

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

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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 approved for the treatment of tardive dyskinesia. Valbenazine is converted to two significant circulating metabolites in vivo, namely, (+)- α -dihydrotrabenazine (R,R,R-HTBZ) and a mono-oxy metabolite, NBI-136110. Radioligand-binding studies were conducted to assess and compare valbenazine, trabenazine, and their respective metabolites in their abilities to selectively and potently inhibit [3 H]-HTBZ 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-HTBZ, and NBI-136110 at >80 receptor, transporter, and ion channel sites. Radioligand binding showed

R,R,R-HTBZ 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-HTBZ. Neither valbenazine, R,R,R-HTBZ, nor NBI-136110 had significant off-target interactions at serotonin (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}) or dopamine (D₁ or D₂) receptor sites. In vivo studies measuring ptosis and prolactin secretion in the rat confirmed the specific and dose-dependent interactions of trabenazine and R,R,R-HTBZ with VMAT2. Evaluations of potency and selectivity of trabenazine 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)].

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, is associated with TD and other movement disorders (Casey, 1991; Mehta et al., 2015). Although 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 is currently only one approved pharmacotherapeutic option, valbenazine.

Several reports and open label studies suggest that inhibition of the presynaptic vesicular monoamine transporter 2 (VMAT2) trabenazine (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.,

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S.R.J.H. and A.M. were employees of Neurocrine Biosciences at the time of the studies.

Configurations of the HTBZ stereoisomers are as follows, along with alternate nomenclature used in the literature and the “NBI” nomenclature used in our laboratory:

- 2R, 3R, 11 β R-HTBZ: R,R,R-HTBZ, (+)- α -HTBZ, NBI-98782
- 2S, 3S, 11 β S-HTBZ: S,S,S-HTBZ, (–)- α -HTBZ, NBI-98771
- 2S, 3R, 11 β R-HTBZ: S,R,R-HTBZ, (+)- β -HTBZ, NBI-98795
- 2R, 3S, 11 β S-HTBZ: R,S,S-HTBZ, (–)- β -HTBZ, NBI-98772

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

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ABBREVIATIONS: ANOVA, analysis of variance; CHO, Chinese hamster ovary; HTBZ, dihydrotrabenazine; DPBS, Dulbecco's phosphate-buffered saline; DRBA, dopamine receptor-blocking agent; EGTA, ethylene glycol-bis[β -aminoethyl ether]-N,N,N',N'-tetraacetic acid; EPS, extrapyramidal symptom; TBZ, trabenazine; TD, tardive dyskinesia; VBZ, valbenazine; VMAT, vesicular monoamine transporter.

1996). VMAT2 distribution in the central nervous system in both mice and humans has been determined using [^{11}C]-methoxytetrabenazine, which demonstrated consistent localization with known monoamine nerve terminal density, particularly in the striatum, lateral septum, and other limbic brain regions (Vander Borgh 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 (HTBZ). These metabolites include four stereoisomers: R,R,R-HTBZ or (+)- α -HTBZ (alternate nomenclature) or NBI-98782 (laboratory nomenclature); S,S,S-HTBZ or (–)- α -HTBZ or NBI-98771; S,R,R-HTBZ or (+)- β -HTBZ or NBI-98795; and R,S,S-HTBZ or (–)- β -HTBZ or NBI-98772 (Kilbourn et al., 1997). 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 with valbenazine (VBZ; NBI-98854) and its metabolites.

VBZ is a novel VMAT2 inhibitor currently in development for the treatment of TD. Structurally, VBZ is the valine ester of the R,R,R-HTBZ isomer (Fig. 1). It is rapidly absorbed after oral administration; following absorption, VBZ levels decline slowly with a half-life of approximately 20 hours, supporting once-daily administration. The primary metabolic clearance pathways of VBZ are hydrolysis (to form R,R,R-HTBZ) and mono-oxidation (to form the metabolite NBI-136110). R,R,R-HTBZ 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 (Luo R, Bozigian H, Jimenez R, Loewen G, and O'Brien CF, 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).

Although both TBZ and VBZ share a common metabolite (R,R,R-HTBZ), 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.

