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Syntheses of 3-Carbomethoxy-4-(aryl)piperidines and In Vitro and In Vivo Pharmacological Evaluation: Identification of Inhibitors of the Human Dopamine Transporter

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Abstract—A series of 3-carbomethoxy-4-(aryl-substituted)piperidines with various aryl groups were synthesized and examined for binding and reuptake inhibition at the human dopamine transporter, the human serotonin transporter, and the human norepinephrine transporter. The binding potency and reuptake inhibition efficacy was compared with that of (−)-cocaine to determine the significance of removing the two-carbon bridge of the cocaine nucleus on the inhibition of transporter binding and reuptake. Of the transporters examined, the substituted piperidines were relatively selective for the human dopamine transporter. In all cases examined, the *cis*-diastereomer of the 3-carbomethoxy-4-(aryl-substituted)piperidine was observed to be a more potent inhibitor of the human dopamine transporter than the *trans* diastereomer. Based on the K_i (binding) and IC_{50} (reuptake inhibition) values obtained, the most potent inhibitor of the series was *cis*-3-carbomethoxy-4-(4′-chlorophenyl)piperidine, and this compound suppressed spontaneous- and cocaine-induced stimulation in non-habituated male Swiss-Webster mice. The conclusion is that substantial portions of the cocaine structure can be dissected away to provide compounds with significant binding and reuptake inhibition of the human dopamine transporter.

Introduction

The plant alkaloid cocaine from the leaves of *Erythroxylon coca* is a potent central nervous stimulant. Cocaine abuse and addiction are serious medical problems in the United States[1] and toxicity due to cocaine overuse includes cardiac arrest, stroke, grand-mal seizures and drug-induced psychosis.[2]

Development of therapies to combat cocaine addiction and abuse is an important goal. While significant advances in the use of enzymes[3–6] and catalytic antibodies[6–8] to protect against the toxicity of cocaine by inactivation have been reported, small-molecule-based approaches for medications development of cocaine antagonists have dominated the field.[9–11] Although compounds that interact with serotonin (5-HT) and norepinephrine (NE) systems are involved in modulating the pharmacological effects of cocaine, it is the dopamine transporter (DAT), controlling the uptake of the neurotransmitter dopamine (DA) that is most often targeted in anti-cocaine abuse medications development.[11] One of the reinforcing (addictive) properties of cocaine stems from the inhibition of binding to and the re-uptake of DA, 5-HT and NE, into the pre-synaptic neurons. In theory, development of compounds that potently and selectively bind to the human DAT and do not inhibit DA re-uptake or only modestly inhibit DA re-uptake may be useful as agents to antagonize or partially antagonize, respectively, the pharmacological properties of cocaine.

Using the structure of cocaine as a lead, molecular dissection of the aryltropane nucleus by removing the 2-carbon bridge showed that 3-carbomethoxy-4-(aryl-substituted)piperidines possessed considerable human DAT affinity and re-uptake inhibition potency. The goal of our work was to examine the effect of aromatic substitution on the binding and reuptake inhibition of 3-carbomethoxy-4-(aryl-substituted)piperidines at the

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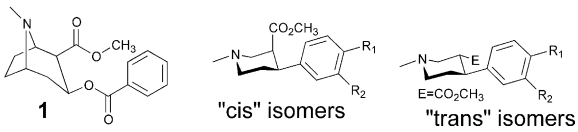


Figure 1. Chemical structure of cocaine, compound **1** and chemical structures of 3-carbomethoxy-4-(aryl)piperidines as *cis* and *trans* diastereomers.

human-DAT, human 5-HT transporter (hSERT) and human NE transporter (hNET). In addition, the stereoselectivity of the process was examined. Highly active compounds identified in vitro were also evaluated in vivo for their ability to alter locomoter activity and compete with the locomoter affects of a parallel dose of cocaine.

