comorbidity. For example, substance/alcohol abuse and dependence appear to be over-represented in adult ADHD.[3,4] Accordingly, prescribing psychostimulants to this population has generated concern.[5] In this context, nothing is known regarding the pharmacology of concomitant **1** and ethanol use or abuse. This concern is justified by numerous recent reports of **1**−ethanol coabuse[6,7] and by emergency department statistics on **1**−ethanol drug combination episodes. In 1997 and 1999, respectively, there were 553 and 422 **1**−ethanol emergency episodes documented.[8]

The metabolic hydrolysis of methyl ester **1** yields inactive[9] ritalinic acid (**3**, Figure 1). This facile process limits the absolute bioavailability of the enantiomers of **1** to <50% for the (+)-isomer and *usually* <5% for the (−)-isomer, and results in the relatively short 2−3 h elimination half-life of **1**.[1] However, consumption of ethanol while receiving **1** results in the methyl ester also being transesterified to yield the ethyl ester ethylphenidate (**2**, Figure 1).[1,10−13] The specific carboxylesterase isoform primarily responsible for the hydrolysis of **1** may similarly catalyze this ethanolysis pathway.[14−16] The formation of **2** in humans dosed with **1** and ethanol occurs enantioselectively.[1,12,13] Further, concomitant ethanol results in significantly elevated plasma concentrations of the parent drug **1**.[1,13]

The separate enantiomers of **2** have not been pharmacologically characterized, and little is known about the activity of racemic **2**. Portoghese and Malspeis[17] reported that (±)-**2** induces locomotor activity in mice with approximately 80% of the potency of (±)-**1**, and Schweri et al.[18] found that (±)-**2** exhibits approximately 50% of the potency of (±)-**1** in inhibiting [^{3}H]-(±)-**1** binding to rat striatal synaptosomal membranes. This inhibition assay models the putative stimulant mode of action of **1**, i.e., uptake inhibition of impulse released dopamine by the presynaptic dopamine transporter (DAT).[19−21]

Figure 1. Left: (+)-(2*R*,2′*R*)-*threo*-methylphenidate [(+)-**1**, R = CH_3)], (+)-(2*R*,2′*R*)-*threo*-ethylphenidate [(+)-**2**, R = CH_2-CH_3], and (2*R*,2′*R*)-*threo*-ritalinic acid [(2*R*,2′*R*)-**3**, R = H]. Right: (−)-(2*S*,2′*S*)-*threo*-methylphenidate [(−)-**1**, R = CH_3)], (−)-(2*S*,2′*S*)-*threo*-ethylphenidate [(−)-**2**, R = CH_2CH_3], and (2*S*,2′*S*)-*threo*-ritalinic acid [(2*S*,2′*S*)-**3**, R = H].

Any potential pharmacodynamic significance regarding the enantioselective formation of metabolite **2** after concomitant **1** and ethanol cannot be assessed without first characterizing the activity of each enantiomer of **2**. Accordingly, enantiomeric **2** and racemic **2** were synthesized and evaluated in vivo for dose−response effects on locomotor activity in C57BL/6J mice, as well as in vitro for monoamine uptake inhibition activity and receptor binding affinity. The behavioral effects of metabolites (+)-**2** and (−)-**2** were gauged against the enantiomers of parent drug **1**.

The receptor pharmacology of (+)-**2** and (−)-**2** was evaluated in context to the prototypic DAT inhibitor cocaine[22] and of racemic **1**.

Results

Chemistry. Enantiomeric and racemic **2** were synthesized by standard methods. Gas chromatography−mass spectrometry (GC−MS) of (*S*)-prolyl diastereomeric derivatives of **2** established the enantiopurity of the synthesized compounds.[19]

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10.1021/jm0490989 CCC: $30.25
Published on Web 03/25/2005

Table 1. In Vitro Monoamine Activities: Ethylphenidate (**2**) Isomers Compared to (±)-Methylphenidate (**1**) and Cocaine[a]

	IC_{50}/K_i (nM)				
	(±)-**1**[b]	(+)-**2**	(−)-**2**	(±)-**2**	cocaine
DAT uptake inhibition	19.9 ± 7.7	27 ± 6	1730± 180	95 ± 18	289 ± 38
DAT binding	121.7 ± 5.7	230 ± 100	2700 ± 540	319 ± 98	367 ± 45
NET uptake inhibition	51 ± 21	290 ± 120	>10 μM	480 ± 200	462 ± 89
NET binding	788 ± 98	3700± 580	>10 μM	>7200	2,990 ± 300
SERT uptake inhibition	ND	ND	>10000	>10000	289 ± 28
SERT binding	>10000	>10000	>9500	7540 ± 400	433 ± 16

[a] DAT, dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter; ND, not determined. [b] Dr. Richard Kline, NIDA, personal communication.

