Published in final edited form as:

AAPS J. ; 20(2): 29. doi:10.1208/s12248-018-0192-y.

New scaffold for lead compounds to treat methamphetamine use disorders

Na-Ra Lee¹, Guangrong Zheng², Peter A. Crooks², Michael T. Bardo³, Linda P. Dwoskin¹

¹Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky, USA

²Department of Pharmaceutical Sciences, College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA

³Department of Psychology, College of Arts and Sciences, University of Kentucky, Lexington, Kentucky, USA

Abstract

Despite increased methamphetamine use worldwide, pharmacotherapies are not available to treat methamphetamine use disorder. The vesicular monoamine transporter-2 (VMAT2) is an important pharmacological target for discovery of treatments for methamphetamine use disorder. VMAT2 inhibition by the natural product, lobeline, reduced methamphetamine-evoked dopamine release, methamphetamine-induced hyperlocomotion and methamphetamine self-administration in rats. Compared to lobeline, lobelane exhibited improved affinity and selectivity for VMAT2 over nicotinic acetylcholine receptors. Lobelane inhibited neurochemical and behavioral effects of methamphetamine; but tolerance developed to its behavioral efficacy in reducing methamphetamine self-administration, preventing further development. The lobelane analog, *R*-*N*-(1,2-dihydroxypropyl)-2,6-*cis*-di-(4-methoxyphenethyl)piperidine hydrochloride (GZ-793A) potently and selectively inhibited VMAT2 function and reduced neurochemical and behavioral effects of methamphetamine. However, GZ-793A exhibited potential to induce ventricular arrhythmias interacting with human-ether-a-go-go (hERG) channels. Herein, a new lead, *R*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propan-1-amine (GZ-11610), from a novel scaffold was evaluated as a VMAT2 inhibitor and potential therapeutic for methamphetamine use disorder. GZ-11610 was 290-fold selective for VMAT2 over dopamine transporters, suggesting that it may lack abuse liability. GZ-11610 was 640 to 3500-fold selective for VMAT2 over serotonin transporters and nicotinic acetylcholine receptors. GZ-11610 exhibited >1000-fold selectivity for VMAT2 over hERG, representing a robust improvement relative to our previous VMAT2 inhibitors. GZ-11610 (3-30 mg/kg, s.c. or 56 mg/kg, oral) reduced methamphetamine-induced hyperactivity in methamphetamine-sensitized rats. Thus, GZ-11610 is a potent and selective inhibitor of VMAT2, may have low abuse liability and low cardiotoxicity, and after oral administration is effective and specific inhibiting the locomotor stimulant effects of methamphetamine, suggesting further investigation as a potential therapeutic for methamphetamine use disorder.

INTRODUCTION

The United Nations reported that methamphetamine (METH) seizures worldwide increased by 158% between 2010 and 2015 (1), revealing recent dramatic increases in METH use. However, there are no pharmacotherapies approved by the US Food and Drug Administration to treat METH use disorder. The abuse liability of METH is the result of its rewarding effects, which are mediated by increases in dopamine (DA) release in the mesocorticolimbic system (2,3). METH penetrates the blood-brain barrier and dopaminergic neuronal cell membranes due to its high lipophilicity ($\text{Log}P = 2.10$) (4) and its ability to act as a substrate for the DA transporter (DAT) (5). Once inside dopaminergic neurons, METH inhibits monoamine oxidase activity, leading to a reduction in DA metabolism and increased cytosolic DA concentrations (6). Moreover, increased cytosolic DA concentrations result from METH-induced inhibition of DA transport from the cytosol into presynaptic storage vesicles via the vesicular monoamine transporter-2 (VMAT2) (7-9). Also, METH stimulates DA release from the vesicles into the cytosol via reverse transport at VMAT2 and via its weak base properties, which reduce the vesicular pH gradient and driving force for VMAT2 function and retention of DA in the storage vesicles (8,10,11). Increased cytosolic DA concentrations are released from the presynaptic terminals into the extracellular space via reversal of DAT, the functional outcome being METH-induced reward (12-14). Support for the concept that METH-induced reward requires an increase in extracellular DA is derived from studies in which bilateral injection of the dopaminergic neurotoxin, 6-hydroxydopamine, into reward-relevant brain regions (e.g., nucleus accumbens) results in decreases in amphetamine self-administration (15). Thus, a reduction in METH-evoked DA release is a desired property of a pharmacotherapy for METH use disorder.

Based on the complex mechanism of action of METH at dopaminergic presynaptic terminals, we identified VMAT2 as a novel target for the discovery of therapeutics for METH use disorder (16). Moreover, we identified lobeline (chemical structure, Fig. 1), the major alkaloidal natural product from *Lobelia inflata*, as a potent inhibitor of VMAT2 function using an isolated synaptic vesicle preparation from rat brain; as such, lobeline was identified from *in vitro* studies as having potential therapeutic efficacy in the treatment of METH use disorder (16,17). Evidence supporting the concept that VMAT2 is a viable pharmacological target comes from studies showing that METH-evoked DA efflux from dissociated cells co-expressing VMAT2 and DAT was reduced by 60% in the presence of either lobeline or dihydrotetabenazene, another VMAT2 inhibitor (18). Furthermore, lobeline decreased METH-evoked DA release from the intact rat brain slice preparation and importantly attenuated METH-induced hyperactivity and METH self-administration in preclinical animal models (19-23).

Although lobeline decreases the neurochemical and behavioral effects of METH by inhibiting VMAT2 function, lobeline has limitations as a therapeutic candidate, in that it acts nonselectively, inhibiting nicotinic acetylcholine receptors (nAChRs) and opioid receptors (17, 24-27). Also, lobeline has aversive side-effects, including conditioned taste avoidance in rats (28) and nausea in humans, resulting from its bitter taste (29). Furthermore, lobeline has a relatively short plasma half-life necessitating multiple daily dosing, which likely would decrease medication compliance (30).

To address these limitations, our research group began a drug discovery program and embarked on structure-activity relationship studies aimed to discover compounds with improved selectivity for VMAT2 and enhanced drug likeness. Lobelane (Fig. 1), a chemically defunctionalized lobeline analog, exhibited increased affinity for VMAT2 and low affinity for nAChRs, i.e., improved selectivity for VMAT2 relative to lobeline (25,31). Lobelane also inhibited VMAT2 function and METH-evoked DA release from rat brain slices, and importantly, decreased METH-induced hyperactivity and METH self-administration in rats (20,31,32). However, tolerance developed to lobelane's behavioral efficacy following repeated administration (32). Upon further iterative investigation of the chemical scaffold, GZ-793A (Fig. 1) was identified as a lead compound, having high affinity and selectivity (> 1000-fold) for VMAT2 over nAChRs (33). GZ-793A inhibited METH-evoked DA release from striatal vesicle preparations and from nucleus accumbens using *in vivo* microdialysis (9,34). Importantly, GZ-793A decreased METH-induced hyperactivity, METH reward in conditioned place preference studies, and METH self-administration without the development of tolerance (35). Further preclinical research showed that GZ-793A decreased METH-induced and cue-induced reinstatement of METH seeking, indicating its potential efficacy in the treatment of relapse of METH seeking (35-37). However, GZ-793A also exhibited affinity for the human-ether-a-go-go related gene (hERG) channel, indicating the potential for cardiotoxicity (33), prohibiting its further development as a medication.

