WS-50030 [7-{4-[3-(1H-inden-3-yl)propyl]piperazin-1-yl}-1,3-benzoxazol-2(3H)-one]: A Novel Dopamine D2 Receptor Partial Agonist / Serotonin Reuptake Inhibitor with Preclinical Antipsychotic-Like and Antidepressant-Like Activity

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WS-50030, A Novel D₂ Partial Agonist / SRI

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Text pages: 53
Tables: 1
Figures: 9
References: 43
Words in Abstract: 254
Words in Introduction: 651
Words in Discussion: 1609

Abbreviations:

6-OHDA – 6-hydroxydopamine
EPS – extrapyramidal side effects
SRI – serotonin reuptake inhibitor
AAALAC – Association for Assessment and Accreditation of Laboratory Animal Care
CSF – cerebrospinal fluid
ANOVA – analysis of variance
LSD – least significant difference
mPFC – medial prefrontal cortex
CI – confidence interval
5-HIAA – 5-hydroxyindole acetic acid
$E_{\text{max}}$ – maximum effect
$I_{\text{max}}$ – maximum inhibition
MED - minimal effective dose
OB – olfactory bulbectomy

Recommended section assignment:

Behavioral Pharmacology
ABSTRACT

The preclinical characterization of WS-50030 [7-{4-[3-(1H-inden-3-yl)propyl]piperazin-1-yl}-1,3-benzoxazol-2(3H)-one] is described. In vitro binding and functional studies reveal highest affinity to the D_2 receptor (D_{2L}, Ki = 4.0 nM) and serotonin transporter (Ki = 7.1 nM), potent D_2 partial agonist activity (EC_{50} = 0.38 nM, E_{max} = 30 %) and complete block of the serotonin transporter (IC_{50} = 56.4 nM). Consistent with this in vitro profile, WS-50030 (10 mg/kg/day, 21 days) significantly increased extracellular 5-HT in the rat medial prefrontal cortex, acute WS-50030 blocked apomorphine-induced climbing (ID_{50} = 0.51 mg/kg) in a dose range that produced minimal catalepsy in mice, and induced low levels of contralateral rotation in rats with unilateral substantia nigra 6-OHDA lesions (10 mg/kg i.p.), a behavioral profile similar to the D_2 partial agonist aripiprazole. In a rat model predictive of antipsychotic-like activity, WS-50030 and aripiprazole reduced conditioned avoidance responding by 42% and 55% at 10 mg/kg, respectively. Despite aripiprazole’s reported lack of effect on serotonin transporters, both chronic aripiprazole and WS-50030 reversed olfactory bulbectomy-induced hyperactivity at doses that did not reduce activity in sham-operated rats, indicating antidepressant-like activity for both compounds. Conclusion: Despite possessing serotonin reuptake inhibitory activity in addition to D_2 receptor partial agonism, WS-50030 displays activity in preclinical models predictive of antipsychotic- and antidepressant efficacy similar to aripiprazole, suggesting potential efficacy of WS-50030 versus positive and negative symptoms of schizophrenia, co-morbid mood symptoms, bipolar disorder, major depressive disorder and treatment resistant depression. Furthermore, WS-50030 provides a tool to further explore how
combining these mechanisms might differentiate from other antipsychotics or antidepressants.
INTRODUCTION

Schizophrenia is a debilitating disease that affects approximately 1% of the world population. The symptoms of schizophrenia can be loosely categorized into 3 main groups: positive symptoms, such as hallucinations and delusions, negative symptoms, such as social withdrawal and anhedonia, and cognitive symptoms, such as deficits in attention and higher-order thought processing (Meltzer, 1999). The neurotransmitter dopamine is known to play a pivotal role in schizophrenia and an excellent correlation exists between the potency of compounds for antagonizing postsynaptic dopamine D2 receptors and their efficacy in treating the positive symptoms of schizophrenia (Seeman et al., 1976; Kapur et al., 2000). It is also known, however, that excessive D2 receptor antagonism in areas of the brain responsible for motor control results in extrapyramidal side effects (EPS) such as Parkinson’s-like movement disorders, a property common to all classical and several atypical antipsychotic agents (Sanberg, 1980; Kapur et al., 2000). Additionally, dopamine has been shown to play a significant role in the reward process and pleasure, such that excessive D2 receptor blockade would be expected to have a less than optimal effect on the negative symptoms of schizophrenia (Bressan and Crippa, 2005). Indeed, currently marketed D2 receptor full antagonists lack efficacy in the treatment of the negative symptoms of schizophrenia.

A dopamine D2 partial agonist, however, can exhibit a dynamic range of functional activity depending upon regional receptor reserve and local endogenous levels of dopamine (Stahl, 2001; Tamminga 2002; Tamminga and Carlsson, 2002). A D2 partial agonist would be expected to behave as an agonist at presynaptic autoreceptors where receptor reserve is high and levels of the neurotransmitter are relatively low, effectively
reducing dopamine transmission. Postsynaptically, a D₂ partial agonist could function either as an agonist or antagonist depending upon its level of intrinsic activity and local dopaminergic tone. A partial agonist should modulate – as opposed to completely block – dopaminergic transmission, thereby controlling positive symptoms with reduced potential for motoric side effects. The potential benefits of a D₂ partial agonist are also important considering evidence that the positive and negative symptoms of schizophrenia may result from dopamine hyperactivity and hypoactivity in mesolimbic and mesocortical brain regions, respectively (Weinberger and Lipska, 1995), such that a modulator of dopamine, instead of a complete blocker, seems appropriate. The clinical benefits of a D₂ receptor partial agonist have been realized with the currently marketed antipsychotic agent aripiprazole (Abilify®). Moreover, in addition to treating positive symptoms, clinical data suggest that aripiprazole significantly improves various negative symptom rating scales as well as having reduced EPS liability (Kane et al., 2002; Swainston and Perry, 2004; Cassano et al., 2007).

In addition to the positive, negative and cognitive symptoms of schizophrenia, there exists a high incidence of co-morbidity of depressive symptoms in schizophrenic patients (Siris et al., 2001). Serotonin reuptake inhibitors (SRIs) are commonly used in the clinic to treat both depression and anxiety, and there is mounting evidence to support the value of SRI augmentation in the treatment of the negative symptoms of schizophrenia (Silver, 2003; Mazeh et al., 2004). SRIs such as fluoxetine, fluvoxamine, citalopram and sertraline have been combined with antipsychotic medications to alleviate these often-refractory symptoms. Therefore, a D₂ receptor partial agonist with low intrinsic activity coupled with serotonin reuptake inhibitory activity would be expected to
have benefit in the treatment of the positive symptoms as well as the co-morbid depressive, and possibly the negative, symptoms of schizophrenia. In addition to their use in treating comorbid depression in schizophrenia, SRIs are often co-administered with antipsychotics for the treatment of bipolar disorder (Emilien et al., 2007). Therefore, a combined D_2 receptor partial agonist/SRI may have utility in the treatment of bipolar disorder. The present studies describe the in vitro and in vivo preclinical characterization of WS-50030 [7-{4-[3-(1H-inden-3-yl)propyl]piperazin-1-yl}-1,3-benzoxazol-2(3H)-one] (Figure 1), a dopamine D_2 receptor partial agonist with serotonin reuptake inhibitor activity. In addition, WS-50030 is compared to the D_2 receptor partial agonist aripiprazole in in vivo functional and efficacy models.
METHODS

Subjects. Male CF-1 mice (22-32 g, Charles River, Wilmington, MA) were used in the antagonism of apomorphine-induced behaviors and cataleptogenic potential assays. Male Sprague-Dawley rats (Charles River, Wilmington, MA) were used for conditioned avoidance test (350-500 g body weight), in vivo microdialysis studies (280-350 g body weight) and olfactory bulbectomy studies (200-225 g body weight). Male Wistar rats (400-500 g, Harlan, Netherlands) were used for unilaterally 6-OHDA – lesioned rat studies. All animals were housed in an AAALAC-accredited facility that was maintained on a 12h light / dark cycle (lights on at 0600h). Animals were group housed with food and water available ad libitum, except for rats used in conditioned avoidance studies. Rats used in conditioned avoidance studies were individually housed with ad libitum access to water but were food restricted to maintain their weight at approximately 80% of their free feeding body weight (15-20 g rodent chow per day). All studies conducted at Wyeth Research were previously approved by the Institutional Animal Care and Use Committee and were performed in accordance to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Pub. 85-23, 1985). All studies conducted at Solvay Pharmaceuticals were in accordance with Dutch law and conformed to local animal care and use committee stipulations.

