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Identification of Potent, Selective, and Peripherally Restricted Serotonin Receptor 2B Antagonists from a High-Throughput Screen

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ABSTRACT

Antagonists of the serotonin receptor 2B (5-HT$_{2B}$) have shown great promise as therapeutics for the treatment of pulmonary arterial hypertension, valvular heart disease, and related cardiopathies. Herein, we describe a high-throughput screen campaign that led to the identification of highly potent and selective 5-HT$_{2B}$ antagonists. Furthermore, selected compounds were profiled for their predicted ability to cross the blood–brain barrier. Two exemplary compounds, VU0530244 and VU0631019, were predicted to have very limited potential for brain penetration in human subjects, a critical profile for the development of 5-HT$_{2B}$ antagonists devoid of centrally-mediated adverse effects.

Keywords: serotonin, 5-HT$_{2B,}$ pulmonary arterial hypertension, high-throughput screen

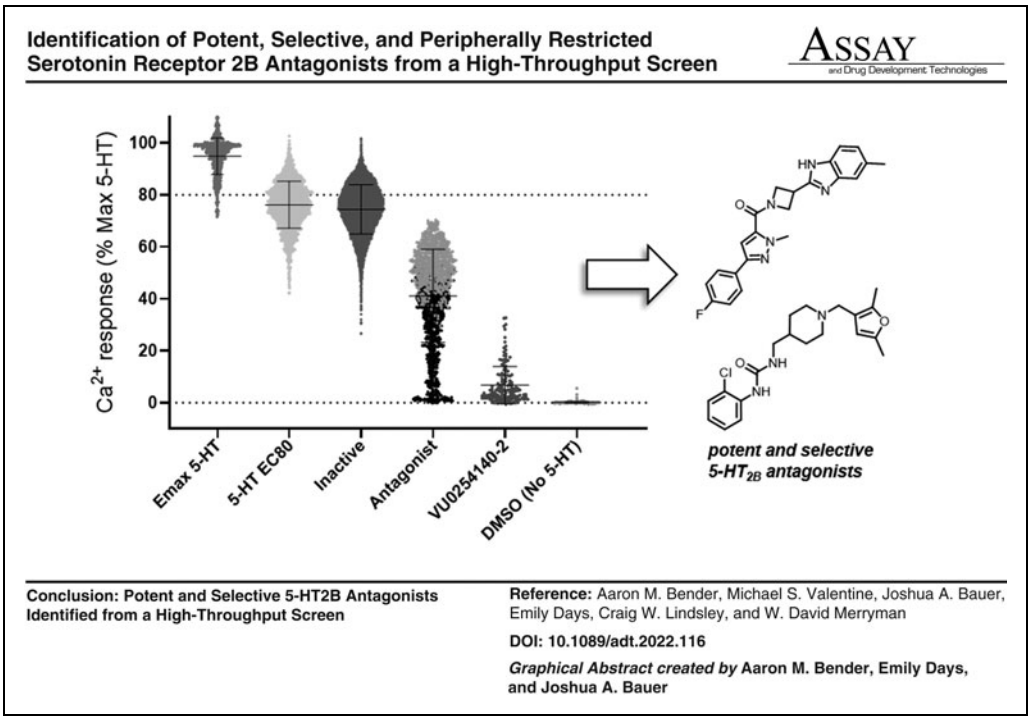
INTRODUCTION

The serotonin receptor 2B (5-HT$_{2B}$) has long been considered an "antitarget" in medicinal chemistry[1]; compounds that activate this G protein-coupled receptor are known to cause serious and sometimes fatal side effects in animal models and human subjects alike.[2–4] Consequently, a routine practice in medicinal chemistry programs is to screen compounds of interest against 5-HT$_{2B}$, and molecules displaying any activity at the receptor are often rapidly deprioritized.[5,6]

That this type of screening is now commonplace in drug discovery is due in no small part to the postclinical failure of fen-phen (fenfluramine/phentermine; *Fig. 1*), a previously approved anti-obesity regimen that was withdrawn from the market due to the observed development of fatal pulmonary arterial hypertension (PAH) and heart valve abnormalities in patients. The resulting legal fallout from the drug's withdrawal (with awarded damages exceeding $20 billion) led to widespread interest regarding the mechanism of action involved in the observed side effects.[7]