Results and Discussion

Molecular dissection of the 2-carbon bridge of cocaine, compound **1**, afforded 3-carbomethoxy-4-(benzoyloxy)-piperidine (Fig. 1). Recognizing that replacement of the benzoyloxy moiety with a phenyl group (as in the case of aryltropanes[12]) in some cases significantly increased the potency of binding to the hDAT and improved the efficacy of inhibition of DA reuptake, a series of 3-carbomethoxy-4-(aryl-substituted)piperidines were stereoselectively synthesized (Fig. 1). The target compounds were tested in binding and uptake inhibition assays in the presence of hDAT, hSERT and hNET. The most active compounds were also evaluated in vivo as inhibitors of mouse locomoter activity.

The 3-carbomethoxy-4-(aryl) piperidines were prepared by combining arecoline free base to the appropriate arylmagnesium bromide in ether/CH_2Cl_2 at -40 °C (Scheme 1). The addition of the aryl group followed by treatment of the resulting product with trifluoroacetic acid in ether at -78 °C and extractive work up provided a mixture of diastereomeric pairs. Using this procedure, generally, a predominance of the *cis*-β-β-compound was obtained. Flash silica gel chromatography readily resolved most of the diastereomeric pairs. The structures of all the compounds were consistent with the proton NMR and mass spectral data.

With the exception of the *p*-methoxy and *m*-methoxy aryl-substituted compounds, each pair of *cis* and *trans* 3-carbomethoxy-4-(aryl-substituted)piperidine diastereomers were examined for potency of inhibition of binding of radiolabelled RTI-55 to the hDAT, hSERT and hNET. As shown in Table 1, compared with cocaine, compound **1**, only compound **2** possessed greater binding affinity for the hDAT. For the hSERT and hNET, inhibition of radiolabelled RTI-55 binding was not significantly more potent than that of cocaine. Inhibition of neurotransmitter uptake by compound **2** was significantly greater than inhibition by cocaine. However, compounds **3**–**12** showed little or no improved efficacy for inhibition of 5-HT or NE reuptake in HEK cells transfected with their corresponding human transporters, as compared with cocaine.

When the IC_{50} value for neurotransporter reuptake inhibition (Table 2) is divided by the binding constant for inhibition of radiolabelled RTI-55 binding (Table 1) to provide a ratio (Table 3) this correlation ratio value can provide insight into the efficacy of a compound as an antagonist of cocaine action. An IC_{50}/K_i correlation ratio of 25 or greater suggests that the

Table 1. Inhibition of radioligand binding in HEK-hDAT, HEK-hSERT and HEK-hNET cells by 3-carbomethoxy-4-(aryl)piperidines[a]

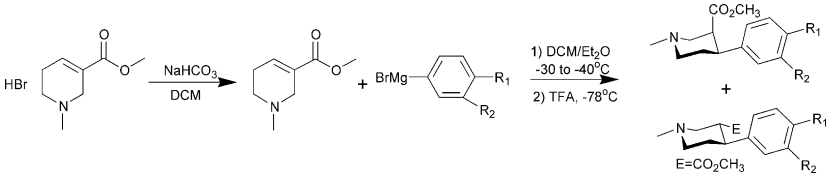
	HEK hDAT		HEK hSERT		HEK hNET	
Compd	K_i (nM)	SEM	K_i (nM)	SEM	K_i (nM)	SEM
1, Cocaine	272	±58	601	±132	830	±147
2	98.88	±6.42	684.03	±205.04	2200.62	±544.54
3	356.21	±17.96	5223.21	±2126.81	2279.26	±842.9
4	339.14	±26.13	2247.74	±330.72	2441.22	±140.85
5	2038.04	±560.75	>10 μM	—	>10 μM	—
6, **7**	4615.01	±894.44	1286.92	±335.13	>10 μM	—
8, **9**	>10 μM	—	2518.3	±688.62	>10 μM	—
10	5134.89	±1462.63	>10 μM	—	>10 μM	—
11	>10 μM	—	>10 μM	—	>10 μM	—
12	2664.96	±604.87	10 μM	—	10 μM	—
13	6830.92	±743.52	>10 μM	—	>10 μM	—

[a]Drug inhibition of [^{125}I]-RTI-55 binding in HEK-hDAT, HEK-hSERT or HEK-hNET cell membranes. Values represent the mean ± SEM for three to four experiments unless the mean of three experiments exceeded 10 μM.

