JPET Fast Forward. Published on February 9, 2006 as DOI: 10.1124/jpet.105.097006

Title Page

Pharmacological and behavioral profile of ACP-103, a novel 5-HT_{2A} receptor inverse agonist

Kimberly E. Vanover, David M. Weiner, Malath Makhay[*], Isaac Veinbergs[$], Luis R. Gardell, Jelveh Lameh, Andria L. Del Tredici, Fabrice Piu, Hans H. Schiffer, Thomas R. Ott, Ethan S. Burstein, Allan K. Uldam[^], Mikkel B. Thygesen, Nathalie Schlienger, Carl Magnus Andersson[%], Thomas Y. Son, Scott C. Harvey[&], Susan B. Powell, Mark A. Geyer, Bo-Ragner Tolf, Mark R. Brann, and Robert E. Davis

ACADIA Pharmaceuticals Inc, 3911 Sorrento Valley Boulevard, San Diego, CA, 92121 (KEV, DMW, MM, IV, LRG, JL, ALDT, FP, HHS, TRO, ESB, AKU, MBT, NS, CMA, TYS, SCH, BRT, MRB, RED), and Department of Psychiatry, University of California San Diego, La Jolla, CA 92037 (SBP, MAG)

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Corresponding Author: Kimberly E. Vanover, Ph.D.

ACADIA Pharmaceuticals Inc.

3911 Sorrento Valley Boulevard

San Diego, CA, 92121

Phone (858) 558-2871

Fax (858) 558-2872

kvanover@acadia-pharm.com

Text Pages: 23

Tables: 3

Figures: 5

References: 35

Word Count: Abstract: 209

Introduction: 577

Discussion: 1,374

Abbreviations used: CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; NMDA, *N*-methyl-D-aspartate; PBS, phosphate buffered saline; PPI, prepulse inhibition; R-SAT®, Receptor Selection and Amplification Technology assay

Section: Neuropharmacology

Abstract

The *in vitro* and *in vivo* pharmacological properties of ACP-103 [*N*-(4-fluorophenylmethyl)-*N*-(1-methylpiperidin-4-yl)-*N'*-(4-(2-methylpropyloxy)phenylmethyl)carbamide (2*R*,3*R*)-dihydroxybutanedioate (2:1)] are presented. A potent 5-HT_{2A} receptor inverse agonist, ACP-103 competitively antagonized the binding of [^{3}H]-ketanserin to heterologously expressed human 5-HT_{2A} receptors with a mean pK_i of 9.3 in membranes and 9.70 in whole cells. ACP-103 displayed potent inverse agonist activity in the cell-based functional assay R-SAT, with a mean pIC_{50} of 8.7. ACP-103 demonstrated lesser affinity (mean pK_i 8.80, membranes, and 8.00, whole cells, as determined by radioligand binding) and potency as an inverse agonist (mean pIC_{50} 7.1 in R-SAT) at human 5-HT_{2C} receptors, and lacked affinity and functional activity at 5-HT_{2B} receptors, dopamine D_2 receptors, and other human monoaminergic receptors. Behaviorally, ACP-103 attenuated head twitch behavior (3 mg/kg, p.o.) and prepulse inhibition deficits (1 – 10 mg/kg, s.c.) induced by the 5-HT_{2A} receptor agonist (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) in rats and reduced the hyperactivity induced in mice by the *N*-methyl-D-aspartate receptor non-competitive antagonist, MK-801, (0.1 and 0.3 mg/kg, s.c.; 3 mg/kg, p.o.) consistent with a 5-HT_{2A} receptor mechanism of action *in vivo* and antipsychotic-like efficacy. ACP-103 demonstrated > 42.6% oral bioavailability in rats. Thus, ACP-103 is a potent, efficacious, orally active, 5-HT_{2A} receptor inverse agonist with a behavioral pharmacological profile consistent with utility as an antipsychotic agent.

JPET Fast Forward. Published on February 9, 2006 as DOI: 10.1124/jpet.105.097006
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Introduction

Most antipsychotic drugs have affinity for a variety of receptors including dopamine D_2 and serotonin 5-HT_{2A} receptors. The dopamine hypothesis of schizophrenia posits that antipsychotic efficacy is mediated by antagonism of dopamine D_2 receptors (Carlsson et al., 1999a; Snyder, 1976). Unfortunately, antagonism of D_2 receptors also causes profound motor, endocrine, and cognitive side effects, which can severely limit the clinical utility of compounds with this property. More recently, high affinity antagonism or inverse agonism at 5-HT_{2A} receptors has been postulated to mediate the efficacy and improved side-effect profile of atypical antipsychotics (Carlsson et al., 1999a; Leysen et al., 1978; Meltzer et al., 1989; Weiner et al., 2001). Such suggestions stimulated efforts to discover selective 5-HT_{2A} receptor antagonists or inverse agonists.

Recently, it was reported that nearly all antipsychotics are 5-HT_{2A} receptor inverse agonists, in that they can attenuate the basal constitutive signaling activity of this receptor, in contrast to neutral antagonists that can only block agonist-induced responses and lack negative intrinsic efficacy (Weiner et al., 2001). Accordingly, a high throughput 5-HT_{2A} receptor inverse agonist screen, followed by an effort in medicinal chemistry, was used to identify potent, selective, and highly efficacious 5-HT_{2A} receptor inverse agonists (Weiner et al., 2001). A prototype 5-HT_{2A} receptor inverse agonist, AC-90179 [2-(4-methoxyphenyl)-*N*-(4-methylbenzyl)-*N*-(1-methylpiperidin-4-yl)acetamide, hydrochloride], was shown to have efficacy in animal models for antipsychotic-like activity and lacked the side effects associated with other antipsychotic drugs, but demonstrated poor oral bioavailability (Vanover et al., 2004; Weiner et al., 2001). Additional synthetic efforts designed to improve metabolic stability resulted in the discovery of

ACP-103 [*N*-(4-fluorophenylmethyl)-*N*-(1-methylpiperidin-4-yl)-*N'*-(4-(2-methylpropyloxy)phenylmethyl) carbamide (2*R*,3*R*)-dihydroxybutanedioate (2:1) (Figure 1).

The pharmacological properties of the novel 5-HT_{2A} receptor inverse agonist ACP-103 were characterized and compared to other 5-HT_2 receptor ligands that have been, or currently are, in development for the treatment of human neuropsychiatric disease. *In vitro*, ACP-103 and comparator compounds were evaluated for potency and efficacy as inverse agonists at 5-HT_{2A} receptors. Selectivity of ACP-103 across monoaminergic and other receptors was assessed in radioligand binding and functional assays.

Behaviorally, ACP-103 was tested for antipsychotic-like efficacy by measuring the ability of ACP-103 to attenuate (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) -induced head twitches in rats. The induction of head twitches by the 5-HT_2 agonist DOI is thought to be mediated by brain 5-HT_{2A} receptors, and attenuation of DOI-induced head twitches is an activity shared by many atypical antipsychotic drugs (Wettstein et al., 1999). ACP-103 also was studied for its ability to restore a DOI-induced disruption of prepulse inhibition (PPI) of the acoustic startle response, an experimental paradigm that measures sensorimotor gating processes in rats. This test is based on the observation that a high intensity stimulus (e.g., an abrupt loud noise) causes a startle response, and that when a stimulus of lesser intensity (e.g., a quieter noise) occurs shortly before the high intensity stimulus, the startle response is diminished. This sensorimotor gating process is disrupted in schizophrenia patients (Braff et al., 1978). Atypical antipsychotic drugs that have high affinity for 5-HT_{2A} receptors have been shown to restore the DOI-induced PPI deficits in rats (Geyer et al., 2001). To extend the antipsychotic-like

behavioral profile beyond direct 5-HT_{2A} receptor interactions, ACP-103 was tested for its ability to attenuate hyperactivity induced by the noncompetitive *N*-methyl-D-aspartate (NMDA) antagonist MK-801 (dizocilpine). Blockade of NMDA antagonist-induced hyperactivity is predictive of antipsychotic-like efficacy (Freed et al., 1984). It was hypothesized that ACP-103 would attenuate DOI-induced head twitches and PPI deficits as well as MK-801-induced hyperactivity. Lastly, the pharmacokinetic profile of ACP-103 was evaluated in rats.

