Characterization of Vabicaserin (SCA-136), a Selective 5-HT$_{2C}$ Receptor Agonist

John Dunlop, Stephanie Watts, James E. Barrett, Joseph Coupet, Boyd Harrison, Hossein Mazandarani, Stanley Nawoschik, Menelas N. Pangalos, Siva Ramamoorthy, Lee Schechter, Deborah Smith, Gary Stack, Jean Zhang, Guoming Zhang and Sharon Rosenzweig-Lipson

Neuroscience Research Unit, Pfizer Global Research and Development (JD, JB, JC, BH, HM, SN, MNP, SR, LS, DS, GS, JZ, GZ, SRL), 445 Eastern Point Road, Groton, CT 06340; Michigan State University (SW), Department of Pharmacology and Toxicology, East Lansing, MI 48824
Running Title: Vabicaserin is a Selective 5-HT2C Receptor Agonist

Corresponding Author:

John Dunlop, Ph.D.

Neuroscience Research Unit, Pfizer, Eastern Point Road, MS8220-4220, Groton, CT 06340; Tel 860 686 9335; Email john.dunlop@pfizer.com

Number of pages: 28
Number of Tables: 2
Number of Figures: 7
Number of References: 21
Abstract: 242 words
Introduction: 413 words
Discussion: 1551 words

ABBREVIATIONS: BW723C86, α-methyl-5-(2-thienylmethoxy)-1H-indole-3-ethanamine hydrochloride; DOCA, deoxycorticosterone acetate; FLIPR, fluorometric imaging plate reader; IP1, inositol monophosphate; LY272015, 6-methyl-1,2,3,4-tetrahydro-1-[3,4-dimethoxyphenylmethyl-9H-pyrido[3,4b]indole] hydrochloride; vabicaserin, (-)-4,5,6,7,9,9a,10,11,12,12a-decahydrocyclopenta[c] [1,4]diazepino[6,7,1-ij]quinoline hydrochloride

Recommended Section Assignment: Cellular and Molecular
ABSTRACT

The 5-HT$_{2C}$ receptor subtype has received considerable attention as a target for drug discovery having been implicated in a wide variety of disorders. Here we describe the in vitro pharmacological profile of the novel 5-HT$_{2C}$ receptor selective agonist vabicaserin ((-)-4,5,6,7,9,9a,10,11,12,12a-decahydrocyclopenta[c][1,4]diazepino[6,7,1-ij]quinoline hydrochloride) (SCA-136), including a comprehensive strategy to assess 5-HT$_{2B}$ receptor selectivity using diverse preparations and assays of receptor activation. Vabicaserin displaced [$_{125}$I]-DOI binding from human 5-HT$_{2C}$ receptor sites, in CHO cell membranes, with a Ki value of 3 nM and was > 50-fold selective over a number of serotonergic, noradrenergic and dopaminergic receptors. Binding affinity determined for the human 5-HT$_{2B}$ receptor subtype using [$_{3H}$]-5HT was 14 nM. Vabicaserin was a potent and full agonist (EC$_{50}$, 8 nM; Emax, 100%) in stimulating 5-HT$_{2C}$ receptor coupled calcium mobilization and exhibited 5-HT$_{2A}$ receptor antagonism and 5-HT$_{2B}$ antagonist or partial agonist activity in transfected cells, dependent on the level of receptor expression. In rat stomach fundus and human colonic longitudinal muscle endogenously expressing 5-HT$_{2B}$ receptors, vabicaserin failed to induce a 5-HT$_{2B}$ receptor dependent contraction and produced a rightward shift of the 5-HT and $\alpha$-methyl-5-HT concentration-response curves in these preparations, respectively, consistent with 5-HT$_{2B}$ competitive antagonism. Similarly, vabicaserin failed to induce a 5-HT$_{2B}$ receptor mediated contraction in arteries from DOCA-salt treated rats, a model of hypersensitized 5-HT$_{2B}$ receptor function, and produced a rightward shift in the 5-HT-induced response consistent with 5-HT$_{2B}$ receptor antagonism. In summary, vabicaserin is a novel, potent and selective 5-HT$_{2C}$ receptor agonist.
Introduction

At least 14 distinct 5-HT receptor subtypes have been cloned and classified based on sequence similarity and common signal transduction pathways (Barnes and Sharp, 1999). The 5-HT$_2$ receptor sub-family accommodates three subtypes designated 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ and these receptors belong to the large family of seven transmembrane domain G-protein coupled receptors. They display high sequence homology with each other and signal transduction initially believed to be principally via activation of phospholipase C (Baxter et al., 1995). More recently, coupling through a wide array of signal transduction pathways has been reported (Berg, 1998; Miller, 2005; Werry et al., 2008).

Pharmacological interest within the 5-HT$_2$ receptor family in the context of drug discovery has largely focused on the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor subtypes. In the case of the 5-HT$_{2A}$ receptor subtype it has been recognized that drugs which are used to treat schizophrenia and depression, in addition to hallucinogenic agents, display affinity for this target (Canton et al., 1990). The 5-HT$_{2C}$ receptor has been implicated in a wide variety of conditions including obesity, anxiety, depression, obsessive compulsive disorder, schizophrenia, migraine and erectile dysfunction (Wacker and Miller, 2008) and as a consequence has received significant attention as a target for drug discovery. Several novel 5-HT$_{2C}$ receptor agonists have recently been described including WAY-163909 (Dunlop et al., 2005; Marquis et al., 2007; Rosenzweig-Lipson et al., 2007) CP-809,101 (Siuciak et al., 2007) and lorcaserin (Thomsen et al., 2008) and shown to have activity in preclinical animal models used to evaluate potential antipsychotic, antidepressant and anti-obesity activity. Moreover, lorcaserin has demonstrated clinical efficacy in obesity
(Smith et al., 2008). As with any small molecule drug discovery effort, selectivity is important and a particular consideration in 5-HT\textsubscript{2C} receptor agonist targeted drug discovery is the selectivity of compounds toward the related 5-HT\textsubscript{2B} receptor subtype, and specifically 5-HT\textsubscript{2B} receptor agonist activity. Activation of the 5-HT\textsubscript{2B} receptor has been implicated in primary pulmonary hypertension (Launay et al., 2002) and valvulopathy (Fitzgerald et al., 2000; Rothman et al., 2000).