Materials and Methods

Radioligand Binding

Studies were conducted to determine the ability of VBZ and its metabolites, R,R,R-HTBZ and NBI-136110, to inhibit the binding of [^3H]-HTBZ to the VMAT2 transporter in rat striatum and human platelet homogenates. Affinity of the four HTBZ stereoisomers

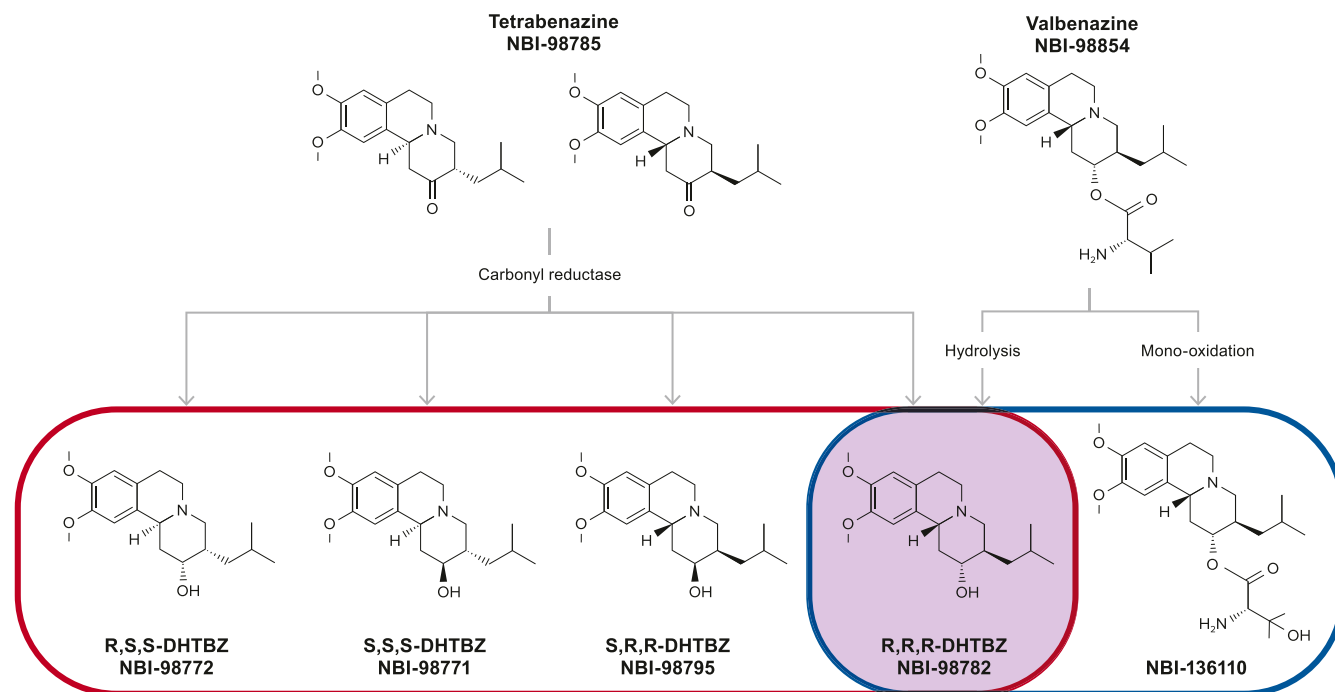


Fig. 1. Primary metabolites derived from TBZ and VBZ. TBZ is metabolized into four independent isomers: R,R,R-HTBZ (NBI-98782); S,R,R-HTBZ (NBI-98795); S,S,S-HTBZ (NBI-98771); and R,S,S-HTBZ (NBI-98772). VBZ is metabolized into two primary metabolites: R,R,R-HTBZ and NBI-136110. R,R,R-HTBZ is the only HTBZ stereoisomer common as a metabolite for both TBZ and VBZ.

(R,R,R-HTBZ, S,R,R-HTBZ, S,S,S-HTBZ, and R,S,S-HTBZ) 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 minutes. 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 (14200-075; Invitrogen, Carlsbad, CA) supplemented with 10 mM MgCl_2 , 2 mM 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 minutes, and then homogenized using a hand-held homogenizer (30,000 rpm on ice or at 4°C for 20 seconds).

Human Platelet Homogenate. One unit of human platelet suspension (approximately 250 ml, sex unknown; San Diego Blood Bank, San Diego, CA) was centrifuged at 3000g for 20 minutes, and the resulting platelet pellet was resuspended in 60 ml ice-cold lysis buffer 1 (50 mM HEPES, 1 mM ethylenediaminetetraacetic acid, pH 7.4; Sigma-Aldrich). The platelet suspension was homogenized using a hand-held homogenizer (30,000 rpm at 4°C for 1 minute) and then left at 4°C for 20 minutes. The platelets were further lysed using nitrogen cavitation at 900 psi for 30 minutes at 4°C , and then the homogenate was decanted.