Methods

In Vitro Pharmacological Experiments

Receptor Cloning. Full-length sequence verified clones of each of the three human 5-HT_2 receptor subtypes were isolated and sub-cloned into a mammalian expression vector as previously described (Weiner et al., 2001). The 5-HT_{2C} receptor is naturally subject to RNA editing which alters the protein sequence of the receptor, generating a multitude of functionally distinct isoforms (Burns et al., 1997). Site directed mutagenesis was utilized to create the various 5-HT_{2C} receptor isoforms as previously described (Marion et al., 2004). All 5-HT_{2C} R-SAT inverse agonist experiments were performed with the constitutively active INI isoform, whereas radioligand-binding experiments utilized the VGV isoform (membranes) or the INI isoform (whole cells). The expression levels for the cells were as follows, 530 ± 264 fmole/mg protein (n = 18) for 5-HT_{2A}, 3251 ± 1457 fmole/mg protein (n = 20) for 5-HT_{2C}, and 277 ± 139 fmole/mg protein (n = 4) for 5-HT_{2B}.

Receptor Selection and Amplification Technology (R-SAT®). R-SAT assays were performed as previously described (Weiner et al., 2001), with the following modifications. Briefly, NIH-3T3 cells were grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (Hyclone; Logan, UT) and 1% penicillin/streptomycin/glutamine (Invitrogen; Carlsbad, CA). Cells were transfected in roller bottles for 18 h with the relevant G-protein coupled receptor gene and the gene for β-galactosidase. After transfection, cells were trypsinized, harvested, and frozen. Aliquots of frozen cell batches were thawed and tested for response to reference agonists and inverse agonists ensuring pharmacologically appropriate responses. To initiate an assay, cells were

thawed rapidly and prepared in DMEM media contained 0.4% calf serum (Hyclone), 30% UltraCulture (Biowhittaker; Rockland, ME), and 1% penicillin/streptomycin/glutamine (Invitrogen), and then added to half-area 96-well microtiter plates containing either test compounds or reference ligands. After five days in culture, media was removed from the wells, and the cells were incubated at room temperature in 200 µl of phosphate buffered saline (PBS), pH 7.4, with 3.5 mM o-nitrophenyl-ß-D-galactopyranoside (Sigma; St. Louis, MO) and 0.5% nonidet P-40 (Sigma). After 2-4 h the plates were read at 420 nm on a plate-reader (Bio-Tek; Winooski, VT).

Radioligand Binding. For the membrane binding, NIH 3T3 cells were grown to 70% confluence in 15 cm^2 dishes, and transfected with 10 µg of receptor plasmid DNA using Polyfect transfection reagent (Qiagen; Valencia, CA) according to manufacturer's protocols. Two days after transfection, cells expressing the desired serotonin receptor were homogenized in 20 mM Hepes / 10 mM EDTA and spun down at 11,000 *g* at 4° C for 30 min. The supernatant was discarded and the pellet resuspended in 20 mM Hepes / 1 mM EDTA and spun down at the same setting. The pellet was then resuspended in 20 mM Hepes / 0.5 mM EDTA and membranes were used for binding assays. Bradford analysis was used to determine total membrane protein. K_d and B_{max} values were derived from 12-point concentration experiments utilizing 1 nM [^{3}H]-ketanserin (Perkin Elmer Life Sciences, Boston, MA) for the 5-HT_{2A} receptor, and 3 nM [^{3}H]-mesulergine (Amersham Biosciences; Piscataway, NJ) for the 5-HT_{2B} and 5-HT_{2C} receptors. Membranes were incubated at room temperature for 3 h with various concentrations of test ligand, in the presence of a fixed concentration of radioligand. The suspension was filtered as explained below for whole cell binding, washed with ice cold buffer, dried, and radioactivity determined using Topcount (Packard, Shelton, CT).

For the whole cell binding, 6 million HEK 293T cells were plated in 10 cm dishes and transfected with 5 μg plasmid DNA using Ployfect (Qiagen) according to manufacturer's instructions. Two days after transfection, cells were harvested with 10 mM EDTA, washed and resuspended in binding buffer (1x DMEM with 0.1%BSA). 60,000 cells transfected with the $5HT_{2A}$ receptor or 20,000 cells transfected with the $5HT_{2C}$-INI receptor were incubated at 37 °C for 3 h in the presence of 5 nM radioligand ([^{3}H]-ketanserin for $5HT_{2A}$ receptors and [^{3}H]-mesulergine for $5HT_{2C}$-INI receptors) and varying concentrations of ligands (total volume 100 μl in a 96 well plate). Cells were filtered onto a 96 well GF/B filterplate (Packard Bioscience; Shelton, CT) and washed with 300 ml wash buffer (25 mM HEPES, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.25M NaCl) using a Filtermate 196 Harvester (Packard Instruments, Downers Grove, IL). The filter plates were dried under a heat lamp before addition of 50 μl of scintillation fluid to each well (Microscint 20, Packard, Shelton, CT). Plates were counted on a Topcount (Packard, Shelton, CT).

Separately, the hydrochloride salt form of ACP-103 (10 uM) was evaluated at MDS Pharma Services (Taipei, Taiwan) for activity in a broad screen of radioligand binding assays at 65 different receptors.

Behavioral Experiments

Animals and Apparatus. Male Sprague Dawley rats, male non-swiss albino (NSA) mice (Harlan; San Diego, CA) were used as subjects. Rats weighed 200- 300 g and mice weighed 20 – 30 g. Animals were housed 2/cage (rats), 8/cage (mice) in a room with controlled temperature

and a 12 h light:dark cycle. Water and standard rodent chow (Harlan Teklad; Madison, WI) were continuously available in the home cage.

The startle testing was performed as previously reported (Mansbach et al., 1988) in commercially available startle chambers (San Diego Instruments; San Diego, CA). Plastic locomotor activity cages (20 x 20 x 30 cm; AccuScan Instruments; Columbus, OH) were equipped with photocell beams for monitoring horizontal activity. Data were collected using Versamax computer software (AccuScan Instruments). Immediately prior to locomotor testing, mice were evaluated for myorelaxation/ataxia using a custom built apparatus consisting of a metal wire (2 mm diameter) suspended 25 cm above the bench top.

Procedure. For DOI head twitch experiments in rats, vehicle or a dose of ACP-103 was administered orally 120 min before DOI administration. DOI HCl (2.5 mg/kg, i.p.) was administered immediately before observations. After injection of DOI, each rat was placed into an empty cage and observed. Latency to the first head twitch and the number of head twitches occurring over 5 min were recorded. Each rat was used only once with 8 – 16 rats per dose group.

