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Title Page

Pharmacologic Characterization of Valbenazine (NBI-98854) and Its Metabolites

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Running Title Page

Running title: Pharmacologic Characterization: Valbenazine and Metabolites

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Abbreviations:

ANOVA	analysis of variance
CHO	Chinese hamster ovary
DAT	dopamine transporter
DHTBZ	dihydrotetrabenazine
DRBA	dopamine receptor blocking agent
EPS	extrapyramidal symptoms
GPCR	G-protein-coupled receptor
HEK	Human embryonic kidney
MTBZ	methoxytetrabenazine
NET	norepinephrine transporter
SERT	serotonin transporter
TBZ	tetrabenazine
TD	tardive dyskinesia
VBZ	valbenazine
VMAT	vesicular monoamine transporter

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Abstract

The vesicular monoamine transporter 2 (VMAT2) is an integral presynaptic protein that regulates the packaging and subsequent release of dopamine and other monoamines from neuronal vesicles into the synapse. Valbenazine (NBI-98854), a novel compound that selectively inhibits VMAT2, is being developed for the treatment of tardive dyskinesia. Valbenazine is converted to two significant circulating metabolites *in vivo*, namely, (+)-α-dihydrotetrabenazine (R,R,R-DHTBZ) and a mono-oxy metabolite, NBI-136110. Radioligand binding studies were conducted to assess and compare valbenazine, tetrabenazine and their respective metabolites in their abilities to selectively and potently inhibit [^{3}H]-DHTBZ binding to VMAT2 in rat striatal, rat forebrain, and human platelet homogenates. A broad panel screen was conducted to evaluate possible off-target interactions of valbenazine, R,R,R-DHTBZ, and NBI-136110 at >80 receptor, transporter, and ion channel sites. Radioligand binding showed R,R,R-DHTBZ to be a potent VMAT2 inhibitor in homogenates of rat striatum (K_i=1.0-2.8 nM), rat forebrain (K_i=4.2 nM), and human platelets (K_i=2.6-3.3 nM). Valbenazine (K_i=110-190 nM) and NBI-136110 (K_i=160-220 nM) also exhibited inhibitory effects on VMAT2, but with lower potency than R,R,R-DHTBZ. Neither valbenazine, R,R,R-DHTBZ, nor NBI-136110 had significant off-target interactions at serotonin (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}) or dopamine (D_1, or D_2) receptor sites. *In vivo* studies measuring ptosis and prolactin secretion in the rat confirmed the specific and dose-dependent interactions of tetrabenazine and R,R,R-DHTBZ with VMAT2. Evaluations of potency and selectivity of tetrabenazine and its pharmacologically active metabolites were also performed. Overall the pharmacologic characteristics of valbenazine appear consistent with the favorable efficacy and tolerability findings of recent clinical studies (KINECT 2 [NCT01733121], KINECT 3 [NCT02274558]).

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Introduction

Dysregulation of dopaminergic neurotransmission is a key component of many central nervous system disorders, including hyperkinetic movement disorders such as tardive dyskinesia (TD) (Muller, 2015). Chronic exposure to dopamine receptor blocking agents (DRBAs), such as neuroleptics or antipsychotics, are associated with TD and other movement disorders (Casey, 1991; Mehta et al., 2015). While side effects of extrapyramidal symptoms (EPS) and TD were generally accepted as unavoidable risks of chronic treatment with first-generation (typical) DRBAs, the hope that second-generation (atypical) DRBAs would dramatically reduce the incidence of TD was never realized (Finley, 2002; Woods et al., 2010). TD can have a significant impact on daily function and quality of life (Othman et al., 2013), and there are currently no approved pharmacotherapeutic options.

Several reports and open label studies suggest that inhibition of the presynaptic vesicular monoamine transporter 2 (VMAT2) tetrabenazine (TBZ) may provide some therapeutic benefit in hyperkinetic movement disorders (Kenney and Jankovic, 2006; Kenney et al., 2007; Jankovic and Clarence-Smith, 2011). VMATs are integral 12-transmembrane proteins found in various cells throughout the body. They play a critical role in the packaging of monoamines into presynaptic vesicles via active-transport within the cytoplasm driven by an ATPase-generated proton gradient (Erickson et al., 1996; Eiden, 2000; Erickson and Varoqui, 2000). VMAT2 (or SLC18A2) is expressed both centrally and peripherally whereas VMAT1 (or SLC18A1) is only expressed in peripheral neuroendocrine cells and sympathetic ganglia (Erickson et al., 1996). VMAT2 distribution in the central nervous system in both mice and humans has been determined using [^{11}C]-methoxytetrabenazine (MTBZ), which demonstrated consistent localization with known monoamine nerve terminal density, particularly in the striatum, lateral

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septum, and other limbic brain regions (Vander Borght et al., 1995). Pharmacologically, there is a distinct profile of inhibitors that can distinguish VMAT1 from VMAT2. Reserpine, an indole alkaloid, inhibits both VMAT1 and VMAT2 in a pseudo-irreversible manner (Schuldiner et al., 1993). In contrast, TBZ and its active metabolites do not bind or functionally inhibit VMAT1 but reversibly inhibit VMAT2, resulting in decreased cytosolic uptake of dopamine, norepinephrine, serotonin and histamine into synaptic vesicles (Wimalasena, 2011).

With the approval of TBZ for the treatment of Huntington's chorea (FDA., 2008), greater attention was directed to how this mechanism and class of drugs might be used to treat a variety of hyperkinetic movement disorders. TBZ is a racemate composed of two ketone enantiomers, (+)-TBZ and (−)-TBZ (Lee et al., 1996). Upon oral administration, TBZ is reduced to form four discrete isomeric secondary alcohol metabolites, collectively referred to as dihydrotetrabenazine (DHTBZ). These metabolites include four stereoisomers: R,R,R-DHTBZ or (+)-α-DHTBZ (alternate nomenclature) or NBI-98782 (laboratory nomenclature); S,S,S-DHTBZ or (-)-α-DHTBZ or NBI-98771; S,R,R-DHTBZ or (+)-β-DHTBZ or NBI-98795; and R,S,S-DHTBZ or (-)-β-DHTBZ or NBI-98772 (Kilbourn et al., 1997).[2] All four isomers can inhibit VMAT2 with varying degrees of potency, but to date, levels of these independent isomers circulating in plasma following an oral dose of TBZ have not been systematically determined. The pharmacological profile and specificity of these metabolites were assessed and compared to valbenazine (VBZ, NBI-98854) and its metabolites.