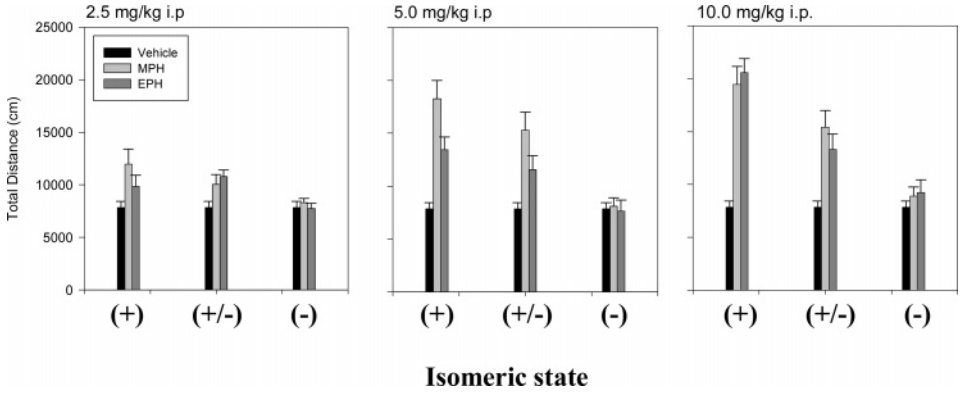

Figure 2. Motor activity effects of enantiomeric [(+) and (−)] and racemic (±) methylphenidate·HCl (**1**; MPH) and ethylphenidate·HCl (**2**; EPH). Values represent cumulative 90 min responses to 2.5 (left), 5 (center), and 10 mg/kg (right) intraperitonal injections in C57B/L mice (n = 10/group). Vehicle was normal saline.

In Vitro Experiments: Monoamine Transporters. The Cocaine Treatment Discovery Program of the National Institute on Drug Abuse (NIDA) performed the binding affinity and uptake inhibition evaluations of (+)-, (−)-, and (±)-**2**. Their monoamine transporter studies used human embryonic kidney (HEK) cell cultures expressing the human transporters for dopamine, norepinephrine (hNET), or serotonin (hSERT). These activities were compared against those of the prototypic DAT inhibitor cocaine.[22]

As with the enantiomers of parent drug **1**,[19] the (*R,R*)-configuration found in metabolite (+)-**2** provided for the greatest in vitro activities in the DAT and NET cellular systems (Table 1). On the basis of NIDA archival values for racemic **1**, the racemate of **2** was less active in these catecholaminergic transporter models. (+)-**2** exhibited approximately an order of magnitude greater potency than cocaine in inhibiting [^{3}H]dopamine uptake: IC_{50} = 27 nM for (+)-**2** versus 235 nM for cocaine. (+)-**2** was marginally more active than cocaine in displacing the potent radiolabeled DAT ligand 3β-(4-iodophenyl)tropan-2β-carboxylic acid methyl ester ([^{125}I]RTl-55) from DAT binding: 230 nM for (+)-**2** versus 367 nM for cocaine. (−)-**2** showed very little activity in these in vitro evaluations, while the racemate of **2** exhibited the anticipated intermediate activity.

The binding selectivity for the hDAT relative to the hNET was approximately 10-fold greater for (+)-**2** than for cocaine (Table 1). [^{3}H]Norepinephrine uptake inhibition assays demonstrated similar potencies for (+)-**2** and cocaine: IC_{50} = 290 nM for (+)-**2** compared to IC_{50} = 462 nM for cocaine.

In HEK-hSERT cells, the binding affinity of (+)-**2** for the SERT was much less than for cocaine; K_i for the displacement of [^{125}I]RTl-55 was >10 μM for (+)-**2** versus 433 nM for cocaine. Uptake assays of **2** were not conducted because K_i was greater than 10 μM (289 ± 28 nM for cocaine).

