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GZ-793A inhibits the neurochemical effects of methamphetamine via a selective interaction with the vesicular monoamine transporter-2

Justin R. Nickell¹, Kiran B. Siripurapu¹, David B. Horton², Guangrong Zheng³, Peter A. Crooks³, Linda P. Dwoskin¹

¹University of Kentucky, College of Pharmacy, 789 South Limestone Street, Lexington, KY 40536

²Medpace, Inc., Cincinnati, OH 45227

³University of Arkansas for Medical Sciences, College of Pharmacy, Little Rock, AR

Abstract

Lobeline and lobelane inhibit the behavioral and neurochemical effects of methamphetamine via an interaction with the vesicular monoamine transporter-2 (VMAT2). However, lobeline has high affinity for nicotinic receptors, and tolerance develops to the behavioral effects of lobelane. A water-soluble analog of lobelane, *R-N*-(1,2-dihydroxypropyl)-2,6-*cis*-di-(4-methoxyphenethyl)piperidine hydrochloride (GZ-793A), also interacts selectively with VMAT2 to inhibit the effects of methamphetamine, but does not produce behavioral tolerance. The current study further evaluated the mechanism underlying the GZ-793A-mediated inhibition of the neurochemical effects of methamphetamine. In contrast to lobeline, GZ-793A does not interact with the agonist recognition site on $\alpha 4\beta 2^*$ and $\alpha 7^*$ nicotinic receptors. GZ-793A (0.3–100 μ M) inhibited methamphetamine (5 μ M)-evoked fractional dopamine release from rat striatal slices, and did not evoke dopamine release in the absence of methamphetamine. Furthermore, GZ-793A (1–100 μ M) inhibited neither nicotine (30 μ M)-evoked nor electrical field-stimulation-evoked (100 Hz/1min) fractional dopamine release. Unfortunately, GZ-793A inhibited [³H]dofetilide binding to human-ether-a-go-go related gene channels expressed on human embryonic kidney cells, and further, prolonged action potentials in rabbit cardiac Purkinje fibers, suggesting the potential for GZ-793A to induce ventricular arrhythmias. Thus, GZ-793A selectively inhibits the neurochemical effects of methamphetamine and lacks nicotinic receptor interactions; however, development as a pharmacotherapy for methamphetamine use disorders will not be pursued due to its potential cardiac liabilities.

Keywords

Methamphetamine; vesicular monoamine transporter; dopamine; lobeline; lobelane; drug discovery

1.0 Introduction

Methamphetamine use disorders negatively impact global health, and approved medications to treat these disorders are not available. The euphoria and high abuse liability of methamphetamine are consequences of dopamine release in brain reward pathways (Rawson et al., 2007). A lipophilic weak base, methamphetamine diffuses across presynaptic plasmalemmal and vesicular membranes, and interacts with the vesicular monoamine transporter-2 (VMAT2), dopamine transporter and monoamine oxidase, leading to increased extracellular dopamine concentrations (Sulzer et al., 1992, 1993, 2005). Considering the integral role that VMAT2 plays in the mechanism of action of methamphetamine to increase extracellular dopamine, VMAT2 represents an important target for novel therapeutics designed to treat methamphetamine use disorders.

Lobeline, a lipophilic non-pyridino alkaloid of *Lobelia inflata* (Indian tobacco), has demonstrated efficacy in attenuating the neurochemical effects of methamphetamine. Lobeline inhibits dopamine uptake at VMAT2 and increases extracellular concentrations of the dopamine metabolite, 3,4 dihydroxyphenylacetic acid (DOPAC), indicating that lobeline does not inhibit monoamine oxidase (Nickell et al., 2010; Teng et al., 1997). Importantly, lobeline also inhibits methamphetamine-evoked dopamine release at both synaptic vesicle and terminal levels (Meyer et al., 2013). Through interactions with VMAT2, lobeline reduces the pool of cytosolic dopamine available for methamphetamine-induced reverse-transport via the dopamine transporter, thereby decreasing dopamine release into the extracellular compartment (Dwoskin and Crooks, 2002). Thus, VMAT2 is implicated as a primary target for the lobeline-mediated inhibition of the pharmacological effects of methamphetamine.

Although VMAT2 is the likely site of action for the lobeline-mediated inhibition of methamphetamine, lobeline also exhibits high affinity for $\alpha 4\beta 2^*$ nicotinic receptors (Nickell et al., 2014). Lobelane, a chemically defunctionalized analog of lobeline, exhibited low affinity for the predominant nicotinic receptor subtypes, while retaining high affinity for VMAT2 (Miller et al., 2004). Furthermore, lobelane decreased methamphetamine-evoked dopamine release (Nickell et al., 2010) and decreased methamphetamine self-administration in rats (Neugebauer et al., 2007). Unfortunately, tolerance developed to the behavioral effects of lobelane.