Multiple studies have since confirmed the excessive activation of 5-HT$_{2B}$ to be a likely culprit; specifically the *N*-des-ethyl metabolite of fenfluramine (**1**) and dexfenfluramine has been shown to be a potent 5-HT$_{2B}$ agonist.[3,4] Numerous follow-up studies[8–10] have since confirmed the link between 5-HT$_{2B}$ activation and PAH (and related disorders), and many such agonists, including 3,4-methylenedioxymethamphetamine (**3**)[11] and pergolide (**4**),[12] have recapitulated the deleterious effects of fen-phen in animal models and human subjects (*Fig. 1*).

Conversely, the development of 5-HT$_{2B}$ *antagonists* has shown great promise for the treatment of PAH and related disorders, including vessel wall stiffness and pulmonary microvasculature cell contractility.[8,9,13] Although there are currently several FDA-approved drugs for the treatment of PAH, all of these treatments are characterized by high cost, low tolerability, and mechanisms of action that are not disease-modifying.



Because PAH is both progressive and often lethal (characterized by pulmonary vascular remodeling and right heart failure), there still exists a great unmet need to develop novel and disease-modifying treatment strategies.[14–17] To this end, deficiencies in both 5-HT_{2B} and the serotonin transporter (5-HTT) have been demonstrated to be protective and therapeutic in the context of hypoxic PAH in mice,[4,18] and the 5-HT_{2B} antagonist SB204741 prevents elevated right ventricular systolic pressure in mouse models of both hypoxia-induced PAH and *Bmpr2* (heritable) PAH.[8,9] In addition, 5-HT_{2B} antagonism can reduce both hypoxia-induced and heritable PAH to normal levels, with treated mice ultimately failing to develop PAH.[8,9]

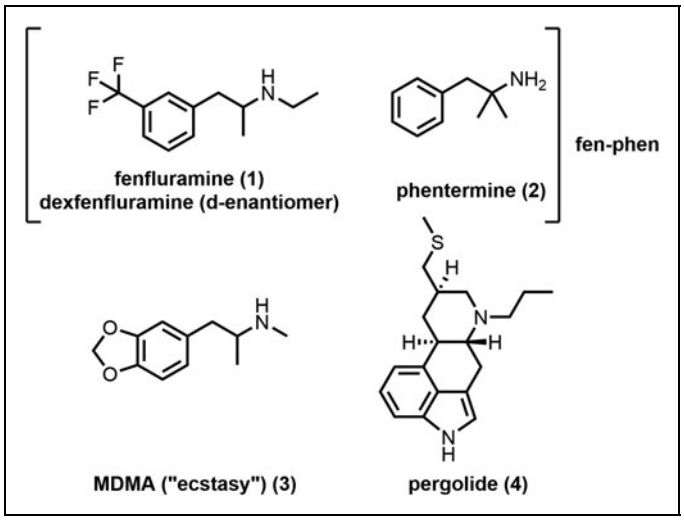


Fig. 1. Fen-phen and additional 5-HT_{2B} agonists. 5-HT_{2B}, serotonin receptor 2B; fen-phen, fenfluramine/phentermine.

An important caveat in the development of 5-HT_{2B} antagonists for the treatment of such disorders is that these compounds must be peripherally restricted (limited potential for permeation through the blood–brain barrier [BBB]). Centrally-mediated 5-HT_{2B} antagonism is associated with a number of consequences in animal models, including impulsivity[19] and impaired sleep.[20] In addition, the presence of a 5-HT_{2B} stop codon in specific human populations has been linked to severe impulsivity, including suicidal ideation.[21] Toward the goal of developing peripherally restricted 5-HT_{2B} antagonists, we undertook a high-throughput screen (HTS) utilizing compounds from the Vanderbilt Institute of Chemical Biology (VICB) Discovery Collection.