VBZ is a novel VMAT2 inhibitor currently in development for the treatment of TD (Muller, 2015). Structurally, VBZ is the valine ester of the R,R,R-DHTBZ isomer (Fig. 1). It is rapidly absorbed after oral administration; following absorption, VBZ levels decline slowly with a half-life of approximately 20 h, supporting once-daily administration. The primary metabolic

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clearance pathways of VBZ are hydrolysis (to form R,R,R-DHTBZ) and mono-oxidation (to form the metabolite NBI-136110). R,R,R-DHTBZ and NBI-136110, the two most abundant circulating metabolites of VBZ, are formed gradually and their plasma concentrations decline with half-lives similar to VBZ, suggesting that the pharmacokinetics of these metabolites are a result of formation rate-limited clearance (manuscript in preparation). Recently, results from two randomized, double-blind, placebo-controlled trials have indicated that once-daily VBZ was generally well-tolerated and effective in adults with moderate-to-severe TD (O'Brien et al., 2015; Hauser et al., 2017).

While both TBZ and VBZ share a common metabolite (R,R,R-DHTBZ), other metabolites are unique and may result in different overall pharmacologic profiles of two drugs. The pharmacologic attributes of the resulting metabolites are key to understanding the safety of TBZ and VBZ and their potential in the treatment of TD.

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Materials and Methods

Radioligand Binding

Studies were conducted to determine the ability of VBZ and its metabolites, R,R,R-DHTBZ and NBI-136110, to inhibit the binding of [^{3}H]-DHTBZ to the VMAT2 transporter in rat striatum and human platelet homogenates. Affinity of the four DHTBZ stereoisomers (R,R,R-DHTBZ, S,R,R-DHTBZ, S,S,S-DHTBZ, R,S,S-DHTBZ) was assessed using a number of tissues expressing VMAT2.

Rat forebrain and striatum homogenates. Stripped whole rat brains (Pelfreeze 56005-2, Rogers, AR; mixed sex) stored at -80°C were thawed at room temperature for 10 min. Forebrain or striatum was dissected and placed in homogenization buffer (Dulbecco's phosphate buffered saline [DPBS]: 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 138 mM NaCl [Invitrogen 14200-075; Carlsbad, CA] supplemented with 10 mM $MgCl_2$, 2 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA), pH 7.4 with NaOH [Sigma-Aldrich; St. Louis, MO]). Tissue from each forebrain (approximately 300 mg wet weight) or three striata (approximately 200 mg wet weight) was placed in 10 ml homogenization buffer, allowed to thaw for an additional 10 min, then homogenized using a hand-held homogenizer (30,000 rpm on ice or at 4°C for 20 s).

Human platelet homogenate. One unit of human platelet suspension (approximately 250 ml, sex unknown; San Diego Blood Bank; San Diego, CA) was centrifuged at 3,000 × g for 20 min, and the resulting platelet pellet was re-suspended in 60 ml ice-cold lysis buffer 1 (50 mM hydroxyethyl piperazineethanesulfonic acid [HEPES], 1 mM ethylenediaminetetraacetic acid, pH 7.4; Sigma-Aldrich; St. Louis, MO). The platelet suspension was homogenized using a hand-held homogenizer (30,000 rpm at 4°C for 1 min), then left at 4°C for 20 min. The platelets

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were further lysed using nitrogen cavitation at 900 psi for 30 min at 4°C, then the homogenate was decanted.

Radiolabeling of homogenates. Homogenates were centrifuged (45,000 × g for 20 min at 4°C) and the supernatant discarded. Forebrain pellets were homogenized in 10 ml fresh homogenization buffer and centrifuged again. Striatum and platelet pellets were re-suspended in lysis buffer 1 (human platelets) or lysis buffer 2 (DPBS with no Ca^{2+} or Mg^{2+}, 10 mM $MgCl_2$, 2 mM EGTA, pH 7.4; rat striatum) and centrifuged once more. The final pellets were resuspended in 2 ml VMAT2 binding buffer (DPBS supplemented with 1 mM ethylenediaminetetraacetic acid, pH 7.4 with NaOH) by homogenization. Protein was quantified using the Coomassie method (Pierce; Rockford, IL) using bovine serum albumin as the standard.

Dilutions of each test compound (50 μl each, with concentrations ranging from 0.32 nM to 32 μM) were made in VMAT2 binding buffer and were placed into wells of a low protein-binding 96-well microtiter plate. A total of 75 μL of [^{3}H]-DHTBZ (American Radiolabeled Chemicals; St. Louis, MO) was placed into each well (final target assay concentration of 6-10 nM), followed by 75 μL of homogenate suspension (50 μg membrane protein/well) for a total final volume of 200 μL. The plate was incubated for 2 h at 25°C. Receptor-bound radioligand was then separated from unbound free radioligand by rapid vacuum filtration as follows: Unifilter GF/C filter plates (6005174; PerkinElmer Life and Analytical Sciences; Boston, MA) were pre-treated with a solution of 0.5% polyethylenimine in distilled water (P3143; Sigma-Aldrich; St. Louis, MO) for at least 30 min. Membranes were collected from the assay plate using a cell harvester (Unifilter-96 Filtermate; PerkinElmer Life and Analytical Sciences) and washed twice with 400 μL of 4°C DPBS. Plates were dried for 30-40 min using an air blower. Finally, each well received 50 μL scintillation fluid (Microscint 20; PerkinElmer Life

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and Analytical Sciences), and the plate was sealed and monitored for [^{3}H] using a TopCount NXT at 35% efficiency (PerkinElmer Life and Analytical Sciences). The exact concentration of [^{3}H]-DHTBZ was determined by counting an aliquot of the radioligand added in the assay using a Packard 1600TR liquid scintillation counter at 55% efficiency (PerkinElmer; Waltham, MA).

Data analysis. Data were analyzed using a one-site competition equation using Prism 4.0 GraphPad Software (La Jolla, CA). IC_{50} values were converted to K_i using the measured radioligand concentration and the K_d of [^{3}H]-DHTBZ using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). For competition experiments, non-specific binding was fit as a variable using the plateau at the highest concentrations of competitor. All compounds inhibited the binding of [^{3}H]-DHTBZ to the same extent.