Radiolabeling of Homogenates. Homogenates were centrifuged (45,000g for 20 minutes at 4°C), and the supernatant was discarded. Forebrain pellets were homogenized in 10 ml fresh homogenization buffer and centrifuged again. Striatum and platelet pellets were resuspended 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 EDTA, 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 [^3H]-HTBZ (American Radiolabeled Chemicals, St. Louis, MO) was placed into each well (final target assay concentration of 6–10 nM), followed by 75 μL homogenate suspension (50 μg membrane protein/well) for a total final volume of 200 μL . The plate was incubated for 2 hours 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 pretreated with a solution of 0.5% polyethylenimine in distilled water (P3143; Sigma-Aldrich) for at least 30 minutes. 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 4°C DPBS. Plates were dried for 30–40 minutes using an air blower. Finally, each well received 50 μL scintillation fluid (Microscint 20; PerkinElmer Life and Analytical Sciences), and the plate was sealed and monitored for ^3H using a TopCount NXT at 35% efficiency (PerkinElmer Life and Analytical Sciences). The exact concentration of [^3H]-HTBZ 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 [^3H]-HTBZ using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). For competition experiments, nonspecific binding was fit as a variable using the plateau at the highest concentrations of competitor. All compounds inhibited the binding of [^3H]-HTBZ 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-HTBZ, NBI-136110, and the other three HTBZ 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, cell surface monoamine transporters, and ion channels.

Radioligand Binding. As a follow-up to the Cerep screening campaign, affinities of the four independent HTBZ stereoisomers for dopamine, serotonin, and adrenergic receptor subtypes were evaluated using direct radioligand-binding assays conducted in membranes from human Chinese hamster ovary (CHO) cells (CHO-K1; American Type Culture Collection, Manassas, VA) expressing the various receptors. Serotonin 5-HT_{1A}, 5-HT_{2B}, 5-HT₇, dopamine D₂, and α_{2A} adrenergic receptors were independently expressed in CHO-K1 cells, whereas the serotonin 5-HT_{2A}, dopamine D₁, and α_1 adrenergic receptors were expressed in human embryonic kidney 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: [^3H]-8-OH-DPAT (5-HT_{1A}), [^3H]-ketanserin (5-HT_{2A}), [^3H]-lysergic acid diethylamide (5-HT_{2B} and 5-HT₇), [^3H]-SCH-23390 (D₁), [^3H]-raclopride (D₂), [^3H]-prazosin (α_1 adrenergic), and [^3H]-rauwolscine (α_{2A} adrenergic).

VMAT1 Inhibition: Uptake Assays. The ability of VBZ and its metabolites to inhibit VMAT1 in mammalian cells was compared with TBZ, its metabolites, and reserpine, the nonselective 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 with the GenBank entry (National Center for Biotechnology Information 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 $\mu\text{g}/\text{ml}$ 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 hours at 37°C and 7.5% CO_2 . The expression construct (0.25 μg DNA) was mixed with 0.75 μL transfection reagent in 25 μL Opti-MEM media (Thermo Fisher Scientific) and then incubated at room temperature for 15 minutes to form the transfection mixtures. This mixture was applied to the CHO-K1 cells in the 24-well plate and further incubated for another 48 hours 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 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 minutes at room temperature. The digitonin solution was aspirated, and the cells were washed once with 1 ml uptake buffer and aspirated. A total of 200 μL uptake buffer (total uptake) or uptake buffer containing 20 μM test compounds (final concentrations 10 μM) was added to individual wells and incubated at 27°C for 10 minutes. Uptake was initiated with the addition of 200 μL uptake buffer with 0.4% bovine serum albumin containing approximately 100 nM [^3H]-serotonin (final concentration 50 nM; specific activity, 27.8 Ci/mmol; Lot 2036964) added to the cells and further incubated for an additional 10 minutes at 27°C . Finally, cells were washed with 2 ml ice-cold uptake buffer without ATP and lysed using 200 μL 10% SDS. The cell lysates were collected and placed in tubes along with 5 ml scintillation

TABLE 1

In vitro VMAT2-binding affinity of VBZ and its metabolites

The affinity of each compound was measured by inhibition of [³H]-HTBZ binding to either human platelets or rat striatal membranes. The affinities relative to R,R,R-HTBZ 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 S.E.M. (*n* = 4 for each compound in each tissue). The K_i value was determined from the mean pK_i as 10^(-pK_i).