Drugs. WS-50030 [7-{4-[3-(1H-inden-3-yl)propyl]piperazin-1-yl}-1,3-benzoazol-2(3H)-one] was prepared by Solvay Pharmaceuticals (Weesp, The Netherlands). WAY-100635 (N-[2-[4-(2-Methoxyphenyl)-1-piperizinyl]ethyl]-N-(2-pyridinyl) cyclohexanecarboxamide) and aripiprazole (except for rotational behavior studies) were
prepared by Wyeth Research (Princeton, NJ). WAY-100635 and aripiprazole used for rotational behavior studies were prepared by Solvay Pharmaceuticals (Weesp, The Netherlands). Desmethyl-imipramine and 6-hydroxydopamine were purchased from Sigma-Aldrich chemicals (Zwijndrecht, The Netherlands). Apomorphine and d-amphetamine were obtained from Sigma (St. Louis, MO). Drugs were dissolved in saline (apomorphine, d-amphetamine), 2% Tween 80®:0.5% Methylcellulose (aripiprazole) or 2% Tween 80®:0.5% Methylcellulose:5% Mannitol (WS-50030) and solutions were administered at a volume of 10 ml/kg to mice and 1-5 ml/kg to rats unless otherwise noted. Dose calculations were based on active moiety. All other materials were analytical grade and were purchased from Sigma-Aldrich chemicals (Milwaukee, WI).

**Procedures.**

*In vitro receptor/transporter binding*

_D2 Receptor Binding_. Affinity for the long form of the human D2 receptor (D_{2L}) was measured in a membrane preparation of CHO-D_{2L} cells by binding studies using \[^{3}\text{H}]\text{spiperone as ligand. The membrane preparation was prepared from CHO-K1 cells transfected with the human D}_{2L} \text{ receptor (CHO-hD}_{2L} \text{ cells, obtained from Dr. D. Grandy, Vollum Institute Oregon, USA, Bunzow et al., 1988). After thawing, the cells were centrifuged for 10 minutes at 2000 g at 4°C. Cell pellets were resuspended in 50 mM Tris-HCl pH 7.7, cell membranes were prepared by two cycles of homogenization (Potter-Elvehjem 10 strokes, 600 rpm) and centrifugation (40,000g for 10 min, 4°C). After the second centrifugation step the pellets were resuspended in assay buffer (50 mM Tris-HCl pH 7.7, 4 mM NaCl, 5 mM KCl, 2 mM CaCl\text{,} 1 mM MgCl\text{,} 0.1% ascorbic
acid). The assay was established so as to achieve steady state conditions and to optimize specific binding. Compounds (10^{-2} M in DMSO) were diluted to 10x test concentrations in aqueous 1% ascorbic acid. Membranes from 5x10^6 cells and test compounds were incubated with 0.2 nM[^3]H spiperone at 37°C for 15 minutes. Nonspecific binding was determined using 10^{-7} M haloperidol. Assays were terminated by rapid vacuum filtration through glass fiber filters (GF/B). Total and bound radioactivity was determined by liquid scintillation counting using Microsint®. Greater than 80% specific binding was achieved in each of these assays. Compounds were tested at a 4-log concentration range; all determinations were performed in triplicate.

**Receptor Selectivity Binding.** Human 5-HT_{1A} receptor binding was determined via the displacement of[^3]H-8-OH-DPAT from human 5-HT_{1A} receptor transfected CHO cells (Dunlop et al., 1998). Human 5-HT_{2A} receptor binding was determined via the displacement of[^125]IDOI from human 5-HT_{2A} transfected CHO cells (Rosenzweig-Lipson et al., 2006). Human 5-HT_{2B} receptor binding was determined via the displacement of[^3]H5-HT from human 5-HT_{2B} transfected CHO cells (Rosenzweig-Lipson et al., 2006). Cortical membranes prepared from male Sprague-Dawley rats incubated with[^3]Hprazosin (0.2 nM) were employed to determine binding to the adrenergic α_1 receptor (U’Prichard and Snyder, 1980). Membranes from stably transfected HEK293 cells expressing the human histamine H_{1} receptor incubated with 3 nM pyridinyl-5-[^3]Hpyrilamine were employed to determine binding. CHO cells expressing the human adrenergic α_{2A} receptor incubated with 2 nM[^3]HMK{912 were used for binding analysis (Pettibone et al., 1989). IC_{50} values were determined by nonlinear regression analysis and inhibition constants (Ki) calculated using the equation
\[ \text{eq 1} \quad K_i = \frac{IC_{50}}{1 + (L/K_d)} \], where \( L \) is the concentration of radioligand in the assay and \( K_d \) the affinity of the radioligand for the receptor (Cheng and Prusoff, 1973). Results are expressed as mean \( K_i \) (± SEM) from at least three separate experiments.

**Transporter Binding.** Binding to crude membrane preparations from HEK293 cells expressing the human serotonin transporter (hSERT) was determined as described by competition with \[^{3}H\]citalopram (Deecher et al., 2006). Binding to crude membrane preparations from MDCK-Net6 cells, stably transfected with the human norepinephrine transporter (hNET) was determined by competition with \[^{3}H\]nisoxetine (Deecher et al., 2006). Binding to crude membrane preparations from CHO cells expressing the human dopamine transporter (DAT) was determined as described by competition with \[^{3}H\]WIN-35,428 (Deecher et al., 2006).

**In vitro functional assays**

**Human D_{2L}.** WS-50030 was tested for functional agonist/antagonist activities using stably expressed recombinant receptors with a published Bmax of 1.30 ± 0.17 (SD) and Kd of 0.064 ± 0.011 (SD) (Oak et al., 2001). CHO-K1 cells transfected with the human D_{2L} receptor were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM glutamine, 200 μg/ml G418 and 10% heat-inactivated fetal bovine serum in 93% air/ 7% CO₂. Cells were cultured two times per week. For the cAMP assay, CHO-hD_{2L} cells were trypsinated and transferred into 96-well plates. Cells were grown for 48h to produce a 100% confluent monolayer.

Functional cAMP assays were performed to assess WS-50030 activity on D_{2L} receptors. Test compounds were diluted in DMEM containing 1mM IBMX and 0.3 μM forskolin. Incubations were initiated by addition of medium in presence or absence of test
compound and performed at 37°C for 20 min. Nucleotides were extracted with lysis-buffer (63% acetonitrile with 10 mM NH₄HCO₃, pH 9) and plates were sealed and stored at 4°C until analysis. Samples were analyzed using HPLC-MS/MS. After separation of ATP and cAMP using HILIC-column, analytes were quantified using a Linear Ion Trap mass spectrometer (LTQ, Thermo). The mass spectrometer was operated in negative electrospray and peaks were quantified using extracted ion chromatography of the daughter ion: m/z 328 and 134 for cAMP, and m/z 506 and 408 for ATP. The conversion of ATP into cAMP was calculated as the ratio of cAMP compared to combined cAMP and ATP. Data was expressed as percentage of control values (forskolin-stimulated cAMP accumulation). The maximal forskolin-induced conversion was taken as maximum value and the maximal inhibition (1µM quinpirole) as minimum. Basal cAMP levels (± SEM) were 1.4 ± 0.4 nM while forskolin (0.3 µM) elevated cAMP levels (± SEM) were 9.5 ± 1.0 nM, producing a suitable assay window. Agonistic effects of compounds are expressed as mean EC₅₀ ± SEM. Antagonistic effects of compounds were determined as percentage inhibition of 1µM quinpirole-inhibited cAMP production and the pA₂ was calculated according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Compounds were tested at a 5-log concentration range, in duplicate and were performed in 3 independent experiments.

**Human 5-HT₁A.** Assays of 5-HT₁A receptor agonism were conducted as described (Hirst *et al*., 2008). Intracellular cAMP levels were measured in human 5-HT₁A CHO cells, grown in 96-well plates. Cells were preincubated at 37°C for 15 min in Krebs’ solution. Functional activity of WS-50030 was assessed by treating the cells with forskolin (10 µM final concentration) followed immediately by the test compound at
concentrations ranging from 0.1 to 10,000 nM. The cells were incubated for an additional
10 min at 37°C and the assay was terminated by the addition of 0.5 M perchloric acid.
The plates were stored at -20°C before the assessment of cAMP formation by a cAMP
scintillation proximity assay according to manufacturers instructions (GE Healthcare).
Data were generated in singlet within each experiment and each experiment was carried
out at least three times. Curve fitting of the mean data were generated by a four-
parameter logistic equation using Prism 5.0 (GraphPad Software, Inc., San Diego, CA)
and results reported as the mean ± SEM.