The PPI of acoustic startle response experiments were conducted in rats at the University of California San Diego. Rats first were matched for levels of acoustic startle magnitude and PPI and assigned to the drug treatment groups. Two days after the baseline session, rats were tested again in the startle chambers to assess the effects of ACP-103 and the 5-HT_{2A} receptor antagonist MDL-100,151 (racemic M100907, as a positive control) on DOI-induced disruptions of PPI. The session used to assess drug effects consisted of a 5 min acclimation period in the chamber

with a constant background noise (65 dB), followed by 62 presentations of acoustic stimuli and 100 subsequent presentations of air puffs to measure acoustic and tactile startle responses, respectively. The 62 acoustic trials consisted of: twenty-four 40-ms presentations of a 120 dB broadband pulse, ten 20-ms presentations of each prepulse intensity (68, 71, 77 dB) 100 ms prior to a 40-ms presentation of a 120 dB broadband pulse, and 8 NOSTIM trials in which no acoustic pulse was delivered in order to assess general motor activation in the rats. Five 120-dB trials, which were not included in the calculation of PPI values, were presented at the beginning of the test session to achieve a relatively stable level of startle reactivity for the reminder of the session (based on the observation that the most rapid habituation of the startle reflex occurs within the first few presentations of the startling stimulus (Geyer et al., 1990). Another 5 120-dB trials, also not included in the calculation of PPI values, were presented at the end of the test session to assess startle habituation. At the end of the 62 acoustic trials, there was a 1 min exposure to a constant 65 dB background noise followed by 100, 30-ms presentations of 40 psi air puffs. Acoustic trials were presented in a pseudo-random order with an average inter-trial interval of 15 s (range 7-23 s), and air puffs were presented every 10 s.

Thirty min before being placed in the startle apparatus, rats were treated with saline (s.c.), MDL-100,151 (1.0 mg/kg, s.c.), or one of three doses of ACP-103 (1.0, 3.0, 10.0 mg/kg, s.c.). Five min after the pretreatment, rats were administered either DOI HCl (0.5 mg/kg, s.c.) or 0.9% saline (s.c.). The acoustic startle session lasted approximately 37min. After one week, rats were tested again in the same acoustic/tactile startle session in the exact order and at the same time as the previous week. The same pretreatment drug or vehicle was administered, and rats were crossed over to receive the treatment opposite to that they received the previous week (e.g, DOI HCl for week 1, 0.9% saline for week 2).

NSA mice were used for locomotor activity experiments. For determination of spontaneous activity, ACP-103 was administered alone (s.c. 60 min before session start or p.o. 60 min before session start). For hyperactivity experiments, mice were treated with 0.3 mg/kg MK-801 (dizocilpine) i.p. 15 min pre-session (the peak dose for producing hyperactivity in an inverted-U dose-effect curve as determined in pilot experiments) in combination with vehicle or ACP-103. Motor activity data were collected during a 15 min session in a lit room. Mice had no prior exposure to the motor cages. Immediately prior to placing the mice in the locomotor chambers, effects on myorelaxation/ataxia were determined by placing each mouse's forepaws in contact with a horizontal wire while holding the mouse by the base of the tail. Mice were required to bring at least one hindpaw in contact with the wire within 10 s to be scored as a "pass" and failure to do so was considered ataxic. Each dose or dose combination was tested in a separate group of mice (n = 8).

Data Analysis. All pharmacological data were analyzed using Prism 4 for Windows (GraphPad; San Diego, CA), and are reported as averaged values derived from multiple experimental replicates (n) ± standard deviation. Inverse agonist potencies are reported as pIC_{50} values, whereas affinity measurements derived from radioligand binding experiments are reported as pK_i values that were corrected according to the following formula ($Ki = (IC_{50})/(1+[L]/Kd)$, in which [L] is the concentration of the test compound and Kd is the dissociation constant of the radioligand).

For DOI head twitch experiments, latency (s) to the first head twitch and the number of head twitches were recorded and averaged across animals in a group. For locomotor experiments,

distance traveled (cm) was calculated and averaged across animals in a group. Standard errors of the means were calculated. An analysis of variance and post-hoc Dunnett's t-test comparisons to vehicle control were conducted for each dose-response function.

For PPI experiments, startle magnitude was quantified from the PULSE-ALONE (120 dB) trials and the PREPULSE + PULSE trials. Percent prepulse inhibition (% PPI) for the three prepulse intensities was calculated according to the following formula: % PPI = 100 – {[(startle response for PREPULSE + PULSE trial)/(startle response for PULSE-ALONE trial)] x 100}. A three-way repeated measures analysis of variance with pretreatment as a between subject factor and treatment and prepulse intensity as within subjects factors was performed on the %PPI data. A separate three-way repeated measures analysis of variance was performed on the %PPI data from the MDL-100,151 comparison. Since there were pretreatment by treatment interactions in both analyses of variance, subsequent two-way repeated measures analyses of variance were performed on the DOI and vehicle-treated groups. The alpha level was adjusted to 0.025 for the two-way pair wise repeated measures analyses of variance, since one within subject factor was dropped from the analysis. The same analyses were performed on the acoustic startle data (startle to the PULSE alone trials) with pretreatment as a between subjects factor and treatment and time as within subjects factors.

Pharmacokinetics

The oral bioavailability of a hydrochloride salt form of ACP-103 was determined at Absorption Systems (Exton, PA) in male Sprague-Dawley rats (Hilltop Lab Animals, Scottdale, PA) weighing between 280 and 310 g. Samples were withdrawn at the following time points: for IV, 0 (pre-dose), 2, 5, 30, 60, 120, 240, 360, 480 minutes; for Oral: 0 (pre-dose), 5, 30, 60, 120, 240,

360, 480 minutes. Approximately 0.50 to 0.75 mL of whole blood was collected by venipuncture from the jugular vein. The blood was transferred to heparinized tubes and placed on ice until centrifuged. Following centrifugation the plasma was placed on ice until frozen at –70°C prior to shipment to the analytical laboratory.

To determine the concentration of ACP-103 (hydrochloride form) in rat plasma samples, standards were prepared with rat plasma in sodium citrate obtained from Lampire Biological Laboratories (Lot #062631954) to contain 1000, 300, 100, 30, 10, 3, 1 and 0.3 ng/mL of ACP-103 (hydrochloride). Plasma standards were treated identically to the plasma samples. Plasma samples were prepared by protein precipitation. A 50 µL aliquot of plasma was combined with 150 µL of acetonitrile. The mixture was then spiked with 10 µL of internal standard, vortexed, and centrifuged for 10 minutes at 10000 rpm. The supernatant (180 µL) was then evaporated to dryness and reconstituted with 100 µL of 66% acetonitrile and 44% deionized water. After vortexing and a second centrifugation at 10000 rpm for 10 minutes, the sample was injected into the LC/MS/MS. The concentration of the internal standard solution was 2 µg/mL of AC-90179. Pharmacokinetic analysis was performed on the average plasma concentration for each time point. The data was subjected to non-compartmental analysis using the pharmacokinetic program WinNonlin v. 3.1 (2).

Drugs. ACP-103, eplivanserin, and M100907 [R(+)-alpha-(2,3-dimethoxyphenyl)-1- [2-(4-fluorophenylethyl)]-4-piperidine-methanol] and its racemate MDL-100,151 were synthesized by ACADIA Pharmaceuticals Inc. Ritanserin, haloperidol, MK-801 (dizocilpine), and (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) were obtained from Sigma (St. Louis, MO),

and clozapine was obtained from Tocris (Ellisville, MO). For the behavioral experiments, ACP-103, MDL-100,151, DOI, and MK-801 were dissolved in 0.9% saline, and haloperidol was dissolved in 10% Tween80 (90% water). All compounds were administered in a volume of 0.1 ml per 10 g body weight and doses were calculated based on the weight of the salt. For the contracted radioligand binding experiments at MDS Pharma Services, a hydrochloride salt form of ACP-103 was used. For the pharmacokinetic experiment, a solution of the hydrochloride salt was prepared using 5% dextrose (w/v) in aqueous PEG-400 (4:1 v/v).