Compound	Structural Description	Rat Striatum			Human Platelets		
		K _i , nM	pK _i Mean (S.E.M.)	Affinity Relative to R,R,R-HTBZ	K _i , nM	pK _i Mean (S.E.M.)	Affinity Relative to R,R,R-HTBZ
VBZ	Parent molecule	110	6.95 (0.02)	39	150	6.82 (0.02)	45
R,R,R-HTBZ	Metabolite formed from hydrolysis of VBZ	1.98	8.70 (0.09)	1.0	3.1	8.52 (0.03)	1.0
NBI-136110	Metabolite formed from mono-oxidation of VBZ	160	6.80 (0.02)	57	220	6.65 (0.04)	67

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 effects of TBZ, R,R,R-HTBZ, and VBZ on norepinephrine depletion via VMAT2, male rats (CD 1GS; Charles River Laboratories Hollister, California) weighing approximately 275 g were observed in clear plexiglass cylinders (6" wide × 12" high) for palpebral ptosis. The total observation time was 3 minutes 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 hours postadministration. An overall ptosis score was assigned to each minute of observation, and the three scores for the observation period were averaged. Ptosis scores were as follows: 0 = no eyelid drooping; 1 = some drooping to half-closed eyelids; and 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 Dunnett's post hoc test for significance.

Prolactin Secretion. Following the ptosis observations, serum prolactin concentrations were measured as a biomarker of dopamine D₂ receptor antagonism. At 90 minutes postadministration, trunk blood was collected from male rats (CD 1GS; Charles River Laboratories; ~300 g) into serum tubes (BD Biosciences, San Jose, CA; 365956), allowed to clot at room temperature for 30 minutes, and then centrifuged at 3800g for 4 minutes at 4°C. Serum was flash frozen and stored at -80°C. Serum prolactin concentrations were determined using MP Biomedicals rPRL enzyme-linked immunosorbent assay (07C-74602; Santa Ana, CA) following the manufacturer's protocol with two exceptions: 1) the lower limit of detection was

expanded to 0.036 ng/ml, and 2) the chromagen substrate incubation time was increased from 30 minutes to 1 hour to increase the sensitivity of the assay for use in male rats. Male rats were used in these studies to minimize the variability of prolactin release due to estrous cycle in females. Prolactin values were analyzed by one-way ANOVA (SigmaStat version 3.0.1; SPSS) with sources of significant differences detected using Dunnett's post hoc test.

Results

VMAT2 Inhibition

VBZ and its metabolites, R,R,R-HTBZ and NBI-136110, were tested for their ability to inhibit the binding of [³H]-HTBZ to VMAT2 in cell lines or native tissues. The primary metabolite, R,R,R-HTBZ, 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-HTBZ. These results were corroborated by the radioligand-binding assay of HTBZ stereoisomers (i.e., TBZ metabolites) in the rat forebrain, which also showed R,R,R-HTBZ to be the most potent inhibitor of VMAT2, followed by S,R,R-HTBZ (Table 2). Comparatively, S,S,S-HTBZ and R,S,S-HTBZ, 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-HTBZ (Table 2).

Off-Target Binding

The multitarget activity screen of more than 80 targets for these compounds (Cerep screen) demonstrated that VBZ and its metabolites, R,R,R-HTBZ 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). In contrast, the other three HTBZ stereoisomers (S,R,R-HTBZ,

TABLE 2

In vitro VMAT2-binding affinity in rat forebrain

The affinity of each compound was measured by inhibition of [³H]-HTBZ binding to rat forebrain membranes. The affinities relative to R,R,R-HTBZ 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 S.E.M. The K_i value was determined from the mean pK_i as 10^(-pK_i).

Compound	VMAT2			
	K _i , nM	pK _i Mean (S.E.M.)	N	Affinity Relative to R,R,R-HTBZ ^a
R,R,R-HTBZ	4.2	8.38 (0.42)	27	1.0
S,R,R-HTBZ	9.7	8.01 (0.32)	6	2.3
S,S,S-HTBZ	250	6.60 (0.22)	4	60
R,S,S-HTBZ	690	6.16 (0.05)	5	160

^aAffinity relative to R,R,R-HTBZ was calculated using the K_i value determined in the same study.