As part of a strategy to develop novel 5-HT\textsubscript{2C} receptor agonists as a potential therapeutic for psychiatric disorders, vabicaserin ((-)-4,5,6,7,9,a,10,11,12,12a-decahydrocyclopenta[c] [1,4]diazepino[6,7,1-ij]quinoline hydrochloride) (SCA-136) has been identified as a potent and selective ligand for the 5-HT\textsubscript{2C} receptor. This report describes its in vitro pharmacological profile determined using in vitro functional assays of 5-HT\textsubscript{2A}, 5-HT\textsubscript{2B} and 5-HT\textsubscript{2C} receptor activation, and a comprehensive strategy for assessing functional activity toward the related 5-HT\textsubscript{2B} receptor subtype. Data demonstrate vabicaserin to be a novel, potent and selective 5-HT\textsubscript{2C} receptor agonist.

**Materials and Methods**

**Drugs.** Vabicaserin (SCA-136; ((9aR*,12aS*)-4,5,6,7,9,a,10,11,12,12a-Decahydro-
cyclopenta[c][1,4]diazepino[6,7,1-ij]quinoline) was synthesized at Wyeth Research. BW723C86 (Tocris, Ellisville, MO, USA), DOCA and 5-HT (Sigma Chemical Co, St. Louis, MO, USA), LY272015 (a gift from Eli Lilly and Co), fluo-3-AM (Invitrogren, Carlsbad, CA) were obtained from external sources.
5-HT2 Receptor Cell Lines. Human 5-HT2C, 5-HT2A and 5-HT2B receptors were expressed in stably transfected Chinese hamster ovary cell lines. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, non-essential amino acids, penicillin/streptomycin, and the following selection markers (maintenance medium): 5-HT2C (50 fmol/mg expression level, checking), 5 μg/ml mycophenolic acid (MPA), 0.25 mg/ml xanthine, 100 μM sodium hypoxanthine and 16 μM thymidine; 5-HT2A (200 fmol/mg), 800 μg/ml neomycin and 500 μg/ml zeocin; 5-HT2B: 400 μg/ml neomycin and 1000 nM methotrexate (MTX) for 5-HT2B receptor low expression line (500 fmol/mg), and 100 nM and 200 nM MTX for the high (5000 fmol/mg) and intermediate (1500 fmol/mg) expression lines, respectively.

5-HT2C Receptor Radioligand Binding. Agonist and antagonist binding experiments were performed in 96 well microtiter plate format using a total volume of 200 μl. Agonist binding studies were conducted using 60 μl of incubation buffer made in 50 mM Tris.HCl buffer, pH 7.4 and containing 4 mM CaCl2; 20 μl of [125I] DOI (S.A., 2200 Ci/mmol, NEN Life Science). The dissociation constant, K_D of [125I] DOI at the human 5-HT2C receptor is 0.4 nM as determined by saturation binding analysis. Antagonist binding studies were conducted using 80 ul of incubation buffer 50 mM Tris.HCl buffer, pH 7.4 and containing 0.1% ascorbic acid, 10 mM pargyline and 4 mM CaCl2, 20 ul of [3H]Mesulergine (S.A., 92.0 Ci/mmol, Amersham Life Science), at a final concentration of 0.5 nM. The dissociation constant, K_D of [3H]Mesulergine at the human serotonin 5-HT2C receptor was 0.8 nM as determined by saturation binding analysis. The reaction was initiated by the addition of 100 μl of tissue suspension. Nonspecific binding was
measured in the presence of 1 μM unlabeled DOI (added in 20 μl volume) or 1 mM mianserin, for agonist and antagonist binding respectively. The reactions proceeded for 60 or 120 min at room temperature. After incubation, the bound ligand-receptor complex was filtered using a 96 well unifilter with a Packard Filtermate 196 Harvester. The bound complex caught on the filter disk was air-dried and the radioactivity was measured in a Packard TopCount equipped with six (6) photomultiplier detectors, after the addition of 40 ul Microscint -20 scintillant to each shallow well. The unifilter plate was heat sealed and counted in a Packard TopCount with a tritium efficiency of 31.0%.

Receptor binding affinity for a range of monoaminergic receptor subtypes including human 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{6}, 5-HT_{7}, dopamine D_{2}, D_{3} and D_{4} and alpha1 adrenergic was assessed using similar protocols to those described for the 5-HT_{2C} receptor studies. In all cases, except the alpha1 adrenergic receptors, recombinant receptors expressed in CHO cells (or HeLa in the case of 5-HT_{6}) were used as receptor source. Rat cortical membrane homogenates were used as a source of alpha1 adrenergic receptors. Table 1 includes the radioligands used and agents for determination of non-specific binding for these receptor subtype selectivity assessments.