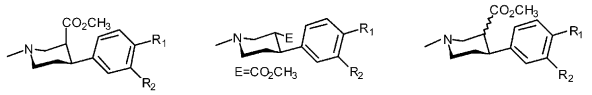
Table 2. Inhibition of radiolabelled neurotransmitter uptake in HEK-hDAT, HEK-hSERT and HEK-hNET cells by 3-carbomethoxy-4-(aryl)piperidines[a]

	HEK hDAT		HEK hSERT		HEK hNET	
Compd	IC_{50} (nM)	SEM	IC_{50} (nM)	SEM	IC_{50} (nM)	SEM
1, cocaine	271	±56	243	±64	188	±48
2	151	±6.88	544	±208	1841	±421
3	325.2	±63.9	3133	±67	2084	±75
4	339.9	±55.9	793	±75	724	±243
5	446.2	±125.7	2868	±931	1127	±387
6, **7**	4736.5	±2048.7	687	±57	>10 μM	—
8, **9**	>10 μM	—	437.6	±224.9	>10 μM	—
10	4657	±2171	2198	±758	1282	±262
11	>10 μM	—	3891	±160	2706	±894
12	1788.7	±580.7	3414	±591	4852	±1852
13	988.44	±465.0	3025	±313	>10 μM	—

[a]Inhibition of [^{3}H]-DA, [^{3}H]-5HT or [^{3}H]-NE in the presence of HEK-hDAT, HEK-hSERT or HEK-hNET cells, respectively. Values are the mean ± SEM for three to four experiments unless the mean of three experiments exceeded 10 μM.



Scheme 1. Synthesis of *cis*- and *trans*-3-carbomethoxy-4-(aryl)piperidines.



"*cis*" isomers			"*trans*" isomers			Mixture of both *cis* and *trans* isomers		
Compound	R_1	R_2	**Compound**	R_1	R_2	**Compound**	R_1	R_2
2	Cl	H	**3**	Cl	H	**6** and **7**	OCH_3	H
4	CH_3	H	**5**	CH_3	H	**8** and **9**	H	OCH_3
10	H	H	**11**	H	H	**13**	F	H
12	F	H						

Figure 2. Chemical structure of *cis*- and *trans*-3-carbomethoxy-4-(aryl)piperidines and corresponding identification numbers.

compound possesses binding potency and reuptake inhibition efficacy of sufficient magnitude to compete with the pharmacological effects exerted by cocaine. As shown in Table 3, however, none of the compounds examined attained the desired level of efficacy.

Compared with cocaine, compounds **2** and **3** were more selective for the hDAT. Thus, the K_i(hSERT)/K_i(hDAT) and K_i(hNET)/K_i(hDAT) values for compounds **2** and **3** were 7, 14.5 and 14.7 and 6.4, respectively. On the basis of the limited number of compounds examined, there does not appear to be a significant electronic substitutent effect on the inhibition of binding to the hDAT or on the inhibition of reuptake of radiolabelled neurotransmitter by HEK cells transfected with hDAT, hSERT or hNET. The *para*-position of the aryl ring may be sensitive to lipophilicity or some other effect because the isoelectronic *cis* compounds **2** and **4** (i.e., *para*-Cl and *para*-CH_3) had among the highest binding and uptake inhibition values. However, there was a significant dependence of pharmacological activity on the stereochemistry of the compound (Fig. 2).

In each example examined, the *cis* diastereomer was more potent an inhibitor of binding and more efficacious an inhibitor of uptake than the corresponding *trans* diastereomer. The preference for *cis* stereoselectivity was pronounced for binding and uptake inhibition at the hDAT, but was less pronounced at the hSERT. There was no apparent stereoselectivity observed for binding at the hNET. The results are in agreement with previous studies that showed that a series of *cis*-3β-carbomethoxy-4β-(*p*-chlorophenyl)piperidine analogues of cocaine exhibited a 2.5-fold increase in affinity at the rat brain DAT compared with cocaine.[13–15]

Table 3. Calculated correlation ratios of uptake inhibition to binding affinity

Compd	HDAT IC_{50}/K_i[a]	hSERT IC_{50}/K_i	HNET IC_{50}/K_i
2	1.53	0.79	0.84
3	0.9	0.6	0.91
4	1.0	0.35	0.296
5	0.22	0.29	0.11
6, **7**	1.03	0.83	1
8, **9**	1.0	0.17	1
10	0.91	0.22	0.13
11	1.0	0.39	0.27
12	0.67	0.34	0.48
13	0.14	0.3	1

[a]The IC_{50} value (Table 2) for potency of uptake inhibition was divided by the K_i value (Table 1) for potency of binding to obtain the correlation ratio listed.