TABLE 3

In vitro activity of VBZ and HTBZ stereoisomers at dopamine, serotonin, and adrenergic receptors
Results expressed as percentage of control-specific binding: (tested compound-specific binding/control-specific binding) \times 100. All compounds were tested at 1 or 10 μ M 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.

Receptor Target	Cerep Broad Panel Screen (Excerpt)			
	VBZ	R,R,R-HTBZ ^a	S,R,R-HTBZ	S,S,S-HTBZ/ R,S,S-HTBZ ^b
Serotonin 5-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

^aMajor metabolite of VBZ.

^bFor the purposes of the broad panel screen, the S,S,S- and R,S,S-metabolites were tested as a 50/50 mixture.

S,S,S-HTBZ, R,S,S-HTBZ), 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 subtypes as well as the transporters for dopamine, serotonin, and norepinephrine for the common metabolite of TBZ and VBZ (R,R,R-HTBZ) and the other relevant metabolites unique to TBZ and VBZ. This detailed analysis revealed the high specificity of R,R,R-HTBZ for the VMAT2 transporter and the nonspecific activities of the other TBZ metabolites, including relatively high affinity for dopamine and serotonin receptor subtypes. Interestingly, the R,R,R-HTBZ metabolite showed the greatest nonselectivity with respect to the monoamine receptors (Table 4). None of the TBZ or VBZ metabolites had any affinity for the monoamine transporters dopamine, serotonin, or norepinephrine (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. Although the nonselective 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-HTBZ or NBI-136110 at concentrations up to 10 μ M (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 (for example, see Fig. 2).

In Vivo Palpebral Ptosis and Prolactin Release

To determine the functional effects of TBZ, VBZ, and their metabolites, rats were administered either TBZ, VBZ, or R,R,R-HTBZ 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; Deniard et al., 1983). In contrast, prolactin release is directly modulated by dopamine D₂ 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-HTBZ-treated groups compared with 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).

TABLE 4

In vitro pharmacological profile of HTBZ 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.

Target	R,R,R-HTBZ ^a			S,R,R-HTBZ			S,S,S-HTBZ			R,S,S-HTBZ		
	K _i , nM	pK _i Mean (S.D.) ^b	n	K _i , nM	pK _i Mean (S.D.) ^b	n	K _i , nM	pK _i Mean (S.D.) ^b	n	K _i , nM	pK _i Mean (S.D.) ^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
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

DAT, dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter.

^aMajor metabolite of VBZ.

^bpK_i is the negative logarithm of K_i. S.D. are only presented for pK_i values >5 .

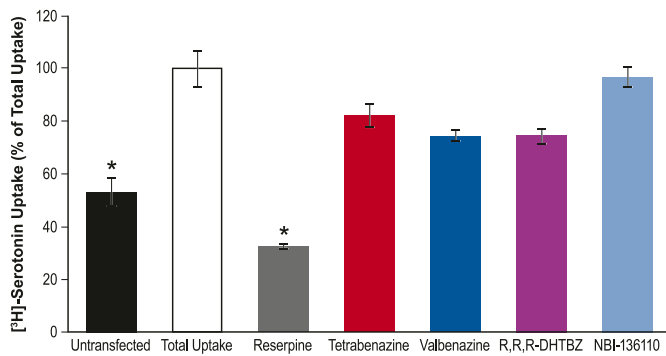


Fig. 2. Inhibition of [³H]-serotonin uptake in CHO-K1 cells expressing VMAT1. Results represent the mean generated from three independent experiments performed in triplicate; error bars represent the S.E.M. *Significance was determined post hoc using a one-way ANOVA with a Dunnett's multiple comparison test at $P < 0.05$.

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. Although 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 were 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 trade-off for treating certain psychiatric conditions.

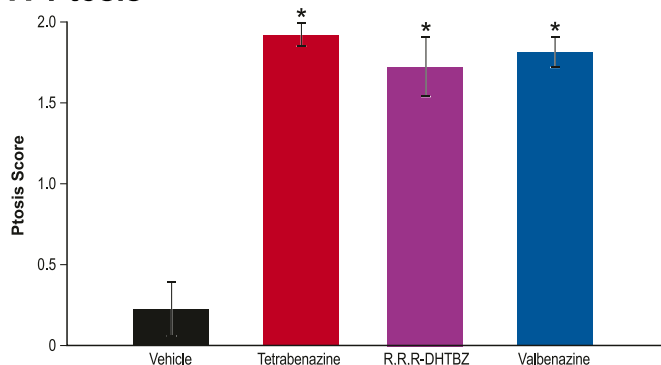
TBZ, a benzoquinolizine derivative that depletes presynaptic dopamine storage and antagonizes postsynaptic dopamine

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 HTBZ contribute to high interpatient variability, erratic systemic exposure after oral administration, and a short half-life; collectively, these characteristics lead to a suboptimal pharmacokinetic profile.