Off-Target Binding

Broad panel screen. A broad panel target screen (Cerep; Paris, France) covering more than 80 targets was conducted to test the affinities of VBZ, R,R,R-DHTBZ, NBI-136110 and the other three DHTBZ stereoisomers from TBZ for targets other than VMAT2. The single concentration screen performed in duplicate included multiple classes of receptor proteins including G-protein-coupled receptors (GPCRs), cell-surface monoamine transporters, and ion channels.

Radioligand binding. As a follow-up to the Cerep screening campaign, affinities of the four independent DHTBZ stereoisomers for dopamine, serotonin and adrenergic receptor subtypes were evaluated using direct radioligand binding assays conducted in membranes from human Chinese hamster ovary cells (CHO-K1; American Type Culture Collection; Manassas, VA) expressing the various receptors. Serotonin 5-HT_{1A}, 5-HT_{2B}, 5-HT_7, dopamine D_2 and α_{2A}

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adrenergic receptors were independently expressed in CHO-K1 cells while the serotonin 5-HT_{2A}, dopamine D_1 and α_1-adrenergic receptors were expressed in human embryonic kidney (HEK) cells (Flp-In™ HEK 293 cell line; Thermo Fisher Scientific; Waltham, MA). Assay methods were the same as for VMAT2 binding, except that the test compounds were diluted in a buffer appropriate for each receptor subtype. The individual radioligands corresponding to each receptor were as follows: [^{3}H]-8-OH-DPAT (5-HT_{1A}), [^{3}H]-ketanserin (5-HT_{2A}), [^{3}H]-lysergic acid diethylamide (5-HT_{2B} and 5-HT_7), [^{3}H]-SCH-23390 (D_1), [^{3}H]-raclopride (D_2), [^{3}H]-prazocin (α_1 adrenergic) and [^{3}H]-rauwolscine (α_{2A} adrenergic).

VMAT1 inhibition: uptake assays. The ability of VBZ and its metabolites to inhibit VMAT1 in mammalian cells was compared to TBZ, its metabolites, and reserpine, the non-selective VMAT1/VMAT2 inhibitor. The coding sequence of VMAT1 (SLC18A1 solute carrier family 18 member 1; GenBank accession number NM_003053) was cloned into pCDNA3.1(+) (Life Technologies; Carlsbad, CA). This expression construct was verified by sequencing and confirmed by comparing to the GenBank entry (NCBI reference sequence NM_003053.3).

CHO-K1 cells were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C and 7.5% CO_2. Transient transfections were performed using X-treme GENE 9 (Roche Diagnostics; Indianapolis, IN) according to manufacturer's instructions. Briefly, on the day prior to transfection, approximately 65,000 CHO-K1 cells in 0.5 ml growth media without penicillin and streptomycin were seeded into a 24-well cell culture plate and incubated for 18 h at 37°C and 7.5% CO_2. The expression construct (0.25 µg DNA) was mixed with 0.75 µl of transfection reagent in 25 µl of Opti-MEM media (Thermo Fisher Scientific; Waltham, MA) and then incubated at room temperature for 15 min to form the

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transfection mixtures. This mixture was applied to the CHO-K1 cells in the 24-well plate and further incubated for another 48 h prior to use in the uptake assay.

For uptake assays, CHO-K1 cells transiently expressing human VMAT1 grown in 24-well culture plates were washed once with DPBS. Cells were permeated using 0.4 ml of 10 μM digitonin in uptake buffer (110 mM K-tartrate, 5 mM glucose, 5 mM $MgCl_2$, 1 mM ascorbic acid, 10 μM pargyline, 5 mM ATP, 20 mM K^+-HEPES, pH 7.6) for 10 min at room temperature. The digitonin solution was aspirated and the cells washed once with 1 ml of uptake buffer and aspirated. A total of 200 μl of uptake buffer (total uptake) or uptake buffer containing 20 μM of the test compounds (final concentrations 10 μM) was added to individual wells and incubated at 27°C for 10 min. Uptake was initiated with the addition of 200 μl of uptake buffer with 0.4% BSA containing approximately 100 nM [^{3}H]-serotonin (final concentration 50 nM; specific activity, 27.8 C_i/mmol; Lot: 2036964) added to the cells and further incubated for an additional 10 min at 27°C. Finally, cells were washed with 2 ml of ice-cold uptake buffer without ATP and lysed using 200 μl of 10% SDS. The cell lysates were collected and placed in tubes along with 5 ml scintillation cocktail ScintiSafe 30% (Fisher Scientific; Pittsburgh, PA) and monitored for radioactivity using a Wallac 1414 Win Spectral Liquid scintillation counter (efficiency 90%). Data were analyzed by one-way analysis of variance (ANOVA) with a Dunnett's multiple comparison post-hoc test.

In Vivo Studies

Palpebral ptosis. Ptosis in rats has been shown to be a measure of norepinephrine depletion induced by reserpine and reversed by adrenergic agonists or compounds that increase brain content of norepinephrine (Deniard et al., 1983; Kato et al., 1986). To investigate the

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effects of TBZ, R,R,R-DHTBZ, and VBZ on norepinephrine depletion via VMAT2, male rats (CD IGS®, Charles River Labs) weighing approximately 275 g were observed in clear plexiglass cylinders (6" wide x 12" high) for palpebral ptosis. The total observation time was 3 min and began when the rat was gently raised so that the forepaws were briefly lifted from the table surface. The handling stimulus facilitated the distinction between resting or sleeping behaviors and pharmacologically-induced ptosis. Observations were made at 1, 2, 3, 6, 9, 12, 18 and 24 h post-administration. An overall ptosis score was assigned to each minute of observation and the 3 scores for the observation period were averaged. Ptosis scores were: 0 = no eyelid drooping, 1 = some drooping to half-closed eyelids, 2 = half-closed to completely closed eyelids. Scores were analyzed by one-way repeated measures ANOVA (SigmaStat version 3.0.1, SPSS, Chicago, IL) followed by the Dunnett's post-hoc test for significance.