Results

Pharmacology Experiments

Functional Antagonism and Inverse Agonism

Transient expression of the three human 5-HT_2 receptors in NIH3T3 cells as part of the R-SAT assay generates robust and pharmacologically appropriate receptor-mediated responses (Figure 2, Table 1). Each of these receptors displays detectable basal levels of constitutive activity that allows for the characterization of the potency and efficacy of 5-HT_2 receptor inverse agonists. Figure 2 displays representative concentration-response curves for the non-selective 5-HT_2 receptor inverse agonist ritanserin, as well as those observed for ACP-103 (Figure 1), the 5-HT_{2A} receptor selective compound M100907 (Kehne et al., 1996), eplivanserin (Rinaldi-Carmona, et al., 1992), and the atypical antipsychotic clozapine, at 5-HT_{2A} (Panel A) and 5-HT_{2C} (Panel B) receptors. Each of these compounds was found to be potent (pIC_{50}'s greater than 8.0) inverse agonists at the 5-HT_{2A} receptor (Fig 2, Table 1). The rank order of potency of these compounds at the 5-HT_{2A} receptor was ritanserin > M100907 = eplivanserin = ACP-103 > clozapine. As expected, all compounds were found to competitively antagonize serotonin-induced 5-HT_{2A} receptor agonist responses in R-SAT (data not shown). Regarding efficacy, all compounds displayed full inverse agonist efficacy (75% or greater) relative to ritanserin (Table 1). In contrast, only ritanserin and clozapine displayed potent inverse agonist activity at the human 5-HT_{2B} receptor (Table 1). The inverse agonist potencies of these compounds at the 5-HT_{2C} receptor were significantly less than those observed at 5-HT_{2A} receptors. As depicted in Figure 2B, ritanserin, ACP-103, M100907, and clozapine display inverse agonist activity, however, eplivanserin was found to lack negative intrinsic efficacy. The rank order of potency at the 5-HT_{2C} receptor was ritanserin > ACP-103 > clozapine > M100907 (Table 1). Each of these

compounds displayed full inverse agonist efficacy (Table 1). All five compounds were found to competitively antagonize serotonin-induced 5-HT_{2C} receptor agonist responses in R-SAT (data not shown). Finally, ACP-103 was tested against 31 of the total 36 human monoaminergic receptors, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_3, 5-HT_{6A}, and 5-HT_{7A} and was found to lack functional activity (pEC_{50}'s or pK_i's less than 6.0) at the 31 monoaminergic receptors and an additional 5 non-monoaminergic (somatostatin SST_1, SST_2, SST_3, SST_4, and SST_5) receptor subtypes when tested as an agonist, inverse agonist, or a competitive antagonist in R-SAT or other functional assays (data not shown).

Radioligand Binding

To further characterize the pharmacology of ACP-103 at human 5-HT_2 receptors, affinity determinations were made using radioligand binding techniques in membranes and whole cells (Table 1). All five compounds displayed high affinity for both receptors. The rank order of affinity for the 5-HT_{2A} receptor was M100907 = eplivanserin = ritanserin > ACP-103 = clozapine for membrane binding and M100907 = ACP 103 > eplivanserin > ritanserin > clozapine for whole cell binding. The rank order of affinity at the 5-HT_{2C} receptor was ritanserin > clozapine = eplivanserin = ACP-103 > M100907 for membrane binding and ACP-103 > ritanserin > M100907 = clozapine = eplivanserin for whole cell binding. The observed affinity values were similar to the observed functional potencies as inverse agonists. Eplivanserin was found to possess high affinity for the 5-HT_{2C} receptor (Table 1), to competitively antagonize serotonin-induced functional responses (data not shown), yet lack inverse agonist activity (Figure 2B, and Table 1), consistent with its designation as a competitive (neutral) antagonist at human 5-HT_{2C} receptors. ACP-103 was found to lack affinity (pK_i less than 6.0) for the human dopamine D_2 receptor as determined by its inability to competitively displace spiperone or

raclopride in radioligand binding assays (data not shown). Finally, a broad receptor binding profiling screen conducted at MDS Pharma Services that included a range of channels, enzymes and receptors, confirmed that out of 65 assays, the only high affinity ($pK_i > 7.0$) interaction of ACP-103 was at 5-HT_2 receptors (Table 2).

Behavioral Experiments

DOI (2.5 mg/kg, i.p.) with a vehicle pretreatment induced an average of 5.2 head twitches (± 0.9 S.E.M.) with the latency to head twitch onset of 183 s (± 25.3). ACP-103 (n = 8-16/group) attenuated DOI-induced head twitches at a dose of 3 mg/kg p.o. after a two h pretreatment (Figure 3). The analysis of variance for number of head twitches [$F(5,71) = 2.795$, $p = 0.02$] was statistically significant and for latency [$F(5,71) = 1.970$, $p = 0.09$] was a statistical trend. The number of head twitches after DOI plus 3 mg/kg ACP-103 was 0.75 ± 0.27. The latency to first head twitch after DOI plus 3 mg/kg ACP-103 was 264.9 ± 11.4 s. No effect on head twitch behavior was seen after treatment with ACP-103 over the same dose range alone (data not shown).

The 3-way repeated measures analysis of variance on the %PPI data from the ACP-103 groups revealed a marginal effect of DOI [$F(1,37) = 3.86$, $p = 0.06$] and a DOI by ACP-103 interaction [$F(3,37) = 3.72$, $p < 0.05$; Figure 4]. DOI disrupted PPI, and ACP-103 was effective in restoring PPI even at the lowest dose tested. ACP-103 did not affect PPI on its own on the %PPI measure [$F(3,37) = 2.31$, n.s.]. Since there was a significant pretreatment by treatment interaction, pair-wise 2-way repeated measures analyses of variance were conducted on the saline- and DOI-treated groups separately. In the saline-treated rats, there was no effect of ACP-103 ($p > 0.025$) on %PPI. In the DOI-treated group, there were significant effects of ACP-103 [$F(3,37) = 4.71$, p

< 0.01] on %PPI. The 2-way repeated measures analysis of variance on startle magnitude from the ACP-103 groups revealed a significant effect of DOI [$F(1,37) = 22.18$, $p < 0.01$] on startle magnitude, but no effect of ACP-103 [$F(3,37) = 1.06$, n.s.] and no DOI by ACP-103 interaction [$F < 1$, n.s.]. The 3-way repeated measures analysis of variance on %PPI from the MDL-100,151 group revealed a significant effect of DOI [$F(1,20) = 12.07$, $p < 0.01$] and a significant interaction between DOI and MDL-100,151 [$F(1,20)=29.39$, $p<0.001$] with MDL-100,581 reversing the PPI disruption produced by DOI. There was no effect of MDL-100,151 on PPI on its own [$F(1,20) = 2.89$, n.s.]. The 2-way repeated measures analysis of variance on startle magnitude from the MDL-100,151 group revealed significant effects of DOI [$F(1,20) = 15.49$, $p < 0.001$] and MDL-100,151 [$F(1,20)=4.84$, $p<0.05$] on startle magnitude, but no DOI by MDL-100,151 interaction [$F(1,20)=3.11$, n.s.].

ACP-103 significantly attenuated MK-801-induced hyperactivity in mice at doses of 0.1 and 0.3 mg/kg s.c. [$F(7,63) = 6.010$, $p < 0.0001$; Figure 5A], consistent with an antipsychotic-like effect. ACP-103 also reduced spontaneous activity at similar doses when administered s.c. [$F(7,63) = 2.741$, $p = 0.0161$]. All mice were able to pull their hind paws up to the horizontal wire after administration of ACP-103, tested just before placing the mice in the locomotor chamber. In a separate experiment, 7 of 8 mice were able to pull their hind paws up to the horizontal wire after administration of 100 mg/kg s.c. ACP-103 alone, indicating a lack of ataxia even at a very high dose.