TBZ itself is a potent inhibitor of human VMAT2, with a K_i for inhibition of [³H]-HTBZ 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 HTBZ (Mehvar et al., 1987), which contains three asymmetric carbon centers (C-2, C-3, and C-11 β ; see Fig. 1), which could hypothetically result in eight stereoisomers (Yao et al., 2011). However, because the C-3 and C-11 β carbons have fixed relative configurations, only four stereoisomers are possible (as listed in Table 2) (Kilbourn et al., 1995). The R,R,R-HTBZ 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 HTBZ stereoisomers (S,R,R-HTBZ, S,S,S-HTBZ, R,S,S-HTBZ) bind to VMAT2 with 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-HTBZ), and VBZ (the parent of R,R,R-HTBZ) to support a mechanism-based novel therapeutic with maximal selectivity and specificity for the VMAT2 transporter. The four HTBZ isomers and VBZ were tested for their ability to inhibit [³H]-HTBZ binding to VMAT2. The affinity of VBZ and its metabolites R,R,R-HTBZ and NBI-136110 for other targets beyond VMAT2 was assessed in an extensive Cerep screen of multiple classes of protein targets, including G

A Ptosis



B Prolactin

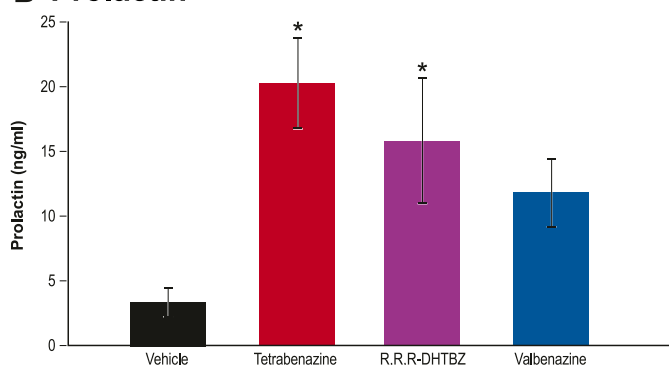


Fig. 3. Effects of TBZ, VBZ, and R,R,R-HTBZ on rat ptosis and prolactin release. Results represent the effects of 10 mg/kg TBZ, VBZ, or R,R,R-HTBZ on ptosis and prolactin release in rats treated orally with a dosing volume of 3 ml/kg, pH = 5.0–5.5. The mean ptosis scores presented (A) in this figure were measured 1 hour following drug administration and were similar for TBZ, VBZ, and R,R,R-HTBZ. Plasma samples from the same animals were collected at 90 minutes following drug administration for assessment of plasma prolactin (B). Both TBZ- and R,R,R-HTBZ-treated rats demonstrated higher prolactin levels (and to a lesser extent, VBZ) than vehicle-treated animals. All values represent mean \pm S.E.M., n = 6/group. * $P < 0.05$ versus vehicle (Dunnett's post hoc test).

protein-coupled receptors, cell surface monoamine transporters, and ion channels, including the cardiac potassium channel, human ether-à-go-go-related gene. Radioligand-binding assays and the broad panel screen indicate that, in addition to varying potency at the VMAT2 transporter, two of the other HTBZ metabolites of TBZ (S,S,S-HTBZ and R,S,S-HTBZ) interact with D₁ and D₂ receptors, which may be undesirable and (in principle) may be contraindicated in terms of managing neuroleptic-induced TD. Because VBZ is not metabolized to either of these HTBZ stereoisomers, its effects on postsynaptic dopamine receptors either directly or indirectly through the metabolites are nonexistent. Moreover, results from the broad panel screen indicate that VBZ and its major metabolites (R,R,R-HTBZ 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-HTBZ 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 with reserpine, a known VMAT1/VMAT2 inhibitor.