In our continued pursuit of a pharmacotherapy to treat METH use disorder, the current study evaluated *in vitro* inhibition of VMAT2 function produced by a novel, but related chemical scaffold represented by the lead compound, *R*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propan-1-amine (GZ-11610; Fig. 1). Selectivity of GZ-11610 for VMAT2 over DAT, the serotonin transporter (SERT), nAChRs, and the hERG channel was determined. In addition, the ability of GZ-11610 to reduce METH-induced hyperactivity in METH-sensitized rats was assessed as an initial *in vivo* preclinical evaluation of its therapeutic potential as a treatment for METH use disorder.

MATERIALS AND METHODS

Animals.

Male Sprague-Dawley rats (200-250 g upon arrival) were purchased from Harlan Inc. (Indianapolis, IN, USA) and individually housed with ad libitum access to food and water. Following arrival, rats acclimated to the environment for 1 week prior to the start of experiments. Experimental protocols involving the animals were in accordance with the 2011 *National Institutes of Health 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.

Radiolabeled dopamine ($[^3\text{H}]\text{DA}$, dihydroxyphenylethylamine, 3,4- $[7\text{-}^3\text{H}]$), specific activity 34.8 Ci/mmol), serotonin ($[^3\text{H}]\text{5-HT}$, 5-hydroxytryptamine creatinine sulfate, 5- $[1,2\text{-}^3\text{H}]\text{[N]}$, specific activity 29.5 Ci/mmol), nicotine ($[^3\text{H}]\text{NIC}$, L-(-)- $[N\text{-methyl-}^3\text{H}]$; specific activity,

80.4 Ci/mmol) and Microscint 20 cocktail were purchased from PerkinElmer (Waltham, MA, USA). [^3H]Dofetilide ([N -methyl- ^3H], specific activity, 80 Ci/mmol) and methyllycaconitine ([^3H]MLA, [$1\alpha,5,6\beta,14\alpha,16\beta$]-20-ethyl-1,6,14,16-tetramethoxy-4-[[[2-([^3H]-[^3H]-methyl-2,5-dioxo-1-pyrrolidinyl)benzoyl]oxy]methyl]-aconitane-7,8-diol; specific activity, 60 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). (+)-Methamphetamine hydrochloride (METH), sucrose, N -[2-hydroxyethyl]piperazine- N' -[2-ethanesulfonic acid] (HEPES), *tris*[hydroxymethyl]-aminomethane hydrochloride (Trizma HCl), *tris*[hydroxymethyl]aminomethane base (Trizma), sodium chloride, magnesium sulfate, α -D-glucose, disodium ethylenediamine tetraacetate (EDTA), ethylene glycol tetraacetate (EGTA), magnesium sulfate, potassium hydroxide, potassium tartrate, adenosine triphosphate (ATP- Mg^{2+}), geneticin, polyethyleneimine (PEI), dopamine hydrochloride, pargyline hydrochloride, catechol, 5-hydroxytryptamine creatinine sulfate (5-HT), amitriptyline, nomifensine maleate (nomifensine), 1-(2-[*bis*(4-fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine dihydrochloride (GBR-12909), 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR-12935), cytosine, and (–)-nicotine hydrogen tartrate salt were purchased from Sigma-Aldrich (St. Louis, MO). Sodium bicarbonate, potassium chloride, calcium chloride, monopotassium phosphate, sodium hydroxide, and hydrogen chloride were purchased from Fisher Scientific Co. (Pittsburgh, PA). Ascorbic acid was purchased from AnalaR-BHD Ltd. (Polle, UK). Scintillation cocktail 3a70B was purchased from Research Products International Corp. (Mount Prospect, IL). Minimum essential medium, 10% fetal bovine serum and Hanks' Balanced Salt solution were purchased from Gibco (Grand Island, NY). Also, 1% non-essential amino acids was purchased from Thermo Fisher Scientific (Waltham, MA). 2-Ethyl-9,10-dimethoxy-3-(2-methylpropyl)-1,3,4,6,7,11b-hexahydrobenzo[*a*]quinolizine-2-ol (RO4-1284) was a kind gift from Hoffman-La Roche, Ltd. (Basel, Switzerland),

Synthesis of GZ-11610.

Step 1: Synthesis of *R*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propanamide (2). To a solution of *R*-amphetamine (1) (3.03 g, 22.41 mmol), 3-(4-methoxyphenyl)propanoic acid (4.44 g, 24.65 mmol), and HOBt (3.63 g, 26.89 mmol) in methylene chloride (80 mL) was added triethylamine (7.81 mL, 56.03 mmol) then EDCI (5.15 g, 26.89 mmol). The resulting mixture was stirred at room temperature overnight. Water was added to the mixture and the aqueous phase was extracted with methylene chloride (2 x 50 mL), and the combined organic layers were washed with water (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4 , filtered and concentrated under reduced pressure. The crude product was chromatographed on silica (hexanes/ethyl acetate 10:1 to 2:1) to afford compound 2 (6.33 g, 95%) as a white solid: ^1H NMR (400 MHz, CDCl_3) δ 7.18–7.34 (m, 2H), 7.05–7.13 (m, 4H),

6.82 (m, 2H), 5.18 (br d, $J=7.2$ Hz, 1H), 3.77 (s, 3H), 2.87 (t, $J=7.6$ Hz, 2H), 2.77 (dd, $J=13.2$, 6.0 Hz, 1H), 2.63 (dd, $J=13.6$, 6.8 Hz, 1H), 2.37 (dt, $J=8.0$, 2.4 Hz, 1H), 1.03 (d, $J=6.8$ Hz) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ 171.6, 158.3, 138.0, 133.1, 129.7, 129.5, 128.5, 126.6, 114.1, 55.5, 46.0, 42.5, 39.2, 31.1, 20.0 ppm; MS (EI) m/z 297.1 (M^+).

Step 2: Synthesis of *R*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propan-1-amine (GZ-11610). To a solution of **2** (5.0 g, 16.81 mmol) in THF (50 mL) was added LiAlH_4 (60 mL, 1.0 M in THF) dropwise at 0 °C. The resulting mixture was heated at reflux for 6 h before being cooled to 0 °C. The reaction was quenched by carefully adding H_2O (2.28 mL), followed by 15% aqueous NaOH (2.28 mL) and H_2O (6.84 mL). The resulting milky suspension was warmed to room temperature. Anhydrous MgSO_4 was added and the mixture was stirred for 1 h, filtered through a pad of Celite, rinsed with ethylacetate and methylene chloride. The combined filtrates were dried, concentrated under vacuum, and the crude product was chromatographed on silica (methylene chloride/methanol, 30:1 to 10:1) to afford GZ-11610 (4.33 gm, 91%) as a white solid with low melting point: ^1H NMR (400 MHz, CDCl_3) δ 7.15-7.34 (m, 5H), 7.01 (d, $J=8.4$ Hz, 2H), 6.80 (dd, $J=8.4$, 2.0 Hz, 2H), 3.77 (s, 3H), 2.87 (m, 1H), 2.47-2.77 (m, 6H), 1.73 (m, 2H), 1.05 (d, $J=6.4$ Hz) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ 157.9, 139.7, 134.2, 129.5, 129.4, 128.6, 126.4, 113.9, 55.4, 54.8, 46.8, 43.8, 32.8, 32.1, 20.4 ppm; MS (EI) m/z 282.2 [$\text{M}-1$] $^+$; Purity: >97% (LC-MS).