**SERT Uptake.**[^3H]-5-HT functional uptake assays using the human placental
choriocarcinoma JAR cell line were conducted as described (Deecher *et al.*, 2006). On
day 1, cells were plated at 75,000 cells/well in 96-well plates containing growth medium
(RPMI 1640 with 10% FBS and 1% Pen/Strep) and 40 nM staurosporine to increase the
expression of the 5-HT transporter. On day 2, growth medium was replaced with 200 μl
of assay buffer (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.2 mM
MgSO₄, and 2 mg/ml glucose, pH 7.4, at 37°C) containing 0.2 mg/ml ascorbic acid and 1
μM pargyline. A stock solution of fluoxetine was prepared in DMSO (10 mM) and
delivered to duplicate wells containing cells for a final test concentration of 10μM. Data
from these wells were used to define nonspecific 5-HT uptake (minimal 5-HT uptake in
the presence of a 5-HT reuptake inhibitor). Test compounds were prepared in DMSO and
diluted in assay buffer according to the test range (1–10,000 nM). Twenty-five
microliters of assay buffer (maximal 5-HT uptake) or test compound was added directly
to duplicate wells containing cells in 200 μl of assay buffer. The cells were incubated
with the test compound for 5 min (37°C). To initiate the reaction,[^3H]hydroxytryptamine
creatine sulfate diluted in assay buffer was delivered in 25 μl aliquots to each well for a final test concentration of 12.5 nM. The cells were incubated with the reaction mixture for 5 min at 37°C. Removing the supernatant from the plates terminated the reaction. The cells were washed twice with 200 μl of assay buffer (37°C) to remove free radioligand. The plates were air-dried for a minimum of 2 hours. Subsequently, the cells were lysed in 25 μl of 0.25 N NaOH (4°C), placed on a shaker table and shaken vigorously for 5 min. After cell lysis, 75 μl of scintillation cocktail was added to the wells. The plates were sealed with film tape and counted in a TopCount scintillation counter to collect the raw counts per minute data. Data were generated in duplicate within each experiment and each experiment was carried out at least three times. Curve fitting of the mean data were generated by a four-parameter logistic equation using Prism 5.0 (GraphPad Software Inc., San Diego, CA) and results reported as the mean ± SEM.

**Human 5-HT\textsubscript{2B}**. WS-50030 was tested up to 10 μM in 5-HT\textsubscript{2B} receptor functional assays that were conducted as described (Dunlop *et al.*, 2005). Stable CHO cell lines expressing the human 5-HT\textsubscript{2B} receptor subtype were used for functional studies using the measurement of agonist-stimulated mobilization of intracellular calcium with the fluorometric imaging plate reader (FLIPR). Cells were maintained and passaged upon reaching approximately 80% confluence. Cells were plated 24 h before the experiment in poly-d-lysine-coated 96-well plates at a density of approximately 60,000 cells per well. In preparation of the assay, the confluent monolayer of cells was washed twice with Hanks' buffered saline solution supplemented with 20 mM HEPES and 2.5 mM probenecid (FLIPR buffer), and then the cells were loaded by adding 4 μM Fluo-4 AM (Molecular Probes, Eugene, OR) in FLIPR buffer for 1 h at 37°C. After loading, the cells were then
rinsed twice with FLIPR buffer, and intracellular calcium increases after agonist application were detected by measuring increases in fluorescence with the FLIPR. For evaluation of antagonist activity, compounds were included during the dye-loading step and subsequently stimulated by the addition of an EC\textsubscript{80} concentration of 5-HT. Curve fitting of the mean data were generated by a four-parameter logistic equation using Prism 5.0 (GraphPad Software Inc., San Diego, CA) and results reported as the mean ± SEM.

Human 5-HT\textsubscript{2A}. WS-50030 was tested in FLIPR-based functional agonist and antagonist assays using the human 5-HT2A receptor expressed recombinantly in HEK293 cells (CEREP, Poitiers, France) as described (Jerman \textit{et al.}, 2001).

\textit{In vivo} microdialysis

Rats were dosed with vehicle (i.p.) or WS 50030 (10 mg/kg i.p.) once a day for 20 days. On day 21, animals were anesthetized with isoflurane (2-3\%) to allow stereotaxic implantation of microdialysis guide cannulae. Two microdialysis guide cannulae (CMA/12; CMA Microdialysis, Sweden) were implanted above the medial prefrontal cortex (mPFC; anterior +3.2 mm and lateral +0.5 mm from bregma, ventral −1.8 mm from dura) and the nucleus accumbens (anterior +2.2 mm and lateral −2.8 mm from bregma, ventral −6.1 mm at a 10° angle; Paxinos and Watson, 1986). Guide cannulae were secured to the skull with two stainless-steel screws (Small Parts, Roanoke, VA) and dental acrylic (Plastics One, Roanoke, VA). Following recovery from surgery, animals were administered the 21st dose of chronic treatment and then individually housed in Plexiglas cages (45 cm x 45 cm) with free access to food and water overnight before the microdialysis experiment. The day after surgery (day 22), microdialysis studies were performed.
As previously described (Beyer et al., 2002), concentric-style microdialysis probes (CMA/12; 20 kD cut-off; CMA/Microdialysis, Sweden) with a 4 mm (mPFC) or a 2 mm membrane (nucleus accumbens) were perfused with artificial CSF (aCSF; 125 mM NaCl, 3 mM KCl, 0.75 mM MgSO4 and 1.2 mM CaCl2, pH 7.4) at a flow rate of 1 μl/min. Microdialysis probes were inserted, via the guide cannulae, and allowed to stabilize for 3 h. Dialysis samples were then collected every 20 min. Initially, 6 dialysate samples were taken to determine the baseline neurotransmitter levels. After the sixth baseline sample, animals were dosed with vehicle or WS-50030 (10 mg/kg, i.p.), and dialysis samples were collected for the following 3 h. The 3 treatment groups were: vehicle (days 1-21) + vehicle (day 22; ‘control’); vehicle (days 1-21) + WS-50030 (day 22; ‘acute’); WS-50030 (days 1-21) + WS-50030 (day 22; ‘chronic’). Group sizes were as follows: 5-HT in nucleus accumbens, vehicle = 3, acute WS-50030 = 4, chronic WS-50030 = 3; DA in nucleus accumbens, vehicle = 7, acute WS-50030 = 6, chronic WS-50030 = 4; 5-HT in mPFC, vehicle = 3, acute WS-50030 = 3, chronic WS-50030 = 5; DA in mPFC, vehicle = 5, acute WS-50030 = 5, chronic WS-50030 = 5.

After completion of microdialysis experiments rats were euthanized to confirm correct probe placement. For the nucleus accumbens studies, probe placement did not discriminate between core and shell. Rats with incorrect probe placement were excluded from the analysis.

**Neurochemical Analysis.** The outlet tubing of the microdialysis probes was connected directly to an ANTEC (Antec, The Netherlands) HPLC-ECD system. Twenty μl dialysate containing serotonin and dopamine was separated by HPLC (C18 ODS3 column, 150 x 3.0 mm, Metachem, Torrance, CA) and detected using an ANTEC
electrochemical detector set at a potential of 0.65V vs. a Ag/AgCl reference electrode. Mobile phase (0.15 M NaH₂PO₄, 0.25 mM EDTA, 1.75 mM 1-octane sulphonic acid, 2% isopropanol and 4% methanol, pH = 4.6) was delivered by a Jasco PU1580 HPLC pump (Jasco Ltd, U.K) at a flow rate of 0.5 ml/min. Neurochemical data were compared to an external standard curve and all data were acquired using the Atlas software package (Thermo Labsystems, Beverley, MA) for the PC. The mean neurotransmitter levels (fmol concentrations) of the baseline samples were calculated and all sample values were expressed as a percent change from this pre-injection baseline value (% change from baseline). Concentrations of neurochemicals were not corrected for probe recovery. Area under the curve (AUC, % basal) was calculated using the trapezoidal equation. AUC data were analyzed using one-way ANOVA with post-hoc LSD test. All statistical calculations were performed using SAS for Excel.