MATERIALS AND METHODS

Calcium Mobilization Assays

Calcium mobilization assays were conducted as previously described.[22] The stable HEK293T cell line with TET-inducible 5-HT_{2B} receptor expression was generously provided by Dr. Bryan Roth (University of North Carolina). Cells were dispensed as follows: 20,000 cells in 20 μL per well in black

384-well amine coated clear bottom plates (Corning) in Dulbecco's Modified Eagle's Medium (Life Technologies) with 1% fetal bovine serum (Dialyzed), 20 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 1% penicillin/streptomycin and 2 μg/mL tetracycline using a Multidrop Combi dispenser (Thermo). Cells were incubated at 37°C under 5% CO_2 and used for screening between 18 and 22 h (not exceeding 24 h).

For screening, medium was removed and replaced with 1 μM Calbryte520 AM (AAT Bioquest®, Sunnyvale, CA, USA) in assay buffer (Hank's Balanced Salt Solution supplemented with 20 mM HEPES) mixed with 10% pluronic acid (Life Technologies) using a BioTek ELx405 plate washer, and plates were incubated 60 min at 37°C under 5% CO_2. Dye was then removed and replaced with 20 μL of fresh assay buffer and equilibrated to ambient temperature for 10 min before loading into the Panoptic (Wavefront Biosciences, Franklin TN, USA) kinetic imaging plate reader.

Compound libraries at a stock of 10 mM in dimethyl sulfoxide (DMSO) were transferred to compound plates using the ECHO OMICS (Labcyte), and diluted to 2× of the final assay concentration. The compound plate, the dye-loaded cell plate, and plate containing EC_{80} serotonin (5-HT) concentrations were placed in the Panoptic kinetic imaging plate reader equipped to measure Ex482/35 Em536/40 fluorescence. Data were collected at 1 frame/s. After 14 s of collecting baseline fluorescence, 20 μL of the compound solutions were added to the cell plate for 133 s.

This was followed by the addition of an EC_{80} concentration of 5-HT along with a maximally effective 5-HT concentration in wells not containing a compound to allow data normalization. The fluorescence signal was collected for a total of 290 s. Each screening set started with an initial plate with a full dose response of 5-HT ranging from 5 μM to 80 pM to measure the suitable 5-HT EC_{80} for the given screen day and to

Table 1. Protocols for High-Throughput Screen Calcium Mobilization Assay

Step	Parameter	Value	Description
1	Plate cells	20 mL	Stable HEK293T cell line with TET-inducible 5-HT$_{2B}$ receptor
2	Calcium indicator dye	20 mL	Exchange media for 1 μM Calbryte520 AM in assay buffer
3	Incubation	60 m	37°C
4	Assay buffer	20 mL	Remove dye and replace with HBSS +20 mM HEPES
5	Library compounds	20 mL	10 mM in DMSO stocks diluted to 2×(20 mM) for live imaging dispense
6	Control	20 mL	10 mM in DMSO stock SB204741 (VU0254140) diluted fresh to 2×(20 mM)
7	5-HT	10 mL	EC_{80} 5-HT or Emax 5-HT
8	Incubation	10 m	ambient equilibration period
9	Panoptic reader	482ex/536em	Live kinetic imaging
Step			**Notes**
1			Corning 384 well amine coated plates, plated with Multidrop Combi
2			Exchange from media to assay buffer using ELX405 plate washer
3			Lidded plates in incubator at 37°C and 5%CO_2
4			Exchange from dye to assay buffer using ELX405 plate washer
5			Create aliquot from DMSO stock library into aqueous 2×working solution using ECHO OMICS
6			Create aliquot from DMSO stock into aqueous 2×working solution using ECHO OMICS
7			Create aliquot from DMSO stock into aqueous 5×working solution using ECHO OMICS
8			Unlidded plate
9			2×plate with compounds, controls; 5×plate with 5-HT; and cell plate load into Panoptic

5-HT$_{2B}$, serotonin receptor 2B; DMSO, dimethyl sulfoxide; HBSS, Hanks' Balanced Salt Solution; HEPES, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid; TET, tetracycline.

monitor the consistency of expression and activity of 5-HT_{2B}. Compound concentration response curves (CRCs) were collected in triplicate within each plate.