Prolactin secretion. Following the ptosis observations, serum prolactin concentrations were measured as a biomarker of dopamine D_2 receptor antagonism. At 90 min post-administration, trunk blood was collected from male rats (CD IGS®, Charles River Labs; ~300 g) into serum tubes (Becton Dickson #365956), allowed to clot at room temperature for 30 min and then centrifuged at 3,800 x g for 4 min at 4ºC. Serum was flash frozen and stored at -80°C. Serum prolactin concentrations were determined using MP Biomedicals rPRL ELISA (07C-74602; Santa Ana, CA) following the manufacturer's protocol with two exceptions; i) the lower limit of detection was expanded to 0.036 ng/ml, and ii) the chromagen substrate incubation time was increased from 30 min to 1 h in order to increase the sensitivity of the assay for use in male rats. Male rats were used in these studies in order to minimize the variability of prolactin release due to estrous cycle in females. Prolactin values were analyzed by one-way ANOVA (SigmaStat

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version 3.0.1, SPSS, Chicago, IL) with sources of significant differences detected using the Dunnett's post-hoc test.

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Results

VMAT2 Inhibition

VBZ and its metabolites, R,R,R-DHTBZ and NBI-136110, were tested for their ability to inhibit the binding of [^{3}H]-DHTBZ to VMAT2 in cell lines or native tissues. The primary metabolite R,R,R-DHTBZ, was the most potent inhibitor of VMAT2 in rat striatum and human platelet homogenates (Table 1). VBZ and NBI-136110 had similar effects on VMAT2 inhibition, but with K_i values that were approximately 40-65 times the K_i values (lower affinity) of R,R,R-DHTBZ. These results were corroborated by the radioligand binding assay of DHTBZ stereoisomers (i.e., TBZ metabolites) in the rat forebrain, which also showed R,R,R-DHTBZ to be the most potent inhibitor of VMAT2, followed by S,R,R-DHTBZ (Table 2). Comparatively, S,S,S-DHTBZ and R,S,S-DHTBZ, the other two primary metabolites of TBZ, were found to be poor VMAT2 inhibitors with affinities approximately 60 and 160 times weaker than R,R,R-DHTBZ (Table 2).

Off-Target Binding

The multi-target activity screen of more than 80 targets for these compounds (Cerep screen) demonstrated that VBZ and its metabolites, R,R,R-DHTBZ and NBI-136110, did not inhibit the binding of cognate ligands to any of the targets by more than 50% at concentrations of 1-10 µM (data not shown[3]). In contrast, the other three DHTBZ stereoisomers (S,R,R-DHTBZ, S,S,S-DHTBZ, R,S,S-DHTBZ), which are metabolites of TBZ but not VBZ, demonstrated >50% inhibition of ligand binding to a number of receptor subtypes including serotonin, dopamine and adrenergic receptors (Table 3). To describe the monoamine systems in greater detail, detailed radioligand binding assays were performed for dopamine, serotonin and adrenergic receptor

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subtypes as well as the transporters for dopamine (DAT), serotonin (SERT), and norepinephrine (NET) for the common metabolite of TBZ and VBZ (R,R,R-DHTBZ) and the other relevant metabolites unique to TBZ and VBZ. This detailed analysis revealed the high specificity of R,R,R-DHTBZ for the VMAT2 transporter and the non-specific activities of the other TBZ metabolites, including relatively high affinity for dopamine and serotonin receptor subtypes. Interestingly, the R,R,R-DHTBZ metabolite showed the greatest non-selectivity with respect to the monoamine receptors (Table 4). None of the TBZ or VBZ metabolites had any affinity for the monoamine transporters DAT, SERT or NET (Table 4). To complete the selectivity profile for VMAT2, the functional activity for the human VMAT1 transporter of these compounds was tested in cells expressing VMAT1. While the non-selective irreversible high-affinity uptake inhibitor of VMAT1, reserpine, substantially inhibited uptake through VMAT1, there was no significant inhibitory activity of TBZ, VBZ, or its metabolites R,R,R-DHTBZ or NBI-136110 at concentrations up to 10 µM (Fig. 2). For both VMAT1 and VMAT2, uptake was measured in the untransfected host cells and was found to be similar to transfected cells in the presence of excess reserpine (example, see Fig. 2).

In Vivo Palpebral Ptosis and Prolactin Release

In order to determine the functional effects of TBZ, VBZ and their metabolites, rats were administered either TBZ, VBZ or R,R,R-DHTBZ and evaluated for their ability to induce ptosis (primarily an adrenergic response; Fig. 3A) and increase plasma prolactin (Fig. 3B) primarily a dopaminergic response. The mechanisms underlying a ptosis response in rats are well documented and have been shown to be induced by central inhibition of presynaptic monoaminergic release and specifically reversed by α-adrenergic agonists (Tedeschi et al., 1967;

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Deniard et al., 1983). On the other hand, prolactin release is directly modulated by dopamine D_2 receptor antagonism in the anterior pituitary (Ben-Jonathan, 1985). All three compounds elicited ptosis to the same degree. There was a significant increase in prolactin in the TBZ- and R,R,R-DHTBZ–treated groups compared to vehicle-treated animals ($P<0.05$ for both) and to a lesser extent with VBZ, which did not reach the level of significance (see Fig. 3).

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Discussion

Involuntary hyperkinetic dyskinesias, which can result from exposure to DRBAs, are a serious limiting factor for chronic antipsychotic drug therapy (Youssef and Waddington, 1987; Browne et al., 1996; Ballesteros et al., 2000). Chronic neuroleptic exposure has long been known to produce EPS and TD (Casey, 1991), and it was hoped that the development of a new class of second generation (or "atypical") antipsychotics would dramatically decrease the risk of EPS and TD. While initial studies provided some evidence that this was indeed the case, a relatively recent prospective cohort study reported that in 352 initially TD-free psychiatric patients, the prevalence and incidence of TD was comparable despite the increased use of atypical antipsychotics (Woods et al., 2010). Several hypotheses for the pathophysiological mechanisms of TD have been proposed (e.g., dopamine hypersensitivity and neurotoxicity), but suitable treatment options targeting the underlying etiology of TD remain largely unrealized. Instead, clinicians must resort to management of the symptoms and accept the risk of TD as a tradeoff for treating certain psychiatric conditions.