Another promising lobelane analog emerged from our iterative drug discovery approach. *R-N*-(1,2-Dihydroxypropyl)-2,6-*cis*-di-(4-methoxyphenethyl)piperidine hydrochloride (GZ-793A), a novel water-soluble analog of lobelane, exhibits high affinity for VMAT2 and is >50-fold more selective for VMAT2 versus the dopamine and serotonin transporters (Horton et al., 2011b). Following both parenteral and oral administration, GZ-793A specifically decreases methamphetamine self-administration in rats (Beckmann et al., 2012; Wilmouth et al., 2013). However, GZ-793A interaction with nicotinic receptors has not been evaluated previously. Thus, the current study determined GZ-793A affinity for the predominant nicotinic receptor subtypes using both radioligand binding and nicotine-evoked dopamine release assays. To further evaluate the selectivity of GZ-793A with respect to inhibition of methamphetamine-evoked dopamine release, inhibition of electrical field-

stimulation-evoked dopamine release was determined. In support of securing an investigational new drug application for GZ-793A, its potential to inhibit cardiac function also was evaluated by assessing affinity for the human-ether-a-go-go related gene (hERG) channel expressed in human embryonic kidney (HEK) cells and by determining action potential duration in rabbit cardiac Purkinje cells following GZ-793A exposure.

2.0 Materials and Methods

2.1 Animals

Male Sprague-Dawley rats (200–250 g upon arrival) were purchased from Harlan (Indianapolis, IN) and housed two per cage with ad libitum access to food and water in the Division of Laboratory Animal Resources in the Biomedical Biological Sciences Research Building at the University of Kentucky (Lexington, KY). Following arrival, rats acclimated to the environment for 1 week prior to the start of experiments, at which rats were approximately 9 weeks of age and weighed 250–275 g. Experimental protocols involving the animals were in accord with the 2011 *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. Also, female New Zealand white rabbits (2.6–3.0 kg upon arrival) were purchased from Myrtle's Rabbitry, Inc. (Thompsons Station, TN), and were housed in an AALAC accredited facility. Studies involving the rabbits were performed in accordance with procedures published in peer-reviewed journals and according to standard operating procedures of ChanTest Corporation (Cleveland, OH).

2.2 Chemicals

[³H]Nicotine (specific activity, 66.9 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA), while [³H]methyllycaconitine (specific activity, 100 Ci/mmol) and [³H]dofetilide (specific activity 80 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). (+)-Methamphetamine hydrochloride, sodium chloride, perchloric acid, disodium ethylenediamine tetra acetate (EDTA), ethyleneglycol tetra acetate (EGTA), L-(+) tartaric acid, *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), dopamine hydrochloride, magnesium sulfate, D-glucose, 1-octanesulfonic acid sodium salt, amitriptyline, polyethyleneimine, ascorbic acid, potassium aspartate, adenosine triphosphate, and ascorbate oxidase were purchased from Sigma-Aldrich (St. Louis, MO). Citric acid, sodium bicarbonate, sodium phosphate, potassium chloride, calcium chloride, potassium phosphate and magnesium chloride were purchased from Fisher Scientific Co. (Pittsburgh, PA).

2.3 Drugs

Lobeline hemisulfate was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Lobelane, a defunctionalized, saturated *meso*-analog of lobeline, was synthesized as previously described (Zheng et al., 2005). The water-soluble analog of lobelane, GZ-793A, was synthesized as previously described (Horton et al., 2011b). Amitriptyline hydrochloride, ketamine hydrochloride, sodium heparin, and xylazine were purchased from Sigma-Aldrich.

2.4 Dopamine release assay

Rat coronal striatal slices (0.5 mm thick) were prepared and incubated in Krebs' buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.0 mM NaH₂PO₄, 1.3 mM CaCl₂, 11.1 mM α -D-glucose, 25 mM NaHCO₃, 0.11 mM L-ascorbic acid and 0.004 mM EDTA, pH 7.4, saturated with 95% O₂/5% CO₂) at 34 °C in a metabolic shaker for 60 min (Teng et al., 1997). Each slice was then transferred to a glass superfusion chamber and superfused at 1 mL/min for 60 min with Krebs' buffer before sample collection. After 1 hr, two 5-min basal samples (1 mL into 50 μ L of 0.1 M perchloric acid) were collected. To determine the ability of GZ-793A to evoke dopamine release in the absence of methamphetamine, each slice was superfused for 30 min in the absence and presence of a single concentration of GZ-793A (1–100 μ M), samples collected and dopamine and DOPAC concentrations in superfusate were determined. To determine the ability of GZ-793A to inhibit methamphetamine-evoked dopamine release, methamphetamine (5 μ M) was added to the superfusion buffer, and slices were superfused for 15 min in the absence and presence of a range of GZ-793A concentrations. Following removal of methamphetamine from the buffer, superfusion continued for 25 min in the absence and presence of GZ-793A. Thus, in each experiment, duplicate slices were superfused with methamphetamine in the absence of GZ-793A, serving as the methamphetamine control condition. Also, in each experiment, slices were superfused in the absence of methamphetamine and GZ-793A, serving as the buffer control condition.

To determine the ability of GZ-793A to inhibit nicotine-evoked dopamine release, slices were prepared and superfused in the absence and presence of GZ-793A (3–100 μ M) as described above. Nicotine (30 μ M) was included in the superfusion buffer for 45 min in the absence and presence of GZ-793A. In each experiment, slices were superfused with nicotine in the absence of GZ-793A, serving as the nicotine control, as well as in the absence of nicotine and GZ-793A, serving as the buffer control.

