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

Dimitri E. Grigoriadis, Evan Smith, Sam R. J. Hoare, Ajay Madan, and Haig Bozigian

Neurocrine Biosciences Inc., San Diego, California (D.E.G., E.S., H.B.); Pharmecanics, Wayne, Pennsylvania (S.R.J.H.); and Crinetics Pharmaceuticals, San Diego, California (A.M.)

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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-dihydrotetrabenazine (R,R,R-HTBZ) 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 [3H]-HTBZ binding to VMAT2 in rat striatal, rat forebrain, and human platelet homogenates. A broad panel screen to two significant circulating metabolites in vivo, namely, (R,S,S-HTBZ, (R,S,S-HTBZ, (R,R,R-HTBZ: R,R,R-HTBZ, 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_2A, 5-HT_3) 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-HTBZ 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)].

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) 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., 2000).
VMAT2 distribution in the central nervous system in both mice and humans has been determined using \[^{11}\text{C}\]-methoxytetrabenazine, which demonstrated consistent localization with known monoamine nerve terminal density, particularly in the striatum, lateral 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 (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, Bozgian 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 \[^{3}\text{H}\]HTBZ to the VMAT2 transporter in rat striatum and human platelet homogenates. Affinity of the four HTBZ stereoisomers...
(R,R,R-HTBZ, S,R,R-HTBZ, S,S,S-HTBZ, and R,S,S-HTBZ) was assessed using a number of tis

**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 KH2PO4, 8.1 mM Na2HPO4, 2.7 mM KCl, 135 mM NaCl (14200-075, Invitrogen, Carlsbad, CA) supplemented with 10 mM MgCl2, 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 ethylenediaminetraacetic 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 Ca2+ or Mg2+, 10 mM MgCl2, 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 (605174; PerkinElmer Life and Analytical Sciences, Boston, MA) were pretreated with a solution of 0.5% polyethyleneimine 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). IC50 values were converted to KI using the measured radioligand concentration and the Kd 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-HT1A, 5-HT3, 5-HT7, dopamine D2, and α2A adrenergic receptors were independently expressed in CHO-K1 cells, whereas the serotonin 5-HT2A, dopamine D1, and α1 adrenergic receptors were expressed in human embryonic kidney cells (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-HT1A), [3H]-ketanserin (5-HT2A), [3H]-lysergic acid diethylamide (5-HT2B and 5-HT7), [3H]-SCH-23390 (D1), [3H]-raclopride (D2), [3H]-prazocin (α1 adrenergic), and [3H]-rauwolscine (α2A 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 reserpin, 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 μg/ml streptomycin at 37°C and 7.5% CO2. Transient transfections were performed using lipofectamine 9000 (Life Technologies, Carlsbad, CA), 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% CO2. 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 MgCl2, 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...
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 IGS; 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 IGS; 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 3800 g for 4 minutes at 4°C. Serum was flash frozen for 4 minutes at 4°C and then centrifuged at 3800 g for 4 minutes at room temperature. Serum was flash frozen 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 Kᵦ values that were approximately 40–65 times the Kᵦ 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 1

| Compound          | Structural Description                           | Rat Striatum | Human Platelets |
|-------------------|--------------------------------------------------|--------------|-----------------|
|                   |                                                  | Kᵦ, nM       | pKi Mean (S.E.M.) | Affinity Relative to R,R,R-HTBZ | Kᵦ, nM       | pKi 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                           |

*affinity relative to R,R,R-HTBZ was calculated using the Kᵦ value determined in the same study.

### Table 2

| Compound         | Kᵦ, nM     | pKi Mean (S.E.M.) | N  | Affinity Relative to R,R,R-HTBZ |
|------------------|------------|-------------------|----|-------------------------------|
| 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                           |
NBI-136110 at concentrations up to 10

Although the nonselective irreversible high-affinity uptake

these compounds was tested in cells expressing VMAT1. (Table 4). To complete the selectivity profile for VMAT2, the

of the TBZ or VBZ metabolites had any affinity for the mono-

dopamine and serotonin receptor subtypes. Interestingly,

other TBZ metabolites, including relatively high affinity for

VMAT2 transporter and the nonspecific activities of the

analysis revealed the high specificity of R,R,R-HTBZ for the

relevant metabolites unique to TBZ and VBZ. This detailed

monoamine systems in greater detail, detailed radioligand-

mine D2 receptor antagonism in the anterior pituitary (Ben-

contrast, prolactin release is directly modulated by dopa-

nists (Tedeschi et al., 1967; Deniard et al., 1983). In

induced by central inhibition of presynaptic monoaminer-

R,R,R-HTBZ and evaluated for their ability to induce ptosis

metabolites, rats were administered either TBZ, VBZ, or

R,R,R-HTBZ or NBI-136110 at concentrations up to 10 μM (Fig. 2). For both

VMAT1 and VMAT2, uptake was measured in the untrans-

fected host cells and was found to be similar to transfected cells in the presence of excess reserpine (for example, see Fig. 2).