**Antagonism of apomorphine-induced climbing and stereotypy in mice**

Male CF-1 mice were acclimated to wire cages (1/2" x 1/2" mesh; cylindrical dimensions 4 1/2"d x 5 1/2"h) for at least 30 min before testing. WS-50030 (0.03 – 30 mg/kg) or aripiprazole (0.1 – 1 mg/kg) was administered i.p. to 6 - 12 mice per dose level. Separate control groups for each study were run simultaneously with drug-treated groups and received vehicle at equal volumes. Thirty minutes later, experimental and control animals were challenged with 1 mg/kg s.c. apomorphine. Five minutes after the apomorphine injection, stereotyped behavior (sniffing-licking-gnawing syndrome; 0 = absent, 1 = present) and climbing behavior (0 = all 4 feet on ground, 1 = 2 feet up on wire cage, 2 = all 4 feet on wire cage) induced by apomorphine were scored and recorded for each animal. Readings were repeated every 5 minutes during a 30-minute test session.
Scores for each animal were totaled over the 30-minute test session for each syndrome (stereotyped behavior and climbing). Mean climbing and stereotypy scores were then expressed as a percent of control values observed in vehicle-treated mice that received apomorphine. ID$_{50}$'s were calculated using nonlinear regression.

**Cataleptogenic potential in mice**

WS-50030 (0.3 – 10 mg/kg), aripiprazole (0.1 – 3 mg/kg) or vehicle was administered i.p. to 6 male CF-1 mice per dose level. Every 30 minutes for 2 hours post dosing, the animal's forelegs were draped over a thin horizontal rod 1 3/4" high. The amount of time (in seconds) for which the animal maintained this awkward position was recorded (60 second maximum). Maintenance of this position is considered catalepsy. Mean seconds spent in the catalepsy position for each dose at each time point was calculated and data were analyzed by two-way repeated measures analysis of variance (ANOVA) with post hoc least significant difference (LSD) test. The time point at which peak catalepsy was observed was expressed graphically. Severity of catalepsy is defined as follows: maintenance of awkward position for 20 sec or less = minimal to no catalepsy, 21-40 sec = moderate catalepsy, and 41-60 sec = high catalepsy.

**Rotational behavior in unilaterally 6-OHDA – lesioned rats**

*Surgery.* Unilateral 6-OHDA lesions of the substantia nigra zona compacta were performed on male Wistar rats using a stereotaxic procedure. One hour prior to surgery, desmethyl-imipramine (20 mg/kg, i.p.) was administered to protect the noradrenergic neurons. Rats were anaesthetized with a 3% halothane + 0.8 l/min N$_2$O + 0.8 l/min O$_2$ - gas mixture at 1013 mbar. During surgery the gas mixture was adjusted to 1.75 - 2% halothane, 0.6 l/min N$_2$O and 0.6 l/min O$_2$. The incisor bar of the stereotaxic instrument
(David Kopf, Tujunga, CA) was set at –3.3 mm, a burr hole was drilled over the substantia nigra pars compacta and 3 μl of a 6-OHDA solution (3.33 mg/ml) was injected (flow rate = 0.75 μl/min; the needle was left in place for 4 minutes prior to withdrawal). Coordinates for this procedure were: +3.2 mm anterior from the interaural line; +1.8 mm lateral from the midline and –8.2 mm ventral from the skull surface.

**Apparatus.** Eight commercially available (TSE systems Bad Homburg, Germany) ‘rotameter’ units (transparent plastic bowls; 57 x 55 x 52 cm) were used for testing. The rats were harnessed and tethered to a rotation sensor interfaced to an IBM compatible personal computer (using the TSE Rotameter Software v. 1.11, TSE systems, Bad Homburg, Germany) that registered clockwise or counterclockwise movement. An internal software rotation filter of 10 was used. After a minimum of 10-14 days post surgery, the rats were habituated (twice) to the apparatus. The patency of the 6-OHDA lesion was checked by testing their sensitivity to direct and indirect dopamine agonism. Following recovery from surgery, rats were challenged with apomorphine (0.1 mg/kg s.c.), amphetamine (0.25 mg/kg s.c.) and a second apomorphine challenge (0.1 mg/kg s.c.) with one week between each challenge. Only those rats which elicited >20 ipsilateral turns following amphetamine in a 5-min time period beginning 25 min after administration and >20 contralateral turns per 5-min measured over one hour in the second apomorphine challenge were used in WS-50030 and aripiprazole studies. In addition, regular testing with apomorphine (0.1 mg/kg s.c.) was carried out to ensure the reliability of the animals in this procedure.

**Protocol.** Following statistical randomization to the treatment groups, rats were treated with WS-50030 (10 mg/kg i.p.; n = 8), aripiprazole (10 mg/kg i.p.; n = 8),
apomorphine (0.1 mg/kg s.c.; n = 16) or vehicle (i.p.; n = 5) and placed in rotometers for 60 minutes. Rotational behavior both ipsilateral and contralateral to the side of lesion was recorded and data are displayed graphically as mean number of contralateral minus ipsilateral turns ± SEM for the 15-min period 45-60 min following drug administration. Data was subjected to a one-way ANOVA with post-hoc LSD test.

**Conditioned avoidance responding in rats**

Shuttlebox test chambers (MED Associates, St Albans, VT) divided into two compartments by an archway were used. Each chamber floor half is composed of thirteen stainless steel grid rods wired for the presentation of an electric foot shock (0.5mA). In addition, each side of the chamber is equipped with a stimulus light, tone generator and two infrared beam source/detectors used to locate the rat within the chamber. Male Sprague-Dawley CD rats trained to avoid the foot shock were placed in the chambers for a 4-min habituation period followed by 50 trials presented on a 15-sec variable interval schedule (range = 7.5 - 22.5 seconds). Each trial consisted of a 10-sec warning tone and stimulus light (conditioned stimulus) followed by a 10-sec shock (unconditioned stimulus), presented through the grid floor on the side where the rat was located, in the presence of the tone and light. If an animal crossed through the archway during the initial 10 seconds of the trial, thereby breaking the beam located 13 cm from the center of the archway, the tone and light were terminated and the response was considered an avoidance response. If an animal crossed through the archway after a foot shock was initiated, the tone, light and shock were terminated and the response was considered an escape response. If a response was made during an inter-trial interval (ITI), the animal was punished with a 0.5-sec shock (0.5mA). A Med Associates computer with
MEDSTATE NOTATION™ software controlled the test session and counted the number of trials in which the animal avoided shock, escaped shock and did not respond. The rats used in these studies were part of a trained colony used repeatedly for antipsychotic screening. Only animals displaying stable performance (approximately 90% avoidance responding on the training session prior to test day) were considered “trained” and included on test day. Training was maintained by at least one non-drug test session each week. On test days, rats were treated with WS-50030 (3 or 10 mg/kg i.p.; 10 mg/kg p.o.) or aripiprazole (1, 3 or 10 mg/kg i.p.; 17 or 30 mg/kg p.o.). Rats were tested 30 minutes post i.p. dosing and 60 minutes post p.o. dosing (within subject design, \( n = 7 - 8 \), separate cohorts for each drug and each route of administration). Doses were administered in ascending order. Data were analyzed using repeated measures ANOVA with *post-hoc* LSD test.

**Olfactory bulbectomy**

*Surgery.* Isoflurane anesthesia (2 - 3%) was administered before animals were secured in a stereotaxic frame with ear and incisor bars (David Kopf, Tujunga, CA). Using aseptic techniques, an incision was made and the skin was retracted to reveal bregma. Bilateral burr holes (2 mm diameter) were drilled above the olfactory bulbs (+8.1 mm anterior from bregma; +/- 2.0 mm lateral from midline) and olfactory bulbs were removed by suction with a vacuum pump attached to a Pasteur pipette. Burr holes were then filled with gel foam (Harvard Apparatus). For animals receiving sham surgery (SHAM), burr holes were drilled and filled but olfactory bulbs were not removed. An antibiotic solution, cefazolin (0.05 ml), was applied locally prior to closing the incision with surgical clips. After surgery, animals were injected s.c. with 5 ml 0.9% sterile saline.
to maintain hydration and were placed in an incubator (85 - 90°C) until fully awake. Animals were then housed with another animal of the same surgery group (e.g. BULB with BULB or SHAM with SHAM) for the duration of the experiment. The day after surgery rats were administered the analgesic, Buprenex (buprenorphine hydrochloride; s.c., 6 μg/rat). All animals were allowed to recover for 14 days following surgery and were handled daily throughout the recovery period. Surgical clips were removed 14 days following surgery.

**Drug Treatment.** Immediately following the 14-day recovery period, animals were each assigned to treatment groups (n = 10 bulbectomized and 8 sham-operated rats/treatment group) and received WS-50030 (3 - 10 mg/kg, i.p.) or aripiprazole (1 - 5.6 mg/kg, i.p.) or vehicle (i.p.) once daily for 2 weeks. Doses were selected based on previous studies using these compounds and limited to a range that produced < 20% reduction in locomotor activity acutely (data not shown).