ACP-103 significantly attenuated MK-801 hyperactivity at dose of 3 mg/kg p.o. [$F(4,39) = 4.681$, $p = 0.0039$; Figure 5B], consistent with oral efficacy. ACP-103 did not reduce spontaneous activity at any dose administered orally [$F(4,39) = 0.9275$, $p = 0.4592$]. All of the

mice “passed” the horizontal wire test at all doses after oral administration of ACP-103 alone, indicating none of the mice exhibited ataxia.

Pharmacokinetics

ACP-103 exhibits a volume of distribution much larger than the total body water of the rat indicating extensive partitioning into fatty tissues (Table 3). The high systemic clearance was consistent with the 59-minute half-life observed. Considering that the AUC after 10 mg/kg is truncated, the oral bioavailability at this dose is higher than 42.6%. After oral administration, ACP-103 showed the elimination phase of the compound with maximum plasma concentrations occurring 60 to 120 minutes post-dosing.

Discussion

A variety of compounds were characterized based on their 5-HT_2 receptor affinity, as determined by competitive radioligand binding assays, and their potency and efficacy as 5-HT_2 receptor inverse agonists using the cell-based functional assay R-SAT. Not surprisingly, all five compounds potently displaced the binding of [^{3}H]-ketanserin to 5-HT_{2A} receptors in both membranes and whole cells, where the absolute and rank order of affinities are similar to those reported previously by our group and others (Bonhaus et al., 1999; Leysen et al., 1985; Kehne et al., 1996; Rinaldi- Carmona et al., 1992; Weiner et al., 2001). Interestingly, the absolute affinities for most compounds were higher in the membrane preparation than in whole cells. Only ritanserin and clozapine had appreciable affinity for the 5-HT_{2B} receptor, consistent with prior reports (Bonhaus et al., 1999; Weiner et al., 2001). In contrast, all compounds competitively antagonized the binding of [^{3}H]-mesulergine to the VGV isoform of the human 5-HT_{2C} receptor in membranes and the INI isoform in whole cells. The affinities observed at the 5-HT_{2C} receptor for ritanserin were similar to those observed by Bonhaus and colleagues (1999). The whole cell binding affinity for clozapine at the 5-HT_{2C} receptor was similar to that previously reported (Canton et al., 1990; Herrick-Davis et al., 2000; Roth et al., 1992), whereas our observed 5-HT_{2C} receptor affinity for clozapine in membranes was about 100-fold lower. The affinity observed for M100907 was slightly higher than previously described (Kehne et al., 1996). Receptor selectivity for 5-HT_{2A} receptors over 5-HT_{2C} receptors depends on the assay, but rank orders for selectivity of 5-HT_{2A} receptors were generally in agreement across the assays. For all compounds, the physiologically relevant assays (the R-SAT functional assay and the whole cell binding assay) showed greater absolute selectivity than the membrane binding assay.

Overall, M100907 displayed the highest 5-HT_{2A}/5-HT_{2C} receptor selectivity amongst this group of compounds, where ACP-103 displayed a 5-HT_{2A}/5-HT_{2C} receptor affinity ratio similar to clozapine. The 5-HT_{2A}/5-HT_{2C} receptor selectivity profiles of ACP-103 and clozapine were similar to that of olanzapine published previously but, in contrast to ACP-103, olanzapine showed similar activity at 5-HT_{2B} receptors as clozapine (Weiner et al., 2001). Risperidone was more potent at 5-HT_{2A} receptors than at 5-HT_{2B} receptors and showed no activity at 5-HT_{2C} receptors (Weiner et al., 2001).

Interestingly, all of the compounds tested demonstrated inverse agonism or negative intrinsic activity at 5-HT_{2A} receptors. Without potent neutral antagonists to test for comparison, it is unclear whether functional differences between inverse agonism and neutral antagonism can be demonstrated *in vivo*. Further, all antispychotic drugs with 5-HT_{2A} receptor affinity are inverse agonists (Weiner, et al., 2001). Whether inverse agonism is necessary for antipsychotic efficacy or not remains uncertain. Given that ACP-103 shares the 5-HT2A receptor inverse agonism with effective antipsychotic drugs, ACP-103 is likely to share any efficacy that is imparted by such a mechanism.

Of the compounds tested, only eplivanserin lacked negative intrinsic activity at 5-HT_{2C} receptors, a property shared by a small number of known antipsychotics (Weiner et al., 2001). The clinical significance of 5-HT_{2C} receptor inverse agonism is currently unknown. The expression of 5-HT_{2C} receptors in dopaminergic neurons in the CNS (Eberle-Wang et al., 1997) and the neurochemical effects of 5-HT_{2C} receptor blockade (Di Matteo et al., 2001) suggest a utility in modifying dopaminergic function, a property that may be advantageous in the treatment of psychosis (Herrick-Davis et al., 2000), Parkinson's disease (Eberle-Wang et al., 1996), affective

disorders (Cremers et al., 2004), and sleep (Frank et al., 2002). Given the relative selectivity for ACP-103 at 5-HT_{2A} receptors over 5-HT_{2C} receptors, it is reasonable to suggest that ACP-103 might show selective blockade for 5-HT_{2A} receptors at low doses, whereas blockade of both receptor subtypes may be possible at higher doses. If the drug has a high degree of safety, the potential therapeutic benefits of 5-HT_{2C} receptor inverse agonism, in addition to 5-HT_{2A} receptor inverse agonism, may be obtained by treatment with ACP-103. The present experiments do not address the issue of receptor selectivity *in vivo*. The challenges of doing so are emphasized by the lack of availability of selective 5-HT_{2A} or 5-HT_{2C} receptor agonists that can be used as tools in conjunction with ACP-103 to understand the *in vivo* selectivity of ACP-103. Furthermore, the lack of commercially available selective radioligands for 5-HT_{2A} or 5-HT_{2C} receptors does not allow for the demonstration of selectivity of this compound *ex vivo*. Thus, additional nonclinical pharmacology experiments as well as the clinical test of the hypotheses are warranted, but not straightforward due to the lack of appropriate tools and are outside of the scope of the present study.

To determine the potential *in vivo* antipsychotic-like activity, ACP-103 was evaluated in several animal models that may predict antipsychotic drug efficacy in humans. ACP-103 potently and robustly attenuated DOI-induced head twitches in rats, with a minimum effective oral dose of 3.0 mg/kg. Interestingly, though, this effect was not seen at a higher dose. Whether this was due to other activity, perhaps 5-HT_{2C}, or just due to variability at the higher dose is unclear. ACP-103 also reversed DOI induced PPI deficits in rats at doses between 1.0-10.0 mg/kg. These doses of ACP-103 did not alter the basal startle response. The reversals of the DOI-induced PPI deficits and head twitches demonstrate that ACP-103 can inhibit the actions of a direct 5-HT_{2A} receptor agonist, consistent with a 5-HT_{2A} mechanism of action *in vivo*. To extend the antipsychotic-like

behavioral profile beyond direct 5-HT_{2A} receptor interactions, ACP-103 attenuated hyperactivity induced by the noncompetitive NMDA antagonist MK-801, with a minimum effective dose of 0.1 mg/kg s.c. or 3 mg/kg p.o. These effects are consistent with previous reports that other 5-HT_{2A} receptor antagonists and inverse agonists, as well as atypical antipsychotics, have been shown to reduce MK-801- and phencyclidine-induced behavioral effects (Carlsson et al., 1999b; Freed et al., 1984; Vanover et al., 2004; Weiner et al., 2001).