To determine the ability of GZ-793A to inhibit electrical field stimulation-evoked dopamine release, slices were prepared and superfused in the absence and presence of GZ-793A (3–100 μ M) as previously described. After 30 min, an electrical field stimulus (100 Hz, 1 min, 60 unipolar rectangular pulses; SD9 stimulator; Grass instruments, Quincy, MA) was applied. Stimulation parameters were chosen to obtain dopamine release comparable to that evoked by nicotine, as determined in pilot studies (data not shown).

Superfusate samples were processed immediately after collection. To each sample (500 μ L), 20 μ L of ascorbate oxidase (168 U/mg; reconstituted to 81 U /1 mL) was added, and samples vortexed for 30 s. An aliquot (100 μ L) of each sample was injected onto the HPLC-EC, equipped with pump and auto-sampler (model 508, Beckman Coulter, Inc., Fullerton, CA), an ODS ultrasphere C18 reverse-phase column (80 $\times$ 4.6 mm, 3- μ m ESA Inc., Chelmsford, MA) and a coulometric-II detector with guard cell (model 5020, ESA, Inc., Chelmsford, MA) maintained at +0.60 V and an analytical cell (model 5011) maintained at E1 = –0.05 V and E2 = +0.35 V. Mobile phase was 0.07 M citrate/0.1 M acetate buffer (175 mg/L octanesulfonic acid-sodium salt, NaCl 650 mg/L, and 7% methanol; pH = 4). HPLC was performed at room temperature at a flow rate of 1.2 mL/min, and 4–5 min was required to process each sample. Retention times of dopamine and DOPAC standards were used to identify peaks. Peak heights were used to quantify amounts of dopamine and DOPAC on the

basis of standard curves. Peak identification, height measurement and analysis were performed using 32 karat software (Beckman Coulter, Inc.).

2.5 [³H]Nicotine and [³H]methyllycaconitine binding assays

GZ-793A inhibition of [³H]nicotine and [³H]methyllycaconitine binding, representing interaction with $\alpha 4\beta 2$ and $\alpha 7$ nicotinic receptors, respectively, was determined using previously published methods (Horton et al., 2011a). Rat whole brain, excluding cortex and cerebellum, was homogenized using a Tekmar polytron (Tekmar-Dohrmann, Mason, OH) in 20 vol ice-cold modified Krebs' -HEPES buffer, containing 2 mM HEPES, 14.4 mM NaCl, 0.15 mM KCl, 0.2 mM CaCl₂·2H₂O and 0.1 mM MgSO₄·7H₂O, pH 7.5. Homogenates were centrifuged at 31,000 g for 17 min at 4 °C (Avanti J-301 centrifuge, Beckman Coulter, Fullerton, CA). Pellets were resuspended by sonication (Vibra Cell, Sonics & Materials Inc., Danbury, CT) in 20 vol Krebs-HEPES buffer and incubated at 37 °C for 10 min (Reciprocal Shaking Bath Model 50, Precision Scientific, Chicago, IL). Suspensions were centrifuged at 31,000 g for 17 min at 4 °C. Resulting pellets were resuspended by sonication in 20 vol buffer, and centrifuged again. Final pellets were stored in incubation buffer, containing 40 mM HEPES, 288 mM NaCl, 3.0 mM KCl, 4.0 CaCl₂·2H₂O, and 2.0 MgSO₄·7H₂O, pH 7.5. Membrane suspensions (100–140 µg protein/100 µl) were added to tubes containing inhibitor (7–9 concentrations, 10 pM–100 µM) and 3 nM [³H]nicotine or [³H]methyllycaconitine for a final assay volume of 250 µl. Nicotine, lobeline and lobelane concentration-response curves were obtained as positive controls. Samples were incubated for 60 min at room temperature. Reactions were terminated by filtration on Unifilter-96 GF/B filter plates presoaked in 0.5% polyethyleneimine using a Packard Filter Mate Harvester (Perkin Elmer, Inc.). Samples were washed 3 times with 350 µl of ice-cold buffer. Filter plates were dried for 60 min at 45 °C, bottom-sealed and each well filled with 40 µl Packard's Microscint 20 cocktail (Perkin Elmer, Waltham, MA). Bound radioactivity was determined via liquid scintillation spectrometry (Top Count NXT scintillation counter; PerkinElmer, Inc.). Nonspecific [³H]nicotine and [³H]methyllycaconitine binding was determined in the presence of 10 µM cytosine and 10 µM nicotine, respectively.