Vesicular [^3H]DA Uptake.

Rat striatal synaptic vesicles were prepared as previously described (17) and used to determine GZ-11610-induced inhibition of [^3H]DA uptake into the isolated presynaptic vesicles. Briefly, striata from individual rats were homogenized in 14 mL of 0.32 M sucrose solution containing 5 mM sodium bicarbonate (pH 7.4) with 10 up-and-down strokes of a Teflon pestle homogenizer (clearance ~0.009 inch) using a Maxima Digital Overhead Stirrer (400 rpm; Fisher Scientific Co., Pittsburgh, PA). Homogenates were centrifuged (2,000 g for 10 min at 4°C), and the resulting supernatants were centrifuged (10,000 g for 30 min at 4°C). Pellets were resuspended in 2 mL of 0.32 M sucrose solution and were subjected to osmotic shock by transferring samples to tubes containing 7 mL of ice-cold MilliQ water. Then, samples were homogenized with 5 up-and-down strokes of the Teflon pestle homogenizer. After 5 min, osmolarity was restored by transferring the samples to tubes containing 900 μL of 0.25 M HEPES and 900 μL of 1.0 M potassium tartrate solution. Samples were centrifuged (20,000 g for 20 min at 4°C) and resulting supernatants centrifuged (55,000 g for 1 h at 4°C), followed by addition of 100 μL of 10 mM magnesium sulfate, 100 μL of 0.25 M HEPES, and 100 μL of 1.0 M potassium tartrate solution, followed by a final centrifugation (100,000 g for 45 min at 4°C). Final pellets were resuspended in 2.4 mL of assay buffer (25 mM HEPES, 100 mM potassium tartrate, 50 μM EGTA, 100 μM EDTA, 1.7 mM ascorbic acid, and 2 mM ATP-Mg^{2+} , pH 7.4). Vesicular suspension (100 μL) was added to tubes containing assay buffer (300 μL), various concentrations (0.1 nM - 0.1 mM; 50 μL) of GZ-11610 and 0.1 μM [^3H]DA (50 μL) to obtain a final assay volume of 500 μL . Nonspecific uptake was determined using RO4-1284 (10 μM). After incubation for 8 min at 37°C, uptake was terminated by rapid filtration. Scintillation cocktail was added to filters (presoaked GF/B filters in 0.5% PEI for 1 h). Radioactivity retained by the filters was determined by liquid scintillation spectrometry.

(TRICARB 2100 TR Packard scintillation counter; Packard BioScience Company, Meriden, CT).

Synaptosomal [³H]DA and [³H]5-HT Uptake.

Inhibition of [³H]DA and [³H]5-HT uptake via DAT and SERT, respectively, was determined using previously published methods (17,38). Briefly, striata from individual rats were homogenized in 20 mL of 0.32 M sucrose containing 5 mM sodium bicarbonate (pH 7.4) with 16 up-and-down strokes of a Teflon pestle homogenizer (clearance ~0.003 inch) using the Maxima Digital Overhead Stirrer (400 rpm). Homogenates were centrifuged (2,000 g for 10 min at 4°C). Resulting supernatants were centrifuged (20,000 g for 17 min at 4°C). Pellets were resuspended in 2.4 mL (DA uptake assay) or 1.4 mL (5-HT uptake assay) of assay buffer (125 mM sodium chloride, 5 mM potassium chloride, 1.5 mM magnesium sulfate, 1.25 mM calcium chloride, 1.5 mM monopotassium phosphate, 10 mM α-D-glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline hydrochloride, and 0.1 mM ascorbic acid, and saturated with 95% O₂/5% CO₂, pH 7.4). To determine [³H]DA uptake, synaptosomal suspension (25 μL) was added to tubes containing assay buffer (375 μL) and various concentrations (0.1 nM – 0.1 mM; 50 μL) of GZ-11610. To determine [³H]5-HT uptake, synaptosomal suspension (50 μL) was added to tubes containing assay buffer (125 μL) and GBR-12935 (25 μL, 100 nM; a DAT inhibitor). After incubation, tubes were placed on ice for 2 min. [³H]DA (50 μL, 100 nM) or [³H]5-HT (25 μL, 100 nM) was added to each tube, and then incubated at 34°C for 10 min. Assays were performed in duplicate in a total volume of 500 μL for the DA uptake assay, or 250 μL for the 5-HT uptake assay. Uptake was terminated by addition of 3 mL of ice-cold assay buffer and subsequent filtration. Nonspecific [³H]DA and [³H]5-HT uptake were determined in the presence of nomifensine (100 μM) and fluoxetine (10 μM), respectively. Radioactivity retained by the filters (presoaked in assay buffer containing 1mM catechol for 1 h) was determined.

[³H]NIC and [³H]MLA binding.

GZ-11610-induced inhibition of [³H]NIC and [³H]MLA binding assesses the interaction with α4β2 and α7 nAChRs, respectively. Binding assays employed previously published methods (39). In brief, rat whole brain excluding cortex and cerebellum was homogenized in 20 vol of ice-cold assay buffer (2 mM HEPES, 14.4 mM sodium chloride, 0.15 mM potassium chloride, 0.2 mM calcium chloride and 0.1 mM magnesium sulfate at pH 7.5) for 90 sec using a Tekmar polytron (Tekmar-Dohrmann, Mason, OH, USA). Homogenates were centrifuged (31,000 g for 17 min at 4°C). Pellets were resuspended in 20 vol of assay buffer by sonication (Vibra Cell, Sonics & Materials Inc., Danbury, CT). Samples were incubated at 37 °C for 10 min (Reciprocal Shaking Bath Model 50, Precision Scientific, Chicago IL, USA). Samples were centrifuged (31,000 g for 17 min at 4°C). Resulting pellets were resuspended in 20 vol assay buffer by sonication, and centrifuged (31,000 g for 17 min at 4°C). Final pellets were resuspended and stored in incubation buffer (40 mM HEPES, 288 mM sodium chloride, 3.0 mM potassium chloride, 4.0 mM calcium chloride and 2.0 mM magnesium sulfate (pH 7.5). Membrane suspensions (100-140 μg protein/100 μL) were added to tubes containing a single concentration of GZ-11610 (7-9 concentrations, 0.1 nM – 0.1 mM), 3 nM (50 μL) [³H]NIC or [³H]MLA, and incubation buffer for a final assay vol of 250 μL. Samples were incubated for 60 min at room temperature. NIC or MLA (10 pM –

100 μ M) concentration-response curves were obtained as positive controls. Nonspecific [3 H]NIC or [3 H]MLA binding was determined using 100 μ M cytosine and 10 μ M nicotine, respectively. Reactions were terminated by filtration on Unifilter-96 GF/B filter plates presoaked in 0.5% PEI using a Packard Filter Mate Harvester (Perkin Elmer, Inc., Waltham, MA). Plates were washed 3 times with 350 μ L of ice-cold assay buffer, dried for 60 min at 45°C, bottom sealed, and each well filled with 40 μ L Microscint 20 cocktail. Bound radioactivity on the filter was determined via liquid scintillation spectrometry (Top Count NXT scintillation counter; PerkinElmer, Inc.).

