Lobeline Analogs with Enhanced Affinity and Selectivity for Plasmalemma and Vesicular Monoamine Transporters

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Abbreviations:  5-HT, 5-hydroxytryptamine, serotonin; 10R-MESP, N-methyl-2R-(2R-hydroxy-2-phenylethyl)6S-(2-phenylethen-1-yl)piperidine; 10S/10R-MEPP, N-methyl-2R-(2R/2S-hydroxy-2-phenylethyl)6S-(2-phenylethyl)piperidine; BSA, bovine serum albumin; DA, dopamine; DAT, dopamine transporter; DTBZ, dihydroxytetrabenazine; EDTA, disodium ethylenediamine tetraacetate; GBR-12909, 1-(2-[bis(4-fluorophenyl)methoxy]ethyl)4-(3-phenylpropyl)piperazine; hDAT, human dopamine transporter; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; HEK, human embryonic kidney 293 cells; HEK-hDAT, HEK-hNET, HEK-hSERT, human
embryonic kidney 293 cells transfected with hDAT, hNET, and hSERT cDNA, respectively; hDAT, human dopamine transporter; hNET, human norepinephrine transporter; hSERT, human serotonin transporter; ketoalkene, $N$-methyl-2$R$-(2-oxo-2-phenylethyl)6$S$-(2-phenylethen-1yl)piperidine; lobeline Tos, lobeline tosylate, lobeline-8-O-tosylate; MLA, methyllycaconitine; MTD, $N$-methyl-2,6-cis-di-trans-styrylpiperidine, meso-transdiene; MTBZ, methoxytetrabenazine; $[^{125}]$RTI-55 ((-)$\beta$-carbomethoxy-$\beta$-(4-iodophenyl)tropane; nAChR, nicotinic receptor; NE, norepinephrine; NET, norepinephrine transporter; PEI, phenylethlenimine; SERT, serotonin transporter; (-)-TTD, $N$-methyl-2$S$,6$S$-di-trans-styrylpiperidine, trans-transdiene; TTX, tetrodotoxin; VMAT2, vesicular monoamine transporter.

**Recommended Section Assignment:** Neuropharmacology
Abstract

Lobeline attenuates the behavioral effects of psychostimulants in rodents and inhibits the function of nicotinic receptors (nAChRs), dopamine transporters (DAT) and vesicular monoamine transporters (VMAT2). Monoamine transporters are considered valid targets for drug development for the treatment of methamphetamine abuse. In the current study, a series of lobeline analogs were evaluated for affinity and selectivity at these targets. None of the analogs were more potent than nicotine at the $[^3\text{H}]$methyllycaconitine binding site ($\alpha_7^*$ nAChR subtype). Lobeline tosylate was equipotent with lobeline inhibiting $[^3\text{H}]$nicotine binding, but 70-fold more potent inhibiting nicotine-evoked $^{86}\text{Rb}^+$ efflux, demonstrating antagonism of $\alpha_4\beta_2^*$ nAChRs. Compared to lobeline, defunctionalized analogs, lobelane, meso-transdiene and (-)-trans-transdiene, showed dramatically reduced affinity at $\alpha_4\beta_2^*$ nAChRs, and 15-100-fold higher affinity (Ki= 1.95, 0.58 and 0.26 $\mu$M, respectively) at DAT. meso-Transdiene and (-)-trans-transdiene competitively inhibited DAT function, whereas lobelane and lobeline acted noncompetitively. $^{10S}/^{10R}$-MEPP and $^{10R}$-MESP were 2-3 orders of magnitude more potent (Ki = 0.01 and 0.04 $\mu$M, respectively) than lobeline inhibiting $[^3\text{H}]$serotonin uptake; $^{10S}/^{10R}$-MEPP showed 600-fold selectivity for this transporter. Uptake results using hDAT and hSERT expressed in HEK-293 cells were consistent with native transporter assays. Lobelane and ketoalkene were 5-fold more potent (Ki= 0.92 and 1.35 $\mu$M, respectively) than lobeline (Ki= 5.46 $\mu$M) inhibiting $[^3\text{H}]$methoxytetrabenazine binding to VMAT2 in vesicle preparations. Thus, structural modification (defunctionalization) of the lobeline molecule markedly decreases affinity for $\alpha_4\beta_2^*$ and $\alpha_7^*$ nAChRs, while increasing affinity for neurotransmitter transporters, affording analogs with enhanced selectivity for these transporters and providing new leads for the treatment of psychostimulant abuse.

Keywords: dopamine, lobeline, lobelane, meso-transdiene, methamphetamine, nicotinic receptors, norepinephrine, serotonin, transporter, vesicular monoamine transporter
Lobeline, an alkaloid from Indian tobacco, inhibits the behavioral and neurochemical effects of psychostimulant drugs of abuse. For example, lobeline attenuates d-amphetamine-, methamphetamine- and nicotine-induced hyperactivity (Green et al., 2001, Miller et al., 2001, 2002), and lobeline inhibits the discriminative stimulus effects of methamphetamine (Miller et al., 2001). Although lobeline is not self-administered, lobeline decreases methamphetamine self-administration in rats, which is not surmounted by increasing methamphetamine unit doses (Harrod et al., 2001, 2003). These results suggest that lobeline lacks abuse liability, while decreasing the stimulant and rewarding effects of methamphetamine via a noncompetitive mechanism of action.

Psychostimulant-induced behavioral activation and reinforcement are mediated, at least in part, via interaction with neurotransmitter transporters, that regulate synaptic dopamine (DA) concentrations (Wise and Bozarth, 1987; Koob, 1992). Methamphetamine is a substrate for the DA transporter (DAT; Sulzer et al., 1995; Johnson et al., 1998) and decreases the activity of the vesicular monoamine transporter (VMAT2; Brown et al., 2000, 2001). Support for a role for VMAT2 in mediating the behavioral effects of stimulant drugs is provided by studies with VMAT2 knockout mice in which amphetamine-induced conditioned place preference is attenuated (Takahashi et al., 1997). While effects on DAT, SERT and/or VMAT2 may not be the only mechanisms responsible for the reinforcing properties of psychostimulants (Rocha et al., 1998; Sora et al., 1998), these neurotransmitter transporters are considered prime targets for developing pharmacotherapies to treat psychostimulant abuse.

Until recently, the pharmacological activity of lobeline was believed to primarily result from its high affinity interaction with nicotinic acetylcholine receptors (nAChRs; Abood et al., 1988; Reavill et al., 1990; Bhat et al., 1991; Court et al., 1994). Lobeline inhibits nAChR
subtypes mediating both nicotine-evoked $[^3H]$DA release and nicotine-evoked $^{86}$Rb$^+$ efflux (Miller et al., 2000). However, lobeline also interacts with VMAT2 and DAT (Dwoskin and Crooks, 2002). Lobeline potently inhibits $[^3H]$dihydroxytetrabenazine (DTBZ) binding to VMAT2 ($IC_{50} = 0.90 \, \mu M$) and inhibits $[^3H]$DA uptake ($IC_{50} = 0.88 \, \mu M$) into rat striatal vesicle preparations (Teng et al., 1997, 1998), and furthermore is ~90-fold less potent ($IC_{50} = 80 \, \mu M$) inhibiting $[^3H]$DA uptake into rat striatal synaptosomes (Teng et al., 1997). In addition to inhibiting DAT and VMAT2 function, high concentrations of lobeline (10-50 $\mu M$) increase $[^3H]$serotonin (5-HT) release from rat hippocampal slices in a mecamylamine-insensitive manner (Lendvai et al., 1996), suggesting that lobeline interacts with SERT. Thus, in addition to interacting with nAChRs, lobeline also inhibits VMAT2 more potently than DAT or SERT, suggesting that VMAT2 may be a critical target for its pharmacological activity.

Consistent with the observation that lobeline is not self-administered in rats (Harrod et al., 2003), lobeline does not evoke DA release, but stimulates dihydroxyphenylacetic acid overflow (Teng et al., 1997), which likely results from alterations in presynaptic DA storage via an interaction of lobeline with VMAT2 (Dwoskin and Crooks, 2002). Furthermore, lobeline inhibits $d$-amphetamine- and methamphetamine-evoked DA release from superfused rat striatal slices (Miller et al., 2001; Krishnamurthy et al., 2004). These results are consistent with the effect of lobeline to inhibit methamphetamine self-administration (Harrod et al., 2001). These preclinical data suggest that lobeline has potential as a pharmacotherapy for psychostimulant abuse.