In Vitro Experiments: Monoamine Receptors. No significant postsynaptic receptor function activity was found for isomers of **2** when evaluated by the standardized NIDA protocols for D_{1-3} and 5-$HT_{1A,2A,2C}$ interactions.

In Vivo Experiments. The effects of a range of (+)-, (−)-, and (±)-**1** and (+)-, (−)-, and (±)-**2** doses on motor activity are summarized in Figure 2. Because motor activity had reached constant low levels of activity by 90 min after injection for all compounds at all doses, only cumulative activity prior to this time point was subjected to analyses. As noted in Figure 2, both the (+)- and (±)-forms of each compound produced dose-dependent increases in motor activity in comparison to vehicle values. No statistically significant effects were produced by the (−)-isomers of **1** and **2**. At the 5.0 mg/kg intraperitoneal dose, motor activity over the 90-min test period was elevated less by (+)- and (±)-**2** than by (+)- and (±)-**1**. At the 10 mg/kg dose, the increases in motor activity were similar for (+)-**1** versus (+)-**2** and for (±)-**1** versus (±)-**2**.

To facilitate behavioral comparisons of the metabolite **2** with the parent drug **1**, motor activity data generated by the isomeric compounds were subjected to independent 2 × 3 (**2** vs **1**) × (isomer) analyses of variance (ANOVAs) for each drug dose. The 2.5 mg/kg doses of the compounds did not elevate motor activity in mice relative to saline controls [$F_{(1,54)} < 1$]. Activity did differ on the basis of isomeric state, with greater activity for the (+)-enantiomer and for racemic modifications in comparison to the (−)-enantiomer [$F_{(2,54)} = 5.863$, $p < 0.01$; Duncan's post hoc tests, $p < 0.05$]. The (+)- and (±)-derivatives elevated motor activity at the 5 mg/kg dose [$F_{(1,53)} = 7.720$, $p < 0.01$], unlike the (−)-isomers

[$F_{(2,53)} = 19.01$, $p < 0.01$; Duncan's post hoc tests, $p < 0.05$]. The effects of **1** and **2** at the 10.0 mg/kg dose did not differ [$F_{(1,54)} < 1$]; however, there were differences associated with the isomeric state [$F_{(2,54)} = 31.045$, $p < 0.001$]. At this dose, the enantiomeric forms differed from each other, with activity being greater for the (+)-enantiomer than for the racemate and the racemate activity being greater than for the (−)-form [Duncan's post hoc tests, $p < 0.05$].

Discussion

Upon development of a preparative-scale method for resolving (±)-**1**,[19] it was established that (+)-**1** was the component enantiomer of the racemic drug responsible for increasing motor activity,[19] elevating blood pressure,[19] and reducing appetite[23] in rats. In mechanistic studies, inhibition of [^{3}H]dopamine uptake into striatal synaptosomes was also limited to the (+)-isomer.[19] Imaging investigations in humans have since reveled that specific binding to ADHD-relevant dopaminergic brain regions is associated with only (+)-**1**.[24] Additional clinical investigations have demonstrated that (+)-**1** produces the therapeutic effects of **1** in ADHD[25] and narcoleptic[26] patients. These, among additional findings,[1] prompted the development of the new "chiral or racemic switch" product (+)-**1** (Focalin, Novartis), released in 2002 as an alternative to the several existing (±)-**1** formulations used to treat ADHD. It now appears that in the absence of (−)-**1**, the pharmacodynamic actions of (+)-**1** are prolonged.[27]

Baldessarini and Cambell[28] recently patented (−)-**1** as an antidote to stimulant overdose based on the finding that (−)-**1** antagonizes behavioral effects of (+)-**1**, cocaine, or apomorphine in rats. In other preclinical studies, Davids et al.[29] reported that (−)-**1** may not merely be a passive isomeric component of racemic **1**. The (−)-enantiomer opposed the actions of (+)-**1** in 6-hydroxydopamine (a catecholaminergic neurotoxin) lesioned rats. Further, examining the behavioral effects of (−)-**1** and (+)-**1** in nonlesioned rats revealed that females were more sensitive than males to some effects of the (−)-isomer (and that females were more sensitive to both isomers in other elements of an observational battery).[30]

Metabolic transesterification of methyl ester **1** to ethyl ester **2** appears to be a biotransformation analogous to the well-established cocaine−ethanol interaction where human esterase(s) transesterify the methyl ester of cocaine to form cocaethylene (the ethyl ester of cocaine).[31] Ethanol has been reported to elevate plasma cocaine concentrations,[32] much as ethanol elevates plasma **1** concentrations.[1,13] X-ray crystallography depicts both (+)-**1** and cocaine displaying a near-superimposable pharmacophore: the amine, phenyl ring, and methyl ester moiety.[33] Such structural similarities between **1** and cocaine may pertain to a common enzymatic transesterification pathway for esters **1** and cocaine, both yielding ethyl esters.