Radioligand binding results were analyzed by constructing log concentration response curves to generate IC_{50} estimates. Ki values were calculated from the equation described by Cheng and Prusoff (Cheng and Prusoff, 1973) Single concentration experiments determined the displacement of specific binding at predetermined drug concentrations. Data are mean values (± SEM) from 2-3 independent experiments.
Measurement of Intracellular Ca\textsuperscript{2+} Mobilization. Mobilization of intracellular calcium upon receptor activation was measured using the fluorometric imaging plate reader (FLIPR). Cells were plated at a density of 50,000 cells/well into black 96-well plates with clear bottoms 16 to 24 hour prior to the assay. Hanks’ balanced salt solution containing 20 mM HEPES and 2.5 mM probenecid was prepared fresh on the day of the assay and was used as assay buffer. The assay buffer was also used to prepare dye-loading buffer which contains a final concentration of 4 \mu M Fluo-3-AM, 0.2% pluronic acid, and 1% fetal bovine serum. 100 \mu l of dye-loading buffer was added to each well after removing the culture media, and the loading lasted 1 hour at 37\degree C in a CO2 incubator. The cells were then washed twice with the assay buffer and kept in the buffer (<1h) at room temperature until transferred to the FLIPR for functional assay. Dye loading was monitored by basal fluorescence signal test in each experiment, with laser intensity adjusted to a suitable level to achieve 8,000 to 15,000 fluorescence units at baseline. The fluorescence was detected with an excitation wavelength of 488 nM and the emission filter at 515 nM. The Argon laser power was adjusted between 0.3 to 1.2 W, and camera F/stop was set at 2, and exposure time, at 0.4 second. Calcium signal acquisitions for each assay plate was approximately 1.5 min, and fluorescence counts were recorded at 1s intervals for the first 60s and every 6s for the remainder of the run. After 10s basal signal readings, drug additions were made by FLIPR equipped 96-well pipettor loaded with black tips to obtain 1:10 dilution, from a 10x compound plate. Generally, 20 \mu l of compound was added to each well containing 180 \mu l of assay buffer, from a height of 150 \mu l and at a rate of 40 \mu l/sec. Agonist elicited increase in fluorescence counts correlated with the increase in intracellular calcium. For antagonist studies, an additional 30 to 60
min pre-incubation period followed the dye-loading procedure. Cells were exposed to the antagonist during pre-incubation time and throughout agonist activation.

**Measurement of Inositol Monophosphate (IP1) Accumulation.** Confluent cells were harvested and plated in 11-mm-diameter wells (24-well plate) in maintenance medium at an initial density of 1.2 x 10^5 cells per well, and labeled with 2 μCi myo-[3H]inositol/ml for 18 to 24 hours. The cells were then preincubated with DMEM containing 25 mM HEPES and 10 mM LiCl for 30 min to inhibit the monophosphatase activity. At the end of the preincubation, the medium was removed, and the cells were incubated with test compounds for an additional 30 min. The reaction was terminated by aspiration of the incubation medium and addition of 0.5 ml ice-cold 5% perchloric acid to extract the accumulated inositol phosphates. After 15 min at 4°C, 200 ml of 0.5 M Tes/1.5 M K2CO3 was added to each sample to neutralize to pH 7, and samples were centrifuged to separate the liquid and the precipitated salt. The supernatant samples were applied to columns (Dowex AG 1-X8 resin – formate form, 100-200 mesh) to elute the [3H]-IP1 fraction. [3H]IP1 in the eluates was quantified with liquid scintillation counting. Compounds undergoing antagonism studies were included both during the 30 min preincubation with DMEM/LiCl and throughout the 30 min agonist exposure. The increase in [3H]-IP1 counts corresponded to the receptor mediated agonist response.

**Native 5-HT_{2B} Receptor Functional Studies** Vabicaserin was evaluated in three isolated tissue bath systems that are 5-HT_{2B} receptor-dependent for the 5-HT-stimulated smooth muscle contraction. Contraction of the rat stomach fundus, the tissue source for
the original cloning of the 5-HT$_{2B}$ receptor, was used to assess the effect of vabicaserin on native rat 5-HT$_{2B}$ receptor functional response. Longitudinal strips of stomach fundus from normal male Sprague-Dawley rats (250-300 gm) were mounted into isolated tissue baths for the measurement of isometric contraction. Tissues were originally challenged with 67 mM KCl and all responses were normalized to this contraction. Responses to cumulative addition of agonists were measured in the fundus. Longitudinal muscle preparations from human colon were used to assess the effect of vabicaserin on native human 5-HT$_{2B}$ receptor functional response. Tissue from 3 donors were mounted in organ baths and evaluated under conditions of sub-maximal electrical field stimulation (EFS) where neuronally mediated contractile response are potentiated by 5-HT in a concentration- and 5-HT$_{2B}$-receptor dependent manner (Borman et al., 2002). EFS-evoked contractions (1-2 Hz, 1 ms pulse width, 10 s duration, 15 v, repeated every 60 s for 3 min) were evaluated in the presence of vabicaserin alone (0.1 and 1 µM) to assess its agonist activity, or in the presence of agonist stimulation with α-methyl-5HT in combination with vabicaserin to assess its antagonist activity. Contractions to α-methyl-5-HT, in the absence or presence of vabicaserin, were expressed as a % of the α-methyl-5-HT maximum response.

Mesenteric artery from the deoxycorticosterone acetate (DOCA) salt hypertensive rat was used to assess the effect of vabicaserin on pathologically up-regulated 5-HT$_{2B}$ receptors. This preparation exhibits a 5-HT receptor pharmacology that is consistent with activation of 5-HT$_{2B}$ receptors, while the 5-HT$_{2A}$ receptor appears to maintain normal functionality in tissue derived from non-hypertensive animals. Superior mesenteric arteries were dissected from sham normotensive rats (systolic blood pressure< 130 mm
Hg) or rats made hypertensive (> 160 mm Hg) by the administration of DOCA and salt (1% NaCl and 0.2% KCl) in their drinking water. Arterial helical strips, denuded of endothelium, were pair-mounted (sham and DOCA artery strip in the same bath) and originally challenged with phenylephrine (PE, 10 µM) and all responses were normalized to this contraction. Responses to cumulative addition of agonists were measured in these tissues.