Structural modification of the lobeline molecule has afforded compounds with differing affinities for nAChRs (Flammia et al., 1999), however, these analogs have not been evaluated for their activity at neurotransmitter transporters. The present study evaluates a series of lobeline
analogs for their activities at $\alpha_4\beta_2^*$ nAChRs, $\alpha_7^*$nAChRs, DAT, SERT, NET and VMAT2, with the aim of identifying analogs with high affinity and selectivity for these target sites. The exact subunit composition, stoichiometry and arrangement of native nAChRs remain to be elucidated, which is indicated by an asterisk (*) following the subunit designation (Lukas et al., 1999). Such analogs may be useful candidates for probing specific targets for elucidating the underlying neurochemical mechanism(s) responsible for lobeline-induced inhibition of the behavioral effects of methamphetamine.
Methods

Animals. Male Sprague-Dawley rats (200-250 g upon arrival) were purchased from Harlan Laboratories (Indianapolis, IN) and were housed two per cage with ad libitum access to food and water in the Division of Laboratory Animal Resources at the College of Pharmacy at the University of Kentucky. Experimental protocols involving the animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Chemicals. [3H]DA (dopamine, dihydroxyphenylethylamine 3,4-ethyl-2-[N-3H]; specific activity, 25.6 Ci/mmol), (±)-[3H]MLA (methyllycaconitine, [1α,4(S),6β,14α,16β]-20-ethyl-1,6,14,16-tetramethoxy-4[[2-([3-3H]methyl-2,5-dioxo-1-pyrrolidinyl)benzoyl]oxy]methyl]-aconitane-7,8-diol); specific activity, 25.4 Ci/mmol), (S)-[3H]nicotine (S(-))[N-methyl-3H]; specific activity, 80 Ci/mmol), [3H]NE (norepinephrine, levo-[7-3H]; specific activity 27.5 Ci/mmol), 86RbCl (specific activity, 55.2 mCi/mmol), [125I]RTI-55 ((S)-2 β-carbomethoxy-3 β-(4-iodophenyl)tropamine; specific activity, 2200 Ci/mmol) and [3H]5-HT (serotonin, hydroxytryptamine creatine sulfate 5-[1,2-3H(N)]; specific activity, 27.5 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston, Massachusetts). [3H]MTBZ ((±)-methoxytetrabenazine [O-methyl-3H]; specific activity 56.8 Ci/mmol) was a generous gift from Dr. Michael Kilbourn (Department of Radiology, University of Michigan Medical School, Ann Arbor, MI). Bovine serum albumin (BSA), catechol, dopamine, disodium ethylenediamine tetraacetate (EDTA), ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), fluoxetine HCl, 1-(2-[bis(4-fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine (GBR-12909 HCl), N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid] (HEPES), S(-)-nicotine ditartrate (nicotine), nomifensine maleate, pargyline HCl, polyethyleneimine (PEI), serotonin, tetrodotoxin (TTX), tris[hydroxymethyl]aminomethane hydrochloride (Trizma HCl), tris[hydroxymethyl]
aminomethane base (Trizma) and tropolone were purchased from Sigma Chemical Co. (St. Louis, MO). α-D-Glucose, L-ascorbic acid and potassium phosphate monobasic were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI), AnalaR- BHD Ltd. (Poole, U.K.) and Mallinckrodt (Paris, KY), respectively. Lobeline hemisulfate [2S,6R,8S-(-)-lobeline] was purchased from ICN Biochemicals Inc. (Aurora, OH). All other commercially obtained chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

The lobeline analogs, ketoalkene (N-methyl-2R-(2-oxo-2-phenylethyl)6S-(2-phenylethen-1yl)piperidine), 10R-MESP (N-methyl-2R-(2R-hydroxy-2-phenylethyl)6S-(2-phenylethen-1yl)piperidine), 10S/10R-MEPP (N-methyl-2R-(2R/2S-hydroxy-2-phenylethyl)6S-(2-phenylethyl)piperidine), lobelanidine, lobelanine, MTD (N-methyl-2,6-cis-di-trans-styrylpiperidine, meso-transdiene), (-)-TTD (N-methyl-2S,6S-di-trans-styrylpiperidine, trans-transdiene), lobelane and lobeline tosylate (lobeline-8-O-tosylate) were synthesized by structural modification of the lobeline molecule (Zheng et al., 2004) and are illustrated in Fig. 1. The structures of the lobeline analogs were verified by 1H- and 13C-NMR spectroscopy, mass spectrometry and in some cases X-ray crystallography.

[3H]Nicotine Binding Assay. Striata from 2-4 rats were homogenized using a Tekmar polytron in 10 vol of ice-cold modified Kreb’s-HEPES buffer (20 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, pH 7.5). Homogenates were incubated (5 min at 37 °C) and centrifuged (29,000 x g for 20 min at 4 °C). Resulting pellets were resuspended in 10 vol of ice-cold MilliQ water, incubated (5 min at 37 °C) and centrifuged (29,000 x g for 20 min at 4 °C). Resulting pellets were again resuspended in 10 vol of ice-cold 10% Kreb’s-HEPES buffer, then incubated and centrifuged as described. Final pellets were stored at -70 °C in fresh 10% Kreb’s-HEPES buffer until use. Upon assay, pellets were resuspended in 10% Kreb’s-HEPES buffer, incubated and centrifuged as previously described. Final pellets were
resuspended in ice-cold MilliQ water (2.0 ml) to provide ~200 µg protein/100 µl membrane suspension. Inhibition of specific [³H]nicotine binding by lobeline and its analogs was assessed using a previously described method (Crooks et al., 1995). Briefly, assays were performed in triplicate in a final vol of 200 µl Kreb’s-HEPES buffer containing 250 mM Tris (pH 7.5, 4 °C). Reactions were initiated by addition of 100 µl of membrane suspension to tubes containing 50 µl Kreb’s-HEPES buffer or 1 of 9 concentrations (0.1 nM – 1 mM, final concentration) of nicotine, lobeline or analog and 50 µl [³H]nicotine (3 nM, final concentration). Nonspecific binding was determined in triplicate in the presence of nicotine (10 µM). Following incubation (90 min at 4 °C), reactions were terminated by dilution of samples with ice-cold Kreb’s-HEPES buffer followed by immediate filtration through Whatman GF/B glass fiber filters (presoaked in 0.5% PEI) using a cell harvester (MP-43RS, Brandel Inc., Gaithersburg, MD). Filters were processed and radioactivity determined by liquid scintillation spectroscopy (B1600TR scintillation counter; Packard/Perkin-Elmer Life Sciences; Boston, MA).

[³H]MLA Binding Assay. Whole rat brain (minus cortex, striatum and cerebellum) was homogenized in 20 vol of ice-cold hypotonic buffer (2 mM HEPES, 14.4 mM NaCl, 0.15 mM KCl, 0.2 mM CaCl₂ and 0.1 mM MgSO₄, pH 7.5). Homogenates were incubated at 37 °C for 10 min and centrifuged (25,000 x g for 15 min at 4 °C). Pellets were washed 3 times by resuspension in 20 vol of buffer, followed by centrifugation. Final pellets were resuspended in incubation buffer to provide ~150 µg protein/100 µl membrane suspension. Binding assays were performed in duplicate, in a final vol of 250 µl incubation buffer, containing 20 mM HEPES, 144 mM NaCl, 1.5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄ and 0.05% BSA, pH 7.5. Assays were initiated by the addition of 100 µl membrane suspension to 150 µl of sample containing 2.5 nM [³H]MLA and 1 of 8 concentrations (30 nM - 100 µM, final concentration) of lobeline or analog, and incubated for 2 hr at room temperature. Nonspecific binding was determined in the
presence of nicotine (1 mM). Assays were terminated by dilution with ice-cold incubation buffer (3 ml) followed by immediate filtration through glass fiber filters (Schleicher & Schuell #32; Keene, NH) presoaked with 0.5% PEI using a cell harvester (MP-43RS, Brandel Inc., Gaithersburg, MD). Filters were processed and radioactivity was determined as described previously.