The fumarate salts of compounds **2** and **4** were prepared and tested for suppression of spontaneous locomoter activity in groups of non-habituated male Swiss-Webster mice. Compounds **2** and **4** were individually tested for suppression of spontaneous locomoter activity at intra-peritoneal doses ranging from 3–100 mg/kg in the period of 0–60 min after dosing. The period of 20–60 or 0–30 min was selected for analysis of dose-response data because these were the time periods that maximal suppression first appeared as a function of dose and this is the time period in which cocaine produces its maximal effects. The mean average horizontal activity (counts/10 min) for these time periods was fit to a linear function of $\log_{10}$ dose of the descending portion of the dose–effect curve. The ID_{50} (dose producing $\frac{1}{2}$ maximal depressant activity where maximal depression is zero counts per 30 min) was calculated as 29.5 and 38.3 mg/kg, respectively, for compounds **2** and **4**, respectively.

An interaction study was also conducted examining the effect of various intra-peritoneal doses of compound **2** (3–100 mg/kg) on the stimulant activity of 20 mg/kg of cocaine. The mean average horizontal counts/10 min on the descending portion of the dose-effect curve (10–100 mg/kg dose range) for the 30-min period were fit to a linear function of $\log_{10}$ dose, and the AD_{50} (the dose attenuating cocaine-induced stimulation by 50%) was calculated to be 47.7 mg/kg. After receiving 100 mg/kg of compound **2**, the locomoter activity of 20 mg/kg cocaine was completely suppressed. However, at a dose of 100 mg/kg **2**, the entire group of eight mice showed tremors during the pretreatment period. It is possible that other central nervous system mechanisms contribute to the pharmacological activity observed for **2**.

Conclusion

In summary, a convenient synthetic procedure was used for the procurement of *cis* and *trans* diastereomers of 3-carbomethoxy-4-(aryl)piperidines. This method was similar to the one previously reported.[13–15] Retrosynthetic analysis of the target compounds showed that the structure was derived from molecular dissection of (−)-cocaine and related (−)-aryltropanes. The significance of this approach is that it could lead to highly potent inhibitors and highly efficacious uptake inhibitors

of hDAT that could be substantially free of the addiction liability inherent to the parent compound, (−)-cocaine. Of the 3-carbomethoxy-4-(aryl)piperidines examined, the most active compounds possessed a *para*-chloro aryl substituent or its isosteric equivalent, a *para*-methyl group. This result is in agreement with work published previously.[13–15] A notable degree of stereoselectivity was observed in the potency of binding or in the efficacy of uptake inhibition of hDAT. The *cis* diastereomer was considerably more active than the corresponding *trans* isomer. This is in keeping with studies of (−)-cocaine or (−)-aryltropanes that also show significantly greater pharmacological efficacy for the beta, beta orientation of the C-2 and C-3 substituents of the tropane nucleus. Molecular dissection as shown in Figure 1 afforded compounds with marked hDAT binding and re-uptake inhibition that were considerably selective for the hDAT. However, examination of the most active compounds in vivo showed that only modest suppression of cocaine-induced locomoter activity was observed following administration of compound **2**. Future studies will be directed at examining the molecular basis for the action of 3-carbomethoxy-4-(aryl) piperidines and developing other agents with greater potency that possesses non-toxic and non-addicting properties.