Results

Receptor Binding Profile of Vabicaserin. Vabicaserin (Fig. 1) displayed high affinity binding at the cloned human 5-HT₃C receptor (Kᵢ = 3 nM) using the agonist radioligand [¹²⁵I]DOI. In contrast, vabicaserin exhibited lower affinity at the 5-HT₃C antagonist binding site (22 nM) labeled with [³H]mesulergine. Additional binding studies indicated that vabicaserin possessed affinity for the 5-HT₂B and 5-HT₁A receptors with Ki values of 14 and 112 nM, respectively. Thus vabicaserin was approximately four-fold selective, in terms of binding affinity, over these related receptors. Vabicaserin was greater than 50-fold selective over other monoamine receptors (5-HT₂A, 5-HT₁B, 5-HT₁D, 5-HT₆, 5-HT₇, dopamine D₂, D₃, D₄ and the alpha-1 adrenergic receptor binding site) (Table 1), and over a range of 76 receptor, ion channel and neurotransmitter uptake sites determined in a Novascreen selectivity profile (Supplemental Table 1).

Functional Assessment of Vabicaserin with Recombinant Receptors. Vabicaserin was a potent and full agonist in stimulating 5-HT₂C receptor coupled calcium
mobilization with an estimated EC$_{50}$ of 8 nM and Emax of 100%, relative to a maximally effective concentration of 5-HT (Fig. 2A). The 5-HT$_{2B}$ receptor potency and efficacy of vabicaserin in transfected cells was found to be highly dependent on receptor expression levels. While vabicaserin exhibited no 5-HT$_{2B}$ receptor agonism in cells expressing approximately 500 fmol/mg or 1500 fmol/mg protein, vabicaserin stimulated calcium mobilization in cells expressing the 5-HT$_{2B}$ receptor at levels of 5000 fmol/mg protein (Fig. 2A, Table 2). In contrast, vabicaserin antagonized responses to 5-HT in both 5-HT$_{2A}$ and 5-HT$_{2B}$ (500 fmol/mg) receptor expressing cells with IC$_{50}$ values of 1650 and 29 nM, respectively (Fig. 2B). The ability of vabicaserin to stimulate the accumulation of IP$_1$ in 5-HT$_{2B}$ receptor transfected cells was also found to be critically dependent on the level of receptor expression. Vabicaserin failed to stimulate IP$_1$ accumulation in 5-HT$_{2B}$ receptor expressing cells at a level of receptor expression of 500 fmol/mg (data not shown), and exhibited partial agonist activity (Emax, 50%) in cells expressing 1500 or 5000 fmol/mg 5-HT$_{2B}$ receptor (Fig. 3). Consistent with the calcium mobilization studies, vabicaserin was a potent (EC$_{50}$, 32 nM) and full 5-HT$_{2C}$ receptor agonist for the stimulation of IP$_1$ accumulation and lacked agonist activity at the 5-HT$_{2A}$ receptor subtype in this assay (Fig. 3). Taken together, these functional studies of 5-HT$_2$ receptor subtype activation in transfected cells demonstrate vabicaserin to be a potent 5-HT$_{2C}$-receptor agonist, 5-HT$_{2A}$ antagonist and 5-HT$_{2B}$ receptor partial agonist, dependent on the level of receptor expression.

**Functional Assessment of Vabicaserin with Native 5-HT$_{2B}$ Receptors.** In order to further understand the activity of vabicaserin at 5-HT$_{2B}$ receptors, a series of studies were
conducted in native systems to assess its 5-HT\textsubscript{2B} receptor functional activity with physiologically and pathophysiologically expressed 5-HT\textsubscript{2B} receptors. In the isolated rat stomach fundus preparation, 5-HT produced a concentration-dependent contraction with a maximum contraction comparable to that observed with 67 mM KCl (Fig. 4A). In contrast, vabicaserin (0.1 nM – 10 µM) did not induce contraction in the rat stomach fundus under identical experimental conditions to those employed for 5-HT (Fig. 4A). In experiments evaluating antagonist activity, vabicaserin (0.1 and 1 µM) produced a concentration dependent rightward shift of a 5-HT concentration effect curve with no suppression of the maximum functional response to 5-HT in rat stomach fundus (Fig. 4B), as did the positive control 5-HT\textsubscript{2B} receptor antagonist LY272015 (Fig. 4C). The \(-\log EC_{50}\) values for 5-HT were as follows: vehicle = 7.80; 0.1 µM vabicaserin = 6.80; 1 µM = 5.97. Taken together, these results indicate that vabicaserin is a competitive antagonist at the rat stomach fundus 5-HT\textsubscript{2B} receptor.

In the isolated longitudinal muscle preparation from human colon, vabicaserin (0.1 and 1 µM) did not induce contraction under conditions of sub-maximal EFS (data not shown), conditions known to support a 5-HT\textsubscript{2B} receptor mediated contractile response. Vabicaserin antagonized the effects of \(\alpha\)-methyl-5-HT on EFS-evoked contractions, causing a parallel rightward shift of the \(\alpha\)-methyl-5-HT concentration-effect curve (Fig 5). The mean pEC\textsubscript{50} of \(\alpha\)-methyl-5-HT was 8.2±0.1 SEM, and the mean dose of antagonist that produced a 2-fold shift of the agonist (pA2) of vabicaserin was 7.5±0.3 SEM. The rightward shift in the agonist concentration-response curve was not associated with a suppression of the maximum contractile response to \(\alpha\)-methyl-5-HT, suggestive of
competitive antagonism, consistent with the profile obtained in the rat stomach fundus preparation.