TBZ, a benzoquinolizine derivative that depletes presynaptic dopamine storage and antagonizes postsynaptic dopamine receptors (by virtue of the four isomeric metabolites derived from TBZ), has been used to treat a wide range of hyperkinetic movement disorders, including TD (Ondo et al., 1999). TBZ inhibits VMAT2, which is the only neuronal transporter that moves dopamine from the cytoplasm into the synaptic vesicles (Yelin and Schuldiner, 2002). Owing to its physicochemical and metabolic characteristics, namely the formation in the body of four independent isomers, the treatment paradigm with TBZ is quite complex. Low bioavailability and variations in human liver enzymes responsible for the primary metabolism of DHTBZ

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contribute to high inter-patient variability, erratic systemic exposure after oral administration and a short half-life; collectively, these characteristics lead to a sub-optimal pharmacokinetic profile.

TBZ itself is a potent inhibitor of human VMAT2, with a K_i for inhibition of [^{3}H]-DHTBZ binding of 10 nM (Cesura et al., 1990; Zucker et al., 2001) and an EC_{50} for inhibition of monoamine uptake of 97 nM (Erickson et al., 1996). Upon oral administration, TBZ is converted to DHTBZ (Mehvar et al., 1987), which contains three asymmetric carbon centers (C-2, C-3, and C-11β; see Fig. 1), which could hypothetically result in eight stereoisomers (Yao et al., 2011). However, because the C-3 and C-11β carbons have fixed relative configurations, only four stereoisomers are possible (as listed in Table 2) (Kilbourn et al., 1995). The R,R,R-DHTBZ stereoisomer binds with the highest affinity to both rat and human VMAT2 (K_i = 1.0 to 4.2 nM). In comparison, the remaining three DHTBZ stereoisomers (S,R,R-DHTBZ, S,S,S-DHTBZ, R,S,S-DHTBZ) bind to VMAT2 with a K_i values of 9.7, 250, and 690 nM, respectively (Table 2). This order of potency is consistent with that described by Kilbourn et al. (1995) and Yao et al. (2011).

The purpose of this study therefore was to characterize the *in vitro* pharmacological profiles of TBZ, its most active metabolite (R,R,R-DHTBZ), and VBZ (the parent of R,R,R-DHTBZ) to support a mechanism-based novel therapeutic with maximal selectivity and specificity for the VMAT2 transporter. The four DHTBZ isomers and VBZ were tested for their ability to inhibit [^{3}H]-DHTBZ binding to VMAT2. The affinity of VBZ and its metabolites R,R,R-DHTBZ and NBI-136110 for other targets beyond VMAT2 was assessed in an extensive Cerep screen of multiple classes of protein targets including GPCRs, cell-surface monoamine transporters, and ion channels including the cardiac potassium channel, human ether-à-go-go-related gene (HERG). Radioligand binding assays and the broad panel screen indicate that in

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addition to varying potency at the VMAT2 transporter, two of the other DHTBZ metabolites of TBZ (S,S,S-DHTBZ and R,S,S-DHTBZ) interact with D_1 and D_2 receptors, which may be undesirable and (in principle) may be contraindicated in terms of managing neuroleptic-induced TD. Since VBZ is not metabolized to either of these DHTBZ stereoisomers, its effects on postsynaptic dopamine receptors either directly or indirectly through the metabolites are non-existent. Moreover, results from the broad panel screen indicate that VBZ and its major metabolites (R,R,R-DHTBZ and NBI-136110) have little to no affinity for more than 80 binding sites, including receptors, monoamine transporters, and ion channels. This profile suggests a low potential for off-target pharmacological effects. In addition, uptake studies using TBZ, VBZ and its metabolites, R,R,R-DHTBZ and NBI-136110, confirmed the selectivity of these compounds for VMAT2 as they had no significant effect on the uptake of monoamines through VMAT1 compared to reserpine, a known VMAT1/VMAT2 inhibitor.

The selectivity and specificity of VBZ was distinctively demonstrated using two *in vivo* surrogate measures of pharmacological effects. Ptosis, known to occur via adrenergic activation and prolactin release from the pituitary, modulated through the D_2 dopamine receptor, demonstrated the difference between treatment with TBZ and VBZ (Fig. 3). TBZ, VBZ and R,R,R-DHTBZ induced ptosis in an equivalent manner. This confirms that the metabolites formed by dosing TBZ or VBZ, or dosing of the active metabolite itself (R,R,R-DHTBZ) all have activity at VMAT2 affecting presynaptic monoamine release, in this case, related to norepinephrine release specifically to induce ptosis. Following similar treatment, but this time using prolactin release as a surrogate for dopaminergic modulation, R,R,R-DHTBZ and VBZ (to a lesser extent) induced a similar increase in serum prolactin levels as TBZ. Thus, along with its metabolic pathway and other *in vitro* results (i.e., inhibition of VMAT2, lack of off-target

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interactions), these findings indicate that VBZ, a novel inhibitor of VMAT2 may be appropriate for the treatment of hyperkinetic movement disorders such as TD.

The limitations of the studies described in this report relate to the nature of *in vitro* expression systems and their direct translation to *in vivo* human physiology. While good correlations exist in terms of *in vitro* affinities and function of compounds for distinct proteins, these may not necessarily reflect *in vivo* pharmacological activity of these compounds. Although the pathophysiology of TD is not completely understood, it is believed that exposure to DRBAs can result in dopamine receptor hypersensitivity (e.g., D_2 receptor hypersensitivity in the striatum), which in turn can lead to the emergence of hyperkinetic movements (Sethi, 2001; Mehta et al., 2015). Inhibition of presynaptic VMAT2 may be an effective strategy for the treatment of hyperkinetic movement disorders, as has been demonstrated for the treatment of chorea associated with Huntington's disease with TBZ, (Jankovic and Clarence-Smith, 2011). VBZ is a novel VMAT2 inhibitor that generates only the R,R,R-isomer of DHTBZ, maintaining its precise selectivity and potency for the VMAT2 transporter. The *in vitro* and *in vivo* studies presented in this report confirm that VBZ is a potent, selective, and specific inhibitor of VMAT2 with no inhibition of the related VMAT1 transporter and minimal off-target interactions at more than 80 other receptors transporters and ion channels. With its gradual metabolism to R,R,R-DHTBZ, its 20-h half-life and low peak to trough concentration ratio, VBZ is appropriate for once-daily dosing as demonstrated thus far in clinical studies (O'Brien et al., 2015; Hauser et al., 2017). These pharmacologic characteristics of VBZ and its metabolites may contribute to an efficacy and safety profile that is favorable for the treatment of TD and other hyperkinetic movement disorders.