$^{86}$Rb$^+$ Efflux Assay. The ability of lobeline and its analogs to evoke $^{86}$Rb$^+$ efflux was determined using a previously published method (Miller et al., 2000). nAChR-mediated $^{86}$Rb$^+$ efflux from preloaded rodent brain synaptosomes has been used to characterize functional interactions of ligands with [3H]nicotine binding sites based on the findings that the response to nAChR agonists in the $^{86}$Rb$^+$ efflux assay is highly correlated with displacement of high affinity [3H]nicotine binding (α4β2* nAChRs) and that nAChR agonist-evoked $^{86}$Rb$^+$ efflux is eliminated when brain from β2-subunit knockout mice is utilized (Marks et al., 1993; 1995, 1999; Sharples et al., 2000). In the current study, thalamus was homogenized and centrifuged (1000 x g for 10 min at 4°C). Supernatants were centrifuged (12,000 x g for 20 min at 4°C) to obtain synaptosomes. Synaptosomes were incubated for 30 min in 35 µl uptake buffer (140 mM NaCl, 1.5 mM KCl, 2.0 mM CaCl$_2$, 1.0 mM MgSO$_4$, 20 mM α-D-glucose, pH 7.5) containing $^{86}$Rb$^+$ (4 µCi). $^{86}$Rb$^+$ uptake was terminated by filtration onto glass fiber filters (6 mm; Type A/E, Gelman Sciences, Ann Arbor, MI) under gentle vacuum (0.2 atm), followed by three washes with superfusion buffer (0.5 ml each). Subsequently, each filter with $^{86}$Rb$^+$-loaded synaptosomes (~40 µg protein/µl) was placed on a glass fiber filter (13 mm, Type A/E) mounted on a polypropylene platform. Synaptosomes were superfused at a rate of 2.5 ml/min with $^{86}$Rb$^+$ efflux assay buffer (125 mM NaCl, 5 mM CsCl, 1.5 mM KCl; 2 mM CaCl$_2$, 1 mM MgSO$_4$, 25 mM HEPES, 20 mM α-D-glucose, 0.1 µM TTX, 1.0 g/l BSA; pH 7.5). TTX and CsCl were included in the buffer to block voltage-gated Na$^+$ and K$^+$ channels, respectively, and to reduce the rate of
basal $^{86}\text{Rb}^+$ efflux. Lobeline- and analog-induced $^{86}\text{Rb}^+$ efflux (intrinsic activity) and lobeline- and analog-induced inhibition of nicotine-evoked $^{86}\text{Rb}^+$ efflux were determined. For these assays, the concentration (1 µM) of nicotine was chosen based on previous observations that this was the lowest concentration producing maximal $^{86}\text{Rb}^+$ efflux (~1.0 percent tissue content; Miller et al., 2000). After 8 min of superfusion, basal samples were collected for 2 min. Subsequently, synaptosomes were superfused for 3 min with 1 of 5 concentrations (1 nM – 100 µM) of lobeline or analog. Nicotine was then added to buffer containing lobeline or analog and superfusion continued for 3 min. Each aliquot part of thalamic synaptosomes was exposed to only 1 concentration of lobeline or analog. In each experiment, one synaptosomal aliquot part was also exposed to nicotine in the absence of lobeline or analog, and one synaptosomal aliquot part was superfused in the absence of lobeline, analog and nicotine to determine basal $^{86}\text{Rb}^+$ efflux during the entire course of the experiment. Samples were analyzed using liquid scintillation spectroscopy as previously described.

**Inhibition of $[^3\text{H}]\text{DA}$ and $[^3\text{H}]\text{5-HT}$ uptake into rat striatal and hippocampal synaptosomes, respectively.** Lobeline- and analog-induced inhibition of $[^3\text{H}]\text{DA}$ and $[^3\text{H}]\text{5-HT}$ uptake into rat striatal and hippocampal synaptosomes, respectively, was assessed using modifications of a previously described method (Teng et al., 1997). Analog-induced inhibition was compared with that induced by the selective DAT and SERT transporter inhibitors, GBR-12909 and fluoxetine, respectively (Fuller et al., 1991; Carroll et al., 2002). Brain regions were homogenized in 20 ml cold 0.32 M sucrose solution containing 5 mM NaHCO$_3$ (pH 7.4) with 12 up-and-down strokes of a Teflon pestle homogenizer (clearance $\approx 0.003''$). Homogenates were centrifuged (2,000 x g for 10 min at 4 °C) and supernatants were centrifuged (20,000 x g for 15 min at 4 °C). Pellets were resuspended in 1.5 ml of Kreb’s buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgSO$_4$, 1.25 mM CaCl$_2$, 1.5 mM KH$_2$PO$_4$, 10 mM $\alpha$-D-glucose, 25 mM HEPES, 0.1 mM
EDTA, 0.1 mM pargyline and 0.1 mM ascorbic acid, saturated with 95% O₂/5% CO₂, pH 7.4). Final protein concentrations were 400 μg/ml, determined by protein-dye binding (Bradford, 1976). Assays were performed in duplicate in a total volume of 500 μl. Aliquot parts of synaptosomal suspension (50 μl) were added to tubes containing 350 μl Kreb's buffer and 50 μl of buffer containing 1 nM – 1 mM final concentrations of lobeline, lobeline analog, GBR-12909, fluoxetine or buffer. Tubes were incubated at 34 °C for 10 min before addition of 50 μl [³H]DA (10 nM final concentration) or 50 μl [³H]5-HT (10 nM final concentration). Accumulation proceeded for 10 min at 34 °C. Reactions were terminated by addition of 3 ml ice-cold Kreb's buffer. Nonspecific [³H]DA and [³H]5-HT uptake was determined in the presence of nomifensine (10 μM) and fluoxetine (10 μM), respectively. Samples were rapidly filtered through a Whatman GF/B filter using a cell harvester (MP-43RS, Brandel Inc., Gaithersburg, MD), and filters were subsequently washed three times with 4 ml ice-cold Kreb’s buffer containing catechol (1 mM). Radioactivity retained by the filters was determined by liquid scintillation spectroscopy (B1600 TR scintillation counter; Packard BioScience Co., Meriden, CT).

**Kinetic analysis of [³H]DA uptake into rat striatal synaptosomes.** To determine if the inhibition of [³H]DA uptake was via a competitive or noncompetitive mechanism, kinetic analyses were performed for lobeline (60 μM), MTD (3 μM), (-)-TTD (1 μM) and lobelane (1 μM). The concentrations used in the kinetic analyses were chosen to approximate the IC₅₀ values from the above concentration-response experiments for analog-induced inhibition of [³H]DA uptake. Experiments were conducted in the absence and presence of lobeline or analog. The absence of lobeline or analog (buffer alone) represented the control condition. Nonspecific uptake was determined in the presence of nomifensine (10 μM). In the absence and presence of nomifensine, aliquot parts of rat striatal synaptosomal suspension (50 μl) were added to tubes containing 350 μl Kreb’s buffer and 50 μl lobeline, analog or buffer alone. Tubes were
incubated for 5 min at 34 °C. Uptake was initiated by the addition of [3H]DA (1 nM – 5 μM final concentration, 50 μl, isotopically diluted with unlabeled DA (0.3-83 μM) to achieve varying DA concentrations and a consistent amount of radioactivity, i.e., 500,000 dpm/per tube). Accumulation proceeded for 10 min at 34 °C. Reactions were terminated by addition of 3 ml ice-cold Kreb’s buffer. Samples were washed three times with 4 ml ice-cold Kreb’s buffer containing catechol (1 mM) and filtered. Radioactivity retained by the filters was determined by liquid scintillation spectroscopy as previously described.

**Inhibition of [125I]RTI-55 binding to hDAT, hSERT and hNET stably expressed in HEK-293 cells.** Lobeline-, lobelane- and MTD-induced inhibition of [125I]RTI-55 binding to hDAT, hSERT and hNET was assessed using a previously described method (Eshleman et al., 1999). HEK-hDAT and HEK-hSERT cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 5% calf bovine serum, 0.05 U penicillin/streptomycin, and purpomycin (2 μg/ml). HEK-hNET cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.05 U penicillin/streptomycin, and geneticin (300 μg/ml). HEK-293 cells stably expressing hDAT, hSERT or hNET were grown to 80% confluence on 150 mm diameter tissue culture dishes in a humidified 10% CO2 environment at 37 °C. Medium was poured off the plates and plates were washed with 10 ml calcium- and magnesium-free phosphate buffered saline, lysis buffer (2 mM HEPES with 1 mM EDTA) added, and the plates placed on ice. After 10 min, cells were scraped from plates and centrifuged (30,000 x g for 20 min). Pellets were resuspended in 12–32 ml 0.32 M sucrose using a Polytron homogenizer (setting 7 for 10 s). Resuspension volumes depended on the density of binding sites within a cell line, providing binding of <10% of the total radioactivity. Analog-induced inhibition of [125I]RTI-55 binding was compared with that induced by cocaine as the standard. Nonspecific binding was determined in the presence of
mazindol (5 μM) for hDAT and hNET assays, or imipramine (5 μM) for the hSERT assays. Competition assays were conducted with duplicate determinations for each point. Aliquot parts of membranes (50 μl, ~10–15 μg protein) were added to tubes containing 15 μl inhibitor (lobeline, lobelane, MTD or cocaine; 20 nM - 10 μM) or Kreb’s-HEPES assay buffer (122 mM NaCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 10 μM pargyline, 100 μM tropolone, 0.2% glucose and 0.02% ascorbic acid, buffered with 25 mM HEPES, pH 7.4). Tubes containing membranes and inhibitor were preincubated at room temperature for 10 min before addition of 25 μl [¹²⁵I]RTI-55 (40-80 pM final concentration) and sufficient Kreb’s-HEPES buffer to obtain a final volume of 250 μl. Tubes were incubated at 25 °C for 90 min. Binding was terminated by filtration over GF/C filters using a Tomtex 96-well cell harvester. Filters were washed for 6 s with ice-cold saline. Scintillation fluid (50 μl) was added to each tube and radioactivity remaining on the filter was determined using a Wallac microBeta or Betaplate scintillation counter (EG & G Wallac; Turku, Finland).