[3 H]Dofetilide binding.

GZ-11610-induced inhibition of [3 H]dofetilide binding to hERG channels assessed potential cardiotoxicity. HEK293 cells stably expressing hERG channels were cultured according to the Millipore protocol (Millipore, Billerica, MA, USA). The method for determining [3 H]dofetilide binding to hERG protein expressed by the cell membranes was described previously (33,40). In brief, frozen cells were thawed at 37°C and immediately transferred into T-75 cm² flasks containing minimum essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 400 μ g/mL geneticin. Cells were allowed to adhere for 4-8 h in a humidified atmosphere with 5% CO₂. Cells were passaged every 6 days, and medium was replaced every 2 days. At least three passages were performed before membrane collection. On the last passage, cells were seeded into 150 x 25 mm dishes at 2.5×10^6 cells per dish and placed at 30 °C, 5% CO₂, for 40-48 h prior to membrane preparation. Cells were rinsed twice with Hanks' Balanced Salt solution at 37 °C and collected by scraping the dishes in ~20 mL of ice-cold 0.32 M sucrose solution containing 5 mM sodium bicarbonate (pH 7.4). Cell membranes were homogenized on ice with a Teflon pestle (~0.003 inch) using a Maximal Digital homogenizer at 280 rpm for 30 sec. Homogenates were centrifuged (300 g and 800 g for 4 min each at 4 °C). Pellets were resuspended in 9 mL of ice-cold MilliQ water, and osmolarity was restored by adding 1 mL of 500 mM Tris buffer (pH 7.4), followed by resuspension and centrifugation (20,000 g for 30 min at 4 °C). Pellets were resuspended in 2 mL assay buffer (50 mM Tris, 10 mM potassium chloride, and 1 mM magnesium chloride, pH 7.4, at 4 °C). Aliquots of cell membrane suspension were stored at -80 °C and thawed the day of the [3 H]dofetilide binding assay. Protein content was determined prior to the assay using a Bradford protein assay with bovine albumin as the standard. On the day of the binding assay, thawed cell membrane suspension (5 μ g) was added to duplicate tubes containing assay buffer (150 μ L), a single concentration (25 μ L; 0.1 nM – 0.1 mM) of GZ-11610 or amitriptyline (0.1 nM – 0.1 mM, as the positive control; 41,42), and 25 μ L of [3 H]dofetilide (5 nM) for a final assay vol of 250 μ L, and incubated for 60 min at room temperature. Amitriptyline (1 mM) was used to determine nonspecific binding. Reactions were terminated by rapid filtration through Whatman GF/B filters presoaked in 0.5% PEI. Filters were washed 3 times with 1 mL ice-cold assay buffer. Radioactivity retained by the filters was determined as described.

METH-induced hyperactivity.

The ability of GZ-11610 to decrease METH-sensitized locomotor activity was determined using a mixed factor design with METH treatment as a between-subjects factor and GZ-11610 as within-subjects factor (36). Briefly, distance traveled was measured in

locomotor activity chambers (42 x 42 x 30 cm) with clear acrylic walls and floor. Chambers contained a horizontal 16 x 16 grid of photo beam sensors located 2.5 cm apart and 7 cm above the chamber floor. Photo beam breaks were recorded automatically and expressed as distance traveled using Versamax and Digipro System software (AccuScan Instruments Inc., Columbus, OH, USA). Rats were assigned randomly to METH treatment or saline control groups. On day 0 (habituation day, no injection), rats were placed in the locomotor activity chamber for 60 min and then returned to their home cages. On days 1-10, METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.) injections were administered based on group assignment, and rats were placed immediately in the chamber for 60 min. On day 11 (first test day), GZ-11610 (either 1, 10 or 30 mg/kg, s.c.) was administered in a randomized order 15 min prior to METH or saline injection, and then, rats were placed immediately into the activity chamber. Between test days, 2-3 washout days occurred in which METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.) was administered in the absence of GZ-11610. To obtain full dose-response curves, additional doses (0, 3 and 5.6 mg/kg) of GZ-11610 were evaluated following s.c. administration using the same group of rats and the same procedures.

In a separate drug-naïve group of rats, the ability of oral GZ-11610 to decrease METH-induced hyperactivity was evaluated using a mixed factor design, with METH treatment as a between-subjects factor and GZ-11610 dose a within-subjects factor. GZ-11610 was administered using an ascending dose order. Initially, rats were habituated to the gavage procedure on 5 consecutive days during the METH-sensitization period (37). Food was removed from the home cage 2 h prior to each oral gavage. GZ-11610 (5.6 - 300 mg/kg or sterile water vehicle) was administered by oral gavage followed 15 min later by either METH (1 mg/kg, s.c.) or saline (1 mg/kg, s.c.) injection, depending on group assignment, and then rats were placed immediately into the activity chamber. As above, on washout days, vehicle (2 mL, oral) was given 15 min prior to METH (1 mg/kg, s.c.) or saline (s.c.) and placement in activity chamber.

Data analysis.

Specific [^3H]DA and [^3H]5-HT uptake as well as [^3H]dofetilide, [^3H]NIC, and [^3H]MLA binding were obtained by subtracting nonspecific uptake or binding from total uptake or binding, respectively. Concentration of GZ-11610 that produced 50% inhibition of uptake or binding (IC_{50} values) was obtained from the concentration-response curves via an iterative curve-fitting program (Prism 4.0; GraphPad Software Inc., San Diego, CA, USA). Inhibition constants (K_i values) were determined using the Cheng-Prusoff equation (43). Distance traveled (in meters) during the last 45 min of the 60 min locomotor activity session was analyzed using two-way or one way ANOVA followed by Tukey's or Dunnett's post hoc analysis, as noted. Data from the first 15 min of the session was considered to be a habituation period and thus, was not included in the analysis. Data analysis was performed using GraphPad Prism 7.03 (GraphPad Software, Inc., La Jolla, CA).

RESULTS

VMAT2 affinity and selectivity.

GZ-11610 potently ($K_i = 8.7$ nM) inhibited [3 H]DA uptake at VMAT2, with a maximal inhibition (I_{max}) of >95% (Fig. 2). GZ-11610 also inhibited [3 H]DA uptake at DAT and [3 H]5-HT uptake at SERT ($K_i = 2.51$ and 5.55 μ M, respectively), with an I_{max} at both transporters of >95% (Fig. 2). GZ-11610 exhibited 288- and 637-fold greater affinity for VMAT2 relative to DAT and SERT, respectively. Thus, GZ-11610 is selective for VMAT2 over DAT and SERT. Also, GZ-11610 inhibited [3 H]dofetilide binding to hERG channels expressed on HEK-293 cell membranes, with a K_i of 9.50 μ M and an I_{max} of >80% (Fig. 2). Thus, GZ-11610 was 1090-fold selective for VMAT2 over hERG. Further, across a wide concentration range (0.1 nM – 0.1 mM), GZ-11610 exhibited an I_{max} of <20% inhibition of [3 H]NIC and [3 H]MLA binding to rat brain membranes (Fig. 2); as such, K_i values could not be obtained. Overall, GZ-11610 exhibited high affinity for VMAT2 and greater than two-orders of magnitude selectivity for VMAT2 over DAT, SERT, hERG, and $\alpha 4\beta 2$ and $\alpha 7$ nAChRs.