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Authorship Contributions

Participated in research design: DEG, ES, SRJH, AM, HB

Conducted experiments and analyzed data: SRJH, ES, DEG

Drafted, reviewed, and/or revised the manuscript: DEG, ES, SRJH, AM, HB

Approved the final manuscript for submission: DEG, ES, SRJH, AM, HB

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Footnotes

The studies presented in this report were supported by Neurocrine Biosciences, Inc., San Diego, California.

Some findings of these studies were previously presented at the 45th Annual Meeting of the American College of Clinical Pharmacology (September 25-27, 2016; Bethesda, Maryland) and the 29th Annual U.S. Psychiatric & Mental Health Congress (October 21-24, 2016; San Antonio, Texas, USA).

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[1]SRJH and AM were employees of Neurocrine Biosciences, Inc., at the time of the studies.

[2]Configurations of the dihydrotetrabenazine (DHTBZ) stereoisomers are follows, along with alternate nomenclature used in the literature and the "NBI" nomenclature used in our laboratory:

- 2R, 3R, 11βR-DHTBZ: R,R,R-DHTBZ, (+)-α-DHTBZ, NBI-98782
- 2S, 3S, 11βS-DHTBZ: S,S,S-DHTBZ, (-)-α-DHTBZ, NBI-98771
- 2S, 3R, 11βR-DHTBZ: S,R,R-DHTBZ, (+)-β-DHTBZ, NBI-98795
- 2R, 3S, 11βS-DHTBZ: R,S,S-DHTBZ, (-)-β-DHTBZ, NBI-98772

[3]Data not shown in this report were made available to journal reviewers upon request.

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Figure Legends

Fig. 1. Primary metabolites derived from tetrabenazine and valbenazine

Tetrabenazine is metabolized into 4 independent isomers R,R,R-DHTBZ (NBI-98782); S,R,R DHTBZ (NBI-98795); S,S,S-DHTBZ (NBI-98771); and R,S,S-DHTBZ (NBI-98772). Valbenazine is metabolized into two primary metabolites, R,R,R-DHTBZ and NBI-136110. R,R,R-DHTBZ is the only DHTBZ stereoisomer common as a metabolite for both tetrabenazine and valbenazine. DHTBZ, dihydrotetrabenazine.

Fig. 2. Inhibition of [^{3}H]-serotonin uptake in CHO-K1 cells expressing VMAT1

Results represent the mean generated from three independent experiments performed in triplicate; error bars represent the standard error of the mean. *Significance was determined post-hoc using a one-way ANOVA with a Dunnett's multiple comparison test at $P<0.05$. ANOVA, analysis of variance; CHO, Chinese hamster ovary; VMAT1, vesicular monoamine transporter 1.

Fig. 3. Effects of tetrabenazine, valbenazine and R,R,R-DHTBZ on rat ptosis and prolactin release

Results represent the effects of 10 mg/kg tetrabenazine, valbenazine, or R,R,R-DHTBZ on ptosis and prolactin release in rats treated orally with a dosing volume of 3 ml/kg pH = 5.0-5.5. The mean ptosis scores presented (A) in this figure were measured 1 hour following drug administration and were similar for tetrabenazine, valbenazine and R,R,R-DHTBZ. Plasma samples from the same animals were collected at 90 minutes following drug administration for assessment of plasma prolactin (B). Both tetrabenazine- and R,R,R-DHTBZ–treated rats demonstrated higher prolactin levels (and to a lesser extent, valbenazine) than vehicle-treated

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animals. All values represent mean ± SEM, n=6/group. *P<0.05 versus vehicle (Dunnett's post-hoc test). DHTBZ, dihydrotetrabenazine; SEM, standard error of the mean.

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Figures

Figure 1.