In the isolated superior mesenteric artery preparation vabicaserin (0.1 nM – 10 µM) did not induce contraction in sham treated rat superior mesenteric artery, but did cause a small, but highly variable contraction in arteries from DOCA-salt rat superior mesenteric artery (Fig. 6A), with a response observed in only half of the tissues. The contraction observed in the DOCA-salt superior mesenteric artery was less than 20% of that observed with 5-HT or the 5-HT2B receptor agonist BW723C86 and was not blocked by the 5-HT2B antagonist LY272015 (Fig. 6B). In antagonist studies, vabicaserin (1 µM) produced an approximate 6-fold rightward shift in the 5-HT concentration-effect curve with no suppression of the maximum response to 5-HT in sham treated rat superior mesenteric artery (Fig. 7A), indicative of competitive 5-HT2A receptor antagonism (Watts et al., 1996). Vabicaserin also produced a rightward shift in the 5-HT concentration-effect curve accompanied by a reduced maximum functional response to 5-HT in DOCA-salt treated rat superior mesenteric artery (Fig. 7B). (Watts et al., 1996; Watts and Fink, 1999). The –logEC50 values for 5-HT were as follows: vehicle = 7.06; 1 µM = 5.97. Taken together, these results suggest that vabicaserin functions as an antagonist at native 5-HT2A and 5-HT2B receptors expressed in the sham normotensive and DOCA-salt hypertensive derived rat superior mesenteric artery, respectively.
Discussion

The 5-HT$_{2C}$ receptor subtype has been implicated as a target for therapeutic intervention in a broad range of disorders leading to intense drug discovery efforts toward the development of 5-HT$_{2C}$ receptor selective agonists (Wacker and Miller, 2008). Most recently, WAY-163909 (Dunlop et al., 2005), CP-809,101 (Siuciak et al., 2007) and lorcaserin (Thomsen et al., 2008) have been reported as potent and selective 5-HT$_{2C}$ receptor agonists with preclinical profiles consistent with their potential therapeutic utility in schizophrenia, depression and obesity. Specifically, WAY-163909 (Marquis et al., 2007) and CP-809,121 (Siuciak et al., 2007) exhibit activity in a broad range of animal models predictive of antipsychotic like activity, and additionally WAY-163909 is efficacious in a number of rodent models of antidepressant-like effects (Rosenzweig-Lipson et al., 2007). One of the greatest challenges for discovery programs aimed at this target is identifying compounds with selectivity over the 5-HT$_{2B}$ receptor, specifically compounds devoid of 5-HT2B receptor agonist activity. This is important because activation of the 5-HT$_{2B}$ receptor has been implicated in both primary pulmonary hypertension (Launay et al., 2002) and valvulopathy (Fitzgerald et al., 2000; Rothman et al., 2000). Based on our expansion of synthetic efforts around WAY-163909 we designed a novel class of tetrahydroquinoline-fused diazepines leading to the discovery of vabicaserin, a potent and selective 5-HT$_{2C}$ receptor agonist without 5-HT$_{2B}$ agonism (Ramamoorthy et al., 2006).

As with any drug discovery effort it is important to appropriately assess the activity and selectivity of the candidate molecule, in particular with respect to closely related receptor targets. In studies using heterologously expressed 5-HT$_2$ receptor subtypes in
stably transfected cell lines, vabicaserin bound with high affinity to the 5-HT$_{2C}$ receptor (Ki = 3 nM), demonstrated >50-fold selectivity vs the 5-HT$_{2A}$ receptor (5-HT$_{2A}$ Ki = 152 nM) and only 4-fold selectivity vs the 5-HT$_{2B}$ receptor (Ki = 14 nM). Initial characterization of the functional activity of vabicaserin at the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor demonstrated that vabicaserin did not demonstrate agonist activity at either receptor subtype (EC$_{50}$ > 5 uM) and functioned as either a potent antagonist at the 5-HT$_{2B}$ receptor (IC$_{50}$ = 29 nM) or a weakly potent antagonist at the 5-HT$_{2A}$ receptor (IC$_{50}$ = 1.65 uM).

Since activation of the 5-HT$_{2B}$ receptor has been implicated in primary pulmonary hypertension and valvulopathy (Rothman et al., 2000), (Fitzgerald et al., 2000), a clear understanding of the functional activity of a compound at this receptor is critical, and our subsequent evaluation of vabicaserin focused on this key question. However, it is important to point out that although we did not evaluate 5-HT$_{2A}$ receptor mediated functional responses at higher heterologous expression levels, we found the 5-HT$_{2A}$ FLIPR assay to be highly predictive of subsequent 5-HT$_{2A}$ receptor-mediated agonist response in the sham superior mesenteric artery, with compounds exhibiting >20% agonist activity in the FLIPR assay inducing sham artery contraction (unpublished observations). Despite the initial demonstration that vabicaserin functioned as an antagonist and not an agonist at the 5-HT$_{2B}$ receptor, the absence of a high level of binding selectivity of vabicaserin for the 5-HT$_{2C}$ receptor relative to the 5-HT$_{2B}$ receptor led to the development and execution of a comprehensive strategy for assessing the activity of a compound at the 5-HT$_{2B}$ receptor using both recombinantly and natively expressed 5-HT$_{2B}$ receptors. Our initial characterization utilized a number of CHO cells...
JPET #179572