Inhibition of [³H]DA, [³H]5-HT and [³H]NE uptake by hDAT, hSERT and hNET, respectively, in stably expressed HEK-293 cells. Lobeline-, lobelane- and MTD-induced inhibition of [³H]DA, [³H]5-HT and [³H]NE uptake by hDAT, hSERT or hNET, respectively, in HEK-293 cells stably expressing these transporters was assessed using a previously described method (Eshleman et al., 1999). HEK-hDAT, HEK-hSERT and HEK-hNET were grown on 150 mm diameter culture dishes as described above. The medium was removed, and cells were washed twice with phosphate buffered saline at room temperature. Following the addition of 3 ml Kreb’s-HEPES buffer, plates were placed in a 25 °C water bath for 5 min. The cells were gently scraped and clusters separated by trituration using a pipette for 5-10 aspirations and ejections. Cells from multiple plates were combined for use in assays. Analog-induced inhibition was compared with that induced by cocaine, as the standard. Nonspecific uptake was
determined in the presence of mazindol (5 \(\mu\)M) for hDAT and hNET assays or imipramine (5 \(\mu\)M) for hSERT assays. Aliquot parts of cell preparation (50 \(\mu\)l) were added to 1 ml vials containing 350 \(\mu\)l Kreb’s-HEPES buffer, 50 \(\mu\)l of inhibitor (lobeline, lobelane, MTD or cocaine; 20 nM - 10 \(\mu\)M) and 50 \(\mu\)l of either mazindol or imipramine to determine nonspecific uptake, in a final assay volume of 500 \(\mu\)l. Tubes were incubated at 25 \(^\circ\)C for 10 min before addition of 50 \(\mu\)l \([^3H]DA, [^3H]5-HT\) or \([^3H]NE\) (final concentration 20 nM). Accumulation of \([^3H]\)neurotransmitter proceeded for 10 min. Reactions were terminated by filtration through Whatman GF/C filters presoaked in 0.05% polyethylenimine. Scintillation cocktail was added and radioactivity remaining on the filter determined as described above for the binding assay.

**Inhibition of \([^3H]MTBZ\) binding to vesicles prepared from rat whole brain.** Lobeline- and analog-induced inhibition of \([^3H]MTBZ\) binding was determined using modifications of a previously described method for \([^3H]DTBZ\) binding (Teng et al., 1998). Nonspecific binding was determined in the presence of tetrabenazine (20 \(\mu\)M). Rat whole brain (excluding cerebellum) was homogenized in 20 ml cold 0.32 M sucrose solution with 7 up-and-down strokes of a Teflon pestle homogenizer (clearance \(\approx\) 0.003”). Homogenates were centrifuged (1,000 x g for 12 min at 4 \(^\circ\)C). Supernatants were centrifuged (22,000 x g for 10 min at 4 \(^\circ\)C). Resulting pellets were incubated in 18 ml cold water for 5 min, and subsequently 2 ml HEPES (25 mM) and potassium-tartrate (100 mM) solution was added. Samples were centrifuged (20,000 x g for 20 min at 4 \(^\circ\)C), and MgSO\(_4\) (1 mM) solution was then added to the supernatants. Solutions were centrifuged (100,000 x g for 45 min at 4 \(^\circ\)C) and re-suspended in cold assay buffer (25 mM HEPES, 100 mM potassium-tartrate, 5 mM MgSO\(_4\), 0.1 mM EDTA and 0.05 mM EGTA; pH 7.5). The final protein concentration was 15 \(\mu\)g of protein/100 \(\mu\)l (Bradford, 1976). Assays were performed in duplicate in 96-well plates. Aliquot parts of vesicular suspension (100 \(\mu\)l) were added to wells containing 50 \(\mu\)l \([^3H]MTBZ\) (final concentration = 3 nM), 50 \(\mu\)l of lobeline or
analog, and 50 µl buffer. Reactions were terminated by filtration (FilterMate Harvester; Packard BioScience Co.) onto Unifilter-96 GF/B filter plates (presoaked in 0.5% polyethylenimine). Filters were subsequently washed five times with 350 µl ice-cold buffer (25 mM HEPES, 100 mM K2-tartrate, 5 mM MgSO4 and 10 mM NaCl, pH 7.5). Filter plates were dried, bottom-sealed and each well filled with 40 µl scintillation cocktail (MicroScint 20, Packard Bioscience Co.). Radioactivity in filters was determined by liquid scintillation spectroscopy (TopCount NXT scintillation counter, Packard Bioscience Co.).

**Data Analysis.** For lobeline- and analog-induced inhibition of [3H]nicotine and [3H]MLA binding, specific binding was determined by subtracting nonspecific binding from total binding. Concentrations of inhibitor that produced 50% inhibition (IC50 values) and 95% confidence intervals were determined from the concentration-effect curves via an iterative curve-fitting program (Prism 3.0, Graph Pad Software, San Diego, CA). Inhibition constants (Ki values) were determined using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

For the 86Rb+ efflux assay, basal rate of efflux was determined via an exponential decay curve used to fit the data points preceding and following superfusion with lobeline or analog and nicotine (Sigma Plot v.8, Systat Software, Inc., Point Richmond, CA). The lobeline-, analog- or nicotine-evoked increase in 86Rb+ efflux was calculated as the fractional increase above baseline. Increases were summed to obtain total 86Rb+ efflux during the period of superfusion with lobeline, analog and/or nicotine and normalized to 86Rb+ content in the corresponding synaptosomal sample to reduce variability within and between experiments. To determine intrinsic activity of lobeline or analog, total 86Rb+ efflux during the 3-min period of superfusion in the absence of nicotine was analyzed by one-way repeated measure analysis of variance (ANOVA) with lobeline or analog concentration as a within-subject factor (SPSS, version 9.0,
To assess the lobeline- and analog-induced inhibition of nicotine-evoked $^{86}\text{Rb}^+$ efflux, total efflux during the 3-min period of superfusion in the presence of nicotine and lobeline or analog was analyzed by one-way repeated measures ANOVA with lobeline or analog concentration as a within-subject factor. Concentrations of lobeline or analog that exhibited intrinsic activity were not included in the analysis to determine inhibition of the effect of nicotine. Additionally, IC$_{50}$ values were determined by nonlinear regression fit of the mean data to sigmoidal concentration-response curves (Prism 3.03, Graph Pad Software, San Diego, CA).

To generate concentration-response curves for inhibition of $[^3\text{H}]$neurotransmitter uptake, specific uptake was determined by subtracting nonspecific uptake from total uptake. Similarly for the competition binding curves for $[^3\text{H}]$MTBZ and $[^{125}\text{I}]$RTI-55, specific binding was determined by subtracting nonspecific from total binding. IC$_{50}$ values were determined from the curves by an iterative curve fitting program (Prism 3.03, Graph Pad Software, San Diego, CA) and Ki values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). For $[^{125}\text{I}]$RTI-55 binding, Hill coefficients were determined. For analysis of $[^3\text{H}]$DA uptake kinetics, Km and Vmax values were determined from concentration-effect curves for specific $[^3\text{H}]$DA uptake (Taylor and Insel, 1990; Kenakin, 1997). Paired two-tailed $t$-tests were performed on the log Km and Vmax values to determine differences ($P < 0.05$) between the absence (control condition) and presence of lobeline, MTD, (-)-TTD or lobelane.
Results

Inhibition of [³H]nicotine binding. Fig. 2 illustrates the competition curves for lobeline and its analogs to inhibit [³H]nicotine binding to rat striatal membranes, and provides the Ki values derived from these competition curves. Lobeline completely inhibited [³H]nicotine binding to rat striatal membranes, and a Ki value of 0.016 µM was obtained. Lobeline was ~3-fold less potent than nicotine in this assay. With the notable exception of MTD, which did not inhibit [³H]nicotine binding, the remaining lobeline analogs inhibited [³H]nicotine binding with wide variation in affinity. The tosyl sulfonic acid ester of lobeline, lobeline tosylate, completely inhibited [³H]nicotine binding and was the most potent (Ki = 0.011 µM) of the lobeline analogs, being equipotent with lobeline. The dehydrated analog ketoalkene and its fully reduced analog 10S/10R-MEPP also completely inhibited binding, but these analogs were ~45-fold less potent than lobeline in this assay. The mono and dihydroxy analogs, lobelanidine and 10R-MESP, respectively, completely inhibited [³H]nicotine binding, and these analogs were more than 2-orders of magnitude less potent than lobeline. The defunctionalized analogs (-)-TTD and lobelane, and the diketo analog lobelanine, provided ~60% maximal inhibition of [³H]nicotine binding at the concentrations examined, and these analogs were more than 3 orders of magnitude less potent than lobeline. The defunctionalized analog MTD did not inhibit [³H]nicotine binding, demonstrating a lack of interaction of this analog with α4β2* nAChRs.