Due to the selectivity at 5-HT_{2A} receptors, ACP-103 was predicted to have an improved side effect profile relative to other antipsychotic drugs. Although ACP-103 decreased spontaneous locomotor behavior after s.c. administration, ACP-103 did not exhibit any myorelaxant or ataxic effects in those same mice as measured by the horizontal wire test and ACP-103 did not reduce spontaneous locomotion at efficacious doses after oral administration. These results would predict less sedation by ACP-103 relative to other antipsychotic drugs in humans.

Clinical data from investigations of 5-HT_{2A} receptor selective compounds, including M100907 (Potkin et al., 2001) and eplivanserin (Meltzer et al., 2004), suggest that they are clinically efficacious compared to placebo in the treatment of acute exacerbation of schizophrenia. In addition, 5-HT_{2A} receptor inverse agonists may find clinical utility as combination therapy with existing antipsychotic drugs. Given that dopamine D_2 receptor antagonism causes extrapyramidal motor side effects and tardive dyskinesia, many antipsychotic drugs are contraindicated for use in sensitive populations such as Parkinson's disease patients. In fact, the low doses of clozapine that have been shown to be effective against treatment-induced psychosis, and tolerated motorically in Parkinson's disease (The French Clozapine Study Group, 1999; The Parkinson Study Group, 1999) are thought to produce high levels of 5-HT_{2A} receptor occupancy

and little D_2 receptor occupancy (Meltzer et al., 1995; Nordstrom et al., 1993). These observations suggest that selective 5-HT_{2A} receptor inverse agonists may be therapeutically beneficial for treatment-induced psychosis in Parkinson's disease (Weiner et al., 2003).

ACP-103 was orally bioavailable in rats. The high oral bioavailability at 10 mg/kg, p.o., appears to be consistent with the behavioral efficacy observed after oral administration of ACP-103 in the animal models. A high volume of distribution and high clearance for ACP-103 also was observed in the pharmacokinetic experiment.

ACP-103 shows antipsychotic-like efficacy in animal models consistent with 5-HT_{2A} receptor mediation. The behavioral effects of ACP-103 are similar to those observed with clozapine (Vanover et al., 2004). Since mice treated with ACP-103 were not sedated or ataxic, ACP-103 is unlikely to exacerbate the motor symptoms in schizophrenic or more susceptible populations such as Parkinson's disease patients. This profile differs from other antipsychotic drugs used to treat psychosis in Parkinson's disease, such as olanzapine, which exacerbate motor symptoms (Breier et al., 2002). Taken together, ACP-103 may address the need for an orally bioavailable and selective 5-HT_{2A} receptor inverse agonist for the treatment of psychosis in Parkinson's disease as well as in schizophrenia.

Acknowledgements

The authors would like to thank the following for their excellent technical assistance: G. Anderson, K. Duncan, D. Hubbard, R. Johnson, K. Schurb, R. Zamora, and M. Buell. Mark Geyer holds an equity interest in San Diego Instruments.

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Footnotes

[*]Current address for M. Makhay: TorreyPines Therapeutics, 11085 N. Torrey Pines Road, Suite 300, La Jolla, CA 92037.

[$]Current address for I. Veinbergs: One Kendall Square, Bldg. 100, Cambridge, MA 02139.

[^]Current address for A. Uldam: Aeblehaven 58, DK-3500 Vaerloese, Denmark.

[%] Current address for C.M. Andersson: Chemistry Department, KaroBio AB, NOVUM, S-141 57 Huddinge, Sweden.

[&]Current address for S. Harvey: Sharp Memorial Hospital, San Diego, CA, 92123.

Figure Legends

Figure 1. Molecular structure of ACP-103. *N*-(4-Fluorophenylmethyl)-*N*-(1-methylpiperidin-4-yl)-*N'*-(4-(2-methylpropyloxy)phenylmethyl)carbamide (2*R*,3*R*)-dihydroxybutanedioate (2:1).

Figure 2. Inverse agonism of ACP-103 at human 5-HT_{2A} and 5-HT_{2C} receptors. Representative concentration-response curves for ACP-103 and various 5-HT_2 compounds as inverse agonists at the human 5-HT_{2A} (Panel A) and 5-HT_{2C} (Panel B) receptors, as determined by R-SAT assays, are reported. Compounds were tested at 8 different concentrations where each point was assayed in duplicate, with error bars depicting the standard error. Data are reported as percent inhibition of basal responses, where -100% represents the maximal response obtained for the reference inverse agonist ritanserin. Compounds tested include ritanserin (filled circles), ACP-103 (filled squares with dashed lines), M100907 (filled diamonds), eplivanserin (filled triangles) and clozapine (open circles). Observed 5-HT_{2A} receptor inverse agonist potencies (pIC_{50}'s) in the reported experiments (Panel A) were ritanserin (10.1), ACP-103 (8.9), M100907 (9.2), eplivanserin (9.1), and clozapine (8.3). Observed 5-HT_{2c} receptor inverse agonist potencies (pIC_{50}'s) in the reported experiments (Panel B) were ritanserin (8.4), ACP-103 (7.3), M100907 (6.3), eplivanserin (no activity), and clozapine (7.0).

Figure 3. Attenuation of DOI-induced head twitches by ACP-103. DOI (2.5 mg/kg i.p.) was administered in combination with vehicle (veh) or various doses of ACP-103. Panel A shows the number of head twitches produced by DOI as a function of ACP-103

dose. Panel B shows the latency in seconds to the first head twitch as a function of ACP-103 dose. Each bar represents the mean (+ standard error of the mean) from a separate group of animals (n = 6/dose). Asterisks indicate statistical significance ($p < 0.05$) compared to DOI + vehicle.

Figure 4. Attenuation of DOI-induced PPI by ACP-103. DOI (0.5 mg/kg s.c.) was administered in combination with vehicle (veh), various doses of ACP-103, or 1.0 mg/kg MDL-100,151 (racemic M100907), s.c. %PPI is shown as a function of ACP-103 dose (+ standard error of the mean). The insert shows that ACP-103 had no effect on basal startle response. Each bar represents the mean (+ standard error of the mean). Asterisks indicate statistical significance ($p < 0.05$) compared to DOI + vehicle.

Figure 5. The effects of ACP-103 on spontaneous locomotion and MK-801-induced hyperactivity in mice. Distance traveled is shown as a function of ACP-103 dose. Panel A shows the effects of ACP-103 after s.c. administration. Panel B shows the effects of ACP-103 after p.o. administration. Each point represents the mean and standard error of a separate group of mice (n = 8/group). Open squares represent spontaneous locomotion whereas filled squares represent MK-801 hyperactivity. Asterisks indicate statistical significance ($p < 0.05$) compared to respective vehicle control.

Table 1. Pharmacological activity of ACP-103 at human 5-HT_{2A} and 5-HT_{2C} receptors. Table 1 reports the potencies and inverse agonist efficacies (relative to ritanserin) of ACP-103, eplivanserin, M100907, ritanserin, and clozapine at human 5-HT_2 receptors determined by R-SAT assays, and their respective receptor affinities as determined by radioligand binding assays in membranes and in whole cells. Inverse agonist potencies are reported as average pIC_{50} values ± standard deviation. Inverse agonist efficacies are reported as percentage (%) inhibition relative to that observed for ritanserin (100%). Affinity determinations are reported as average pK_i values ± standard deviation. Abbreviations used: n denotes the number of experiments performed, ND denotes not determined, and NA denotes no activity (< 20% inhibition).