Bourland and co-workers[34] incubated (±)-**1** with ethanol in a rat liver preparation and reported the detection of the product **2**. Subsequently, Markowitz et al.[10] quantified **2** in postmortem blood and liver from two suicide cases involving concomitant **1** and ethanol. The lethal drug doses were unknown. This prompted controlled clinical studies of **1**−ethanol metabolic[11−13] and pharmacodynamic (unpublished) interactions. The metabolite **2** was found in human plasma primarily as the (−)-isomer, where it reached low ng/mL values while (+)-**2** concentrations were in the pg/mL range.[1,13] These studies also demonstrated that ethanol significantly elevates maximum plasma concentrations of (+)-**1** and the area under the plasma concentration−time profile of (+)-**1**.[13] The clinical implications of these findings are under active investigation.

Ethyl ester **2** has served as an internal standard in pharmacokinetic studies of **1**.[35] However, in view of the slower rate of chemical hydrolysis of **2** relative to **1**,[17] it cannot control for possible hydrolytic loss of **1** during sample processing. This concern with differential rates of **1** vs **2** hydrolysis in biological samples, in addition to former problems of baseline chromatographic resolution between **1** and **2**,[36] has been overcome through the utilization of piperidine deuterated **1** as an internal standard and analytical separation by mass spectrometry.[13,36] Owing to **2** now being recognized as a metabolite of **1**, its role as an internal standard becomes even more problematic.

In the present study, the human metabolite (+)-**2** produced potent effects on DAT model systems (Table 1). Racemic **2** was marginally less active than racemic **1** in these tests, though (+)-**2** was approximately an order of magnitude more potent than cocaine in inhibiting [^{3}H]dopamine uptake. (+)-**2** also exhibited greater selectivity than cocaine in inhibiting transmitter uptake at the DAT relative to the NET.

Both (+)-**1** and (+)-**2** elevated motor activity with respect to saline controls (Figure 2). The effect of these eutomers differed at the middle dose, 5 mg/kg, with **2** inducing approximately half the activity recorded for **1**. Neither (−)-**1** nor (−)-**2** was associated with significant behavioral activity. The dose−response evaluation yielded floor and ceiling effects; i.e., differences between the drugs were not observed at the low (2.5 mg/kg) and high (10 mg/kg) doses.

The results of the motor activity experiments are in concordance with the results of the in vitro studies. (−)-**2** was inactive, (+)-**2** was highly active, and (±)-**2** was of intermediate activity. The effects of (+)-**1** and (+)-**2** on motor activity may be driven through activation of dopaminergic systems in the nucleus accumbens, particularly its core.[37] Our observations of stereotypic behavior at the high 10 mg/kg dose are consistent with a dose-dependent breakthrough of (+)-**2** effects into striatal dopaminergic systems.[38]

The present study provides evidence for a molecular level basis/mechanism underlying the behavioral effects induced by the new metabolite **2**. Further characterization of behavioral profiles for **1** and **2** is in progress. These analyses, when complemented with measurements of plasma and brain concentrations, should yield meaningful pharmacokinetic/pharmacodynamic correlations. Exploration of the rewarding and discriminative properties of metabolite **2** could contribute to a fundamental understanding of the abuse liability associated with **1** combined with ethanol. These studies are ultimately directed toward the rational optimization of drug therapy for adult ADHD patients with or without comorbid alcohol use disorder. Ironically, metabolite **2**

has recently been patented as an ADHD medication with diminished abuse potential.[39]