Data were imported and analyzed using custom-built Vanderbilt HTS software (Waveguide) and exported for plotting data in GraphPad Prism. First, each compound and control well's data were normalized to the initial baseline fluorescence. Then, each well value was calculated as maximal values within the 5-HT stimulation time window (145–290 s) subtracting the prestimulation timeframe minimum (135–140 s). Normalized values were then calculated as a percentage of maximal 5-HT response for each screen plate.

Likewise, the activity for compound stimulation alone was calculated as maximal values within the compound stimulation time window (10–125 s) subtracting the prestimulation timeframe minimum (1–5 s) with a subset for putative agonist peak (15–60 s). For CRCs, this percentage of maximum response was plotted against log[compound] and fit to a four parameter logistical equation to determine log(IC_{50}) (see *Table 1* for further details).

Radioligand Binding Assays

Radioligand binding assays for 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} were performed through contract by Eurofins Discovery (Taiwan) in human recombinant CHO-K1 cells with 0.5 nM [^{3}H]ketanserin, 1.2 nM [^{3}H]lysergic acid diethylamide (LSD), and 1.0 nM [^{3}H]mesulergine as the respective specific ligands in accordance with their established standard assay methodology. 5-HT_{2B} IC_{50s} were generated across five concentration points (0.1 nM, 1 nM, 10 nM, 0.1 μM, and 10 μM), and 5-$HT_{2A/2C}$ counterscreening was assessed as % inhibition at a single-point concentration of 10 μM.

P-Glycoprotein Efflux

Initial determination of test compounds' potential for efflux by human P-glycoprotein (P-gp) at a single-point concentration (5 μM; in duplicate) was performed through contract by Absorption Systems (Exton, PA, USA) using a bidirectional permeability assay with MDCK-MDR1 cells in accordance with their established standard assay methodology.

Compounds

VU0530244, VU0544894, and VU0631019 were purchased from Life Chemicals, Inc. (Ontario, Canada) and assigned Vanderbilt-specific ID numbers for purposes of chemical inventory (*see Supplementary Data*). Compounds were determined to have >95% purity by liquid chromatography-mass-spectrometry analysis at 215 nm, and structures were confirmed by proton nuclear magnetic resonance analysis. Low-resolution mass spectra were obtained on a Waters QDa (Performance) SQ mass spectrometer (MS) with ESI source. MS parameters were as follows: cone voltage: 15 V, capillary voltage: 0.8 kV, probe temperature: 600°C. Samples were introduced through an Acquity I-Class PLUS UPLC comprising a binary solvent manager, fixed loop sample manager, column compartment, and photodiode array detector. UV absorption was generally observed at 215 and 254 nm; 4 nm bandwidth.

Column: Phenomenex EVO C18, 1.0 × 50 mm, 1.7 μm. Column temperature: 55°C. Flow rate: 0.4 mL/min. Default gradient: 5%–95% CH_3CN (0.05% trifluoroacetic acid [TFA]) in H_2O (0.05% TFA) over 1.4 min, hold at 95% CH_3CN for 0.1 min. Nuclear magnetic resonance spectra were recorded on a 400 MHz Brüker AV-400 instrument. ^{1}H chemical shifts are reported as δ values in ppm relative to the residual solvent peak ($CDCl_3$ = 7.26, DMSO = 2.50). Data are reported as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, dd = doublet of doublets, ddd = doublet of doublet of doublets, td = triplet of doublets, dt = doublet of triplets, m = multiplet), coupling constant, and integration.

RESULTS AND DISCUSSION

Preparation for screening was first conducted using a series of validation experiments to demonstrate feasibility for HTS. Tet-induced 5-HT_{2B} HEK293T cells were tested at multiple tetracycline concentrations and incubation times for optimal 5-HT_{2B} expression and response in the calcium mobilization assay, finding 2 μg/mL and 18–24 h to be optimal. Cells were then stimulated with a dose response of 5-HT with or without tetracycline preincubation, which indicated that increasing 5-HT concentration stimulated calcium response only with induced expression of 5-HT_{2B} (see *Supplementary Fig. S1A*).