	R-SAT Functional Data								
Receptor	5-HT_{2A}			5-HT_{2B}			5-HT_{2C}		
Compound	n	pIC_{50}	% Inhibition	n	pIC_{50}	% Inhibition	n	pIC_{50}	% Inhibition
Ritanserin	25	10.16±0.09	100%	2	8.6±0	99±0%	8	8.35±0.16	100%
M100907	4	8.94±0.14	101±2%	2	NA	NA	4	6.27±0.15	76±5%
Eplivanserin	2	8.98±0.38	87±7%	2	ND	35±12%	6	NA	NA
ACP-103	19	8.73±0.3	93±6%	3	ND	NA	17	7.04±0.15	110±3%
Clozapine	5	7.87±0.16	95±6%	5	7.00±0.5	100%	3	6.70±0.23	88±5%

	Radioligand Binding Data							
Receptor	Membrane Binding pK_i				Whole Cell Binding pK_i			
Compound	n	5-HT_{2A}	n	5-HT_{2C}	n	5-HT_{2A}	n	5-HT_{2C}
Ritanserin	3	10.08±0.14	4	9.36±0.12	7	8.88±0.14	9	7.61±0.12
M100907	4	10.52±0.08	4	8.42±0.09	4	9.76±0.10	6	6.35±0.18
Eplivanserin	3	10.34±0.17	5	8.87±0.17	2	9.40±0.10	3	6.13±0.29
ACP-103	3	9.3±0.14	2	8.80±0.07	6	9.70±0.19	7	8.00±0.14
Clozapine	3	9.26±0.33	3	8.88±0.25	3	8.23±0.10	3	6.16±0.22

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Table 2. Broad screen receptor profiling for ACP-103 in radioligand binding. If ACP-103 (10 μM) demonstrated greater than 50% inhibition in receptor binding, the percent inhibition is listed in the table. The IC_{50} and K_i were only determined at those receptors for which ACP-103 (10 μM) demonstrated 100% inhibition. ACP-103 (10 μM) demonstrated less than 50% inhibition in receptor binding assays at the following receptors: Adenosine A_1, Adensosine A_{2A}, Adenosine A_{2B}, Adrenergic α_{1A}, Adrenergic α_{1B}, Adrenergic α_{1D}, Adrenergic α_{2A}, Adrenergic α_{2B}, Adrenergic β_2, Bradykinin B_1, Bradykinin B_2, Calcium Channel Type N, Dopamine D_1, Dopamine D_{2L}, Dopamine $D_{4,2}$, Dopamine Transporter, Endothelin ET_A, Endothelin ET_B, Epidermal Growth Factor (EGF), Estrogen ERα, GABA Transporter, $GABA_A$ Agonist Site, $GABA_A$ Benzodiazepine Central, $GABA_B$, Glucocorticoid, Glutamate Kainate, Glutamate NMDA Agonism, Glutamate NMDA Glycine, Glutamate NMDA Phencyclidine, Histamine H_1 Central, Histamine H_2, Histamine H_3, Imidazoline I_2 Central, Interleukin IL-1α, Leukotriene B_4, Leukotriene D_4, Neuropeptide Y_1, Neuropeptide Y_2, Nicotinic Acetylcholine Central, Opiate δ, Opiate κ, Opiate μ, Phorbol Ester, Platelet Activating Factor (PAF), Potassium Channel [K_{ATP}], Purinergic P_{2X}, Purinergic $P_{2Y,}$ Serotonin 5-HT_{1A}, Serotonin 5-HT_3, Serotonin 5-HT_4, Serotonin Transporter, Tachykinin NK_1, Testosterone.

Receptor	**% Inhibition at 10μM**	**IC_{50}**	**K_i**
Adrenergic Norepinephrine Transporter	64%	ND	ND
Calcium Channel Type L, Benzothiazepine	101%	0.34 μM	0.31 μM
Calcium Channel Type L, Dihydropyridine	88%	ND	ND
Dopamine D_3*	60%	ND	ND
Muscarinic M_1*	87%	ND	ND
Muscarinic M_2*	69%	ND	ND
Muscarinic M_3*	68%	ND	ND
Serotonin 5-HT_{2A}	100%	0.46 nM	0.13 nM
Sigma σ1	67%	ND	ND
Sigma σ2	75%	ND	ND
Sodium Channel Site 2	103%	1.41 μM	1.29 μM

* ACP-103 showed no activity up to 100 nM at these receptors as either an agonist or an inverse agonist in R-SAT (data not shown).

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Table 3. Bioavailability and pharmacokinetic parameters of ACP-103 in rats.

Route	AUC (min.ng/mL)	$T_{1/2}$ (min)	Bioavailability (%)	Volume of Distribution* ($L.kg^{-1}$)	CLs ($mL.min^{-1}.kg^{-1}$)*
Intravenous 1 mg/kg	2106	58.8	NA	29.91	473.1
Oral 10 mg/kg	8978	ND	**42.6**	NA	ND

**Normalized to the average weight of the rats*

NA: Not applicable

ND: Not determined

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Figure 1

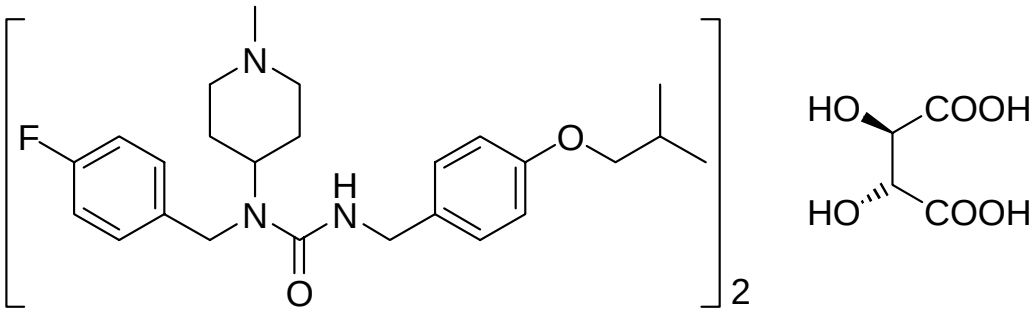

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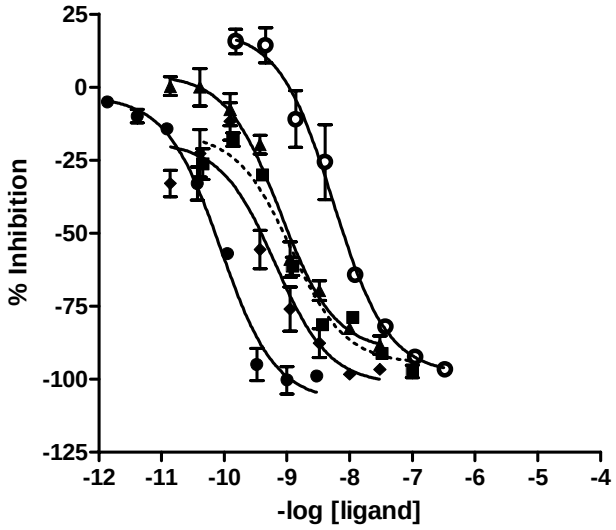
Figure 2