Experimental Section

Chemistry. GC−MS was performed using an Agilent 6890 GC-5973N MS (Wilmington, DE) instrument. The GC used a 5% phenylpolysiloxane 30 m × 0.25 mm, 0.25 μm film thickness fused-silica column (J & W Scientific, Palo Alto, CA), with the helium linear velocity at 42 cm/s. For nonderivatized samples, the GC oven was held at 150 °C for 6 min, followed by a 15 °C/min ramp, then held at 240 °C. MS ionization was by electron impact at −70 eV, acquiring *m/z* 50−700. Chiral derivatized samples were treated with (*S*)-*N*-trifluoroacetylprolyl (TFP) chloride (0.1 M in dichloromethane, 97% ee, Aldrich, Milwaukee, WI) at 40 °C for 45 min. The GC oven was held at 125 °C for 1.5 min, followed by a 20 °C/min ramp to 280 °C while acquiring *m/z* 277 (*N*-trifluoroprolylpiperidyl fragment).[12,19] The minor contribution of the trace impurity (*R*)-TFP chloride to the chromatographically separated diasteriomeric products from (+)-**2** or (−)-**2** was corrected for by analysis of enantiopure methamphetamine derivatized with TFP chloride, followed by subtraction of the resulting ∼1% peak area ratio of (*R*)-TFP-/(*S*)-TFP-methamphetamine as described previously.[19]

Both proton and carbon NMR spectra were obtained on a Varian INOVA spectrometer operating at 400 and 100 MHz, respectively. Proton assignments were made by employing the double quantum filtered COSY (DQ-COSY) experiment acquired in the phase-sensitive mode, and 2 × 256 free induction decays (FIDs) were acquired. Digital resolution in F1 was increased by linear prediction to 1024 points, processed using the Gaussian weighting function and then Fourier transformed. The chemical shifts of unresolved multiplets were based on the chemical shifts of the cross-peaks. Carbon resonances were assigned using gradient versions of the heteronuclear single quantum coherence (gHSQC) and heteronuclear multibond correlation (gHMBC) experiments. In the gHSQC, 128 FIDs were acquired, then use of linear prediction increased the points in F1 to 512. After Gaussian weighting, the FIDs were then Fourier transformed to produce the 2D contour plot. In the gHMBC, 400 FIDs were acquired, then linear prediction increased the points in F1 to 1200. Upon sine-bell weighting, the FIDs were then Fourier transformed to produce the 2D contour plot.

Elemental analyses, optical rotations, and melting points were performed by Quantitative Technologies Inc. (Whitehouse, NJ). (+)- and (−)-**1**·HCl were generous gifts from Celgene Corporation (Warren, NJ). (±)-**1**·HCl was provided by NIDA. Ethanol for synthesis was from AAPER (Shelbyville, KY) and that for GC was from Aldrich (Milwaukee, WI). Diethyl ether and HCl gas were also from Aldrich.

(+)-(2*R*,2*R*′)-*threo*-Ethylphenidate Hydrochloride [(+)-2·HCl, Figure 1]. (+)-**1**·HCl (627 mg, 2.32 mmol) was dissolved in 10% aqueous HCl (60 mL) and refluxed with stirring for 24 h. The solution was evaporated to dryness under reduced pressure, then the flask was purged with nitrogen and desiccated in vacuo overnight. The product (+)-**3**·HCl was used without further purification. Ethanolic HCl (100 mL) was added to the flask containing (2*R*,2′*R*)-**3**·HCl. After refluxing for 24 h under nitrogen, the solution was evaporated to dryness under reduced pressure. Crystallization from ethanol−diethyl ether yielded white solid (+)-**2**·HCl (66%): mp 224−226 °C; as the TFP derivative, t_R = 11.0 min (98% ee); ^{1}H NMR (400 MHz, CD_3OD) δ 7.40 (m, 2H, H-3″,5″), 7.38 (m, 1H, H-4″), 7.30 (m, 2H, H-2″,6″), 4.20 (m, 2H, CH_2), 3.83 (m, 1H, H-2), 3.82 (m, 1H, H-2′), 3.43, 3.10 (m, 2H, H-6′), 1.87, 1.67 (m, 2H, H-5′), 1.51, 1.35 (m, 2H, H-3′), 1.79, 1.28 (m, 2H, H-4′), 1.16 (t, 3H, *J* = Hz, CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 172.9 (C-1), 135.4 (C-1″), 130.6 (C-3″,5″), 129.9 (C-4″), 129.7 (C-2″,6″), 63.2 (CH_2), 59.3 (C-2′), 55.6 (C-2), 46.7 (C-6′), 27.8 (C-3′), 23.5 (C-5′), 22.8 (C-4′), 14.2 (CH_3); MS-EI *m/z* 246 (M − 1, <1%), 84 (M − 163, 100%), 91 (M − 156, 30%), 55 (M − 192, 6.0%), 164 (M − 83, 6.0%), 56 (M − 191, 5.0%), 85 (M − 162, 5.0%), 65 (M − 182, 3.0%), 115 (M − 132, 3.0%); $[\alpha]^{25}_D$ +65.10 (*c* 1.08, methanol). Anal. ($C_{15}H_{22}Cl\ NO_2$) C, H, N.