**Locomotor Activity.** Four hours after administration of the final dose, rats were placed in an open field (90x90 cm Plexiglas box with 30 cm walls) where locomotor activity was recorded for 5 minutes using a video camera suspended over the open field and Ethovision tracking software (Noldus Ethovision, Leesburg, VA). Total distance (cm) moved in the 5-min period in vehicle-treated sham operated rats was compared to vehicle-treated bulbectomized rats using a t-test (p<0.05) for each experiment. Subsequently, separate one-way ANOVAs compared the effect of dose of drug on total distance moved in sham and bulbectomized subjects, followed by Dunnett's post-hoc comparison to the vehicle treated group (p<0.05).
RESULTS

Receptor binding profile

WS-50030 binds with high affinity to the serotonin transporter (mean Ki ± SEM = 7.1 ± 0.1 nM) and the dopamine D2 receptor (D2L, Ki ± SEM = 4.0 ± 0.1 nM). In addition, WS-50030 had moderate affinity for the serotonin 5-HT1A (Ki ± SEM = 22.2 ± 7.7 nM), 5-HT2A (Ki ± SEM = 85.5 ± 23.5 nM) and 5-HT2B (Ki ± SEM = 38 ± 23 nM) receptors, adrenergic α1 receptor (Ki ± SEM = 15.4 ± 5.1 nM) and histamine H1 receptor (Ki ± SEM = 98.8 ± 34.5 nM) and low affinity, hNET (Ki ± SEM = 3.6 ± 0.1 μM) and hDAT (Ki ± SEM = 2.9 ± 0.1 μM).

In vitro functional activity

The functional activities of WS-50030 against the primary target proteins (D2, SERT) were assessed in vitro. WS-50030 displayed potent D2 partial agonist activity (mean EC50 ± SEM = 0.38 ± 0.02 nM, Emax = 30 ± 3%) by attenuating forskolin-induced accumulation of cAMP in CHO cells expressing D2L (Fig. 2A) while quinpirole exhibited full agonist activity (EC50 = 10 nM; data not shown). In a corresponding antagonist assay (Fig. 2A), WS-50030 partially attenuated quinpirole-induced blockade of forskolin-stimulated cAMP accumulation in a dose-dependent manner [mean IC50 ± SEM = 2.2 ± 0.2 nM (pA2 = 10.7), Imax = 73 ± 3%]. Complete serotonin transporter blockade was exhibited by WS-50030 (IC50 = 56.4 ± 0.2 nM) in 3H-5-HT uptake assays using Jar cells (Fig. 2B). In cAMP accumulation assays using CHO cells transfected with the human serotonin 5-HT1A receptor, WS-50030 was found to be an agonist (EC50 = 67 ± 1.1 nM, Emax = 90 ± 3%, Fig. 2C). WS-50030 (10 μM) displayed no detectable 5-HT2B agonist activity in measurements of intracellular calcium accumulation (data not shown).
FLIPR-based functional agonist and antagonist assays using the human 5-HT$_{2A}$ receptor expressed recombinantly in HEK293 cells, WS-50030 was determined to be an antagonist ($pA_2 = 6.65 \pm 0.15$). No detectable 5-HT2A agonist activity was detected.

**In vivo microdialysis**

Basal levels of 5-HT and dopamine (mean ± SEM) in either the mPFC or nucleus accumbens were not affected by chronic WS-50030 treatment (10 mg/kg i.p. daily for 22 days): mPFC, 5-HT (pM): 679.78 ± 280.84 vehicle; 482.13 ± 106.69 chronic WS-50030; mPFC, dopamine: 84.06 ± 22.89 vehicle; 175.12 ± 50.92 chronic WS-50030; nucleus accumbens, 5-HT: 105.99 ± 41.54 vehicle; 186.07 ± 20.98 chronic WS-50030; nucleus accumbens, dopamine: 901.31 ± 234.44 vehicle; 898.83 ± 109.67 chronic WS-50030. These findings are consistent with previous studies showing that chronic treatment with an SSRI (Dawson *et al.*, 2000) or aripiprazole (Jordan *et al.*, 2004) do not significantly change basal levels of 5-HT or dopamine in the rat frontal cortex. WS-50030 treatment significantly elevated extracellular levels of 5-HT in the rat mPFC (Fig. 3A) [$F(2,10) = 5.02$, $p < 0.05$]. Following 21 days of WS-50030 treatment (10 mg/kg i.p.), a challenge injection of WS-50030 (10 mg/kg, i.p.) elicited a significant increase in the extracellular levels of 5-HT in the rat mPFC ($p = 0.01$ relative to vehicle treatment). Additional post hoc analyses revealed that in rats treated chronically with vehicle an acute injection of WS-50030 (10 mg/kg, i.p.) elicited a non-significant trend ($p = 0.096$) towards elevating 5-HT relative to vehicle. Additionally, no significant differences were observed between rats treated acutely or chronically with WS-50030 ($p = 0.40$). In contrast, neither acute nor chronic treatment with WS-50030 altered levels of dopamine in the mPFC following a dose of 10 mg/kg (Fig. 3B) [$F(2,13) = 0.01$, $p = 0.98$]. Following 21 days of treatment
with either vehicle or WS-50030 (10 mg/kg i.p.), a challenge injection of WS-50030 (10 mg/kg, i.p.) did not elicit significant changes in levels of 5-HT [F(2,10) = 0.01, p = 2.52] or dopamine [F(2,14) = 1.84, p = 0.83] in the rat nucleus accumbens (Fig. 4A and 4B).

**Antagonism of apomorphine-induced climbing and stereotypy in mice**

Figure 5 shows the effects of WS-50030 and aripiprazole on apomorphine-induced climbing (effects on apomorphine-induced stereotypy not shown). Apomorphine (1 mg/kg s.c.) induced consistent levels of climbing and stereotypy in vehicle-treated mice (climbing [mean ± SEM] = 10.89 ± 0.21; stereotypy = 5.78 ± 0.22). WS-50030 (0.03 – 30 mg/kg i.p.) produced a dose-dependent decrease in apomorphine-induced climbing more potently than apomorphine-induced stereotypy [ID$_{50}$ climbing = 0.51 mg/kg (95% CI, 0.39 – 0.66); ID$_{50}$ stereotypy = 1.02 mg/kg (95% CI, 0.83 – 1.27)]. Similarly, aripiprazole (0.1 – 1 mg/kg i.p.) produced a dose-dependent decrease in both apomorphine-induced behaviors [ID$_{50}$ climbing = 0.14 mg/kg (95% CI, 0.06 – 0.30); ID$_{50}$ stereotypy = 0.58 mg/kg (95% CI, 0.48 – 0.71)].

**Cataleptogenic potential in mice**

While WS-50030 (0.3 – 10 mg/kg i.p.) demonstrated statistically significant catalepsy [MED = 3 mg/kg at 120 min time point; treatment x time interaction F(12,75) = 2.15; p < 0.05, repeated measures ANOVA; p < 0.01, LSD post hoc, Table 1], it was minimal (<20 sec). The peak catalepsy exhibited was at the 10 mg/kg dose at 90 minutes post dosing, when mice displayed a mean (± SEM) of 14.33 ± 7.29 seconds in the catalepsy position out of a maximum possible 60 seconds (Fig. 6). Aripiprazole (0.1 – 3 mg/kg i.p.) also demonstrated statistically significant catalepsy [MED = 1 mg/kg at 120 min time point; treatment x time interaction F(12,75) = 2.67; p < 0.01, repeated measures
ANOVA; p < 0.05, LSD post hoc, Table 1]. Peak catalepsy following aripiprazole administration was also exhibited at the 90-min test point, when the 1 and 3 mg/kg i.p. dose groups displayed means (± SEM) of 16.83 ± 9.25 and 30.33 ± 9.06 seconds in the catalepsy position, respectively, out of a maximum possible 60 seconds (minimal and moderate catalepsy, respectively).

**Rotational behavior in unilaterally 6-OHDA – lesioned rats**

Contralateral minus ipsilateral rotations recorded in the 15-min period occurring 45-60 minutes post dosing are displayed in Figure 7. Apomorphine (0.1 mg/kg s.c.) produced an expected amount of contralateral rotation (contralateral – ipsilateral rotations [mean ± SEM] = 74.59 ± 13.24). WS-50030 (10 mg/kg i.p.) produced contralateral rotation that was significantly less than that produced by apomorphine (WS-50030 contralateral – ipsilateral rotations = 22.23 ± 5.38; post hoc p = 0.01 vs. apomorphine). Aripiprazole produced a similar amount of contralateral rotation (contralateral – ipsilateral rotations = 37.21 ± 17.55) to WS-50030, demonstrating a non-significant trend to be less than that produced by apomorphine (post hoc p = 0.52 vs. WS-50030; post hoc p = 0.07 vs. apomorphine). Mean number (± SEM) of contralateral and ipsilateral rotations were as follows: apomorphine: 78.24 ± 13.40 contralateral, 3.65 ± 1.53 ipsilateral; WS-50030: 23.62 ± 5.39 contralateral, 1.39 ± 0.55 ipsilateral; aripiprazole: 38.86 ± 17.38 contralateral, 1.65 ± 0.39 ipsilateral.