lines exhibiting different expression levels of the 5-HT_{2B} receptor. In these studies vabicaserin exhibited 5-HT_{2B} receptor antagonist activity in CHO cells where the receptor expression level was 500 fmol/mg, and in cells where the receptor was expressed at higher levels (1500 and 5000 fmol/mg), vabicaserin exerted partial agonist activity. Significantly, in the calcium mobilization functional assay, vabicaserin was a potent 5-HT_{2B} receptor antagonist, in cells with the lowest level of 5-HT_{2B} receptor expression, with an IC_{50} value determined as 29 nM, in excellent correlation with its measured binding affinity in ligand displacement studies for the 5-HT_{2B} receptor. In studies of IP1 accumulation in 5-HT_{2B} receptor expressing cells, vabicaserin lacked activity in cells with the low level of receptor expression and was a partial 5-HT_{2B} receptor agonist in cells expressing 1500 and 5000 fmol/mg, respectively. The explanation for the mixed 5-HT_{2B} receptor pharmacology of vabicaserin in transfected cells is likely based on the well established property in GPCR receptor pharmacology that over-expression of receptors can frequently lead to increases in apparent agonist potency and/or efficacy, providing highly sensitive assays of agonist functional responses. However, this has led to some caution in the interpretation of agonist-dependent effects in transfected systems, especially when taken in the absence of effects in native systems. Since our studies in the recombinant cell system with the lowest level of receptor expression supported the pharmacological activity of vabicaserin as a 5-HT_{2B} receptor antagonist, while partial agonist activity was apparent in cells with higher levels of receptor expression, we adopted a strategy using a number of systems with native expression of the receptor to further investigate these findings.
Native expression systems where receptors are expressed in a physiologically relevant environment allow for an evaluation of agonist intrinsic activity in the absence of receptor over-expression. In the case of the 5-HT$_{2B}$ receptor subtype, the isolated rat stomach fundus preparation, a key tissue source for the 5-HT$_{2B}$ receptor has been well validated as a model of 5-HT dependent receptor activation that is mediated by the 5-HT$_{2B}$ receptor (Baxter et al., 1994). Using this model we found that while 5-HT produced a concentration-dependent contraction, vabicaserin failed to exhibit agonist activity, but behaved as a classical competitive 5-HT$_{2B}$ receptor antagonist producing parallel rightward shifts in the 5-HT concentration-response curve. In order to assess the effect of vabicaserin on the human 5-HT$_{2B}$ receptor in native tissue, we utilized the isolated colonic longitudinal muscle preparation. Recent studies employing mRNA and protein localization, in combination with functional receptor pharmacology have provided evidence for a predominant 5-HT$_{2B}$ receptor dependent functional contraction in this preparation (Borman et al., 2002). Similar to our data obtained using the rat stomach fundus preparation, α-methyl-5-HT, but not vabicaserin, produced a concentration-dependent contraction of the colon isolated longitudinal muscle demonstrating a lack of agonist activity of vabicaserin in this native model of human 5-HT$_{2B}$ receptor activation. In contrast, vabicaserin produced parallel rightward shifts in the α-methyl-5-HT concentration response curve consistent again with its property as a 5-HT$_{2B}$ receptor competitive antagonist.

In the rat mesenteric artery, 5-HT receptors mediating contractile responses have been shown, based on pharmacological characterization, to represent a 5-HT$_{2A}$ receptor-dependent response under physiological conditions. In contrast, under pathophysiological
conditions arising from the administration of DOCA and salt (1% NaCl and 0.2% KCl) in
their drinking water, rats become hypertensive, and the contractile response measured in
isolated mesenteric artery from DOCA-salt treated animals represents a 5-HT$_{2B}$ receptor-
dependent response (Watts et al., 1996). The role of the 5-HT$_{2B}$ receptor in the
pathophysiology of hypertension in these animals is further supported by the
demonstration of the anti-hypertensive activity of the 5-HT$_{2B}$ receptor antagonist LY-272015 in DOCA-salt treated rats (Watts and Fink, 1999). Using this model we were
able to evaluate vabicaserin for functional effects on native 5-HT$_{2A}$ receptors under
physiological conditions and functional effects on up-regulated 5-HT$_{2B}$ receptors under
pathophysiological conditions. Vabicaserin failed to induce contractions in sham
normotensive mesenteric artery, and produced small variable contractions in the DOCA-
salt hypertensive mesenteric artery in a fraction of the preparations studied. Follow up
studies indicated these responses to be small in magnitude compared to the 5-HT$_{2B}$
receptor agonist BW723C86 and to be relatively unresponsive to the presence of the 5-
HT$_{2B}$ receptor antagonist LY272015. In experiments evaluating antagonist activity,
vabicaserin produced a rightward-shift in the 5-HT concentration-response curve in both
the sham and DOCA-salt derived mesenteric artery consistent with 5-HT$_{2A}$ and 5-HT$_{2B}$
receptor antagonism, respectively. In the latter case, this effect was accompanied by a
suppression in the maximum response to 5-HT, in contrast to the profiles observed in rat
stomach fundus and human colon, where the vabicaserin profile was more reminiscent of
a competitive 5-HT$_{2B}$ receptor antagonist. The reason for the different profile observed in
the DOCA-salt mesenteric artery preparation are unclear, but a speculation is that the
enhanced efficacy of 5-HT in causing arterial contraction is purely 5-HT$_{2B}$ receptor
mediated, hence the significant reduction in 5-HT-induced contraction in arteries from the DOCA-salt but not sham animals. Finally, it is important to highlight that while the studies in rat mesenteric artery provide a robust in vitro assessment of the functional activation of 5-HT_{2B} receptors in this cardiac preparation, they do not definitively address activation of 5-HT_{2B} receptors in situ in the heart, studies beyond the scope of the current study.