METH sensitization.

For Day 0, 1 and 10, data from the two experiments determining effects of s.c. and oral administration of GZ-11610 were combined to evaluate effects of acute and repeated administration of METH or saline, since these data were obtained prior to GZ-11610 administration and groups were handled identically in the two experiments. Two-way ANOVA revealed a significant treatment $\times$ day interaction [$F_{2,14} = 36.44$, $p < 0.0001$]. On Day 0 (habituation, prior to METH or saline administration), no differences in distance traveled between the METH-treated and saline-injected groups were found (Fig. 3). On Day 1, acute METH (1 mg/kg, s.c.) increased ($p < 0.05$) distance traveled compared to saline control (Fig. 3). On Day 10, repeated administration of METH (1 mg/kg, s.c., once daily for 10 days) increased distance traveled ($p < 0.05$) compared to 10 consecutive daily saline injections (Fig. 3). On Day 10, distance traveled by the METH group was greater than distance traveled by this same group on Day 1 ($p < 0.05$; Fig. 3), indicative of sensitization.

Effect of GZ-11610 (s.c.) on METH-sensitized locomotor activity.

After 10 consecutive daily administrations of METH or saline, GZ-11610 ($1 - 30$ mg/kg) or vehicle (sterile water) was administered s.c. to both groups of rats 15 min prior to injection (s.c.) of METH or saline, respectively, followed by placement in the activity chamber. Two-way ANOVA revealed a GZ-11610 pretreatment $\times$ METH treatment interaction [$F_{5,48} = 68.81$, $p < 0.0001$] on distance traveled. Compared to vehicle injection, GZ-11610 dose-dependently reduced the distance traveled following an injection of METH in the METH-sensitized group (Fig. 4). Tukey's test revealed that GZ-11610 at doses from 3 to 30 mg/kg were different from vehicle in the METH group. Although GZ-11610 appeared to decrease activity in the saline group, this decrease did not reach statistical significance as determined by the two-way ANOVA followed by Tukey's test, likely due to the high variability following the vehicle injection (Fig. 4, inset). Additionally, pair-wise comparison of the distance traveled between the METH group and the saline group after each dose of GZ-11610 revealed a greater distance traveled in the METH group ($p < 0.05$, Tukey's test), with the

exception of the highest dose (30 mg/kg) of GZ-11610. However, further evaluation using one-way ANOVA of the effect of GZ-11610 in the saline control group revealed a significant dose effect [$F = 5.55$, $p < 0.005$], and Dunnett's test revealed a significant decrease in locomotor activity following GZ-11610 3-30 mg/kg (Fig. 4, inset). Thus, GZ-11610 (s.c.) decreased METH-induced hyperactivity in METH-sensitized rats and in the saline control group, such that the effect of GZ-11610 to decrease the METH sensitized behavior was not specific. The decrease in activity induced by GZ-11610 in saline group did not appear to be due to lethargy or pain, based on experimenter observation.

Effect of GZ-11610 (oral) on METH-sensitized locomotor activity.

After 10 consecutive daily administrations of METH or saline, GZ-11610 (5.6 – 300 mg/kg) or vehicle (sterile water) was administered orally to rats in both groups 15 min prior to injection (s.c.) of METH or saline, respectively, and placement in the activity monitor. Two-way ANOVA revealed a GZ-11610 pretreatment x METH treatment interaction [$F_{7,63} = 7.403$, $p < 0.0001$] on distance traveled. Compared to vehicle injection, GZ-11610 dose dependency reduced the distance traveled following an injection of METH in the METH-sensitized group (Fig. 5). Tukey's test revealed that GZ-11610 at doses from 56 – 300 mg/kg were different from vehicle in the METH group; whereas, GZ-11610 did not decrease significantly the distance traveled in the saline group compared to vehicle injection (Fig. 5, inset). Furthermore, pair-wise comparison of the distance traveled between the METH group and the saline group after each dose of GZ-11610 revealed a greater distance traveled in the METH group. Using one-way ANOVA to assess the effect of GZ-11610 in the saline group also revealed that there were no significant GZ-11610 effects on locomotor activity in saline group [$F_{7,32} = 0.572$, $p > 0.05$]. Thus, oral GZ-11610 specifically decreased METH-induced hyperactivity in METH-sensitized rats.

Of note, there was a 2-fold greater amount of locomotor activity in the saline group following oral administration of vehicle (Fig. 5, insert) relative to that after s.c. administration of vehicle (Fig. 4, insert). Despite the habituation to the oral gavage, this procedure may have produced some stress, resulting in greater activity. However, the standard errors of the mean in the oral and s.c. vehicle conditions overlap, suggesting that there were not significant differences in locomotor activity between these control groups and that the apparent difference is reflective of variability in the data.

DISCUSSION

Using an iterative drug discovery approach targeting VMAT2, the current study identified a new *N*-butyl(1-methyl-2-phenylethyl)amine, amphetamine-like scaffold, containing an important chiral center, and moreover, evaluated the neurochemical and behavioral effects of GZ-11610, the pure *R*-(-)-enantiomer. GZ-11610 was found to selectively inhibit VMAT2 function and to specifically attenuate METH-sensitized locomotor activity when given by the oral route, suggesting that this lead compound has potential as a candidate pharmacotherapy for METH use disorder.

The current in vitro neurochemical results demonstrate that GZ-11610 exhibited high affinity ($K_i = 8.7$ nM) for VMAT2 and high selectivity (290 to 3500-fold) for VMAT2 over

DAT, SERT, hERG, $\alpha 4\beta 2$ nAChRs and $\alpha 7$ nAChRs. The high selectivity for VMAT2 over DAT suggests that GZ-11610 may lack abuse liability. Inhibition of DAT function results in increased extracellular DA concentrations and stimulation of postsynaptic DA receptors leading to reward. Inhibition of DAT function is highly correlated with psychostimulant-induced behaviors, reward and abuse liability (45-48).

The 4-orders of magnitude selectivity for VMAT2 over hERG suggests reduced risk for untoward cardiac arrhythmias at concentrations of GZ-11610 interacting with VMAT2 and providing therapeutic efficacy. Interaction at hERG channels has been associated with cardiac arrhythmias due to the role of these inward rectifying potassium channels during depolarization of the heart muscle and propagation of cardiac rhythm (49-51). The robust >1000-fold selectivity for VMAT2 over hERG in the current study is particularly compelling, because previous lead compounds acting as VMAT2 inhibitors, including lobeline, lobelane and GZ-793A, provided only 3 to 26-fold selectivity for VMAT2 over hERG (20,33).

Importantly, the greater than 4-orders of magnitude selectivity for VMAT2 over the most abundant nicotinic receptor subtypes ($\alpha 4\beta 2$ and $\alpha 7$) supports the interpretation that these nAChR protein targets are not responsible for the decrease in METH's behavioral effects nor their potential therapeutic efficacy. Furthermore, robust selectivity of GZ-11606 for VMAT2 will allow validation of VMAT2 as an important pharmacological target in the discovery for METH use disorder therapeutics.