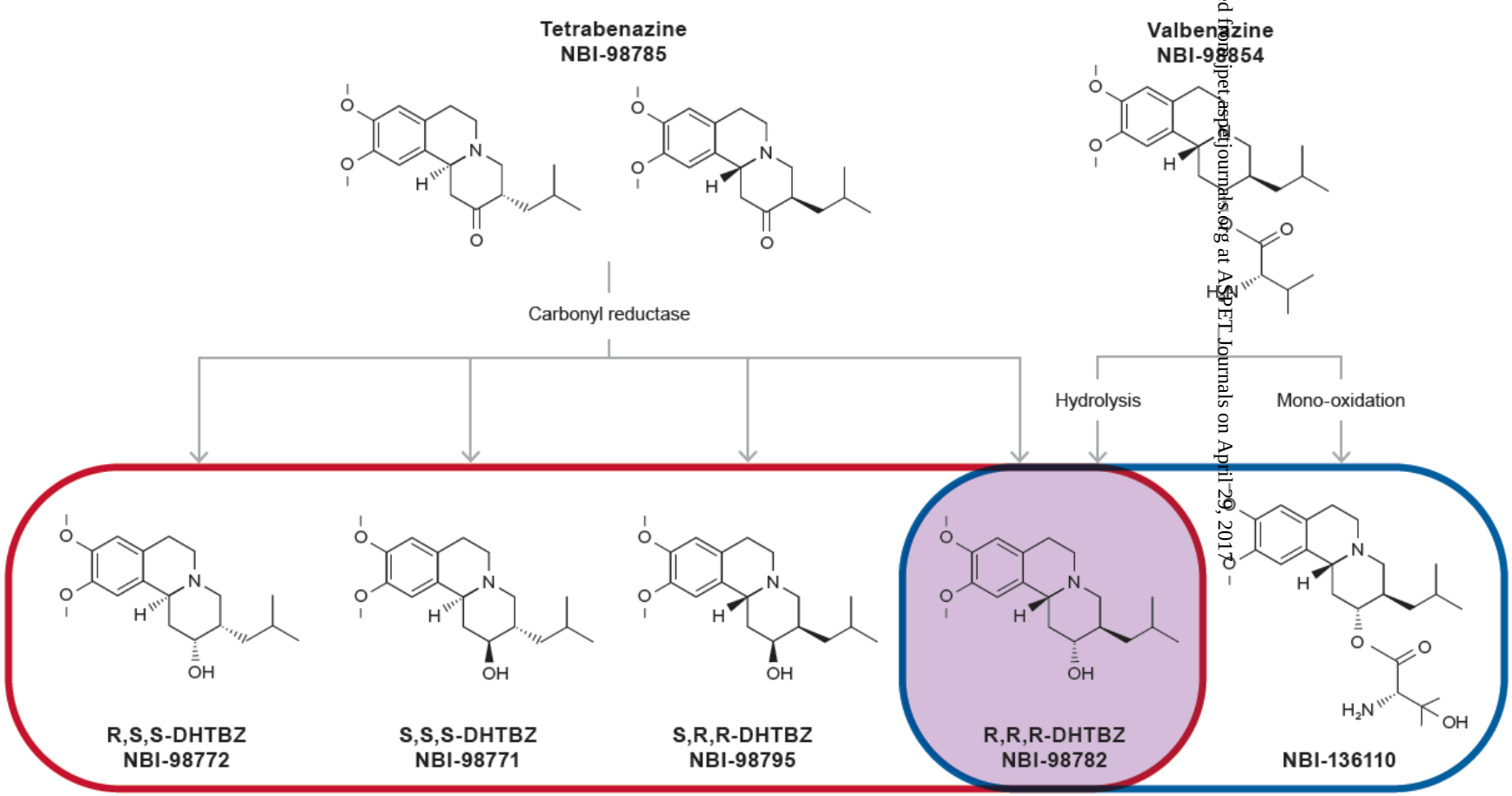

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Figure 2.

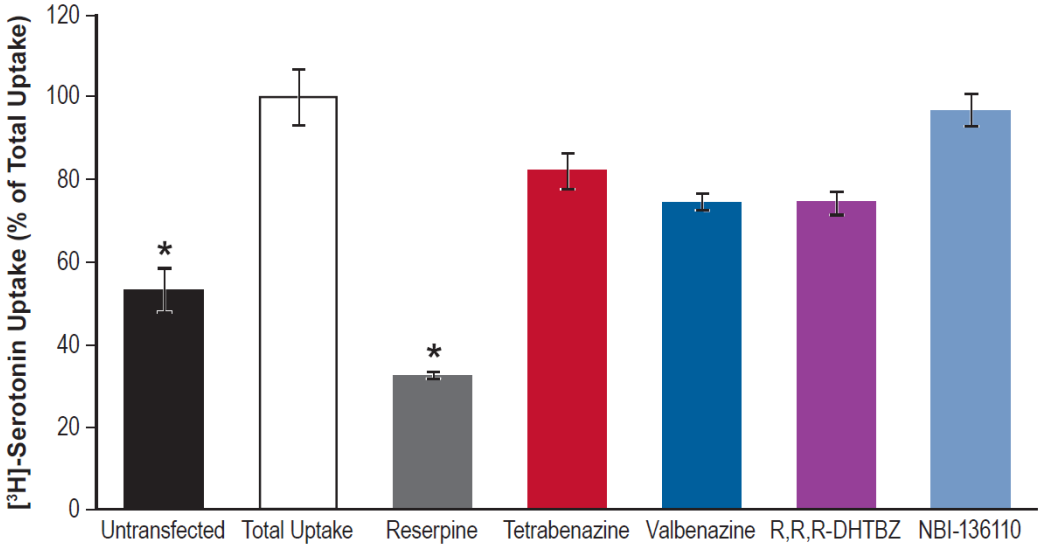

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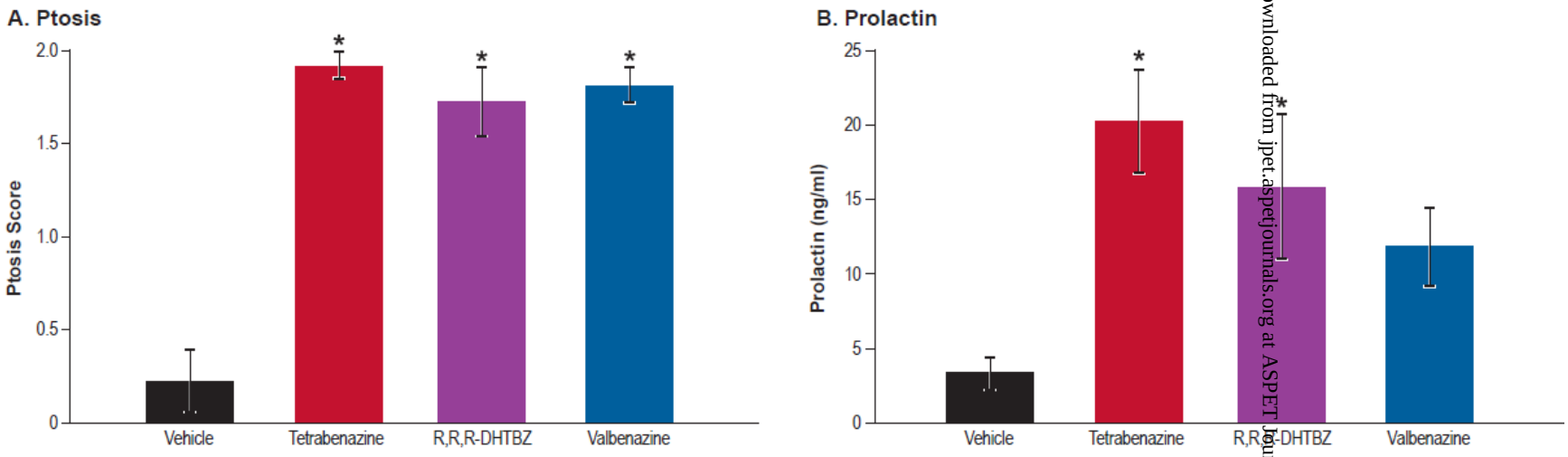
Figure 3.



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Tables

Table 1. *In vitro* VMAT2 binding affinity of valbenazine and its metabolites

The affinity of each compound was measured by inhibition of [^{3}H]-DHTBZ binding to either human platelets or rat striatal membranes. The affinities relative to R,R,R-DHTBZ were also calculated and are presented. Data are reported as both the negative logarithm of the K_i (pK_i) for statistical calculation with the normally distributed binding parameter used to determine the mean and SEM (n=4 for each compound in each tissue). The K_i value was determined from the mean pK_i as $10^{(-pKi)}$.