Taken in combination, the functional studies with native 5-HT_{2B} receptors in a number of different preparations indicate vabicaserin to be a 5-HT_{2B} receptor antagonist, in agreement with studies performed in 5-HT_{2B} receptor transfected cells expressing 500 fmol/mg receptor. In 5-HT_{2B} receptor transfected cells with higher levels of receptor expression vabicaserin exhibited 5-HT_{2B} receptor partial agonist activity. Ultimately, the pharmacology of vabicaserin will depend on many characteristics of the tissue and cells with which it interacts including receptor expression level, the complement and expression level of components of the receptor signaling pathways and the efficiency of receptor and effector coupling. In summary, we developed a comprehensive strategy for assessment of agonist selectivity of 5-HT_{2C} receptor agonists with respect to the related 5-HT_{2B} receptor subtype, and describe vabicaserin as a novel and selective 5-HT_{2C} receptor agonist.
Authorship Contributions

Participated in research design: Dunlop, Watts, Barrett, Pangalos, Coupet, Schechter, Rosenzweig-Lipson

Conducted experiments: Mazandarani, Nawoschik, Smith, J Zhang, G Zhang

Contributed new reagents or analytic tools: Harrison, Ramamoorthy, Stack

Wrote or contributed to writing of the manuscript: Dunlop, Watts, Rosenzweig-Lipson
References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochemical Pharmacology* 22:3099-3108.


Footnotes

1John Dunlop and Stephanie Watts contributed equally to this work
Figure Legends

Fig. 1. Chemical structure of the 5-HT$_{2C}$ receptor selective agonist vabicaserin ((-)-4,5,6,7,9a,10,11,12,12a-decahydrocyclopenta[c][1,4]diazepino[6,7,1-ij]quinoline hydrochloride)

Fig. 2. Effects of vabicaserin on intracellular calcium mobilization in 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptor transfected CHO cells in a FLIPR assay. Vabicaserin was added to cells expressing the 5-HT$_{2C}$, 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors, the latter with 3 different cell lines representing different receptor densities for 5-HT$_{2B}$ receptor expression (low, 500 fmol/mg; intermediate, 1500 fmol/mg; high, 5000 fmol/mg) (A). Estimated EC$_{50}$ values for vabicaserin stimulation of intracellular calcium mobilization were determined from the log-concentration response curves shown and presented in Table 2. The ability of vabicaserin to antagonize 5-HT stimulated responses in the 5-HT$_{2A}$ and 5-HT$_{2B}$ (low receptor expression at 500 fmol/mg) receptor expressing cell lines was evaluated (B). Vabicaserin antagonized 5-HT responses in both 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor expressing cells with IC$_{50}$ derived from the log-concentration response curves shown of 1650 and 29 nM, respectively. For quantification of agonist responses, the magnitude of calcium responses following agonist receptor activation were calculated as a maximum minus minimum relative fluorescence units and these were then expressed as a percentage of the measured FLIPR response to a maximally effective concentration of 5-HT.

Fig. 3. Effects of vabicaserin on IP$_1$ accumulation in 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptor transfected CHO cells. Vabicaserin was added to cells expressing the 5-HT$_{2C}$, 5-
HT2A and 5-HT2B receptors, the latter with 2 different cell lines representing different receptor densities for 5-HT2B receptor expression (intermediate, 1500 fmol/mg; high, 5000 fmol/mg). Estimated EC50 values for vabicaserin stimulation of IP1 accumulation, derived from the log-concentration response curves shown, were 32, 12, 102 and >10,000 nM for the 5-HT2C, 5-HT2B (high expression, 5000 fmol/mg), 5-HT2B (intermediate expression, 1500 fmol/mg) and 5-HT2A receptor subtypes, respectively. Preliminary experiments determined a lack of functional response to vabicaserin, using IP1 measurements, in cells expressing a low level of expression (500 fmol/mg) of the 5-HT2B receptor (not shown).

Fig. 4. Effect of vabicaserin on contraction of the isolated rat stomach fundus.

Vabicaserin or 5-HT was added to tissue baths with mounted strips of isolated rat stomach fundus and isometric contractions were measured in response to agonist application (A). All agonist responses were normalized to the contraction observed with an initial addition of 67 mM KCl. Vabicaserin was also evaluated for its effect on 5-HT contractions by constructing concentration-response curves for 5-HT in the absence (open squares) and presence of 0.1 µM (filled circles) or 1 µM (filled squares) vabicaserin (B). Positive control for the antagonism of 5-HT induced contractions in rat stomach fundus by the 5-HT2B receptor antagonist LY272015 (C). Points represent mean +/- SEM from 4-7 animals. *Denotes significant differences between concentration-response curves of both 0.1 µM and 1 µM vabicaserin, and 10 nM LY272015, from vehicle responses.
Fig. 5. Effect of vabicaserin on contractile responses to α-methyl-5-HT, under conditions of low electric field stimulation, in isolated human colonic longitudinal muscle. α-methyl-5-HT, in the absence and presence of vabicaserin, was added to tissue baths with mounted strips of isolated human colon longitudinal muscle and isometric contractions were measured in response to agonist application, under conditions of continual low frequency electrical field stimulation. Contractions to α-methyl-5-HT, in the absence or presence of vabicaserin, were expressed as a % of the α-methyl-5-HT maximum response.

Fig. 6. Effect of vabicaserin on contraction of the isolated rat superior mesenteric artery. Vabicaserin was added to tissue baths with mounted endothelium denuded strips of isolated rat superior mesenteric artery obtained from sham normotensive and DOCA-salt hypertensive rats and isometric contractions were measured in response to drug application (A). All agonist responses were normalized to the contraction observed with an initial addition of 10 µM phenylephrine. Contractions observed with vabicaserin in DOCA-salt animal derived superior mesenteric artery were compared in magnitude to 5-HT and the 5-HT$_{2B}$ receptor agonist BW723C86, and were also measured in the combined presence of the 5-HT$_{2B}$ receptor antagonist LY272015 (B). Vabicaserin-stimulated contractions were small in magnitude compared to 5-HT and BW723C86, and were not significantly altered by 10 nM LY272015.