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, dopa-

mine, 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 nonselect-

tivity with respect to the monoamine receptors (Table 4). None

of the TBZ or VBZ metabolites had any affinity for the mono-

amine 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 μM (Fig. 2). For both

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 re-

response. The mechanisms underlying a ptosis response in rats are well documented and have been shown to be

induced by central inhibition of presynaptic monoaminer-

nic release and specifically reversed by α-adrenergic ago-

nists (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).

In vitro pharmacological profile of TBZ 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 Ki values were below 500 nM, assays were performed in 3

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Dopamine D1

Dopamine D2(s)

Serotonin

5-HT7

Receptor Target

SERT

DAT

Table 4

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) × 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.

| **Target** | **K<sub>i</sub>, nM** | **pK<sub>i</sub> Mean (S.D.)<sup>a</sup>** | **n** | **K<sub>i</sub>, nM** | **pK<sub>i</sub> Mean (S.D.)<sup>a</sup>** | **n** | **K<sub>i</sub>, nM** | **pK<sub>i</sub> Mean (S.D.)<sup>a</sup>** | **n** | **K<sub>i</sub>, nM** | **pK<sub>i</sub> Mean (S.D.)<sup>a</sup>** | **n** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VMAT2 | 4.2 | 8.38 (0.42) | 27 | 9.7 | 8.01 (0.32) | 6 | 250 | 6.60 (0.22) | 4 | 650 | 6.18 (0.05) | 5 |
| Dopamine D<sub>1</sub> | >5000 | <5 | 2 | >5000 | <5 | 2 | >5000 | <5 | 2 | >5000 | <5 | 2 |
| Dopamine D<sub>2</sub>(<i>al</i>) | >5000 | <5 | 2 | >5000 | <5 | 2 | 180 | 6.73 (0.23) | 5 | 53 | 7.27 (0.18) | 6 |
| Serotonin 5-HT<sub>1A</sub> | 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<sub>2A</sub> | >5000 | <5 | 2 | >5000 | <5 | 2 | >5000 | <5 | 2 | 2200 | 5.66 (0.00) | 2 |
| Serotonin 5-HT<sub>2B</sub> | >5000 | <5 | 2 | >5000 | <5 | 2 | 600 | 6.22 (0.33) | 3 | 460 | 6.34 (0.40) | 4 |
| Serotonin 5-HT<sub>7</sub> | >5000 | <5 | 2 | >5000 | <5 | 2 | 71 | 7.15 (0.12) | 2 | 5.9 | 8.23 (0.08) | 2 |
| α<sub>1A</sub> Adrenergic | >5000 | <5 | 2 | >5000 | <5 | 2 | >5000 | <5 | 2 | 980 | 6.01 (0.15) | 4 |
| α<sub>2A</sub> Adrenergic | >5000 | <5 | 2 | >5000 | <5 | 2 | 1700 | 5.76 (0.08) | 2 | 220 | 6.65 (0.36) | 2 |
| DAT<sup>b</sup> | >5000 | <5 | 2 | >5000 | <5 | 2 | >5000 | <5 | 2 | >5000 | <5 | 2 |
| NET<sup>b</sup> | >5000 | <5 | 2 | >5000 | <5 | 2 | >5000 | <5 | 2 | >5000 | <5 | 2 |
| SERT<sup>b</sup> | >5000 | <5 | 2 | >5000 | <5 | 2 | >5000 | <5 | 2 | >5000 | <5 | 2 |

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

a Major metabolite of VBZ.

b pK<sub>i</sub> is the negative logarithm of K<sub>i</sub>. S.D. are only presented for pK<sub>i</sub> values >5.
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 Ki for inhibition of [3H]-HTBZ binding of 10 nM (Cesura et al., 1990; Zucker et al., 2001) and an EC50 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-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-HTBZ stereoisomer binds with the highest affinity to both rat and human VMAT2 (Ki = 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 Ki 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

**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

**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.

**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 ± 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-a-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 D_1 and D_2 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. Ptosis, known to occur via adrenergic activation and prolactin release from the pituitary, demonstrated through the D_2 dopamine receptor, demonstrated the difference between treatment with TBZ and VBZ (Fig. 3). TBZ, VBZ, and R,R,R-HTBZ 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-HTBZ), 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-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 translational 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., 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 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 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 pharmacological 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: Grigoriadis, Smith, Hoare, Madan, Bozigan.
Conducted experiments: Hoare, Smith, Grigoriadis.
Performed data analysis: Hoare, Smith, Grigoriadis.
Wrote or contributed to the writing of the manuscript: Grigoriadis, Smith, Hoare, Madan, Bozigan.

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Address correspondence to: Dr. Dimitri E. Grigoriadis, Neurocrine Biosciences Inc., 12780 El Camino Real, San Diego, CA 92130. E-mail: dgrigoriadis@neurocrine.com