Experimental

Synthesis

General. Chemicals, reagents, buffers and solvents used in this study were of the highest purity available from commercial sources. Solvents used in the reaction (i.e., tetrahydro furan, ether, CH_2Cl_2) were freshly distilled prior to use. Cocaine was provided by the National Institute on Drug Abuse Drug Supply Program, National Institute of Health (Bethesda, MD, USA). RTI-55 was a kind gift from Dr. Ivy Carroll (RTI, Research Triangle Park, NC, USA). Other buffers, reagents and solvents were obtained from VWR (San Diego, CA, USA). Chromatography was done with silica gel 60 (230–400 mesh) from Acros Chemical (Pittsburgh, PA), and thin layer chromatography was done with silica gel F_{254} plates from E. Merck (Darmstadt, Germany). [^{3}H]-DA, [^{3}H]-5HT, [^{3}H]-NE and [^{125}I]RTI-55 were purchased from DuPont—New England Nuclear (Boston, MA, USA). The preparation of the hDAT used was described previously.[16] The hSERT cDNA and HEK cells transfected with hNET was supplied by Dr. Randy Blakely (Vanderbilt University, Nashville, TN, USA). ^{1}H NMR spectra were recorded on a Bruker spectrometer operating at 500 MHz with TMS as an internal standard. Mass spectra were obtained on a HP 1100 MSD (Palo Alto, CA, USA).

General procedure for synthesis of *cis* and *trans* 3-carbomethoxy-4-(aryl) piperidines

4.99 g (1 equiv) of arecoline free base in 40 mL of Et_2O, and 50 mL of CH_2Cl_2 was cooled to −30 to −40 °C and was treated with 64.4 mL (2 equiv) of a 1 M ethereal solution of the arylmagnesium bromide was added to the flask drop-wise. The reaction was stirred at −30 °C to −40 °C for 3 h and then cooled to −78 °C for 30 min. 10 mL of TFA in 10 mL of Et_2O was added to the reaction drop-wise. The reaction was allowed to warm to 0 °C and 30 mL of 1N HCl was added to the mixture followed by enough saturated NH_4OH to raise the pH of the reaction to >12. The aqueous layer was extracted three times with 40 mL of EtOAc. The combined organic extracts were dried over Na_2SO_4 and concentrated to a thick oil. A mixture of diastereomeric products were crudely separated with flash silica chromatography (R_f = 0.3 and 0.2) using Et_3N/hexanes 25/75, v:v) to afford in approximately 40% yield a mixture of *cis* and *trans* compounds in a ratio of 2:1, respectively. Each diastereomer was further purified by silica chromatography ($MeOH/CH_2Cl_2$ 5:95, v:v) to afford highly purified *cis* and *trans* 3-carbomethoxy-4-(aryl)piperidines.

General procedure for preparing the fumarate salts of selective piperidines

To an ethereal solution of the 3-carbomethoxy-4-(aryl)-piperidine (1g, 6.5 mmol) was added 1 equivalent of fumaric acid (0.75 g, 6.5 mmol) in ether. After stirring for 30 min, the reaction mixture was evaporated to dryness to afford a crystalline salt of the desired piperidine.

***cis* 3-Carbomethoxy-4-(4′-chlorophenyl)piperidine, 2.** ^{1}H NMR ($CDCl_3$) δ 1.80 (dd, 1H, J = 2.8, 13.4 Hz), 2.08 (dt, 1H, J = 2.4, 11.2 Hz), 2.29 (s, 3H), 2.35 (dd, 1H, J = 3.2, 11.5 Hz), 2.67 (dq, 1H, J = 3.5, 11.8 Hz), 2.78–2.81 (m, 1H), 2.97–3.00 (m, 2H), 3.18 (dd, 1H, J = 1.0, 11.4 Hz), 3.52 (s, 3H), 7.20–7.26 (m, 5H); EI–MS m/z 268/270 ($M^+ + 1$), 234/235, 202/203, 174/175, 70.

***trans* 3-Carbomethoxy-4-(4′-chlorophenyl)piperidine, 3.** ^{1}H NMR ($CDCl_3$) δ 1.78–1.82 (m, 2H), 2.06.2.11 (m, 1H), 2.16 (t, J = 11.2 Hz), 2.34 (s, 3H), 2.74 (m, 1H), 2.88 (dt, 1H, J = 3.8, 11.2 Hz), 2.93 (br d, 1H, J = 11.7 Hz), 3.07 (dd, 1H, J = 2.6, 7.6 Hz), 3.44 (s, 3H), 7.11–7.26 (m, 5H); EI–MS m/z 268/270 ($M^+ + 1$), 208/210.