In the current study, GZ-11610 decreased the psychomotor response to METH in rats that previously had been sensitized to repeated METH administration. Based on the current findings, GZ-11610 appears to have improved potency and efficacy reducing METH-induced locomotor activity relative to our previous lead compounds, lobeline, lobelane and GZ-793A (21,32,36). However, the behavioral effects of the previous leads were evaluated in these earlier studies with respect to their ability to reduce the acute, dose-related hyperactivity induced by METH. Behavioral sensitization induced by repeated exposure to psychostimulants has been established as an animal model of human addiction due to the associated enduring alterations in nucleus accumbens DA neurochemistry leading to reward and persistent drug seeking (14,52-54). Thus, the GZ-11610-induced decrease in METH-stimulated locomotor activity in METH-sensitized animals provides greater face validity with respect to the clinical situation than does a reduction in the acute behavioral response to METH.

The assertion that the GZ-11610-induced decrease in METH-stimulated locomotor activity in METH-sensitized rats was specific is based on the observation that GZ-11610, following oral administration, did not significantly alter locomotor activity in the control group repeatedly administered saline. However, following s.c. administration, GZ-11610 was found to decrease locomotor activity in the saline control group. Thus, the GZ-11610-induced decrease in METH sensitized activity was specific only following oral administration of GZ-11610. This is important because the oral route is the preferred clinical route of administration.

Consistent with previous findings regarding our earlier lead compounds (21,55), GZ-11610 significantly decreased METH-sensitized locomotor activity following either s.c. or oral administration. The GZ-11610-induced decrease in METH's behavioral response supports the interpretation that GZ-11610 penetrates the blood-brain barrier and accesses the brain and that the lead compound is sufficiently orally bioavailable to demonstrate efficacy. However, an approximately 10-fold higher dose was required following oral administration relative to s.c. administration. Further, the maximal effect after oral administration of GZ-11610 was about 50% of that following s.c. administration. Likely, the physicochemical characteristics (e.g., solubility, logP) and/or pharmacokinetic properties (e.g., half-life, clearance, plasma protein binding, metabolism) of GZ-11610 may be responsible for the observed reduced potency and efficacy following oral versus s.c. administration. Interestingly, it is possible that GZ-11610 (*R*-enantiomer) may be metabolized via oxidative N-CH₂ bond cleavage to *R*(-)-amphetamine, which has relatively lower psychostimulant effects compared with S-(+)-amphetamine (44). Future pharmacokinetic and drug metabolism studies will determine the ADME profile of GZ-11610 and also identify its metabolites after oral administration, in order to assess the limitations and potential abuse liability of this promising lead compound.

CONCLUSION

GZ-11610, a representative compound from a new VMAT2-selective inhibitor scaffold (*N*-butyl(1-methyl-2-phenylethyl)amine), was identified and its effects on METH-stimulated locomotion in METH-sensitized rats were evaluated following s.c. and oral administration. GZ-11610 exhibited high affinity ($K_i = 8.7$ nM) and high selectivity (290- to 3500-fold) for VMAT2 over hERG, DAT, SERT, and nAChRs. Of note, GZ-11610 exhibited robustly improved selectivity for VMAT2 over the hERG channel (>1000-fold) compared to previously reported VMAT2 inhibitors (3- to 26-fold). Oral administration of GZ-11610 specifically reduced METH-stimulated locomotor activity in METH-sensitized rats. Further studies aimed at improving the bioavailability of GZ-11610 would contribute to the development of this lead compound as a treatment for METH use disorders.

ACKNOWLEDGMENTS

This work was supported by funding from the National Institute of Health grants U01 DA013519 and UL1 TR001998.

REFERENCES

1. 2015 World Drug Report from the United Nations Office on Drugs and Crime (UNODC) [Internet]. Available from: <http://www.unodc.org/>
2. Wise RA, Rompre PP. Brain dopamine and reward. *Annu Rev Psychol.* 1989;40:191–225. [PubMed: 2648975]
3. Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci U A.* 1988 7;85(14):5274–8.
4. Gulaboski R, Cordeiro MNDS, Milhazes N, Garrido J, Borges F, M Jorge, et al. Evaluation of the lipophilic properties of opioids, amphetamine-like drugs, and metabolites through electrochemical studies at the interface between two immiscible solutions. *Anal Biochem.* 2007 2 15;361(2):236–43. [PubMed: 17178092]

5. Johnson RA, Eshleman AJ, Meyers T, Neve KA, Janowsky A. [³H]substrate- and cell-specific effects of uptake inhibitors on human dopamine and serotonin transporter-mediated efflux. *Synapse*. 1998 9;30(1):97–106.
6. Mantle TJ, Tipton KF, Garrett NJ. Inhibition of monoamine oxidase by amphetamine and related compounds. *Biochem Pharmacol*. 1976 9 15;25(18):2073–7. [PubMed: 985546]
7. Peter D, Jimenez J, Liu Y, Kim J, Edwards RH. The chromaffin granule and synaptic vesicle amine transporters differ in substrate recognition and sensitivity to inhibitors. *J Biol Chem*. 1994 3 11;269(10):7231–7. [PubMed: 8125935]
8. Sulzer D, Sonders MS, Poulsen NW, Galli A. Mechanisms of neurotransmitter release by amphetamines: a review. *Prog Neurobiol*. 2005 4;75(6):406–33. [PubMed: 15955613]
9. Horton DB, Nickell JR, Zheng G, Crooks PA, Dwoskin LP. GZ-793A, a lobeline analog, interacts with the vesicular monoamine transporter-2 to inhibit the effect of methamphetamine. *J Neurochem*. 2013 10;127(2): 177–86. [PubMed: 23875622]
10. Schuldiner S, Steiner-Mordoch S, Yelin R, Wall SC, Rudnick G. Amphetamine derivatives interact with both plasma membrane and secretory vesicle biogenic amine transporters. *Mol Pharmacol*. 1993 12;44(6): 1227–31. [PubMed: 7903417]
11. Sulzer D, Rayport S. Amphetamine and other psychostimulants reduce pH gradients in midbrain dopaminergic neurons and chromaffin granules: a mechanism of action. *Neuron*. 1990 12;5(6):797–808. [PubMed: 2268433]
12. Sulzer D, Chen TK, Lau YY, Kristensen H, Rayport S, Ewing A. Amphetamine redistributes dopamine from synaptic vesicles to the cytosol and promotes reverse transport. *J Neurosci Off J Soc Neurosci*. 1995 5; 15(5 Pt 2):4102–8.
13. Fleckenstein AE, Volz TJ, Riddle EL, Gibb JW, Hanson GR. New insights into the mechanism of action of amphetamines. *Annu Rev Pharmacol Toxicol*. 2007;47:681–98. [PubMed: 17209801]
14. Volkow ND, Wang G-J, Fowler JS, Tomasi D, Telang F. Addiction: beyond dopamine reward circuitry. *Proc Natl Acad Sci U S A*. 2011 9 13; 108(37): 15037–42. [PubMed: 21402948]
15. Lyness WH, Friedle NM, Moore KE. Destruction of dopaminergic nerve terminals in nucleus accumbens: effect on d-amphetamine self-administration. *Pharmacol Biochem Behav*. 1979 11;11(5):553–6. [PubMed: 531077]
16. Dwoskin LP, Crooks PA. A novel mechanism of action and potential use for lobeline as a treatment for psychostimulant abuse. *Biochem Pharmacol*. 2002 1 15;63(2):89–98. [PubMed: 11841781]
17. Teng L, Crooks PA, Sonsalla PK, Dwoskin LP. Lobeline and nicotine evoke [³H]overflow from rat striatal slices preloaded with [³H]dopamine: differential inhibition of synaptosomal and vesicular [³H]dopamine uptake. *J Pharmacol Exp Ther*. 1997 3;280(3):1432–44. [PubMed: 9067333]
18. Wilhelm CJ, Johnson RA, Lysko PG, Eshleman AJ, Janowsky A. Effects of methamphetamine and lobeline on vesicular monoamine and dopamine transporter-mediated dopamine release in a cotransfected model system. *J Pharmacol Exp Ther*. 2004 9;310(3):1142–51. [PubMed: 15102929]
19. Wilhelm CJ, Johnson RA, Eshleman AJ, Janowsky A. Lobeline effects on tonic and methamphetamine-induced dopamine release. *Biochem Pharmacol*. 2008 3 15;75(6): 1411–5. [PubMed: 18191815]
20. Nickell JR, Krishnamurthy S, Norrholm S, Deaciuc G, Siripurapu KB, Zheng G, et al. Lobeline inhibits methamphetamine-evoked dopamine release via inhibition of the vesicular monoamine transporter-2. *J Pharmacol Exp Ther*. 2010 2;332(2):612–21. [PubMed: 19855096]
21. Miller DK, Crooks PA, Teng L, Witkin JM, Munzar P, Goldberg SR, et al. Lobeline inhibits the neurochemical and behavioral effects of amphetamine. *J Pharmacol Exp Ther*. 2001 3;296(3): 1023–34. [PubMed: 11181937]
22. Harrod SB, Dwoskin LP, Crooks PA, Klebaur JE, Bardo MT. Lobeline attenuates d-methamphetamine self-administration in rats. *J Pharmacol Exp Ther*. 2001 7;298(1):172–9. [PubMed: 11408539]
23. Harrod SB, Dwoskin LP, Green TA, Gehrke BJ, Bardo MT. Lobeline does not serve as a reinforcer in rats. *Psychopharmacology (Berl)*. 2003 2;165(4):397–404. [PubMed: 12459929]
24. Teng L, Crooks PA, Dwoskin LP. Lobeline displaces [³H]dihydrotetrabenazine binding and releases [³H]dopamine from rat striatal synaptic vesicles: comparison with d-amphetamine. *J Neurochem*. 1998 7;71(1):258–65. [PubMed: 9648873]