Inhibition of nicotine-evoked ⁸⁶Rb⁺ efflux. ⁸⁶Rb⁺ efflux from preloaded rat thalamic synaptosomes has been used as a functional assay for α4β2* nAChRs to determine if compounds act as agonists or antagonists at this site (Marks et al., 1995; Miller et al., 2000). Lobeline has been previously shown to act as a nAChR antagonist in this assay (Miller et al., 2000). Functional effects of the most potent analogs (lobeline tosylate and ketoalkene) as well as the effect of an analog (MTD) which had no affinity for the [³H]nicotine binding site were
assessed in the $^{86}\text{Rb}^+$ efflux assay. Neither lobeline nor any of the above analogs evoked $^{86}\text{Rb}^+$ efflux (Table 1), demonstrating no agonist activity at $\alpha 4\beta 2^\ast$ nAChRs. As expected, nicotine (1 $\mu$M) evoked an increase in $^{86}\text{Rb}^+$ efflux of $\sim$1.0% of total $^{86}\text{Rb}^+$ tissue content (Fig. 3). Lobeline inhibited ($IC_{50} = 0.73$ $\mu$M) nicotine-evoked $^{86}\text{Rb}^+$ efflux ($F_{(6,24)} = 15.01, P < 0.001$), consistent with previous results (Miller et al., 2000). Post hoc analysis revealed that 1 and 10 $\mu$M lobeline decreased nicotine-evoked $^{86}\text{Rb}^+$ efflux compared to control (nicotine alone). Lobeline tosylate, which was equipotent to lobeline in the $[^3\text{H}]\text{nicotine binding assay}$ (Fig. 2), was nearly 70-fold more potent ($IC_{50} = 0.011$ $\mu$M) than lobeline inhibiting nicotine-evoked $^{86}\text{Rb}^+$ efflux ($F_{(5,20)} = 10.76, P < 0.01$). Post hoc analysis revealed that lobeline tosylate (10 nM - 10 $\mu$M) inhibited the effect of nicotine. Ketoalkene also inhibited ($IC_{50} = 0.30$ $\mu$M) nicotine-evoked $^{86}\text{Rb}^+$ efflux ($F_{(5,20)} = 4.48, P < 0.05$), and had a similar potency to lobeline in this assay. Post hoc analysis revealed that 1 and 10 $\mu$M ketoalkene significantly inhibited the effect of nicotine. In contrast, MTD, which did not interact with the $[^3\text{H}]\text{nicotine binding site}$ (Fig. 2), also did not inhibit nicotine-evoked $^{86}\text{Rb}^+$ efflux (Fig. 3).

**Inhibition of $[^3\text{H}]\text{MLA binding}$.** Although completely inhibiting $[^3\text{H}]\text{MLA binding}$ to membranes prepared from whole rat brain, lobeline exhibited low affinity ($K_i = 11.6$ $\mu$M) for $\alpha 7^\ast$ nAChRs (Fig. 4), and was $\sim$20-fold less potent than nicotine in this assay. The 10-hydroxy analogs, $10R$-MESP and $10S/10R$-MEPP, were $\sim$4-8-fold more potent than lobeline in this assay. Lobelanidine was 3-fold more potent than lobeline. Lobeline tosylate, ketoalkene lobelanine and lobelane had similar affinity to lobeline at the $[^3\text{H}]\text{MLA binding site}$. MTD and (-)-TTD did not inhibit $[^3\text{H}]\text{MLA binding}$ at the concentrations examined, demonstrating no affinity for $\alpha 7^\ast$ nAChRs. Thus, defunctionalization of lobeline decreases affinity for $\alpha 7^\ast$ nAChRs.
**Inhibition of [³H]DA uptake.** Lobeline and each of the lobeline analogs completely inhibited specific [³H]DA uptake into rat striatal synaptosomes, but with varying affinity (Fig. 5). GBR 12909 inhibited [³H]DA uptake (Ki = 18 nM), consistent with previously reported results (Carroll et al., 2002). Also in agreement with previous reports (Teng et al., 1997), lobeline inhibited (Ki = 29.4 µM) [³H]DA uptake, but had low affinity for this site. The highly functionalized analogs, lobelanidine (a dihydroxy analog), lobeline tosylate (the O-8-tosyl sulfonic acid ester) and lobelanine (a diketo analog) were equipotent (Ki = 16-33 µM) with lobeline inhibiting [³H]DA uptake. Ketoalkene, lobelane, 10S/10R-MEPP and 10R-MESP were 5-34-fold more potent than lobeline. Although an order of magnitude less potent than GBR 12909, the defunctionalized and unsaturated C-6 epimers, MTD and (-)-TTD were the most potent analogs inhibiting [³H]DA uptake, being 50-100-fold more potent inhibiting DAT compared to lobeline and exhibiting a surprising lack of stereoselectivity. Thus, removal of the functionalities from the C-2 and C-6 side chains of lobeline and introduction of unsaturation (MTD and (-)-TTD) afforded the most potent analogs in the [³H]DA uptake assay.

**Kinetic analysis of the inhibition of [³H]DA uptake.** To determine whether lobeline and selected lobeline analogs inhibited [³H]DA uptake competitively or noncompetitively, kinetic analyses were performed and the results are illustrated in Fig. 6 and Table 2. Concentrations of lobeline (60 µM), MTD (3 µM), (-)-TTD (1 µM) and lobelane (1 µM) were chosen to approximate their IC₅₀ values obtained from the inhibition curves illustrated in Fig. 5. Lobeline decreased Vmax compared to control (t₅(5) = 3.16, P < 0.05), without an alteration in Km (t₅(5) = 0.39, P = 0.72; Fig. 6A, Table 2), indicating that lobeline noncompetitively inhibits [³H]DA uptake into striatal synaptosomes. Similarly, lobelane inhibited [³H]DA uptake in a noncompetitive manner, decreasing the Vmax (t₅(5) = 2.57, P = 0.05), without altering the Km (t₅(5) = 1.38, P = 0.23; Fig. 6B, Table 2). In contrast to lobeline and lobelane, the defunctionalized unsaturated
stereoisomers, MTD and (-)-TTD, increased the Km compared to control ($t_{(5)} = 4.49, P < 0.05$ and $t_{(6)} = 6.65, P < 0.001$, respectively; Fig. 6C and 6D, Table 2), without altering Vmax $t_{(5)} = 0.28, P = 0.79$ and $t_{(6)} = 0.33, P = 0.76$, respectively), indicating competitive inhibition of DAT.

Inhibition of $[^3H]$5-HT uptake. Lobeline and its analogs completely inhibited specific $[^3H]$5-HT uptake into rat hippocampal synaptosomes, but with varying affinity (Fig. 7). Consistent with previous reports (Fuller et al., 1991), fluoxetine inhibited specific $[^3H]$5-HT uptake with a Ki of 41 nM. Lobeline and lobelanidine were the least potent analogs, with Ki values of ~25 µM. Loberanine, lobelane, lobeline tosylate and ketoalkene were 8-16-fold more potent than lobeline inhibiting $[^3H]$5-HT uptake. (-)-TTD was 24-fold more potent than its C-6 epimer MTD inhibiting $[^3H]$5-HT uptake, and both epimers were more potent than lobeline in this assay. Both C-10 monohydroxy analogs, 10S/10R-MEPP and 10R-MESP had high affinity for SERT (Ki = 10 and 44 nM, respectively), constituting the most potent analogs in the series. Furthermore, 10S/10R-MEPP and 10R-MESP were equipotent with fluoxetine. Importantly, these monohydroxy analogs were ~2-3 orders of magnitude more potent than lobeline.