Cells were tested in optimal conditions for evaluating HTS standard metrics of 5-HT response with DMSO alone (neutral control) versus antagonist SB204741 (VU0254140, positive control) in a checkerboard pattern, for demonstrating HTS readiness ($Z' > 0.5$, coefficients of variations <10%), in single-well formatted 384-well plates (*Supplementary Fig. S1C*). DMSO tolerance was tested and evaluated to be suitable for testing the small molecule library up to 1% v/v. The Selleck Chemical's FDA drug set library was used for a pilot screen to evaluate the ability to identify hits selected from single-point 10 μM screening and retest in our calcium mobilization assay.

Identified hits were revealed as known 5-HT receptor antagonists, such as pizotifen malate. Overall, the pilot screen

included 1,181 drugs, from which was found 14 putative agonists, 59 antagonists, and 6 undesignated hits (see *Supplementary Fig. S1*). After these validation experiments, the primary screen included 24,592 compounds from the VICB Discovery Collection at 10 μM single-point concentration. Compounds that significantly decreased the 5-HT EC_{80} responses were selected as hits when the measured response was 3 standard deviations or 3 median absolute deviations from the plate compound population mean or median response, respectively (*Fig. 2*).

Discovery of 1,207 putative antagonists and 206 agonist candidates were identified. In addition, 78 undesignated hits were found to respond independent of 5-HT, had increased 5-HT response, or were flagged as an active fluorescent compound. All screening plates were required to have an acceptable Z', with overall mean Z' 0.74 ± 0.04. Hit compounds were replated from a source tube, rather than cherry-picked from the library plate and retested in duplicate at 10 μM with 478 of 576 (83%) compounds retested positive for antagonist activity.

Compounds that reduced the 5-HT EC_{80} response but did not modify calcium response alone were triaged for further studies ($n = 411$). Candidate compounds were then profiled in 10-point CRCs in the calcium mobilization assay to calculate potencies (IC_{50} values calculated from the change in 5-HT EC_{80} responses). Thirty-six compounds had IC_{50} values <1 μM. Of these, 15 were reordered as dry powders from Life Chemicals (Ontario, Canada) and retested in counterscreens using 5-HT$_{2A}$ or 5-HT$_{2C}$ receptor expressed cell lines (Eurofins Discovery; see Materials and Methods section).

After the generation of CRCs for 315 of these antagonist hits, compounds with potencies ranging from 9.4 nM to >10 μM were ultimately identified, with many hits displaying 5-HT$_{2B}$ potencies in the low nanomolar range (*Fig. 2C*). From