25. Zheng G, Dwoskin LP, Deaciuc AG, Norrholm SD, Crooks PA. Defunctionalized lobeline analogues: structure-activity of novel ligands for the vesicular monoamine transporter. *J Med Chem*. 2005 8 25;48(17):5551–60. [PubMed: 16107155]
26. Nickell JR, Siripurapu KB, Vartak A, Crooks PA, Dwoskin LP, Torres B, et al. The vesicular monoamine transporter-2: an important pharmacological target for the discovery of novel therapeutics to treat methamphetamine abuse. *Adv Pharmacol*. 2014 2 14;69(21):71–106. [PubMed: 24484975]
27. Miller DK, Lever JR, Rodvelt KR, Baskett JA, Will MJ, Kracke GR. Lobeline, a potential pharmacotherapy for drug addiction, binds to mu opioid receptors and diminishes the effects of opioid receptor agonists. *Drug Alcohol Depend*. 2007 7 10;89(2–3):282–91. [PubMed: 17368966]
28. Harrod SB, Dwoskin LP, Bardo MT. Lobeline produces conditioned taste avoidance in rats. *Pharmacol Biochem Behav*. 2004 5;78(1):1–5. [PubMed: 15159128]
29. Glover ED, Rath JM, Sharma E, Glover PN, Laflin M, Tonnesen P, et al. A multicenter phase 3 trial of lobeline sulfate for smoking cessation. *Am J Health Behav*. 2010 2;34(1):101–9. [PubMed: 19663757]
30. Miller DK, Harrod SB, Green TA, Wong M-Y, Bardo MT, Dwoskin LP. Lobeline attenuates locomotor stimulation induced by repeated nicotine administration in rats. *Pharmacol Biochem Behav*. 2003 1;74(2):279–86. [PubMed: 12479946]
31. Miller DK, Crooks PA, Zheng G, Grinevich VP, Norrholm SD, Dwoskin LP. Lobeline analogs with enhanced affinity and selectivity for plasmalemma and vesicular monoamine transporters. *J Pharmacol Exp Ther*. 2004 9;310(3): 1035–45. [PubMed: 15121762]
32. Neugebauer NM, Harrod SB, Stairs DJ, Crooks PA, Dwoskin LP, Bardo MT. Lobeline decreases methamphetamine self-administration in rats. *Eur J Pharmacol*. 2007 9 24;571(1):33–8. [PubMed: 17612524]
33. Nickell JR, Siripurapu KB, Horton DB, Zheng G, Crooks PA, Dwoskin LP. GZ-793A inhibits the neurochemical effects of methamphetamine via a selective interaction with the vesicular monoamine transporter-2. *Eur J Pharmacol*. 2017 1 15;795:143–9. [PubMed: 27986625]
34. Meyer AC, Neugebauer NM, Zheng G, Crooks PA, Dwoskin LP, Bardo MT. Effects of VMAT2 inhibitors lobeline and GZ-793A on methamphetamine-induced changes in dopamine release, metabolism and synthesis in vivo. *J Neurochem*. 2013 10; 127(2): 187–98. [PubMed: 23875705]
35. Beckmann JS, Denehy ED, Zheng G, Crooks PA, Dwoskin LP, Bardo MT. The effect of a novel VMAT2 inhibitor, GZ-793A, on methamphetamine reward in rats. *Psychopharmacol Berl*. 2012 3;220(2):395–403.
36. Alvers KM, Beckmann JS, Zheng G, Crooks PA, Dwoskin LP, Bardo MT. The effect of VMAT2 inhibitor GZ-793A on the reinstatement of methamphetamine-seeking in rats. *Psychopharmacol Berl*. 2012 11;224(2):255–62.
37. Wilmouth CE, Zheng G, Crooks PA, Dwoskin LP, Bardo MT. Oral administration of GZ-793A, a VMAT2 inhibitor, decreases methamphetamine self-administration in rats. *Pharmacol Biochem Behav*. 2013 11; 112:29–33. [PubMed: 24075974]
38. Norrholm SD, Horton DB, Dwoskin LP. The promiscuity of the dopamine transporter: implications for the kinetic analysis of [³H]serotonin uptake in rat hippocampal and striatal synaptosomes. *Neuropharmacology*. 2007 12;53(8):982–9. [PubMed: 18022203]
39. Horton DB, Siripurapu KB, Norrholm SD, Culver JP, Hojahmat M, Beckmann JS, et al. meso-Transdiene analogs inhibit vesicular monoamine transporter-2 function and methamphetamine-evoked dopamine release. *J Pharmacol Exp Ther*. 2011 3;336(3):940–51. [PubMed: 21177475]
40. Sviripa VM, Zhang W, Balia AG, Tsodikov OV, Nickell JR, Gizard F, et al. 2',6'-Dihalostyrylanilines, pyridines, and pyrimidines for the inhibition of the catalytic subunit of methionine S-adenosyltransferase-2. *J Med Chem*. 2014 7 24;57(14):6083–91. [PubMed: 24950374]
41. Teschemacher AG, Seward EP, Hancox JC, Witchel HJ. Inhibition of the current of heterologously expressed HERG potassium channels by imipramine and amitriptyline. *Br J Pharmacol*. 1999 9;128(2):479–85. [PubMed: 10510461]