Inhibition of $[^{125}I]$RTI-55 binding and of $[^3H]$DA, $[^3H]$5-HT and $[^3H]$NE uptake by hDAT, hSERT and hNET, respectively, in stably expressed HEK-293 cells. Similar to what has been previously shown for a number of uptake inhibitors (Eshleman et al., 1999), the potency of lobeline and its analogs to inhibit hDAT and hSERT function ($[^3H]$DA and $[^3H]$5-HT uptake, respectively) was correlated with potency to inhibit $[^{125}I]$RTI-55 binding in the cell lines expressing the respective transporters. The relationship between neurotransmitter uptake and binding was less apparent for hNET in both the previous and current studies. In binding and uptake assays, lobeline generally exhibited low affinity for hDAT, hSERT and hNET compared with cocaine; and similar to cocaine, lobeline did not exhibit selectivity at these transporter sites (Table 3). In both $[^{125}I]$RTI-55 binding and $[^3H]$DA uptake assays, the defunctionalized saturated
analog, lobelane (Ki = 97 and 87 nM, respectively) was ~50-fold more potent than lobeline inhibiting hDAT. In contrast to lobeline, lobelane demonstrated high nM affinity for hSERT. Whereas lobelane was equipotent to lobeline in the \([^{125}I]RTI-55\) binding assay probing hNET, lobelane was 33-fold more potent than lobeline inhibiting \([^3H]NE\) uptake via hNET expressed in HEK-293 cells. Furthermore, lobelane showed only a 6-10-fold selectivity for hDAT over hSERT. However, lobelane was 18-fold more selective for hDAT compared to hNET in the binding assay, but demonstrated no selectivity between hDAT and hNET in the uptake assay. The defunctionalized unsaturated analog MTD was at least 100-fold more potent than lobeline at hDAT and hSERT in both \([^{125}I]RTI-55\) binding and uptake assays. With respect to hNET, MTD was 7-14-fold more potent than lobeline in binding and uptake assays. Generally, the affinity of MTD for these three transporters was not different from that obtained for lobelane in these assays (≤ 7-fold difference between MTD and lobelane, Table 3). In the \([^{125}I]RTI-55\) binding assays, MTD was 44-fold more selective for hDAT over hSERT, and 7-fold more selective for hDAT over hSERT. Similarly, MTD was 40-fold more selective for hDAT over hSERT in the uptake assays, whereas this analog showed no selectivity for hDAT compared to hNET in the uptake assay. Generally, the Hill coefficients for all of the compounds approximated unity, suggesting competition with \([^{125}I]RTI-55\) at a single binding site.

**Inhibition of \([^3H]MTBZ\) binding.** Lobeline and its analogs inhibited \([^3H]MTBZ\) binding to vesicle membranes prepared from rat whole brain (Fig. 8). With the exception of lobelanidene, this series of compounds exhibited a narrow range of Ki values (0.92-8.8 µM). Lobelane was the most potent of the analogs in this series (Ki = 920 nM), and lobelanidene was the least potent (Ki = 26 µM). In contrast to the results obtained for inhibition of \([^3H]DA\) and \([^3H]5-HT\) uptake in rat brain synaptosomes, lobeline and its analogs were equipotent inhibiting \([^3H]MTBZ\) binding to vesicle membranes, with the exception of ketoalkene and lobelane, which
were both more potent at VMAT2 than lobeline. Furthermore, MTD was significantly more potent than its epimer, (-)-TTD, inhibiting [3H]MTBZ binding to vesicle membranes.
Discussion

The current SAR analysis investigated effects of defunctionalized, esterified, reduced and oxidized lobeline analogs on α4β2* and α7* nAChRs, and on monoamine plasmalemma and vesicular monoamine transporters. As previously reported, lobeline has high affinity for [3H]nicotine binding sites in rodent striatal membrane preparations (Ki = 4–20 nM; Abood et al., 1998; Reavill et al., 1990; Bhat et al., 1991; Court et al., 1994; Flammia et al., 1999). Defunctionalized lobeline analogs, lobelane, MTD and (-)-TTD, generally exhibited decreased affinity for [3H]nicotine binding sites (α4β2* nAChRs). MTD, an unsaturated, cis-defunctionalized analog of lobeline, did not interact with α4β2* sites; however, its C-6 epimer (-)-TTD showed enhanced interaction with the α4β2* site, indicating that stereochemical factors play a role in binding site recognition. However, the affinity of (-)-TTD for the [3H]nicotine binding site was 3-orders of magnitude less than that of lobeline. Partially defunctionalized analogs, ketoalkene and 10S/10R-MEPP, exhibited intermediate affinity for α4β2* nAChRs, with 30-fold lower affinity than lobeline. Structurally-related analogs, lobelanine and lobelanidine, had 2-3 orders of magnitude lower affinity than lobeline in the [3H]nicotine binding assay, consistent with results from previous studies (Flammia et al., 1999). Lobeline and lobeline tosylate were equipotent in the [3H]nicotine binding assay, which assesses interaction with high affinity α4β2* nAChRs. The latter results suggest that increases in molecular volume and steric bulk adjacent to the 8-hydroxy group by addition of the tosyl sulfonate moiety, can be accommodated at the high affinity α4β2* site. Although a correlation between inhibition of [3H]nicotine binding and inhibition of nicotine-evoked 86Rb+ efflux was expect, surprisingly tosylation of the 8-hydroxy group provided an analog (lobeline tosylate) with higher potency than lobeline in the 86Rb+ efflux assay. This lack of correlation may be the result of different modes of interaction of these compounds at the high affinity α4β2* nAChRs (e.g., competitive vs. noncompetitive).
The $^{86}$Rb$^+$ efflux assay assesses functional response at $\alpha 4\beta 2^* \text{nAChRs}$ (Marks et al., 1995; Miller et al., 2000). Effects of fully and partially defunctionalized analogs were evaluated in this assay to ascertain if these analogs act as nAChR agonists or antagonists. Neither lobeline nor its analogs evoked $^{86}$Rb$^+$ efflux; demonstrating that these compounds are not agonists at $\alpha 4\beta 2^* \text{nAChRs}$. Lobeline acts as an $\alpha 4\beta 2^* \text{nAChR}$ antagonist (Miller et al., 2000), and the current findings are consistent with these previous results. Ketoalkene and lobeline tosylate both inhibited $[^3H] \text{nicotine}$ binding to striatal membranes and inhibited nicotine-evoked $^{86}$Rb$^+$ efflux from thalamic synaptosomes, demonstrating antagonism of $\alpha 4\beta 2^* \text{nAChRs}$. MTD, which did not inhibit $[^3H] \text{nicotine}$ binding, also did not inhibit nicotine-evoked $^{86}$Rb$^+$ efflux, indicating that it does not interact with $\alpha 4\beta 2^*$ sites either competitively or noncompetitively.

In general, the members of this series of lobeline analogs have low affinity for $\alpha 7^* \text{nAChRs}$. The C-10 hydroxy analogs, 10S/10R-MEPP and 10R-MESP were the most potent in the series, but were 5-fold less potent than nicotine. As lobeline is progressively defunctionalized, affinity for $\alpha 7^* \text{nAChRs}$ is decreased, and selectivity between $\alpha 4\beta 2^*$ and $\alpha 7^*$ is diminished or eliminated, mainly due to the marked decrease in affinity for $\alpha 4\beta 2^* \text{nAChRs}$. Conversely, lobeline and lobeline tosylate are nearly 3-orders of magnitude more selective for $\alpha 4\beta 2^* \text{nAChRs}$ than $\alpha 7^* \text{nAChRs}$.

Lobeline interacts nonselectively with monoamine transporters (DAT, SERT, NET and VMAT2), consistent with previous findings (Teng et al., 1997, 1998; Dwoskin and Crooks, 2002). Lobeline exhibited highest affinity, albeit in the low $\mu$M range, for VMAT2. Generally, defunctionalization of the lobeline molecule provided analogs with higher affinity for the plasmalemma transporters. Specifically, two compounds, MTD and (-)-TTD, were 1-2-orders of magnitude more potent than lobeline inhibiting DAT function. Lobelane (a defunctionalized,
fully-saturated analog), and $10R$-MESP and ketoalkene (partially defunctionalized analogs) were 15-30-fold more potent than lobeline inhibiting DAT function. Both lobeline and lobelane inhibited DAT function noncompetitively. Interestingly, the unsaturated, defunctionalized epimers, MTD and $(-)$-TTD, competitively inhibited DAT. Taken together, it appears that this series of analogs interact with at least two different sites on DAT. The more rigid unsaturated epimers, MTD and $(-)$-TTD, compete with the substrate site, whereas the flexible fully-saturated analog lobelane appears to interact with an alternative site on DAT.

With respect to inhibition of SERT, $(-)$-TTD was 24-fold more potent than its C-6 epimer MTD, indicating that SERT is sensitive to the stereochemistry at C-6, whereas DAT and VMAT2 are not. Partially defunctionalized analogs, $10S/10R$-MEPP and $10R$-MESP, were 2-3 orders of magnitude more potent than lobeline inhibiting SERT function. These analogs had affinity similar to fluoxetine, a drug which selectively inhibits SERT (Fuller et al., 1991). Defunctionalization of lobeline at C-8 and introduction of a C-10 hydroxy group affords $10S/10R$-MEPP and $10R$-MESP, both of which exhibit high affinity for SERT. Removal of the C-10 hydroxy group from $10S/10R$-MEPP and $10R$-MESP afforded lobelane and MTD, respectively, both of which exhibited ~200-fold lower affinity at SERT. Thus, the completely defunctionalized analogs (lobelane and MTD) have low affinity for and little or no selectivity between DAT and SERT. Moreover, $10S/10R$-MEPP showed 600-fold more selectivity inhibiting SERT over DAT. Therefore, the C-10 hydroxy group appears to be a critical functionality for selective interaction with SERT. Taken together, the results suggest that $10S/10R$-MEPP may be a potential lead compound for development of new therapeutic agents for the treatment of mood disorders.