The selectivity and specificity of VBZ were distinctively demonstrated using two in vivo surrogate measures of pharmacological effects. Ptoxis, known to occur via adrenergic activation and prolactin release from the pituitary, modulated through the D₂ dopamine receptor, demonstrated the difference between treatment with TBZ and VBZ (Fig. 3). TBZ, VBZ, and R,R,R-HTBZ induced ptoxis 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-HTBZ), all have activity at VMAT2 affecting presynaptic monoamine release, in this case, related to norepinephrine release specifically to induce ptoxis. Following similar treatment, but this time using prolactin release as a surrogate for dopaminergic modulation, R,R,R-HTBZ 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 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. Although 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., D₂ 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 HTBZ, 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 receptor transporters and ion channels. With its gradual metabolism to R,R,R-HTBZ, its 20-hour half-life, and low peak to trough concentration ratio, VBZ is appropriate for once-daily dosing, as demonstrated to date 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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Wrote or contributed to the writing of the manuscript: Grigoriadis, Smith, Hoare, Madan, Bozigian.

References

- Ballesteros J, González-Pinto A, and Bulbena A (2000) Tardive dyskinesia associated with higher mortality in psychiatric patients: results of a meta-analysis of seven independent studies. *J Clin Psychopharmacol* **20**:188–194.
- Ben-Jonathan N (1985) Dopamine: a prolactin-inhibiting hormone. *Endocr Rev* **6**: 564–589.
- Browne S, Roe M, Lane A, Gervin M, Morris M, Kinsella A, Larkin C, and Callaghan EO (1996) Quality of life in schizophrenia: relationship to sociodemographic factors, symptomatology and tardive dyskinesia. *Acta Psychiatr Scand* **94**:118–124.
- Casey DE (1991) Neuroleptic drug-induced extrapyramidal syndromes and tardive dyskinesia. *Schizophr Res* **4**:109–120.
- Cesura AM, Bertocci B, and Da Prada M (1990) Binding of [3H]dihydrotrabenzazine and [125I]azidoiodoketanserin photoaffinity labeling of the monoamine transporter of platelet 5-HT organelles. *Eur J Pharmacol* **186**:95–104.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.
- Deniard MJ, Meignen J, and DeFeudis FV (1983) Reversal of reserpine-induced ptoxis in the mouse by alpha-adrenoceptor-agonists. *Psychopharmacology (Berl)* **80**:243–248.
- Eiden LE (2000) The vesicular neurotransmitter transporters: current perspectives and future prospects. *FASEB J* **14**:2396–2400.
- Erickson JD, Schafer MK, Bonner TI, Eiden LE, and Weihe E (1996) Distinct pharmacological properties and distribution in neurons and endocrine cells of two isoforms of the human vesicular monoamine transporter. *Proc Natl Acad Sci USA* **93**:5166–5171.
- Erickson JD and Veroqui H (2000) Molecular analysis of vesicular amine transporter function and targeting to secretory organelles. *FASEB J* **14**:2450–2458.
- FDA (2008) (Press release, August 15, 2008) *FDA Approves First Drug for Treatment of Chorea in Huntington's Disease*. Food and Drug Administration, Silver Spring, MD.
- Finley PR (2002) Atypical antipsychotics and tardive dyskinesia. *J Manag Care Pharm* **8**:291–292.
- Hauser RA, Factor SA, Marder SR, Knesevich MA, Ramirez PM, Jimenez R, Burke J, Liang GS, and O'Brien CF (2017) KINECT 3: a phase 3 randomized, double-blind, placebo-controlled trial of valbenazine (NBI-98854) for tardive dyskinesia. *Am J Psychiatry* DOI: 10.1176/appi.ajp.2017.16091037 [published ahead of print].
- Jankovic J and Clarence-Smith K (2011) Tetrabenazine for the treatment of chorea and other hyperkinetic movement disorders. *Expert Rev Neurother* **11**:1509–1523.
- Kato T, Katsuyama M, Karai N, Hirose A, Nakamura M, and Katsube J (1986) Reversal of the reserpine-induced ptoxis by L-threo-3,4-dihydroxy-phenylserine (L-threo-DOPS), a (-)-norepinephrine precursor, and its potentiation by imipramine or nialamide. *Naunyn Schmiedeberg's Arch Pharmacol* **332**:243–246.
- Kenney C, Hunter C, and Jankovic J (2007) Long-term tolerability of tetrabenazine in the treatment of hyperkinetic movement disorders. *Mov Disord* **22**:193–197.
- Kenney C and Jankovic J (2006) Tetrabenazine in the treatment of hyperkinetic movement disorders. *Expert Rev Neurother* **6**:7–17.
- Kilbourn M, Lee L, Vander Borgh T, Jewett D, and Frey K (1995) Binding of alpha-dihydrotrabenzazine to the vesicular monoamine transporter is stereospecific. *Eur J Pharmacol* **278**:249–252.