2.6 hERG assays

Membranes from HEK-293 cells, which specifically and stably express hERG channel protein (Millipore, Billerica, MA), were prepared and harvested, and binding assays conducted as previously described (Sviripa et al., 2014). Briefly, cell membrane suspension (5 µg) was added to duplicate tubes containing assay buffer (50 mM Tris, 10 mM KCl, and 1 mM MgCl₂, pH 7.4), 25 µL of a single concentration (10 nM–100 µM) of GZ-793A or amitriptyline (positive control; Teschemacher et al., 1999; Jo et al., 2000), and 25 µL of [³H]dofetilide (5 nM, final concentration) for a final assay vol of 250 µL. Samples were incubated for 60 min at 25 °C and reactions terminated by rapid filtration through Whatman GF/B filters pre-soaked in 0.5% polyethyleneimine. Filters were washed 3 times with 1 mL of ice-cold assay buffer. Radioactivity retained by the filters was determined as previously described. Assays were conducted in duplicate, and three independent assays were performed for each compound. Amitriptyline (1 mM) was used to determine nonspecific binding.

HEK-293 cells, which specifically and stably express hERG channel protein (Millipore, Billerica, MA), were transferred to a recording chamber and superfused with buffer (130 mM potassium aspartate, 5 mM MgCl₂, 5 mM EGTA, 4 mM adenosine triphosphate, 10 mM HEPES, pH 7.2). Onset and steady state inhibition of hERG potassium current were measured using a pulse pattern with fixed amplitudes (conditioning pulse: +20 mV for 2 sec; test pulse: -50 mV for 2 sec) repeated at 10 sec intervals, from a holding potential of -80 mV. Peak tail current was measured during the 2 sec step to -50 mV. Steady state was maintained for at least 30 sec before GZ-793A application. GZ-793A concentrations were applied sequentially in ascending order. Peak current was monitored until a new steady state was achieved or following 12 min of GZ-793A exposure.

Interaction of GZ-793A with hERG channels also was evaluated using rabbit cardiac Purkinje cells, which were selected due to the similarity of action potential waveform, underlying ionic currents, ion channel pharmacological properties and drug sensitivity relative to human cardiac cells (Lu et al., 2001). Rabbits were anesthetized with xylazine (2.5 mg/kg) and ketamine (37 mg/kg), and heparinized (1,000 U/kg, i.v.), and the heart was obtained rapidly and placed in oxygenated, chilled, cardioplegic buffer (131 mM NaCl, 22 mM KCl, 2 mM CaCl₂, 0.5 mM MgCl₂, 18 mM NaHCO₃, 1.8 mM NaH₂PO₄, 55 mM glucose, aerated 95% O₂/5% CO₂, pH 7.2). All viable Purkinje fibers from both ventricles were removed along with muscle attachments and stored at room temperature in cardioplegic buffer until use. Purkinje fibers were placed within recording chambers mounted on a heated platform, and superfused at 5 mL/min. Bath temperature was maintained at 37 °C. Intracellular membrane potentials were recorded using conventional intracellular microelectrodes pulled from borosilicate glass capillary tubing on a micropipette puller (Sutter Instruments P-97, Novato, CA). Microelectrodes were filled with 3 M KCl solution and connected via Ag-AgCl wire to an intracellular electrometer amplifier (Warner Instruments IE 210, Hamden, CT). Purkinje fibers were paced continuously at a cycle length of 0.5–1 sec for at least 30 min (recovery and stabilization) before obtaining control action potentials. Only fibers with resting potentials more negative than -80 mV and normal action potential morphology (action potential duration₉₀ > 150 ms) were used. Fibers were stimulated continuously with a cycle length of 1 sec for at least 5 min. Then, baseline action potential duration rate-dependence was determined using stimulus pulse trains consisting of ~50 pulses at basic cycle length of 1.0 and 0.5 sec. The lowest concentration of GZ-793A was applied for at least 20 min to allow for equilibration, and stimulus trains repeated. The sequence was repeated at cumulatively increasing GZ-793A concentrations. Average responses from the last 5 action potentials from each stimulus train were analyzed.

2.8 Data analysis

For dopamine release assays, fractional release was defined as the concentration of dopamine or DOPAC in each 1 mL superfusate sample collected at 5 min intervals divided by slice weight. Basal release was calculated from the average fractional release during the initial 10 min superfusion period prior to addition of GZ-793A to the superfusion buffer. GZ-793A-evoked fractional release was determined during the initial 30 min period of superfusion with GZ-793A in the absence of methamphetamine. Fractional release data were analyzed by two-way repeated-measures ANOVAs, with concentration and time as within-

subject factors. Post hoc analyses using Bonferroni adjusted pair-wise comparisons determined the time points at which GZ-793A inhibited methamphetamine-evoked, nicotine-evoked or electrical field stimulus-evoked fractional release relative to the respective control. Total DOPAC overflow was calculated as the sum of the increases in fractional release above basal outflow during superfusion with GZ-793A. Overflow data were analyzed by one-way repeated-measures ANOVA, and when appropriate followed by Dunnett's post hoc test to determine differences between GZ-793A exposure and control

Specific [^3H]nicotine, [^3H]methyllycaconitine and [^3H]dofetilide binding were determined by subtracting the nonspecific from total binding. GZ-793A concentration producing 50% inhibition of specific binding (IC_{50} values) was determined from concentration-effect curves via an iterative curve fitting program (Prism 5.0; GraphPad Software Inc., San Diego, CA). Inhibition constants (K_i values) were determined using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

For HEK-293 cell-based electrophysiology assays, steady state was defined by the limiting constant rate of change with time (linear time dependence). Action potential duration (APD) was defined as the duration of the action potential in milliseconds, measured from the initial upstroke to the point of return to either 60% (APD_{60}) or 90% (APD_{90}) of the initial resting potential. Steady state before and after GZ-793A application was used to calculate percentage of current inhibited at each GZ-793A concentration. Concentration-response curves were generated utilizing a nonlinear least squares fit. For Purkinje cell electrophysiology assays, data were pooled for control baseline, GZ-793A concentration and stimulus frequency. Changes in action potential parameters at each GZ-793A concentration were evaluated using one-way ANOVA followed by Dunnett's test. For all assays, $p < 0.05$ was considered significant.