Lobeline, lobelane and MTD were investigated for their interaction with DAT, SERT and NET expressed in HEK-293 cells to further investigate their selectivity for specific transporters. Consistent with previous findings (Eshelman et al., 1999), the current study generally shows
similar results (i.e., the same rank order of transporter inhibition for the compounds) in the hDAT and hSERT expression systems compared to rat DAT and rat SERT in brain synaptosomal preparations. By and large, higher affinity for these analogs was observed in the expression systems than in native tissues, but this may be a consequence of comparison between human and rat transporters. In contrast, more variable results comparing binding and uptake assays using cell expression systems were obtained with respect to NET. Interaction of lobelene, lobelane and MTD with NET was not determined in rat brain; however, results from HEK-293 cells suggest that lobelane and MTD should inhibit \([^{3}\text{H}]\text{NE}\) uptake in brain more potently than does lobeline.

Lobelane and ketoalkene were significantly more potent than lobeline inhibiting binding of \([^{3}\text{H}]\text{MTBZ}\) to VMAT2 in whole brain synaptic vesicle preparations. Although there was only a 5-fold difference in affinity between Ki values for lobeline and these two analogs, the confidence intervals for lobelane and ketoalkene did not overlap with that for lobeline, indicating significant differences in affinity for VMAT2. Similarly, MTD exhibited a significantly higher affinity for VMAT2 compared to (-)-TTD, although only a 4-fold difference in affinity was observed. These results indicate a modest enhancement of affinity at VMAT2 for these analogs over lobeline. The SAR trends for VMAT2 interaction indicate that introduction of a 10\(R\) hydroxy group into the lobeline molecule reduces affinity for VMAT2, and complete defunctionalization of the lobeline molecule affords analogs with the highest affinity for VMAT2 in the series.

The series of lobeline analogs assessed in the current study were generally more potent than lobeline at DAT, SERT, NET and VMAT2, demonstrating that appropriate modification of lobeline affords compounds with higher affinity at these sites. These transporters have been implicated in the behavioral activation induced by methamphetamine and cocaine (e.g., Kuhar et al., 1991; Sulzer et al., 1995; Johnson et al., 1998; Brown et al., 2000, 2001).
across transporter assays provides an indication of the selectivity exhibited by these analogs. Lobeline was significantly more potent at VMAT2 than at plasmalemma transporters, although only a 5-fold difference in selectivity was observed. The structurally related Lobelia alkaloid lobelanidine showed no selectivity at these transporter sites; whereas lobelanine, another Lobelia alkaloid, exhibited 5-7-fold selectivity for SERT and VMAT2 over DAT. Lobeline tosylate was 6-17-fold more selective for SERT over DAT and VMAT2; however, affinity of this compound for SERT was in the low µM range. Although lobelane and ketoalkene were amongst the most potent inhibitors at VMAT2, these analogs showed no selectivity between transporters. (-)-TTD showed significantly greater (14-20-fold) selectivity for the plasmalemma transporters over VMAT2; whereas its C-6 epimer, MTD, showed greater selectivity for DAT over SERT, but was not selective with regards to DAT and VMAT2. 10R-MESP, which exhibited high affinity at SERT (Ki = 44 nM), showed 20-fold selectivity at SERT over DAT and 200-fold selectivity at SERT over VMAT2. The epimeric mixture 10S/10R-MEPP exhibited the highest affinity (Ki = 10 nM) at SERT, and was 660-fold selective for SERT over DAT, and 260-fold selective at SERT over VMAT2. Thus, selectivity between plasmalemma transporters can be achieved through structural modification of the lobeline molecule, since 10S/10R-MEPP demonstrated good selectivity at SERT and was devoid of nAChR activity.

Findings from this initial SAR study demonstrate that defunctionalization of lobeline markedly decreases affinity for α4β2* and α7* nAChRs, while increasing affinity and selectivity for monoamine neurotransmitter transporters. These results suggest that the oxygen functionalities in the lobeline molecule are critical for interaction with nAChRs, but are not for interaction with neurotransmitter transporters. Monoamine transporters are considered valid targets for drug development for the treatment of methamphetamine abuse. In this respect, little is known regarding the VMAT2 pharmacophore. Drug discovery targeting VMAT2 may
provide a unique opportunity to probe the underlying neurochemical mechanisms responsible for psychostimulant abuse, and may provide novel approaches for treatment. Subsequent SAR studies will be directed at enhancing selectivity of lobeline analogs for individual transporters.
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Figure Legends

Fig. 1. Chemical structures of lobeline and lobeline analogs.

Fig. 2. Lobeline and lobeline analogs inhibit [³H]nicotine binding to rat striatal membranes. Nicotine was used as a standard for comparison. Nonspecific binding was determined in the presence of nicotine (10 μM). Ki values for lobeline and its analogs are provided in brackets. Data are the mean (± SEM) specific binding presented as a percentage of the control condition (mean ± SEM, 51.4 ± 2.4 fmol/mg). (n = 4 rats/compound).

Fig 3. Lobeline, ketoalkene and lobeline tosylate, but not MTD, inhibit nicotine (1 μM)-evoked ⁸⁶Rb⁺ efflux from superfused rat thalamic synaptosomes. Thalamic synaptosomes were superfused with buffer containing lobeline or lobeline analog for 3 min. Subsequently, nicotine (1 μM) was added to buffer and superfusion continued for an additional 3 min. Data are presented as the mean (± SEM) of the percentage of ⁸⁶Rb⁺ tissue content during the latter 3-min period of superfusion (n = 5 – 7 rats/experiment). *P < 0.05, different from control condition.

Fig. 4. Lobeline and lobeline analogs inhibit [³H]MLA binding to rat whole brain membranes. Nicotine was used as a standard for comparison. Nonspecific binding was determined the presence of nicotine (1 mM). Ki values for lobeline and its analogs are provided in brackets. Data are the mean (± SEM) specific binding presented as a percentage of the control condition (mean ± SEM, 89.6 ± 2.5 fmol/mg). (n = 4 rats/compound).
Fig. 5. Lobeline and lobeline analogs inhibit specific \[^3H\]DA uptake into rat striatal synaptosomes. GBR 12909, a specific DAT inhibitor, was used as a standard for comparison. Nonspecific uptake was determined in the presence of nomifensine (10 µM). Ki values for lobeline and its analogs are provided in brackets. Data are the mean \((± SEM)\) specific \[^3H\]DA uptake presented as a percentage of the control condition \((mean ± SEM, 43.5 ± 1.9 \text{ fmol/mg})\). \((n = 4 - 6 \text{ rats/compound})\).

Fig. 6. Kinetic analysis of the inhibition of specific \[^3H\]DA uptake by lobeline, lobelane, MTD and (-)-TTD. Concentrations of lobeline \((60 \mu\text{M}; \text{Panel A})\), lobelane \((3 \mu\text{M}; \text{Panel B})\), MTD \((1 \mu\text{M}; \text{Panel C})\) and (-)-TTD \((3 \mu\text{M}; \text{Panel D})\) were chosen from the concentration response curves illustrated in Fig. 5. Nonspecific uptake was determined in the presence of nomifensine \((10 \mu\text{M})\). Km and Vmax values are presented in Table 2 \((n = 6–7 \text{ rats/compound})\).

Fig. 7. Lobeline and lobeline analogs inhibit specific \[^3H\]5-HT uptake into rat hippocampal synaptosomes. Fluoxetine, a specific SERT inhibitor, was used as a standard for comparison. Nonspecific uptake was determined in the presence of fluoxetine \((10 \mu\text{M})\). Ki values are provided in brackets. Data are the mean \((± SEM)\) specific \[^3H\]5-HT uptake presented as a percentage of the control condition \((mean ± SEM, 1.39 ± 0.08 \text{ pmol/min/mg})\). \((n = 4 - 6 \text{ rats/compound})\).