**Conditioned avoidance responding in rats**

Aripiprazole and WS-50030 both significantly decreased avoidance responding following i.p. and p.o. dosing with no effect on the number of trials in which escape failures occurred. WS-50030, at 3 and 10 mg/kg i.p., produced 17 and 42% reductions
in avoidance responding, respectively $[F(2,14) = 8.82; p < 0.01; \text{Fig. 8}]$. Following oral administration, 10 mg/kg WS-50030 produced a 40% reduction in avoidance $[F(1,7) = 13.10; p < 0.01; \text{data not shown}]$. Aripiprazole produced 5, 36 and 55% reductions in avoidance responding at 1, 3 and 10 mg/kg i.p. $[F(3,18) = 26.33; p < 0.001; \text{Fig. 8}]$, respectively, and 21 and 49% reductions in avoidance at 17 and 30 mg/kg p.o. $[F(2,14) = 14.41; p < 0.001; \text{data not shown}]$, respectively.

**Olfactory bulbectomy**

The effect of surgery on locomotor activity was observed in both experiments as a significant increase in locomotor activity in the vehicle treated bulbectomized rats compared to the vehicle-treated sham operated rats ($\text{WS:50030: } t = 2.677, \text{df} = 12, p<0.05$; aripiprazole: $t = 3.650, \text{df} = 16, p<0.01$, Fig. 9). Administration of WS-50030 for 14 days completely reversed bulbectomy-induced hyperactivity ($F(3, 31) = 3.65, p < 0.001$) while producing a smaller effect in sham subjects ($F(3, 25) = 6.28, p<0.01$). Post-hoc analysis revealed a selective effect of WS-50030 at 3 mg/kg i.p. to normalize bulbectomy-induced hyperactivity without significantly affecting locomotor activity in sham subjects (Fig. 9A). The 5.6 and 10 mg/kg doses of WS-50030 also reversed bulbectomy-induced hyperactivity relative to vehicle-treated bullectomized subjects, however these doses also caused a significant reduction in activity in sham-operated animals. Administration of aripiprazole (1 - 5.6 mg/kg i.p.) for 14 days also significantly reversed bulbectomy-induced hyperactivity ($F(3, 35) = 9.76, p < 0.0001$) without significantly reducing activity in sham operated rats ($F(3, 24) = 0.963, p > 0.05$) (Figure 9B). Administration of 1 mg/kg i.p. aripiprazole for 14 days partially reversed the bulbectomy-induced hyperactivity (66% reversal vs. vehicle-treated bullectomized; Fig.
9B). Higher doses of aripiprazole (3 and 5.6 mg/kg i.p.) completely reversed bulbectomy-induced hyperactivity without reducing locomotor activity in sham-operated animals.
DISCUSSION

Our objective was to develop a novel antipsychotic agent with broad therapeutic efficacy by combining D2 receptor partial agonist activity with SRI properties in a single molecule. We have characterized WS-50030 binding and function \textit{in vitro} as well as in a variety of \textit{in vivo} functional and efficacy models. When appropriate, we compared the effects of WS-50030 directly to the D2 receptor partial agonist, aripiprazole, which reportedly lacks significant SRI activity.

The present data indicate that WS-50030 \([7-\{4-[3-(1H-inden-3-yl)propyl]piperazin-1-yl\}-1,3-benzoxazol-2(3H)-one]\) is a combined D2 receptor partial agonist and serotonin reuptake inhibitor. WS-50030 demonstrates both antipsychotic- and antidepressant-like activity in preclinical \textit{in vivo} models with a low propensity for extrapyramidal side effects as measured by mouse catalepsy, a profile similar to aripiprazole.

Receptor binding studies demonstrate that WS-50030 binds with high affinity to the human D2 receptor (Ki = 4.0 nM) and human serotonin transporter (Ki = 7.1 nM) and with moderate affinity to other receptors. \textit{In vitro} functional studies demonstrated that WS-50030 behaved as a D2 receptor partial agonist, partially attenuating forskolin-induced accumulation of cAMP in CHO cells expressing the long form of the dopamine D2 receptor and partially attenuating a dopamine agonist-induced blockade of forskolin-induced accumulation of cAMP. This \textit{in vitro} characterization of partial agonism in cells recombinantly expressing the D2L receptor has the potential to overestimate agonist activity due to the fact that receptor density is higher than might be expected \textit{in vivo}. 


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Therefore, it is important to evaluate D₂ agonist-like and antagonist-like activity in vivo (see below).

WS-50030 exhibited complete serotonin transporter blockade in ³H-5-HT uptake assays using JAR cells and behaved as an agonist in CHO cells expressing the human 5-HT₁A receptor. While WS-50030 exhibited moderate binding affinity to 5-HT₂B receptors (Ki = 38 nM), no detectable agonist activity was observed, thereby limiting the likelihood of cardiovascular effects that have been demonstrated to occur in response to 5-HT₂B receptor agonists (Huang et al., 2009).

Aripiprazole is reportedly a D₂ receptor partial agonist as well as a partial agonist at 5-HT₁A receptors and an antagonist at 5-HT₂A receptors (Stark et al., 2007). Consistent with preliminary in house data (not shown), literature reports demonstrate that aripiprazole binds more potently to the long form of the D₂ receptor (Ki = 0.74 nM; Shapiro et al., 2003) than WS-50030. Unlike WS-50030, which binds equipotently at D₂ receptors and the serotonin transporter, aripiprazole binds with much greater selectivity for D₂ receptors (serotonin transporter Ki = 1080 nM, Shapiro et al., 2003).

To examine the effects of WS-50030 on extracellular levels of serotonin and dopamine, in vivo dual probe microdialysis studies were performed in the rat. These studies were designed to investigate the neurochemical effects of WS-50030 in both the medial prefrontal cortex (mPFC) and nucleus accumbens in the same animal. Consistent with the serotonin reuptake properties of this molecule, chronic treatment with WS-50030 elicited a significant increase in the extracellular levels of serotonin in the rat mPFC. This response was qualitatively similar in nature to that previously reported for chronic treatment with the SRI fluoxetine (Dawson et al., 2000). A similar trend towards
increased serotonin was also observed following acute WS-50030 in this brain region; however this latter effect did not reach statistical significance. It is likely that additional receptor functional efficacy, such as agonism at 5-HT$_{1A}$ receptors, acts to limit the acute effects of WS-50030 on this neurotransmitter, and upon repeated administration, these receptors desensitize allowing higher levels of serotonin to occur. In contrast, neither acute nor chronic treatment with WS-50030 altered levels of serotonin or dopamine in the rat nucleus accumbens, nor dopamine in the mPFC. The lack of dopaminergic effects found in both of these brain regions is consistent with the D$_2$ receptor partial agonist activity of WS-50030. For instance, the D$_2$ receptor partial agonist aripiprazole does not alter the extracellular levels of serotonin (Zocchi et al., 2005) or dopamine in the rat mPFC or striatum following acute treatment (Jordan et al., 2004). Similarly, Jordan et al. (2004) demonstrated that chronic aripiprazole treatment – unlike chronic olanzapine treatment – failed to significantly alter extracellular concentrations of 5-HIAA (5-hydroxyindole acetic acid; a metabolism product of serotonin) or dopamine in the rat striatum. These in vivo microdialysis studies support the conclusion that WS-50030 modulates serotonin transporters whereas aripiprazole does not.