3.0 Results

3.1 GZ-793A inhibited methamphetamine-evoked dopamine release

GZ-793A (1–100 μM) inhibited methamphetamine (5 μM)-evoked fractional dopamine release in a concentration-dependent manner (Fig. 1). During the initial 30-min superfusion with GZ-793A in the absence of methamphetamine, basal dopamine release was not altered. In the absence of GZ-793A, addition of 5 μM methamphetamine to the superfusion buffer resulted in a rapid increase in extracellular dopamine, which returned to basal levels within 10 min after removal of methamphetamine from the buffer. A GZ-793A concentration $\times$ time interaction ($F_{136,1088} = 1.7$, $p < 0.001$) was found, and post hoc analysis revealed inhibition of methamphetamine-evoked dopamine release from 50–55 min with 10 μM , from 50–60 min with 30 μM and from 45–65 min with 100 μM ($p < 0.001$). Additionally, a one-way, repeated measures ANOVA revealed a main effect ($F_{5,59} = 9.41$, $p < 0.0001$) of GZ-793A concentration on DOPAC overflow during the initial 30 min period prior to addition of methamphetamine to the buffer (Table 1). Post hoc analysis revealed that GZ-793A at high concentrations (30 and 100 μM) increased DOPAC overflow ($p < 0.0001$) compared to the buffer control condition. Thus, GZ-793 inhibited methamphetamine-evoked dopamine release, and at high concentrations increased DOPAC overflow.

3.2 GZ-793A did not inhibit [³H]nicotine and [³H]methyllycaconitine binding

GZ-793A was evaluated and compared to nicotine, lobeline and lobelane for ability to inhibit [³H]nicotine and [³H]methyllycaconitine binding to whole rat brain membranes (Fig. 2). Nicotine and lobeline exhibited high affinity for the [³H]nicotine binding site, having K_i values of 3.0 ± 0.2 nM and 4.0 ± 0.1 nM, respectively. Compared to lobeline, lobelane was 3700-fold less potent ($K_i = 14.8 \pm 1.68$ μ M) inhibiting [³H]nicotine binding. Nicotine displayed relatively high affinity ($K_i = 0.37 \pm 0.08$ μ M) for the [³H]methyllycaconitine binding site. Lobeline and lobelane exhibited K_i values of 6.26 ± 1.30 μ M and 26.1 ± 6.58 μ M, respectively, for the [³H]methyllycaconitine binding site. At the highest concentrations employed, GZ-793A (1 mM) decreased [³H]nicotine binding by ~40% and GZ-793A (30 μ M) decreased [³H]MLA binding ~20%. GZ-793A exhibits poor solubility above 1 mM, precluding evaluation of higher concentrations and generation of a full concentration-response curve. The K_i value for VMAT2, the primary pharmacological target, is 30 nM (Horton et al., 2011b), while a 50% decrease in [³H]nicotine and [³H]MLA binding was not found at 1 mM. Thus, GZ-793A exhibits ~4–5 orders of magnitude greater selectivity for VMAT2 versus the $\alpha 4\beta 2$ and $\alpha 7$ nicotinic receptor subtypes.

3.3 GZ-793A did not inhibit nicotine-evoked dopamine release

GZ-793A (3–100 μ M) did not inhibit nicotine-evoked fractional dopamine release (Fig. 3). After 30 min of superfusion with GZ-793A alone, the addition of nicotine (30 μ M) to the superfusion buffer resulted in an immediate increase in fractional dopamine release. Nicotine-evoked fractional dopamine release was not sustained during the period of nicotine exposure, but returned to baseline by 20 min of exposure. A main effect of time ($F_{60,15} = 22.58$; $p < 0.001$) was found; however the main effect of concentration ($F_{60,4} = 0.346$, $p = 0.845$) and the concentration $\times$ time interaction ($F_{60,420} = 0.47$, $p = 0.999$) were not significant. Thus, GZ-793A did not inhibit nicotine-evoked fractional dopamine release.

3.4 GZ-793A did not inhibit electrical field-stimulation evoked dopamine release

GZ-793A (3–100 μ M) did not inhibit electrical field-stimulation-evoked fractional dopamine release (Fig. 4). Following superfusion of striatal slices with GZ-793A for 30 min, electrical field-stimulation was applied (S1, 60 pulses at 100 Hz); fractional dopamine release rapidly increased above basal levels. Only a main effect of time ($F_{44,11} = 33.40$, $p < 0.001$) was found; main effect of concentration ($F_{44,4} = 0.300$, $p = 0.876$) and the concentration $\times$ time interaction ($F_{44,407} = 0.998$, $p = 0.480$) were not significant. Thus, GZ-793A did not inhibit field-stimulation evoked dopamine release.