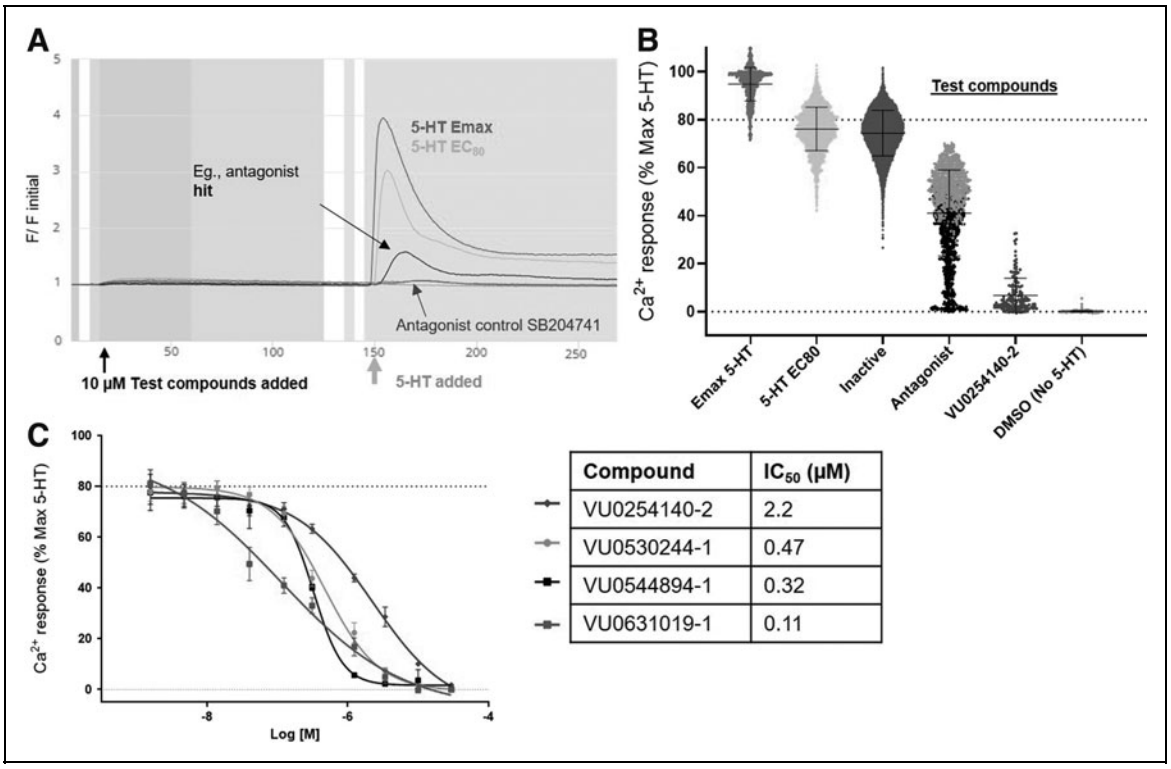
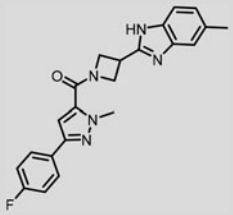
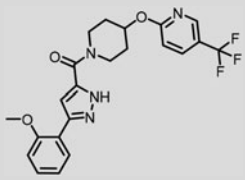
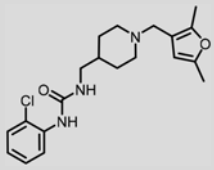


Fig. 2. Results of screening HEK-5-HT$_{2B}$ cells. **(A)** Example traces from the calcium assay showing representative wells of a 384-well screen plate over time (s); test compound addition (14 s), 5-HT stimulation (147 s). Fluorescence values are normalized as a ratio of initial value at time 0 s (F/F initial). Control maximal response of serotonin ("5-HT Emax"), EC_{80} stimulation with 5-HT ("5-HT EC_{80}"), example hit compound, antagonist control, SB204741 (VU0254140-2). **(B)** Screening results of 24,592 small molecules shown as individual well values in presence of EC_{80} 5-HT, unless otherwise indicated. Values normalized to percentage of maximal 5-HT response. Putative antagonist hit candidates identified as values decreasing 3SD or 3MAD below the mean or median response per 384-well plate tested. Five hundred seventy-six hits have completed testing in duplicate and confirmed hits have undergone CRCs. Additional 631 antagonist hits have not been further tested. Additional hits not shown: putative agonists (206) identified as increasing calcium response 3SD or 3MAD above mean or median before stimulation with 5-HT as well as other undesignated hits or flagged as fluorescent (78) were identified. **(C)** Selected antagonist candidates measured in triplicate compared with control antagonist VU0254140-2; all activity shown in presence of EC_{80} 5-HT and values normalized to maximal 5-HT response. Values are mean and SD ($n = 3$) modeled with four parameter logistic curve-fitting, least squares regression (GraphPad Prism v.9.2). CRCs, concentration response curves; MAD, median absolute deviation; SD, standard deviation.

Table 2. Structure, Serotonin Subtype Potency, and Predicted P-Glycoprotein Efflux for Hit Compounds VU0530244, VU0544894, and VU0631019

Compound	Structure	5-HT_{2B} IC_{50} (nM)[a]	5-HT_{2A} % inh. (10 μM)[b]	5-HT_{2C} % inh. (10 μM)[c]	P-gp efflux ratio (P_{appA-B}, 10^{-6} cm/s)[d]
VU0530244 (5)		17.3	<50%	<50%	12.3 (5.4)
VU0544894 (6)		5.3	<50%	<50%	0.59 (6.7)
VU0631019 (7)		29	<50%	<50%	25 (2.6)