***cis* 3-Carbomethoxy-4-(*p*-toluene) piperidine, 4.** ^{1}H NMR ($CDCl_3$) δ 1.81–1.86 (m, 1H), 2.07–2.13 (m, 1H), 2.29 (s, 3H), 2.34 (s, 3H), 2.72–2.76 (m, 1H), 2.88-2.96 (m, 2H), 3.07-3.10 (m, 1H), 3.44 (s, 3H), 7.08 (s, 4H), EI–MS m/z 248 ($M^+ + 1$).

***trans* 3-Carbomethoxy-4-(*p*-toluene)piperidine, 5.** ^{1}H NMR ($CDCl_3$) δ 1.82 (m, 1H), 2.15 (m, 1H), 2.29 (s, 3H), 2.30 (s, 3H), 2.40 (m, 1H), 2.68 (m, 1H), 2.92 (m, 1H), 3.00 (m, 2H), 3.14 (m, 1H), 3.51 (s, 3H), 7.10 (m, 2H), 7.17 (m, 2H), EI–MS m/z 248 ($M^+ + 1$).

***cis*- And *trans* 3-carbomethoxy-4-(4′-methoxyphenyl)-piperidine, 6 and 7.** ^{1}H NMR ($CDCl_3$): δ 2.29 (s, 3H), 3.52 (s, 3H), 7.20-7.26 (m, 5H); EI–MS m/z 263 ($M^+ + 1$), 232, 204, 98.

***cis*- And *trans* 3-carbomethoxy-4-(3′-methoxyphenyl)-piperidine, 8 and 9.** ^{1}H NMR ($CDCl_3$): δ 2.29 (s, 3H),

3.52 (s, 3H), 7.20–7.26 (m, 5H); EI–MS m/z 264 ($M^+ + 1$), 232, 192, 77.

***cis* 3-Carbomethoxy-4-(4′-phenyl)piperidine, 10.** ^{1}H NMR ($CDCl_3$) δ 1.84 (dd, 1H, $J = 3.0$, 12.6 Hz), 2.10 (dt, 1H, $J = 2.2$, 11.2 Hz), 2.29 (s, 3H), 2.38 (dd, 1H, $J = 3.1$, 11.4 Hz), 2.70 (dq, 1H, $J = 2.9$, 12.0 Hz), 2.86 (m, 1H), 2.98–3.02 (m, 2H), 3.18 (dd, 1H, $J = 1.0$, 6.5 Hz), 3.50 (s, 3H), 7.17–7.30 (m, 5H); EI–MS m/z 234 ($M^+ + 1$), 202, 173, 131, 70.

***trans* 3-Carbomethoxy-4-(4′-phenyl)piperidine, (11).** ^{1}H NMR ($CDCl_3$) δ 1.82–1.87 (m, 2H), 2.10 (dt, 1H, $J = 5.6$, 11.2 Hz), 2.19 (t, $J = 11.2$ Hz), 2.34 (s, 3H), 2.75 (dt, 1H, $J = 5.6$, 11.2 Hz), 2.91 (dd, 1H, $J = 3.9$, 11.3 Hz), 2.95 (dd, 1H, $J = 4.8$, 9.2 Hz), 3.10 (dd, 1H, $J = 2.4$, 11.3 Hz), 3.42 (s, 3H), 7.16–7.28 (m, 5H); EI–MS m/z 234 ($M^+ + 1$), 202, 174, 131, 79.

***cis* 3-Carbomethoxy-4-(4′-fluorophenyl)piperidine, (12).** ^{1}H NMR ($CDCl_3$) δ 1.79–1.82 (br d, 1H), 2.09 (br d, 1H), 2.28 (s, 3H), 2.29 (s, 3H), 2.37 (m, 1H), 2.67 (dq, 1H, $J = 2.0$, 12.0 Hz), 2.80 (m, 1H), 2.95–3.00 (m, 2H), 3.16 (m, 1H), 3.51 (s, 3H), 6.90 (m, 2H), 7.25 (m, 2H); EI–MS m/z 252 ($M^+ + 1$), 113.