3.5 GZ-793A exhibited high affinity for the hERG channel

Evaluation of GZ-793A for potential cardiac liabilities revealed that GZ-793A inhibited with high affinity ($IC_{50} = 0.81 \pm 0.11$ μ M) [³H]dofetilide binding to hERG channels expressed by HEK-293 cells (Fig. 5). Affinity of amitriptyline ($IC_{50} = 10.7 \pm 2.25$ μ M) for the [³H]dofetilide binding site on hERG channels was consistent with previous reports (Jo et al., 2000). Lobeline exhibited moderate affinity ($IC_{50} = 1.50 \pm 0.483$ μ M) for hERG, while lobelane exhibited the highest affinity ($IC_{50} = 0.238 \pm 0.0389$ μ M) of this series of compounds. Also, GZ-793A inhibited ($IC_{50} = 0.07$ μ M; Hill coefficient = 1.1) hERG

channel current flow in the HEK-293 cell model (Fig. 6). In rabbit cardiac Purkinje cells, GZ-793A (0.03 – 0.3 μ M) prolonged ($p < 0.05$) action potential duration (APD₆₀ and APD₉₀) at 0.5 sec and 1 sec cycle lengths relative to time-matched vehicle control cells, but did not alter resting membrane potential, action potential amplitude nor the maximum rate of depolarization (Table 2). Thus, GZ-793A inhibits binding to the hERG channel in HEK cells and channel function in cardiac Purkinje cells.

4.0 Discussion

GZ-793A has emerged as a lead compound from an iterative drug discovery program aimed at identifying lobelane analogs that decrease methamphetamine self-administration. Results show that GZ-793A decreased methamphetamine-evoked dopamine release from superfused rat striatal slices. At relatively low concentrations (10 μ M), GZ-793A inhibited methamphetamine-evoked dopamine release without increasing DOPAC overflow, whereas at higher concentrations (30 and 100 μ M), GZ-793A inhibited methamphetamine-evoked dopamine release and increased DOPAC overflow. The current results are consistent with our previous findings showing that lobeline and lobelane inhibit methamphetamine-evoked dopamine release from striatal slices, while increasing extracellular DOPAC (Nickell et al., 2010), and that lobeline and GZ-793A increase extracellular DOPAC in in vivo microdialysates (Meyer et al., 2013). Collectively, our results suggest that GZ-793A inhibits the neurochemical effects of methamphetamine through an interaction with VMAT2, leading to a reduction in the pool of dopamine available for methamphetamine-induced reverse-transport by the dopamine transporter.

Lobeline exhibits high affinity for nicotinic receptors, whereas lobelane, which lacks the hydroxyl and keto moieties of lobeline, exhibited markedly reduced affinity for nicotinic receptors (Miller et al., 2004). GZ-793A, which further includes methoxy and *N*-dihydroxypropyl groups, exhibits little to no affinity for the agonist recognition site on $\alpha 4\beta 2$ and $\alpha 7$ nicotinic receptor subtypes. However, studies had not previously determined the affinity of GZ-793A for $\alpha 6$ -containing nicotinic receptors, which play a major role in mediating nicotine-evoked dopamine release (Pons et al., 2008; McIntosh et al., 2009; Brunzell, 2012; Crooks et al., 2014; Beckmann et al., 2015). The finding that GZ-793A did not inhibit nicotine-evoked dopamine release suggests that GZ-793A also does not interact with $\alpha 6$ -containing nicotinic receptors that modulate nicotine-evoked dopamine release.

While the current results reveal that GZ-793A selectively inhibits methamphetamine-evoked dopamine release, and not nicotine-evoked dopamine release, additional evidence that GZ-793A also did not inhibit electrical field stimulation-evoked dopamine release further supports the interpretation that GZ-793A inhibits methamphetamine effects via selective interaction with VMAT2. Application of an electrical stimulus elicited a rapid dopamine release, which was comparable in magnitude to that evoked by exposure of the striatal slices to 30 μ M nicotine. Also, GZ-793A did not inhibit field-stimulation-evoked dopamine release at concentrations found to inhibit methamphetamine-evoked dopamine release. The current findings are consistent with our previous results showing that low concentrations of lobeline did not inhibit field-stimulation-evoked dopamine overflow (Miller et al., 2001). Only lobeline concentrations 30-fold greater than that required to inhibit methamphetamine-

evoked dopamine release inhibited electrically-evoked dopamine release. The current results clearly demonstrate that concentrations of GZ-793A which inhibit methamphetamine-evoked dopamine release do not alter either nicotine-evoked or field-stimulation-evoked dopamine release. Thus, GZ-793A selectively inhibits methamphetamine-induced dopamine release.