Fig. 7. Antagonism of 5-HT-evoked contractions of sham- and DOCA-salt animal derived superior mesenteric artery by vabicaserin. Vabicaserin was evaluated for its
effect on 5-HT contractions in both sham normotensive (A) and DOCA-salt hypertensive (B) derived tissue by constructing concentration-response curves for 5-HT in the absence and presence of 1 µM vabicaserin. Vabicaserin produced a rightward shift of the 5-HT concentration response curve with no change in the maximum response to 5-HT in the sham normotensive derived tissue (A) and a rightward shift of the 5-HT concentration response curve accompanied by a decrease in the maximum response to 5-HT in the DOCA-salt hypertensive derived tissue (B). Points represent mean +/- SEM from 6 animals. *(A), denotes significant differences (p < 0.05) from sham responses.
Table 1. Vabicaserin receptor binding affinities for a range of monoaminergic receptor subtypes

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Ligand</th>
<th>NSB</th>
<th>Ki, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT2C</td>
<td>[$^{125}$I]DOI</td>
<td>1 µM DOI</td>
<td>3</td>
</tr>
<tr>
<td>5-HT2C</td>
<td>[$^3$H]mesulergine</td>
<td>10 µM mesulergine</td>
<td>22</td>
</tr>
<tr>
<td>5-HT1A</td>
<td>[$^3$H]5-HT</td>
<td>10 µM 8-OH-DPAT</td>
<td>112</td>
</tr>
<tr>
<td>5-HT1B</td>
<td>[$^3$H]5-HT</td>
<td>10 µM 5-HT</td>
<td>31%</td>
</tr>
<tr>
<td>5-HT1D</td>
<td>[$^3$H]5-HT</td>
<td>10 µM 5-HT</td>
<td>307</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>[$^{125}$I]DOI</td>
<td>1 µM DOI</td>
<td>152</td>
</tr>
<tr>
<td>5-HT2B</td>
<td>[$^3$H]5-HT</td>
<td>10 µM 5-HT</td>
<td>14</td>
</tr>
<tr>
<td>5-HT6</td>
<td>[$^3$H]LSD</td>
<td>10 µM methiothepin</td>
<td>481</td>
</tr>
<tr>
<td>5-HT7</td>
<td>[$^3$H]LSD</td>
<td>10 µM methiothepin</td>
<td>287</td>
</tr>
<tr>
<td>D2</td>
<td>[$^3$H]spiperone</td>
<td>1uM Butaclamol</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>D3</td>
<td>[$^3$H]spiperone</td>
<td>1uM Butaclamol</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>D4</td>
<td>[$^3$H]spiperone</td>
<td>10uM Clozapine</td>
<td>4220</td>
</tr>
<tr>
<td>α1 adrenergic</td>
<td>[$^3$H]prazosin</td>
<td>10 µM phenolamine</td>
<td>935</td>
</tr>
</tbody>
</table>

Serotonin and dopamine receptors were recombinantly expressed in CHO cells (or HeLa cells for 5-HT6) and membranes prepared from these cells were used as receptor source.

Rat cortical membrane preparation was used as a source of α1 adrenergic receptors. NSB, non-specific binding; 1% displacement at 1 µM vabicaserin.
Table 2. Functional potency for vabicaserin and 5-HT stimulated calcium mobilization in 5-HT$_{2B}$ receptor transfected cell lines as a function of receptor expression level

<table>
<thead>
<tr>
<th></th>
<th>500 fmol/mg</th>
<th>1500 fmol/mg</th>
<th>5000 fmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50, nM</td>
<td>Emax, %</td>
<td>EC50, nM</td>
</tr>
<tr>
<td>Vabicaserin</td>
<td>&gt;10,000</td>
<td>N/A</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.2</td>
<td>100</td>
<td>0.15</td>
</tr>
</tbody>
</table>

N/A, not applicable
Figure 1
Figure 2

(A)

![Graph A]

- EC50's (nM):
  - 2A: n/a
  - 2B-high: 1.5
  - 2B-intmt: n/a
  - 2B-L: n/a
  - 2C: 8

(B)

![Graph B]

- IC50's (nM):
  - 2A: 1650
  - 2B-low: 29
Figure 3

![Graph showing EC50's for different 5-HT receptors]

- **EC50's**:
  - 2C: 32 nM
  - 2A: 41 µM
  - 2B-high: 12 nM
  - 2B-M: 102 nM

**Graph Details**:
- **X-axis**: LOG [Vabicaserin] M
- **Y-axis**: % Maximum 5-HT Response
- **Legend**:
  - 2C
  - 2A
  - 2B-H
  - 2B-M
Figure 4 (revised)

A.

Rat Stomach Fundus

Percent KCl (67 mM) Contraction

log Agonist [M]

(N=6)

- 5-HT
- Vabicaserin

B.

Rat Stomach Fundus

Percent Maximum Contraction

log 5-HT [M]

(N=4-7)

- Vehicle
- Vabicaserin (0.1 μM)
- Vabicaserin (1 μM)
Figure 4 (revised)

C.

Rat Stomach Fundus

Percentage of KCl (67 mM) contraction

(N=6)

- Vehicle
- LY272015 (10 nM)

log 5-HT [M]
Figure 5

Vabicaserin: pA₂ = 7.5
Figure 6

A.

Rat Mesenteric Artery -E

(N=6)

Percentage PE
(10^-5 M) Contraction

log Vabicaserin [M]

Sham

DOCA-salt

B.

DOCA Mesenteric Artery -E

(N=6-10)

Percentage PE
(10^-5 M) Contraction

log Agonist [M]

5-HT

BW723C86

Vabicaserin

Vabicaserin +
LY272015 (10 nM)
Figure 7

A. 
Sham Rat Superior Mesenteric Artery -E
(N=6)

B. 
DOCA-salt Rat Superior Mesenteric Artery -E
(N=6)