In vivo behavioral studies showed that both WS-50030 and aripiprazole antagonize dopamine agonist-induced behaviors in mice, namely, climbing and stereotypy. As expected based on in vitro potency at D$_2$ receptors, aripiprazole was slightly more potent than WS-50030 in this model. However, neither compound elicited a strong cataleptogenic response in mice, consistent with a D$_2$ partial agonist profile and predictive of low extrapyramidal side effects. Other receptor activities of aripiprazole and WS-50030, such as 5-HT$_{1A}$ agonism, may also contribute to their low extrapyramidal...
side effect liability. *In vivo* dopamine agonist activity was demonstrated using rats with unilateral 6-OHDA – lesions of the substantia nigra zona compacta. In lesioned rats, post-synaptic dopamine receptors become supersensitive on the side of the lesion. Systemic injection of a post-synaptic agonist such as apomorphine will induce rotational behavior contralateral to the side of the lesion. Both WS-50030 and aripiprazole produced modest yet significant contralateral rotations in 6-OHDA – lesioned rats. As expected given the low intrinsic activity of WS-50030, the amount of contralateral rotation induced by WS-50030 was significantly less than that produced by the D₂ receptor full agonist apomorphine. Thus it can be surmised that WS-50030 demonstrated less *in vivo* D₂ agonist activity than the corresponding full agonist. While aripiprazole produced contralateral rotational behavior that was similar in magnitude to WS-50030, it did not differ significantly from apomorphine. This result is inconsistent with previous studies in 6-OHDA – lesioned rats by Kikuchi *et al.* (1995) in which no contralateral rotation was observed following acute aripiprazole (10 mg/kg, p.o.).

Overall, the results described thus far support the conclusion that WS-50030 demonstrates *in vitro* and *in vivo* D₂ receptor partial agonism and *in vivo* functional effects on serotonin reuptake inhibition while literature characterizes aripiprazole as a D₂ receptor partial agonist. WS-50030 and aripiprazole were therefore compared in *in vivo* models predictive of antipsychotic and antidepressant efficacy. WS-50030 demonstrated, at the same dose (10 mg/kg), an effect similar in magnitude to aripiprazole in the rat conditioned avoidance test, a standard screening model for antipsychotic efficacy (Arnt, 1982). In this model, all clinically active antipsychotics reportedly reduce the number of trials in which a rat will avoid a footshock by crossing to the opposite side
of the test chamber. In our hands, aripiprazole produced a 55% reduction in avoidance responding and WS-50030 a 42% reduction in avoidance responding (both at 10 mg/kg) and both increased escape responding without producing escape failures. This data suggests that WS-50030 may provide antipsychotic efficacy similar to that of aripiprazole.

As previously stated, there exists a high incidence of comorbidity of depressive symptoms in schizophrenic patients (Siris et al., 2001) and there is mounting evidence to support the value of SRI augmentation in the treatment of the negative symptoms of schizophrenia (Silver, 2003; Mazeh et al., 2004). Therefore, doses of WS-50030 which were active in rat conditioned avoidance and increase extracellular 5-HT were evaluated in a preclinical model of depression, specifically the olfactory bulbectomy-induced hyperactivity (OB) model in rats. It has previously been shown that a bilateral olfactory bulbectomy induces increased locomotor activity compared to sham-operated rats that is reversed by chronic administration of SRIs (Kelly et al., 1997). Chronic WS-50030 (3 mg/kg once daily for 14 days) was efficacious in this model, completely reversing bulbectomy-induced hyperactivity without reducing locomotor activity in sham-operated rats. Efficacy in this model suggests that WS-50030 may provide benefit in the treatment of mood disorders associated with schizophrenia and co-morbid depression.

Surprisingly, chronic aripiprazole was also effective in this model, behaving similarly to WS-50030 despite a reported lack of effect on serotonin transporters. Therefore, WS-50030 failed to distinguish itself from aripiprazole using the olfactory bulbectomy model. In preliminary studies, aripiprazole has been reported to be active in the olfactory bulbectomy model (Pistovcakova et al., 2008), and while antidepressant
efficacy of aripiprazole treatment alone has not yet been demonstrated in the clinic, clinical data indicate that aripiprazole augments the effectiveness of standard antidepressant treatment in patients with major depressive disorder and treatment-resistant depression (Pae et al., 2008). In addition, aripiprazole has been reported to produce improvement on various clinical negative symptom rating scales in schizophrenic patients (Swainston and Perry, 2004; Cassano et al., 2007).

Given the similar results in the OB model with WS-50030 and aripiprazole, further studies are required to definitively elucidate the mechanisms of action responsible for their effect in the model and to determine the degree to which serotonin reuptake inhibition activity may be contributing to the efficacy of WS-50030 in this model. Other mechanisms besides serotonin reuptake inhibition have been reported to normalize olfactory bulbectomy-induced hyperactivity (Kelly et al., 1997). It may be that the D₂ receptor partial agonist properties of WS-50030 and aripiprazole are sufficient to demonstrate efficacy in this model. Millan et al. (2004) have reported preclinical efficacy with the D₃/D₂ receptor agonists ropinirole and S32504 in depression models such as the forced swim test, learned helplessness and chronic mild stress models that were blocked by D₂ but not D₃ receptor antagonists.

In summary, despite possessing serotonin reuptake inhibitory activity in addition to D₂ receptor partial agonism, WS-50030 displays activity in preclinical models predictive of antipsychotic- and antidepressant efficacy similar to aripiprazole, thus suggesting potential efficacy of WS-50030 versus positive and negative symptoms of schizophrenia, co-morbid mood symptoms and bipolar disorder as well as major depressive disorder and treatment resistant depression. Furthermore, WS-50030 provides
a tool to further explore how combining these mechanisms might differentiate from other antipsychotics or antidepressants.
ACKNOWLEDGEMENTS

The authors would like to thank Thomas A. Comery and Dr. Terrance H. Andree for their critical review of this manuscript and Virginia Pulito for assistance with the manuscript preparation.

The authors would also like to thank the following people for their expert technical assistance: Paul van de Feer, Jan van de Kieft and Juan Mercado.

We would also like to thank Dr. Jan-Hendrik Reinders for his technical and intellectual contributions. Sadly, Dr. Reinders passed away unexpectedly on February 4, 2008.
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FOOTNOTES

All studies were funded by Wyeth Research and Solvay Pharmaceuticals Research Laboratories.
LEGENDS FOR FIGURES

Figure 1. WS-50030

Chemical structure of WS-50030 [7-{4-[3-(1H-inden-3-yl)propyl]piperazin-1-yl}-1,3-benzoxazol-2(3H)-one].

Figure 2. The functional effects of WS-50030 on D2 receptors, hSERT and h5-HT1A

A) D2 receptor partial agonist activity of WS-50030: In a concentration-dependent manner, WS-50030 partially attenuated forskolin-induced accumulation of cAMP in CHO cells transfected with the D2 long receptor (EC50 = 0.38 nM, Emax = 30%). As a control, quinpirole at 1 µM completely blocked forskolin induced cAMP accumulation. Data were analyzed and plotted using Prism 3.0 (GraphPad) and represent mean ± SEM % of control (10 µM quinpirole) from n = 6 individual experiments.

D2 receptor partial antagonist activity of WS-50030: In a concentration-dependent manner, WS-50030 partially blocked quinpirole (1 µM)-induced blockade of forskolin-induced accumulation of cAMP in CHO cells transfected with the D2 long receptor (IC50 = 2.2 nM (pA2 10.7), Imax = 73%). Data were analyzed and plotted using Prism 3.0 (GraphPad) and represent mean ± SEM % of control (10 µM quinpirole) from n = 4 individual experiments. Symbols: square = WS-50030; triangle = WS-50030 + quinpirole.

B) hSERT uptake inhibition activity of WS-50030: In Jar cells expressing the hSERT, WS-50030 blocked [3H]5-HT uptake in a concentration dependent manner (IC50 = 56.4 nM). Data were analyzed and plotted using Prism 3.0 (GraphPad) and represent mean ±
SEM from \( n = 2 \) individual experiments. Symbols: square = WS-50030; circle = fluoxetine.

C) \( \text{h5-HT}_{1A} \) partial agonist activity of WS-50030: In cAMP accumulation assays using CHO cells transfected with the human serotonin \( \text{5-HT}_{1A} \) receptor, WS-50030 was found to be a partial agonist (\( \text{EC}_{50} = 67 \text{ nM}, \text{Emax} = 90\% \)). Curve fitting of the mean data were generated by a four-parameter logistic equation using Prism 5.0 (GraphPad).

**Figure 3. Acute and chronic dual-probe microdialysis in the mPFC**

Chronic treatment with WS-50030 (10 mg/kg, i.p.) significantly elevated extracellular levels of serotonin in the rat medial prefrontal cortex (Panel A) without altering dopamine levels in the medial prefrontal cortex of the same animals (Panel B). In animals treated chronically with vehicle, an acute challenge injection of WS-50030 (10 mg/kg, i.p.) resulted in a non-significant (\( p = 0.09 \)) trend to elevate cortical serotonin. *\( p < 0.05 \) compared to vehicle-treated animals. Data are means ± SEM. \( n = 3-5 \) per treatment group.