A. 5-HT_{2A}



B. 5-HT_{2C}

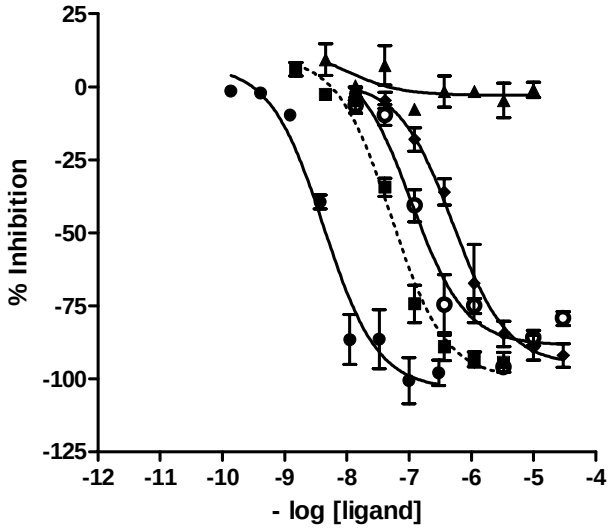


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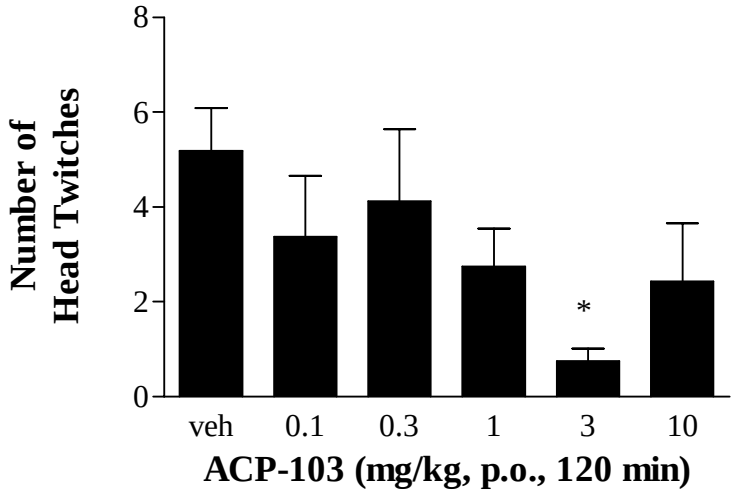
Figure 3

A.



B.

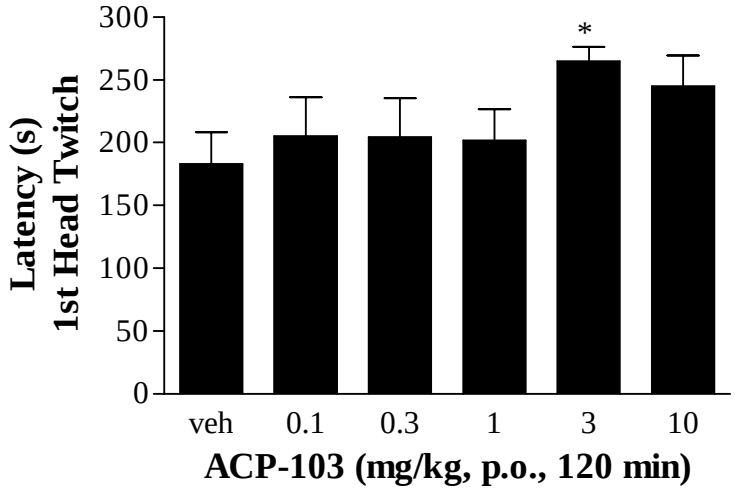


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Figure 4

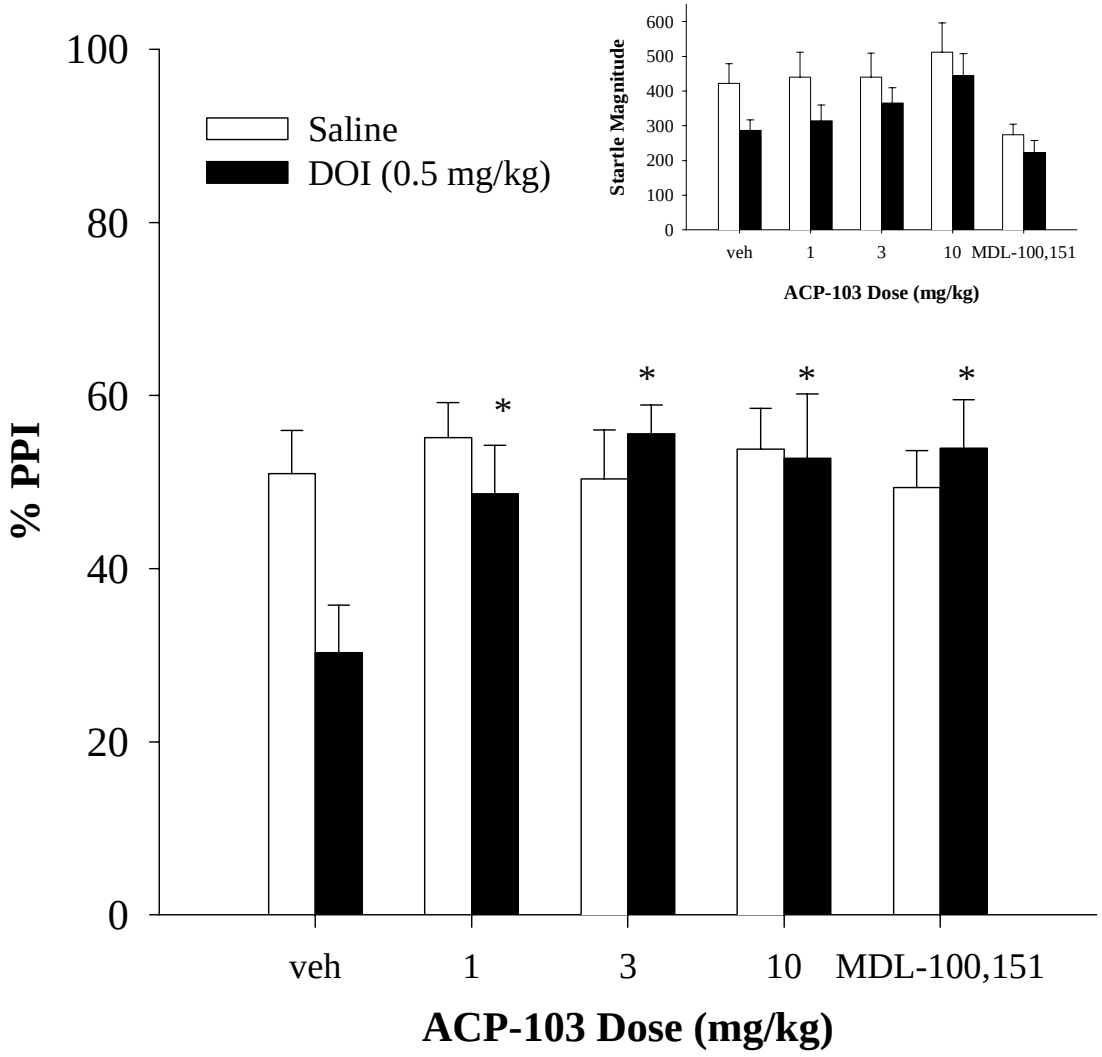

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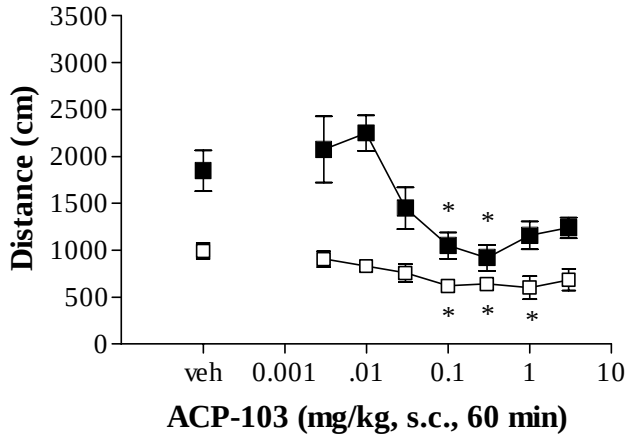
Figure 5

A.



B.