Fig. 8. Lobeline and lobeline analogs inhibit specific \[^3H\]MTBZ binding to vesicles prepared from rat whole brain. Nonspecific binding was determined in the presence of tetrabenazine \((20 \mu\text{M})\). Ki values are reported in brackets. Data are the mean \((± SEM)\) specific \[^3H\]MTBZ binding presented as a percentage of the control condition \((mean ± SEM, 226 ± 10.8 \text{ fmol/mg})\). \((n = 4 - 6 \text{ rats/compound})\).
TABLE 1

Intrinsic activity of lobeline, lobeline tosylate, ketoalkene and MTD on $^{86}$Rb$^+$ efflux from preloaded rat thalamic synaptosomes. Synaptosomes were superfused for a 3-min period in the absence or presence of lobeline or lobeline analog before addition of nicotine to buffer. Data from the period of superfusion following addition of nicotine to buffer are presented in Fig. 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>1 nM</th>
<th>10 nM</th>
<th>100 nM</th>
<th>1 µM</th>
<th>10 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lobeline</td>
<td>0.13 ± 0.04$^a$</td>
<td>-0.16 ± 0.22$^b$</td>
<td>0.03 ± 0.07</td>
<td>-0.02 ± 0.08</td>
<td>0.44 ± 0.20</td>
<td>0.18 ± 0.08</td>
</tr>
<tr>
<td>Lobeline Tosylate</td>
<td>0.11 ± 0.06</td>
<td>0.46 ± 0.27</td>
<td>0.14 ± 0.13</td>
<td>0.30 ± 0.32</td>
<td>-0.06 ± 0.06</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Ketoalkene</td>
<td>0.17 ± 0.14</td>
<td>0.36 ± 0.07</td>
<td>0.16 ± 0.13</td>
<td>0.16 ± 0.10</td>
<td>0.16 ± 0.12</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>MTD</td>
<td>-0.08 ± 0.10</td>
<td>0.03 ± 0.02</td>
<td>0.21 ± 0.03</td>
<td>0.30 ± 0.10</td>
<td>0.31 ± 0.15</td>
<td>0.27 ± 0.11</td>
</tr>
</tbody>
</table>

$^a$ Data are mean (± SEM) percent $^{86}$Rb$^+$ efflux tissue content.

$^b$ Negative values indicate that $^{86}$Rb$^+$ efflux was below that obtained at baseline; $^{86}$Rb$^+$ efflux induced by a compound was calculated as the fractional change from baseline.
TABLE 2
Lobeline (60 μM) and lobelane (3 μM) inhibit DAT function via a noncompetitive mechanism, whereas MTD (3 μM) and (-)-TTD (1 μM) competitively inhibit DAT function in rat striatal synaptosomes. Data are presented as (mean ± SEM) values for Km and Vmax. Nonspecific [3H]DA uptake was determined in the presence of nomifensine (10 μM). No significant differences were found in Vmax or Km values between control groups (parameters determined in the absence of drug) in the four series of experiments. Control values for specific [3H]DA uptake were combined for tabular presentation. Concentration-response curves are presented in Fig. 6.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Km (μM)</th>
<th>Vmax (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.270 ± 0.138a</td>
<td>35.7 ± 5.75</td>
</tr>
<tr>
<td>Lobeline</td>
<td>0.312 ± 0.145</td>
<td>19.0 ± 3.95*</td>
</tr>
<tr>
<td>Lobelane</td>
<td>0.135 ± 0.078</td>
<td>26.8 ± 4.74*</td>
</tr>
<tr>
<td>MTD</td>
<td>0.507 ± 0.126*</td>
<td>33.0 ± 12.5</td>
</tr>
<tr>
<td>(-)-TTD</td>
<td>0.398 ± 0.161*</td>
<td>37.0 ± 3.78</td>
</tr>
</tbody>
</table>

aValues are presented as mean (± SEM) Km or Vmax values. n = 6 – 7 rats/group. *P < 0.05 different from control.
TABLE 3
Lobeline, lobelane and MTD inhibit [\(^{125}\)I]RTI-55 binding and [\(^3\)H]neurotransmitter uptake into recombinant hDAT, hSERT and hNET stably expressed in HEK-293 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>[(^{125})I]RTI-55 Binding Ki (µM)</th>
<th>Hill Coefficient</th>
<th>[(^3)H]Neurotransmitter Uptake IC(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HEK-hDAT cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lobeline</td>
<td>5.40 ± 1.30 (^a)</td>
<td>-1.03 ± 0.10</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>Lobelane</td>
<td>0.097 ± 0.033</td>
<td>-0.92 ± 0.06</td>
<td>0.087 ± 0.14</td>
</tr>
<tr>
<td>MTD</td>
<td>0.043 ± 0.015</td>
<td>-0.80 ± 0.07</td>
<td>0.117 ± 0.033</td>
</tr>
<tr>
<td>Cocaine</td>
<td>0.469 ± 0.037</td>
<td>-1.19 ± 0.12</td>
<td>0.524 ± 0.055</td>
</tr>
<tr>
<td><strong>HEK-hSERT cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lobeline</td>
<td>&gt; 10</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Lobelane</td>
<td>0.530 ± 0.130</td>
<td>-0.930</td>
<td>0.830 ± 0.320</td>
</tr>
<tr>
<td>MTD</td>
<td>1.89 ± 0.29</td>
<td>-0.99 ± 0.09</td>
<td>4.80 ± 1.20</td>
</tr>
<tr>
<td>Cocaine</td>
<td>0.353 ± 0.030</td>
<td>-1.07 ± 0.03</td>
<td>0.419 ± 0.037</td>
</tr>
<tr>
<td><strong>HEK-hNET cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lobeline</td>
<td>1.87 ± 0.82</td>
<td>-0.58 ± 0.05</td>
<td>2.79 ± 0.480</td>
</tr>
<tr>
<td>Lobelane</td>
<td>1.80 ± 0.47</td>
<td>-0.97 ± 0.14</td>
<td>0.085 ± 0.006</td>
</tr>
<tr>
<td>MTD</td>
<td>0.277 ± 0.082</td>
<td>-0.79 ± 0.04</td>
<td>0.198 ± 0.070</td>
</tr>
<tr>
<td>Cocaine</td>
<td>2.42 ± 0.12</td>
<td>-0.87 ± 0.04</td>
<td>0.370 ± 0.056</td>
</tr>
</tbody>
</table>

\(^a\) Values are presented as mean (± SEM) from at least 3 independent experiments, each experiment was conducted in duplicate (binding) or triplicate (uptake). \(^b\) ND, not determined.
Fig. 1; JPET #68098

Lobeline

Ketoalkene

10R-MESP

10S/10R-MEPP

Lobelanidine

Lobelanine

MTD

(-)-TTD

Lobelane

Lobeline Tosylate
Specific $[^3]H$Nicotine Binding (% control)

log [Inhibitor] M

Control -10 -9 -8 -7 -6 -5 -4 -3

[Ki (µM)]
- Nicotine [0.005]
- Lobeline [0.016]
- LOB Tos [0.011]
- Ketoalkene [0.44]
- 10S/10R-MEPP [0.51]
- Lobelanidine [3.1]
- 10R-MESP [7.6]
- (-)-TTD [12]
- Lobelainine [37.1]
- Lobelanine [77.3]
- MTD [>100]
Fig. 3; JPET #68098

The figure shows a graph of $^8$Rb$^+$ efflux (percent tissue content) against the logarithm of the inhibitor concentration (log [Inhibitor] M) for different compounds:

- Lobeline
- LOB Tos
- Ketoalkene
- MTD

The graph illustrates the inhibitory effects of these compounds on $^8$Rb$^+$ efflux.
Fig. 4; JPET #68098

Specific [³H]MLA Binding
(% control)

[Note: The diagram includes various compounds and their corresponding IC₅₀ values. The compounds are labeled with their respective inhibitory potencies.]

--Nicotine [0.32]
--Lobelane [43.1]
--LOB Tos [10.8]
--Ketoalkene [34.2]
--10S/10R-MEP [1.46]
--10R-MESP [1.60]
--Lobelanline [22.7]
--Lobeline [11.6]
--Lobelanidine [3.29]
--(-)-TTD [>100]
--MTD [>100]

[Ki (µM)]

Specific [³H]MLA Binding
(% control)

Fig. 4; JPET #68098

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Fig. 5; JPET #68098
Fig. 6; JPET #68098

A

Specific $[^3H]$DA Uptake (pmol/min/mg)

- Control
- Lobeline (60 µM)

[Dopamine] µM

B

Specific $[^3H]$DA Uptake (pmol/min/mg)

- Control
- Lobelane (3 µM)

[Dopamine] µM

C

Specific $[^3H]$DA Uptake (pmol/min/mg)

- Control
- MTD (3 µM)

[Dopamine] µM

D

Specific $[^3H]$DA Uptake (pmol/min/mg)

- Control
- (-)-TTD (1 µM)

[Dopamine] µM

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Fig. 7; JPET #68098

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Specific [3H]5-HT Uptake (% control) vs. log [Inhibitor] M

- Fluoxetine [0.041]
- Lobelianine [3.31]
- Lobelanine [3.31]
- Lobelanidine [25.6]
- Lobeline [25.7]
- MTD [8.94]
- (-)-TTD [0.38]
- 10R-MESP [0.044]
- 10S/10R MEPP [0.01]
- Ketoalkene [1.86]
- Lobelane [1.59]
- LOB Tos [1.67]

[Ki (µM)]

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Specific $[^3$H]$MTBZ$ Binding (% control) vs. log [Inhibitor] (M)

- (-)-TTD [5.15]
- Lobeline [5.46]
- Ketoalkene [1.35]
- Lobelanidine [25.9]
- Lobelanine [2.41]
- MTD [1.29]
- Lobelane [0.92]
- 10S/10R-MESP [8.78]
- LOB Tos [4.33]

Fig. 8; JPET #68098

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