- Kilbourn MR, Lee LC, Heeg MJ, and Jewett DM (1997) Absolute configuration of (+)-alpha-dihydrotetrabenazine, an active metabolite of tetrabenazine. *Chirality* **9**:59–62.
- Lee LC, Vander Borgh T, Sherman PS, Frey KA, and Kilbourn MR (1996) In vitro and in vivo studies of benzoquinoline ligands for the brain synaptic vesicle monoamine transporter. *J Med Chem* **39**:191–196.
- Mehta SH, Morgan JC, and Sethi KD (2015) Drug-induced movement disorders. *Neurol Clin* **33**:153–174.
- Mehvar R, Jamali F, Watson MW, and Skelton D (1987) Pharmacokinetics of tetrabenazine and its major metabolite in man and rat: bioavailability and dose dependency studies. *Drug Metab Dispos* **15**:250–255.
- Müller T (2015) Valbenazine granted breakthrough drug status for treating tardive dyskinesia. *Expert Opin Investig Drugs* **24**:737–742.
- O'Brien CF, Jimenez R, Hauser RA, Factor SA, Burke J, Mandri D, and Castro-Gayol JC (2015) NBI-98854, a selective monoamine transport inhibitor for the treatment of tardive dyskinesia: a randomized, double-blind, placebo-controlled study. *Mov Disord* **30**:1681–1687.
- Ondo WG, Hanna PA, and Jankovic J (1999) Tetrabenazine treatment for tardive dyskinesia: assessment by randomized videotape protocol. *Am J Psychiatry* **156**:1279–1281.
- Othman Z, Ghazali M, Razak AA, and Husain M (2013) Severity of tardive dyskinesia and negative symptoms are associated with poor quality of life in schizophrenia patients. *Int Med J* **20**:677–680.
- Schuldiner S, Liu Y, and Edwards RH (1993) Reserpine binding to a vesicular amine transporter expressed in Chinese hamster ovary fibroblasts. *J Biol Chem* **268**:29–34.
- Sethi KD (2001) Movement disorders induced by dopamine blocking agents. *Semin Neurol* **21**:59–68.
- Tedeschi DH, Fowler PJ, Fujita T, and Miller RB (1967) Mechanisms underlying reserpine-induced ptosis and blepharospasm: evidence that reserpine decreases central sympathetic outflow in rats. *Life Sci* **6**:515–523.
- Vander Borgh TM, Kilbourn MR, Koeppe RA, DaSilva JN, Carey JE, Kuhl DE, and Frey KA (1995) In vivo imaging of the brain vesicular monoamine transporter. *J Nucl Med* **36**:2252–2260.
- Wimalasena K (2011) Vesicular monoamine transporters: structure-function, pharmacology, and medicinal chemistry. *Med Res Rev* **31**:483–519.
- Woods SW, Morgenstern H, Saksa JR, Walsh BC, Sullivan MC, Money R, Hawkins KA, Gueorgieva RV, and Glazer WM (2010) Incidence of tardive dyskinesia with atypical versus conventional antipsychotic medications: a prospective cohort study. *J Clin Psychiatry* **71**:463–474.
- Yao Z, Wei X, Wu X, Katz JL, Kopajtic T, Greig NH, and Sun H (2011) Preparation and evaluation of tetrabenazine enantiomers and all eight stereoisomers of dihydrotetrabenazine as VMAT2 inhibitors. *Eur J Med Chem* **46**:1841–1848.
- Yelin R and Schuldiner S (2002) Vesicular neurotransmitter transporters, in *Neurotransmitter Transporters: Structure, Function, and Regulation* (Reith MEA ed) pp 313–354, Humana Press, Totowa, NJ.
- Youssef HA and Waddington JL (1987) Morbidity and mortality in tardive dyskinesia: associations in chronic schizophrenia. *Acta Psychiatr Scand* **75**:74–77.
- Zucker M, Weizman A, Harel D, and Rehavi M (2001) Changes in vesicular monoamine transporter (VMAT2) and synaptophysin in rat Substantia nigra and prefrontal cortex induced by psychotropic drugs. *Neuropsychobiology* **44**:187–191.

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