(−)-(2*S*,2*S*′)-*threo*-Ethylphenidate Hydrochloride [(−)-2·HCl, Figure 1]. (−)-**2**·HCl was synthesized as above, yielding a white solid (86%): mp 220−222; GC (TFP derivative), t_R = 11.6 min (98+% ee); $[\alpha]^{25}_D$ −66.87 (*c* 1.24, methanol). Anal. ($C_{15}H_{22}Cl\ NO_2$) C, H, N.

(±)-*threo*-Ethylphenidate Hydrochloride [(±)-2·HCl, Figure 1]. (±)-*threo*-Ritalinic acid [(±)-**3**,[9] Figure 1] was esterified by the above method used to synthesize the enantiomers of **2**, yielding a white solid: mp 197−199 °C; GC t_R = 10.1 min underivatized (99%). Anal. ($C_{15}H_{22}ClNO_2$) C, H, N.

In Vitro Pharmacology. All in vitro testing was conducted by NIDA through their standard Cocaine Treatment Testing Program screening.

Biogenic Amine Transporter Binding. (+)-, (−)-, and (±)-**2**·HCl in DMSO (10 mM) was diluted to 50 μM in assay buffer for binding (or to 1 mM for uptake). Further dilutions gave DMSO concentrations of 0.1−0.25%.

Inhibition of [^{125}I]RTI-55 Binding. HEK293 cells expressing hDAT, hSERT, or hNET inserts were grown to 80% confluence. Cell membranes were washed with phosphate-buffered saline (10 mL). Lysis buffer (10 mL, 2mM HEPES with 1 mM EDTA) was added, then cells were centrifuged (30000*g*). The pellet was resuspended in 0.32 M sucrose (12−32 mL) to reflect binding of 10% or less of the total radioactivity. Each assay tube contained 50 μL of membrane preparation (10−15 μg of protein), 25 μL of test compound or compound used to define nonspecific binding or buffer (Krebs-HEPES, pH 7.4, 122 mM NaCl, 2.5 mM $CaCI_2$, 1.2 mM $MgSO_4$, 10 μM pargyline, 100 μM tropolone, 0.2% glucose, and 0.02% ascorbic acid, buffered with 25 mM HEPES), 25 μL of [^{125}I]RTI-55 (40−80 pM), and additional buffer sufficient to bring up the final volume to 250 μL was added. Membranes are preincubated with test compounds for 10 min prior to the addition of [^{125}I]-RTI-55, then incubated at 25 °C for 90 min. Binding was terminated by filtration over GF/C filters using a Tomtec 96-well cell harvester, and when the sample was washed, scintillation fluid was added to each square and filter radioactivity determined using a Wallac μ- or β-plate reader. Specific binding was defined as the difference in binding observed in the presence and absence of 5 μM mazindol (HEK-hDAT and HEK-hNET) or 5 μM imipramine (HEK-hSERT). Two or three independent competition experiments were conducted with duplicate determinations. GraphPAD Prism was used to analyze the data, with IC_{50} values converted to K_i values using the Cheng−Prusoff equation [$K_i = IC_{50}/(1 + ([\text{RTI-55}]/K_d(\text{RTI-55})))$].

Filtration Assay for Inhibition of [^{3}H]Neurotransmitter Uptake in HEK293 Cells Expressing Recombinant Biogenic Amine Transporters. Cells were grown to confluence as described above and then washed. Krebs-HEPES buffer was added, and then the mixture was triturated. Specific uptake was defined as the difference in uptake observed in the presence and absence of 5 μM mazindol (HEK-hDAT and HEK-hNET) or 5 μM imipramine (HEK-hSERT). Cells (50 μL) were added and preincubated with the unknowns for 10 min. The assay was initiated by the addition of [^{3}H]-dopamine, [^{3}H]serotonin, or [^{3}H]norepinephrine (50 μL, 20 nM final concentration). Filtration through Whatman GF/C filters presoaked in 0.05% polyethylenimine was used to terminate uptake after 10 min. IC_{50} values were calculated by applying the GraphPAD Prism program to triplicate curves made up of six test compound concentrations each. Two or three independent determinations of each curve were made.