[a]Radioligand binding assay with 1.20 nM [^{3}H]lysergic acid diethylamide (LSD) and 10 μM serotonin (5-HT) as the nonspecific ligand across five concentration points (0.1 nM, 1 nM, 10 nM, 0.1 μM, 10 μM).
[b]Radioligand binding assay with 0.5 nM [^{3}H]ketanserin and 1.0 μM mianserin as the nonspecific ligand at single-point 10 μM concentration.
[c]Radioligand binding assay with 1.0 nM [^{3}H]mesulergine and 1.0 μM mianserin as the nonspecific ligand at single-point 10 μM concentration (Eurofins Discovery).
[d]BBB penetration potential using Madin Darby canine kidney cells with overexpression of MDR1 gene (encoding for P-gp) in cell monolayers; ER is defined as $P_{app(B-to-A)}$/$P_{app(A-to-B)}$; see Materials and Methods section for further details.
BBB, blood–brain barrier; ER, efflux ratio.

this set, VU0530244 (**5**), VU0544894 (**6**), and VU0631019 (**7**) (*Table 2*) were among the top potency compounds chosen for selectivity profiling relative to the serotonin receptor 2A and 2C subtypes (5-HT_{2A} and 5-HT_{2C}), as well as a preliminary assessment for predicted ability to cross the BBB and induce centrally-mediated antagonism (*Table 2*). Encouragingly, all three hits were found to be robustly selective for 5-HT_{2B} relative to 5-HT_{2A} and 5-HT_{2C} in radioligand binding assays, and are among the most potent and selective 5-HT_{2B} antagonists yet detailed in the drug discovery literature.

In addition, VU0530244 and VU0631019 are predicted to have very limited potential for brain penetration in human subjects, as both are predicted to be substrates for P-gp-mediated efflux in humans (*Table 2*). A strong correlation exists between the measured efflux ratios and membrane permeability in such assays with the observed *in vivo* brain to plasma exposure.[23] Although some structural similarity is noted between VU0530244 and VU0544894, the latter is predicted to be highly brain-penetrant; consequently, VU0544894 may find use as a tool to study the effects of centrally mediated 5-HT_{2B} antagonism. Follow-up studies to rigorously characterize the potential for central penetration will be necessary to further de-risk these and related analogs for clinical development.

CONCLUSIONS

In summary, we have conducted a successful HTS campaign for the purpose of detailing new chemical matter for the

development of potent, selective, and peripherally restricted 5-HT$_{2B}$ antagonists. From the list of most potent antagonists after full CRC profiling, three compounds are detailed in this article. It is our hope that the hit compounds described herein will prove useful as starting scaffolds for medicinal chemists interested not only in the development of selective 5-HT$_{2B}$ tool compounds, but also for the development of next-generation and disease-modifying agents for PAH, valvular heart disease, and related disorders.

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AUTHORS' CONTRIBUTIONS

Data curation, formal analysis, investigation, project supervision, validation visualization, and writing—review and editing (medicinal chemistry) by A.M.B. Data curation, formal analysis, investigation, methodology, validation, and visualization (molecular pharmacology) by M.S.V. Data curation, formal analysis, investigation, methodology, project administration, supervision, validation, visualization, and writing—review and editing (HTS lead) by J.A.B. Data curation, formal analysis, methodology, validation, visualization, and writing—review and editing (HTS supporting) by E.D. Project administration and supervision by C.W.L and W.D.M.

DISCLOSURE STATEMENT

No competing financial interests exist.

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SUPPLEMENTARY MATERIAL

Supplementary Data
Supplementary Figure S1

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Abbreviations Used

5-HT_{2B} = serotonin receptor 2B
BBB = blood–brain barrier
DMSO = dimethyl sulfoxide
ER = efflux ratio
fen-phen = fenfluramine/phentermine
HBSS = Hanks' Balanced Salt Solution
HEPES = N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HTS = high-throughput screen
MAD = median absolute deviation
MS = mass spectrometry
PAH = pulmonary arterial hypertension
P-gp = P-glycoprotein
SD = standard deviation
TFA = trifluoroacetic acid
VICB = Vanderbilt Institute of Chemical Biology