		Rat Striatum			Human Platelets		
Compound	**Structural Description**	**K_i, nM**	**pK_i mean (SEM)**	**Affinity Relative to R,R,R-DHTBZ**	**K_i, nM**	**pK_i mean (SEM)**	**Affinity Relative to R,R,R-DHTBZ**
Valbenazine	Parent molecule	110	6.95 (0.02)	39	150	6.82 (0.02)	45
R-R-R-DHTBZ	Metabolite formed from hydrolysis of valbenazine	1.98	8.70 (0.09)	1.0	3.1	8.52 (0.03)	1.0
NBI-136110	Metabolite formed from mono-oxidation of valbenazine	160	6.80 (0.02)	57	220	6.65 (0.04)	67

DHTBZ, dihydrotetrabenazine; SEM, standard error of the mean; VMAT2, vesicular monoamine transporter 2.

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Table 2. *In vitro* VMAT2 binding affinity in rat forebrain

The affinity of each compound was measured by inhibition of [^{3}H]-DHTBZ binding to rat forebrain membranes. The affinities relative to R,R,R-DHTBZ were also calculated and are presented. Data are reported as both the negative logarithm of the K_i (pK_i) for statistical calculation with the normally distributed binding parameter used to determine the mean and SEM. The K_i value was determined from the mean pK_i as $10^{(-pKi)}$.

	VMAT2			
Compound	**K_i, nM**	**pK_i mean (SEM)**	**N**	**Affinity Relative to R,R,R-DHTBZ[a]**
R,R,R-DHTBZ	4.2	8.38 (0.42)	27	1.0
S,R,R-DHTBZ	9.7	8.01 (0.32)	6	2.3
S,S,S-DHTBZ	250	6.60 (0.22)	4	60
R,S,S-DHTBZ	690	6.16 (0.05)	5	160

[a]Affinity relative to R,R,R-DHTBZ was calculated using the K_i value determined in the same study.

DHTBZ, dihydrotetrabenazine; SEM, standard error of the mean; VMAT2, vesicular monoamine transporter 2.

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Table 3. *In vitro* activity of valbenazine and DHTBZ stereoisomers at dopamine, serotonin, and adrenergic receptors

Results expressed as percent of control specific binding: (tested compound specific binding / control specific binding) x 100. All compounds were tested at 1 or 10 μM final concentration and results are an excerpt of a larger 80 target panel performed as an initial screen at Cerep (n=2 for each compound at each target). Bolded results (>50%) indicate activity at target receptor; full dose response for these results are shown in Table 4.

	Cerep Broad Panel Screen (Excerpt)			
Receptor Target	**Valbenazine**	**R,R,R-DHTBZ[a]**	**S,R,R-DHTBZ**	**S,S,S-DHTBZ/ R,S,S-DHTBZ[b]**
Serotonin5-HT_{1A}	26	17	**69**	**96**
Serotonin 5-HT_{2A}	1	-4	3	**84**
Serotonin 5-HT_7	4	3	**80**	**98**
Dopamine D_1	8	-6	-5	**82**
Dopamine $D_{2(s)}$	2	6	25	**89**

[a]Major metabolite of valbenazine.

[b]For the purposes of the broad panel screen, the S,S,S- and R,S,S- metabolites were tested as a 50/50 mixture.

DHTBZ, dihydrotetrabenazine.

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Table 4: *In vitro* pharmacological profile of DHTBZ isomers

Dopamine, serotonin, and adrenergic receptors were assessed through radioligand binding and monoamine transport sites were assessed through radioligand uptake. All assays were performed in two independent experiments and in triplicate. Where the K_i values were below 500 nM, assays were performed in 3-5 independent experiments.

	R,R,R-DHTBZ[a]			S,R,R-DHTBZ			S,S,S-DHTBZ			R,S,S-DHTBZ		
Target	**K_i, nM**	**pK_i mean (SD)[b]**	**n**	**K_i, nM**	**pK_i mean (SD)[b]**	**n**	**K_i, nM**	**pK_i mean (SD)[b]**	**n**	**K_i, nM**	**pK_i mean (SD)[b]**	**n**
VMAT2	4.2	8.38 (0.42)	27	9.7	8.01 (0.32)	6	250	6.60 (0.22)	4	690	6.16 (0.05)	5
Dopamine D_1	>5000	<5	2	>5000	<5	2	>5000	<5	2	4000	5.40 (0.05)	2
Dopamine $D_{2(s)}$	>5000	<5	2	>5000	<5	2	180	6.73 (0.23)	5	53	7.27 (0.18)	6
Serotonin 5-HT_{1A}	6100	5.21 (0.07)	2	7500	5.13 (0.05)	2	750	6.13 (0.23)	2	1500	5.82 (0.41)	2
Serotonin 5-HT_{2A}	>5000	<5	2	>5000	<5	2	>5000	<5	2	2200	5.66 (0.00)	2
Serotonin 5-HT_{2B}	>5000	<5	2	>5000	<5	2	600	6.22 (0.33)	3	460	6.34 (0.40)	4
Serotonin 5-HT_7	>5000	<5	2	970	6.01 (0.03)	2	71	7.15 (0.12)	2	5.9	8.23 (0.08)	2
α_{1A} Adrenergic	>5000	<5	2	>5000	<5	2	>5000	<5	2	980	6.01 (0.15)	4
α_{2A} Adrenergic	>5000	<5	2	>5000	<5	2	1700	5.76 (0.08)	2	220	6.65 (0.36)	2

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DAT	>5000	<5	2	>5000	<5	2	>5000	<5	2	>5000	<5	2
NET	>5000	<5	2	9200	5.03 (0.00)	2	9500	5.02 (0.00)	2	>5000	<5	2
SERT	>5000	<5	2	>5000	<5	2	>5000	<5	2	>5000	<5	2

[a]Major metabolite of valbenazine.

[b]pK_i is the negative logarithm of K_i. Standard deviations are only presented for pK_i values >5.

DAT, dopamine transporter; DHTBZ, dihydrotetrabenazine; NET, norepinephrine transporter; SD, standard deviation; SERT, serotonin transporter; VMAT2, vesicular monoamine transporter 2.

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