**Figure 4. Acute and chronic dual-probe microdialysis in the nucleus accumbens**

Neither acute nor chronic treatment with WS-50030 (10 mg/kg, i.p.) elevated extracellular levels of serotonin (Panel A) or dopamine (Panel B) in the rat medial prefrontal cortex or nucleus accumbens. Data are means ± SEM of area under the curve from 0 to 180 min post drug expressed as a percentage of baseline. \( n = 3-7 \) per treatment group.
**Figure 5. Antagonism of apomorphine-induced behaviors in mice**

A) Mean climbing scores (± SEM) are expressed as a percent of control values observed in vehicle-treated mice that received apomorphine. ID$_{50}$’s were calculated using nonlinear regression. WS-50030 (0.03 – 30 mg/kg i.p.) produced a dose-dependent decrease in apomorphine-induced (1 mg/kg s.c.) climbing [ID$_{50}$ = 0.51 mg/kg (95% CI, 0.39 – 0.67)]. $n$ = 12 for vehicle and 1 mg/kg groups, $n$ = 6 for all other treatment groups. Aripiprazole (0.1 – 1 mg/kg i.p.) produced a dose-dependent decrease in apomorphine-induced (1 mg/kg s.c.) climbing [ID$_{50}$ = 0.14 mg/kg (95% CI, 0.06 – 0.30)]. $n$ = 6 per treatment group. Symbols: square = WS-50030; diamond = aripiprazole.

**Figure 6. Cataleptogenic potential in mice**

Mean seconds spent in the catalepsy position (± SEM) for the time point at which peak catalepsy was observed are shown. Peak catalepsy following treatment with WS-50300 (0.3 – 10 mg/kg i.p.) was exhibited at the 90-minute test point. At 10 mg/kg, mice displayed a mean (± SEM) of 14.33 ± 7.29 seconds in the catalepsy position out of a maximum possible 60 seconds. Peak catalepsy following aripiprazole administration (0.1 – 3 mg/kg i.p.) was also exhibited at the 90-minute test point. At 3 mg/kg, mice displayed a mean of 30.33 ± 9.06 seconds in the catalepsy position out of a maximum possible 60 seconds. Severity of catalepsy is defined as follows: maintenance of awkward position for 20 sec or less = minimal to no catalepsy, 21-40 sec = moderate catalepsy, and 41-60 sec = high catalepsy. $n$ = 6 per treatment group. Symbols: square = WS-50030; diamond = aripiprazole.
Figure 7. Rotational behavior in unilaterally 6-OHDA – lesioned rats

Contralateral minus ipsilateral rotations recorded in the 15-min period occurring 45-60 minutes post dosing are displayed. Data was subjected to one-way ANOVA with subsequent post-hoc LSD test and are displayed as mean ± SEM. Apomorphine was administered s.c.; WS-50030 and aripiprazole were administered i.p. n = 5 vehicle, n = 16 apomorphine, n = 8 for all other treatment groups. *p < 0.05 vs. apomorphine.

Figure 8. Conditioned avoidance responding in rats

Data is expressed as mean number of trials (± SEM) in which the animals avoid shock (Panel A), escape shock (Panel B) and do not respond (within subject design, n = 7-8). WS-50030 significantly decreased avoidance and increased escape responding with no effect on the number of trials in which escape failures occurred (escape failures not shown). Doses of 3 and 10 mg/kg produced 17 and 42% reductions in avoidance responding, respectively [repeated measures ANOVA; F(2,14) = 8.82; *p = 0.003]. Aripiprazole significantly decreased avoidance and increased escape responding with no effect on the number of trials in which escape failures occurred (escape failures not shown) producing 5, 36 and 55% reductions in avoidance responding at 1, 3 and 10 mg/kg [repeated measures ANOVA; F(3,18) = 26.33; *p < 0.001], respectively. Solid black bars = WS-50030, solid gray bars = aripiprazole.
Figure 9. Olfactory bulbectomy-induced hyperactivity

A) Bulbectomized and sham-operated rats received WS-50030 (3 - 10 mg/kg, i.p.) or vehicle (i.p.) once daily for two weeks. Total distance (cm) moved in the 5-min period in vehicle-treated sham operated rats was compared to vehicle-treated bulbectomized rats using a t-test (#p<0.05) for each experiment. Subsequently, separate one-way ANOVAs compared the effect of dose of drug on total distance moved in sham and bulbectomized subjects, followed by Dunnett's post-hoc comparison to the vehicle treated group (*p<0.05). Data are mean (± SEM). n = 8-10 per group.

B) Bulbectomized and sham-operated received aripiprazole (1 - 5.6 mg/kg i.p.) or vehicle (i.p.) once daily for two weeks. Total distance (cm) moved in the 5-minute period in vehicle treated sham operated rats was compared to vehicle treated bulbectomized rats using a t-test (#p<0.05) for each experiment. Subsequently, separate one-way ANOVAs compared the effect of dose of drug on total distance moved in sham and bulbectomized subjects, followed by Dunnett's post-hoc comparison to the vehicle treated group (*p<0.05). Data are mean (± SEM). n = 8-10 per group.
Table 1. Catalepsy Induced by WS-50030 or Aripiprazole in Mice: Effect of Dose and Pretreatment Interval.

Data are means ± SEM in male CF-1 mice evaluated repeatedly at different intervals (min) following the administration of test compound intraperitoneally (n = 6 per dose level). Time spent (in seconds) with forelimbs positioned on an elevated bar was measured with a maximum cut-off of 60 seconds. Severity of catalepsy is defined as follows: maintenance of awkward position for 20 sec or less = minimal to no catalepsy, 21-40 sec = moderate catalepsy, and 41-60 sec = high catalepsy.

<table>
<thead>
<tr>
<th>Pretreatment (min)</th>
<th>Dose (mg/kg, i.p.)</th>
<th>0</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>WS-50030</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0 ± 0</td>
<td>0.5 ± 0.5</td>
<td>0.8 ± 0.5</td>
<td>4.7 ± 2.7</td>
<td>5.3 ± 5.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0 ± 0</td>
<td>1.3 ± 0.9</td>
<td>2.2 ± 1.5</td>
<td>3.0 ± 1.1</td>
<td>14.3 ± 7.3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>0 ± 0</td>
<td>3.5 ± 2.2</td>
<td>2.2 ± 1.0</td>
<td>13.0 ± 6.1*</td>
<td>10.3 ± 4.8*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Aripiprazole      |                    |      |      |      |      |      |      |
| 30                | 0 ± 0              | 0.8 ± 0.8 | 0 ± 0 | 2.3 ± 2.3 | 0.0 ± 0.0|
| 60                | 0.7 ± 0.7          | 0.5 ± 0.5 | 2.2 ± 1.4 | 12.2 ± 7.5 | 13.8 ± 7.4|
| 90                | 0 ± 0              | 1.5 ± 1.0 | 5.2 ± 3.6 | 16.8 ± 9.2* | 30.3 ± 9.0*|
| 120               | 0.3 ± 0.3          | 1.3 ± 1.0 | 7.0 ± 3.0 | 24.8 ± 9.8* | 25.2 ± 9.6*|

* p<0.05 vs. vehicle-treated mice at same time point by post-hoc LSD test.
Figure 1
Figure 2

A

\[
\text{% Control Response} \quad \log [\text{WS-50030}] \text{ (M)}
\]

- WS-50030
- WS-50030 + 1 \mu M Quinpirole

B

\[
\text{[^3]H}5\text{-HT uptake (cpm)} \quad \log [\text{Compound}] \text{ (M)}
\]

- WS-50030
- Fluoxetine

C

\[
\text{% FSK-Stimulated cAMP Production} \quad \log [\text{WS-50030}] \text{ (M)}
\]
Figure 3

A

B
Figure 4

A

B
Figure 5

![Graph showing dose-response curve for W5-50030 and aripiprazole.][1]

[1]: https://jpet.aspetjournals.org/content/10.1124/jpet.109.157388.full
Figure 6

The graph shows the relationship between dose (mg/kg, i.p.) and the seconds in catalepsy position for two drugs: WS-50030 and aripiprazole. The y-axis represents seconds in catalepsy position ranging from 0 to 60, and the x-axis represents dose (mg/kg, i.p.) ranging from 0.03 to 10.
Figure 7
Figure 8

A

![Bar chart showing number of trials at different doses of WS-50030 and aripiprazole.](chart_A.png)

B

![Bar chart showing number of trials at different doses of WS-50030 and aripiprazole.](chart_B.png)
Figure 9

A

B