Considerations of efficacy, potency and selectivity alone are not sufficient to drive drug discovery efforts. A large number of lead compounds have been withdrawn recently from the drug discovery and development pipeline due to their interaction with the hERG channel located on cardiac myocytes (Shah 2006). Inhibition of the hERG channel has the potential to induce prolongation of the QT interval in the electrical rhythm of the heart, which can lead to the development of ventricular arrhythmias and increase the potential for sudden death (Pollard et al., 2010). As such, interaction of novel compounds with the hERG channel is investigated routinely early in the pathway for drug discovery. In the current study, the ability of GZ-793A to inhibit [³H]dofetilide binding to the hERG channel expressed on HEK-293 membranes served as the initial screen for potential cardiac liability. Compounds exhibiting affinity for the [³H]dofetilide binding site within the range of 1–10 μ M are classified as moderate inhibitors of the hERG channel (Guth and Rast, 2010). GZ-793A exhibited an affinity of ~800 nM for the [³H]dofetilide binding site. Consistent with results from the hERG binding assay, GZ-793A inhibited (IC₅₀ = ~70 nM) current flow in voltage clamped mammalian cells expressing hERG channels, and GZ-793A (30 nM) prolonged action potential duration in rabbit cardiac Purkinje fibers. Thus, GZ-793A is associated with an elevated risk of ventricular arrhythmias.

While the current findings of GZ-793A interaction with hERG channels are disheartening, structure-activity relationships have been generated for interaction with the hERG channel. Specifically, positively charged moieties bind to the hERG channel via π -interactions at tyrosine residues lining the channel pore. Thus, a reduction in basicity of a molecule may reduce hERG affinity (Mitcheson and Perry, 2003; Pearlstein et al., 2003). Substitution of the piperidine ring of GZ-793A for a piperazine ring may reduce interactions with the hERG channel, while maintaining activity at VMAT2. Alternatively, increasing the polarity of the molecule through halogen substitution of the lipophilic phenyl rings (Meyers et al., 2007) or replacing the phenyl rings with pyridine rings (Berglund et al., 2009) also may reduce interactions with hERG channel. Of note, lobelane analogs which exhibit a number of these structural modifications potently inhibit VMAT2 function (Nickell et al., 2011, 2014, 2016), and such alterations may enhance hydrophilicity.

GZ-793A has emerged from an iterative drug discovery approach and has the ability to inhibit the neurochemical and behavioral effects of methamphetamine. GZ-793A selectively inhibits VMAT2 versus the dopamine and serotonin transporters, inhibits methamphetamine-evoked dopamine release in vitro, and decreases the reinforcing and rewarding properties of methamphetamine. The current results further elucidate the neurochemical mechanism of GZ-793A action, in that inhibition of the pharmacological effects of methamphetamine is not the result of an interaction with nicotinic receptors. Also, GZ-793A does not alter the response of the dopaminergic system to physiological stimuli. However, GZ-793A is precluded from further development as a treatment for methamphetamine use disorders due

to its interaction with the cardiac hERG channel. Future research will introduce structural moieties into the chemical scaffold of the GZ-793A molecule in order to reduce hERG channel interactions, while retaining affinity for VMAT2 and inhibiting the neurochemical and behavioral effects of methamphetamine.

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Abbreviations:

ANOVA	analysis of variance
APA	action potential amplitude
APD₆₀ and APD₉₀	action potential duration measured at 60% and 90% repolarization
DOPAC	3,4-dihydroxyphenylacetic acid
EGTA	ethyleneglycol tetraacetate
EDTA	disodium ethylenediamine tetraacetate
GZ-793A	<i>R-N</i> -(1,2-dihydroxypropyl)-2,6- <i>cis</i> -di-(4-methoxyphenethyl)piperidine hydrochloride
HEK	human embryonic kidney
HEPES	<i>N</i> -[2-hydroxyethyl]piperazine- <i>N'</i> -[2-ethanesulfonic acid]
hERG	human-ether-a-go-go related gene
HPLC-EC	high performance liquid chromatography with electrochemical detection
mV	absolute millivolt change from baseline
RMP	resting membrane potential
VMAT2	vesicular monoamine transporter-2
V_{max}	maximum rate of depolarization
%	percent change from baseline

6.0 References

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Fig. 1: GZ-793 inhibits methamphetamine-evoked fractional dopamine release in a concentration-dependent manner.

Arrow denotes time point at which striatal slices were superfused with GZ-793A (0, 1–100 μM), which remained in the buffer throughout the experiment. Bracketed line indicates time during which superfusion buffer contained methamphetamine (5 μM). Slices superfused with methamphetamine only represent the control condition. Asterisk indicates that 10, 30 and 100 μM GZ-793A inhibited methamphetamine-evoked dopamine release from 50–55, 50–60, and 45–65 min, respectively ($p < 0.001$). Data are expressed as pg/ml/mg slice weight, and represent mean $\pm$ SEM ($n = 7$ rats).