42. Jo SH, Youm JB, Lee CO, Earm YE, Ho WK. Blockade of the HERG human cardiac K(+) channel by the antidepressant drug amitriptyline. *Br J Pharmacol*. 2000 4;129(7):1474–80. [PubMed: 10742304]
43. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K₁) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol*. 1973 12 1;22(23):3099–108. [PubMed: 4202581]
44. Heal DJ, Smith SL, Gosden J, Nutt DJ. Amphetamine, past and present--a pharmacological and clinical perspective. *J Psychopharmacol Oxf Engl*. 2013 6;27(6):479–96.
45. Stathis M, Scheffel U, Lever SZ, Boja JW, Carroll FI, Kuhar MJ. Rate of binding of various inhibitors at the dopamine transporter in vivo. *Psychopharmacology (Berl)*. 1995 6;119(4):376–84. [PubMed: 7480516]
46. Swanson JM, Volkow ND. Serum and brain concentrations of methylphenidate: implications for use and abuse. *Neurosci Biobehav Rev*. 2003 11;27(7):615–21. [PubMed: 14624806]
47. Volkow ND, Swanson JM. Variables that affect the clinical use and abuse of methylphenidate in the treatment of ADHD. *Am J Psychiatry*. 2003 11;160(11): 1909–18. [PubMed: 14594733]
48. German CL, Baladi MG, McFadden LM, Hanson GR, Fleckenstein AE. Regulation of the Dopamine and Vesicular Monoamine Transporters: Pharmacological Targets and Implications for Disease. *Pharmacol Rev*. 2015 10;67(4): 1005–24. [PubMed: 26408528]
49. Trudeau MC, Warmke JW, Ganetzky B, Robertson GA. HERG, a human inward rectifier in the voltage-gated potassium channel family. *Science*. 1995 7 7;269(5220):92–5. [PubMed: 7604285]
50. Abbott GW, Sesti F, Splawski I, Buck ME, Lehmann MH, Timothy KW, et al. MiRP1 forms IKr potassium channels with HERG and is associated with cardiac arrhythmia. *Cell*. 1999 4 16;97(2): 175–87. [PubMed: 10219239]
51. Doggrell SA, Hancox JC. Cardiac safety concerns for domperidone, an antiemetic and prokinetic, and galactagogue medicine. *Expert Opin Drug Saf*. 2014 1;13(1):131–8. [PubMed: 24147629]
52. Vezina P, Lorrain DS, Arnold GM, Austin JD, Suto N. Sensitization of midbrain dopamine neuron reactivity promotes the pursuit of amphetamine. *J Neurosci Off J Soc Neurosci*. 2002 6 1;22(11):4654–62.
53. Wang Q, Li D, Bubula N, Campioni MR, McGehee DS, Vezina P. Sensitizing exposure to amphetamine increases AMPA receptor phosphorylation without increasing cell surface expression in the rat nucleus accumbens. *Neuropharmacology*. 2017 5 1;117:328–37. [PubMed: 28223211]
54. Kalivas PW, Stewart J. Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. *Brain Res Brain Res Rev*. 1991 12; 16(3):223–44. [PubMed: 1665095]
55. Neugebauer NM, Harrod SB, Stairs DJ, Crooks PA, Dwoskin LP, Bardo MT. Lobelane decreases methamphetamine self-administration in rats. *Eur J Pharmacol*. 2007 9 24;571(1):33–8. [PubMed: 17612524]

Figure 1. Chemical structures of lobeline, lobelane, GZ-793A and GZ-11610.

Figure 2. GZ-11610 potently inhibits $[^3\text{H}]\text{DA}$ uptake at VMAT2 and is selective for VMAT2 over DAT, SERT, hERG, $\alpha 4\beta 2$ nAChRs and $\alpha 7$ nAChRs.

K_i values and selectivity ratios are provided in the symbol legend. Data are mean $\pm$ SEM specific uptake or binding expressed as % of the respective control (CON) uptake or binding in the absence of GZ-11610. Control values (mean $\pm$ SEM) are the following: for specific $[^3\text{H}]\text{DA}$ uptake at VMAT2, 49.6 ± 8.35 pmol/mg of protein/min; for specific $[^3\text{H}]\text{DA}$ uptake at DAT, 12.2 ± 0.54 pmol/mg of protein/min; for specific $[^3\text{H}]\text{5-HT}$ uptake at SERT, 7.89 ± 0.51 pmol/mg of protein/min; for specific hERG binding, 1540 ± 178 fmol/mg; for specific $[^3\text{H}]\text{NIC}$ binding at $\alpha 4\beta 2$ nAChRs, 22.6 ± 3.38 fmol/mg; and for specific $[^3\text{H}]\text{MLA}$ binding at $\alpha 7$ nAChRs, 36.4 ± 2.11 fmol/mg. ($n=4$ rats for neurotransmitter uptake assays, $n=3$ cell batches for hERG assays, and $n=3$ for nAChR binding assays).

Figure 3. Locomotor sensitization to repeated METH administration.

Day 0 (habituation day) represents distance traveled before METH or saline injection. Day 1 represents distance traveled following acute METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.) injection. Day 10 represents distance traveled following 10 daily injections of METH or saline. Data are mean $\pm$ SEM distance traveled in meters. Data were analyzed using two-way mixed factor ANOVA followed by Tukey's post hoc tests. (* $p < 0.05$ compared to respective saline group; # $p < 0.05$ compared to METH group on Day 0; \$ $p < 0.05$ compared to METH group on Day 1; $n=10$ /group; however, due to a computer problem, data for $n=2$ from each of the METH and saline groups are not included in the analysis).

Figure 4. GZ-11610 (s.c.) decreased METH-induced hyperactivity in METH-sensitized and control rats.

GZ-11610 (1 – 30 mg/kg) or vehicle (Veh, sterile water) was administered s.c. to the METH-sensitized group and the saline group (inset) 15 min before METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.), respectively, followed by placement in the activity chamber. Data are mean $\pm$ SEM distance traveled in meters. Dotted line represents 50% of the distance traveled after vehicle injection. Data were analyzed by two-way mixed factor ANOVA followed by Tukey's post hoc tests (* $p < 0.05$ compared to vehicle within each group; $n=5$ /group) or by one-way ANOVA followed by Dunnett's test (# $p < 0.05$ compared to vehicle).

Figure 5. GZ-11610 (oral) specifically decreased METH-induced hyperactivity in METH-sensitized rats.

GZ-11610 (5.6 – 300 mg/kg) or vehicle (Veh, sterile water) was administered orally to the METH-sensitized group and the saline group (inset) 15 min before METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.), respectively, followed by placement in the activity chamber. Data are mean $\pm$ SEM distance traveled in meters. Dotted line represents 50% of the distance traveled after vehicle injection. Data were analyzed by two-way mixed factor ANOVA followed by Tukey's post hoc tests. (* $p < 0.05$ compared to vehicle within each group; $n=5/\text{group}$), or by one-way ANOVA for the data in the saline control group.