Numbers represent the mean ± SEM from at least three independent experiments, each conducted with duplicate (for binding assays) or triplicate (for uptake assays) determinations. When the K_i or the IC_{50} for the test compound was greater than 10 μM, only two experiments were conducted and no standard error was reported.

In Vivo Pharmacology. Animals. Male C57BL/6 mice were obtained from the Jackson Laboratories (Bar Harbor, ME) at 49 days of age. They were individually housed in a

colony room on a 12-h light/dark cycle (light: 0700−1900) for at least 4 days prior to the start of behavioral tests. All experiments were approved by and conducted within the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina and followed the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

Drugs and Treatment Groups. Animals were assigned to 1 of 18 treatment groups ($n = 10$/group). Treatment groups consisted of low (2.5 mg/kg), medium (5.0 mg/kg), and high (10.0 mg/kg) doses of each of the six compounds [(+)-, (−)-, and (±)-**1**·HCl and (+)-, (−)-, and (±)-**2**·HCl]. All drugs were dissolved in 0.9% saline. An additional control group ($n = 20$) received injections of saline and provided basal levels of motor activity during 3 h of motor activity tests. All drugs were administered at 0.01 mL/g body weight by the intraperitoneal route immediately before testing.

Apparatus. Motor activity was assessed with a Digiscan animal activity monitor system, model RXYZCM (8) TAO with a two-animal option (Omnitech Electronics, Columbus, OH). Each activity chamber contained a total of 16 photobeams spaced 5 cm apart, with 8 beams located on the *x*-axis and 8 on the *y*-axis. Photocells were activated when the photobeams on the wall directly opposite the cells were interrupted. The Digiscan analyzer recorded the interruption of each beam and provided the total distance (in cm) each animal traveled during testing. Each activity chamber was partitioned into 20 cm × 20 cm quadrants with acrylic dividers to allow simultaneous testing of two mice. Three activity chambers allowed testing of six mice per session. Each activity chamber was enclosed in 90 cm × 54 cm × 35 cm sound-attenuated box. The Digiscan analyzer was interfaced with a PC-type computer using ILAM software (Coulbourn Instruments, Lehigh Valley, PA).

Procedures for Motor Activity Assessment. Activity was assessed for a 3 h period 4 days per week between 1200 and 1600 h over a 10 week period. One animal from each of the 18 treatment groups was tested in each week of the experiment, with the exception of the saline group in which 2 animals were tested each week**.** Since a subject from each of the 19 groups could not be represented during each day of testing, the order for each of the treatment groups to be tested within each week and the particular test chamber in which the animal was tested were counterbalanced. Thus, data generated by mice in each treatment group came from all of the different activity chambers and occurred with equal frequency on each day of the test week. The counterbalancing eliminated any contribution of possible differences in activity monitors or test day during the week to observed drug effects on motor activity. Each animal was subjected to only one drug and dose and 1 day of testing to minimize confounds that may result from multiple exposures to **1**·HCl or **2**·HCl or from differences in the extent of habituation to the activity chambers. Mice received an intraperitoneal injection of the assigned drug and were placed in the activity chamber. Horizontal movements (measured in centimeters traveled) were recorded in 5 min bins for the entire 3 h session.

Acknowledgment. The authors appreciate the generous donation of (+)- and (−)-**1**·HCl by the Celgene Corporation, with special thanks to Dr. Steve Teo and Ms. Elizabeth Pensabene. We thank NIDA for supplying (±)-**1** and especially for the involvement of their Cocaine Treatment Discovery Program investigators Drs. Jane Acri and Richard Kline and the associated laboratories of Drs. Aaron Janowsky (transporter testing) and Nathan Apple (receptor function). We are grateful for the support from the Medical University of South Carolina NMR Resource Facility, for Institutional Research Funds, and for the sponsorship by NIH/NIDA (Grant No. RO1 DA-15797).

Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0490989