Transporter binding assays: [^{125}I]RTI-55 binding

HEK-hDAT,-hSERT, or-hNET cells were grown until confluent as described previously.[17] Cells were scraped from plates and centrifuged for 20 min at 30,000g and the pellet was re-suspended in 0.32 M sucrose with a Polytron at a setting of 1 for 5 s. Assays contained 50 μL of membrane preparation, 25 μL of the test compound and 25 μL of [^{125}I]RTI-55 (40–80 pM final concentration) in a final volume of 250 μL Krebs HEPES buffer (25 mM HEPES, 122 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 μM pargyline, 100 μm tropolone, 2 mg glucose/mL and 0.2 mg ascorbic acid/mL at pH 7.4). Membranes were pre-incubated with test compounds for 10 min before addition of [^{125}I]RTI-55. Specific binding was determined as the difference in binding observed in the presence and absence of 5 μM mazindol (HEK-hDAT and hNET) or 5 μM imipramine (HEK-hSERT). The incubations were done in the dark and terminated by filtration onto a Whatman GF/C filter (Whatman, Inc., Clifton, NI, USA) using a 96-well Tomtech cell harvester (Tomtech, Orange, CT, USA). Scintillation fluid was added to each filter spot and radioactivity remaining on the filter was determined using a Wallace β-plate reader (Wallace Labs, Cranbury, NJ, USA).

Inhibition of substrate uptake

HEK-hDAT,-hSERT or -hNET cells were grown as above. Cells were scraped from the plates and suspended cells were added to a 96-well plate containing test compounds and Krebs-HEPES buffer in a final assay volume of 0.5 mL. After a 10-min preincubation in a 25 °C water bath, [^{3}H]-labeled neurotransmitter (50 μL, 20 nM final concentration) was added and the assay was initiated. After 10 min, the incubation was terminated by filtration onto GF/C filters presoaked with 0.05% polyethylenimine using a Tomtech call harvester and scintillation counting as described above. Specific uptake was defined as the difference in uptake in the presence and absence of 5 μM mazindol (hDAT and hNET) or 5 μM imipramine (hSERT).

In vivo studies

Separate non-habituated male Swiss-Webster mice were used in the conduct of this work. Fully approved animal protocols were used and the studies were done in keeping with the NIH standards for use of experimental animals.

Data analysis

GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used to determine the saturation and binding kinetic data. IC_{50} values were converted to K_i values using the Cheng–Prusoff equation.

In vivo evaluation of selected piperidines. Dose–response study. A dose–response study of compounds **2**- and **4**-induced locomotor activity was conducted according to the locomotor activity studies standard operation procedure of the Intramural Research Program of the National Institute on Drug Abuse (Baltimore, MD, USA). The study was conducted using 16 Digiscan locomotor activity testing chambers (40.5×40.5×30.5 cm). Panels of infrared beams (16 beams) and corresponding photodetectors were located in the horizontal direction along the sides of each activity chamber. Separate groups of eight non-habituated male Swiss-Webster mice were injected via the intraperitoneal (IP) route with either vehicle (saline) or the test compounds (3, 10, 30, or 100 mg/kg), 20 min prior to locomotor activity testing. Just prior to placement in the apparatus, all mice received a saline injection IP. In all studies, horizontal activity (interruption of one photocell beam) was measured for 1-h within 10-min periods. Testing was conducted with one mouse per activity chamber.

Interaction study

The interaction study was conducted using 16 Digiscan locomotor activity testing chambers as described in the previous section. Twenty minutes following IP vehicle or compound **2** injections (3, 10, 30, 100 mg/kg), groups of eight non-habituated male Swiss-Webster mice were injected with either 0.9% saline or 20 mg/kg cocaine IP and placed in the Digiscan apparatus for a 1-h session. IC_{50} values were estimated from a linear regression against log doses. A two-way analysis of variance was conducted on horizontal activity counts/10 min of treatment, 10-min periods and the interaction of periods and treatment.

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