Fig. 2: GZ-793A does not inhibit $[^3\text{H}]$ nicotine or $[^3\text{H}]$ methyllycaconitine binding. Concentration-response curves for GZ-793A-, nicotine-, lobeline- and lobelane-inhibition of $[^3\text{H}]$ nicotine (top panel) and $[^3\text{H}]$ methyllycaconitine (bottom panel) binding to rat whole brain membranes. GZ-793A exhibited poor solubility at concentrations greater than 1 mM, precluding generation of full concentration-response curves. Nonspecific $[^3\text{H}]$ nicotine and $[^3\text{H}]$ methyllycaconitine binding was determined in the presence of 10 μM cytosine and 10 μM nicotine, respectively. Control (CON) represents $[^3\text{H}]$ nicotine and $[^3\text{H}]$ methyllycaconitine binding in the absence of compound ($45.4 \pm 5.83 \text{ fmol/mg}$ protein and $63.2 \pm 8.11 \text{ fmol/mg}$ protein, respectively; $n = 3-4$ rats/compound).

Fig. 3: GZ-793A does not inhibit nicotine-evoked fractional dopamine release.

Arrow indicates the time at which striatal slices were superfused with GZ-793A (0, 3–100 μM), which remained in the buffer throughout the experiment. Bracketed line indicates time during which the superfusion buffer contained nicotine (30 μM). Slices superfused with only nicotine represent the control condition. Data are expressed as pg/ml/mg slice weight, and represent mean ± SEM (n = 5 rats).

Fig. 4: GZ-793A does not inhibit electrical field stimulation-evoked fractional dopamine release. First arrow denotes time during which GZ-793A (0, 3–100 μ M), which remained in the buffer throughout the experiment, was added to the superfusion buffer. Second arrow indicates 1 min period of electrical field stimulation (60 pulses, 100 Hz). Slices superfused in the absence of GZ-793A represent the control condition. Data are expressed as pg/ml/min, and represent mean $\pm$ SEM (S1: n = 5–6 rats/concentration).

Fig. 5: In a concentration-dependent manner, GZ-793A inhibits [³H]dofetilide binding to hERG channel protein expressed by HEK-293 cells.

Nonspecific [³H]dofetilide binding was determined using 1 mM amitriptyline. Compounds listed in the legend are presented in order of highest to lowest affinity. Control (CON) represents [³H]dofetilide binding in the absence of compound. Data are mean ($\pm$ SEM) specific [³H]dofetilide binding expressed as a percentage of control (2550 ± 298 fmol/mg protein; $n = 3-8$ individual preparations of HEK-293 stock/compound).

Fig. 6: In a concentration-dependent manner, GZ-793A inhibits hERG channel current in HEK-293 cells.

Data are mean ($\pm$ SEM) inhibition of hERG channel potassium currents, expressed as a percentage of control in the absence of GZ-793A ($n = 3$).

Table 1.

GZ-793A increases DOPAC overflow from superfused striatal slices.

GZ-793A (μ M)	Total DOPAC overflow (pg/30 ml/mg)
0	45.7 ± 15.7^a
1	93.8 ± 32.2
3	155 ± 48.6
10	159.4 ± 29.4
30	$621 \pm 184^*$
100	$1080 \pm 315^*$

^aData are mean $\pm$ S.E.M.

*
 $p < 0.0001$ different from buffer control (n = 7 rats)

Table 2.

Effects of GZ-793A on action potential parameters for rabbit cardiac Purkinje fibers at 1 and 0.5 sec cycle duration

Parameters ^a at 1 sec Cycle					
GZ-793 Concentration	ADP60 (%)	APD90 (%)	RMP (mV)	APA (mV)	Vmax (%)
0.03 μ M	13.6 $\pm$ 2.2 *	10.4 $\pm$ 0.8 *	-0.2 $\pm$ 0.3	1.2 $\pm$ 2.4	-1.3 $\pm$ 0.9
0.1 μ M	26.7 $\pm$ 4.9 *	23.8 $\pm$ 1.4 *	0.5 $\pm$ 0.6	-0.1 $\pm$ 3.6	3.9 $\pm$ 4.7
0.3 μ M	33.6 $\pm$ 5.7 *	33.9 $\pm$ 4.8 *	1.4 $\pm$ 1.3	-1.0 $\pm$ 4.4	1.4 $\pm$ 3.3
Parameters at 0.5 sec Cycle					
GZ-793 Concentration	ADP60 (%)	APD90 (%)	RMP (mV)	APA (mV)	Vmax (%)
0.03 μ M	12.3 $\pm$ 3.0 *	9.7 $\pm$ 0.9 *	-0.9 $\pm$ 0.7	2.4 $\pm$ 3.8	2.9 $\pm$ 2.1
0.1 μ M	21.4 $\pm$ 5.0 *	19.4 $\pm$ 1.8 *	0.1 $\pm$ 1.4	0.6 $\pm$ 5.6	1.2 $\pm$ 4.7
0.3 μ M	26.8 $\pm$ 5.5 *	27.9 $\pm$ 3.1 *	1.6 $\pm$ 1.7	-1.6 $\pm$ 5.8	-2.5 $\pm$ 4.5

^a APD₆₀ and APD₉₀, action potential duration measured at 60% and 90% repolarization in rabbit cardiac Purkinje fibers; %, percent change from baseline; RMP, resting membrane potential; mV, absolute millivolt change from baseline; APA, action potential amplitude; Vmax, maximum rate of depolarization.

* $p < 0.05$ compared to the vehicle control.