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Pharmacological and Behavioral Profile of N-(4-Fluorophenylmethyl)-N-(1-methylpiperidin-4-yl)-N′-(4-(2-methylpropyloxy)phenylmethyl) Carbamide (2R,3R)-Dihydroxybutanedioate (2:1) (ACP-103), a Novel 5-Hydroxytryptamine2A Receptor Inverse Agonist

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ABSTRACT

The in vitro and in vivo pharmacological properties of N-(4-fluorophenylmethyl)-N-(1-methylpiperidin-4-yl)-N′-(4-(2-methylpropyloxy)phenylmethyl)carbamide (2R,3R)-dihydroxybutanedioate (2:1) (ACP-103) are presented. A potent 5-hydroxytryptamine (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 receptor selection and amplification technology (R-SAT), with a mean pIC$_{50}$ of 8.7. ACP-103 demonstrated lesser affinity (mean pK_i of 8.80 in membranes and 8.00 in 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 in rats and reduced the hyperactivity induced in mice by the N-methyl-D-aspartate receptor noncompetitive antagonist 5H-dibenzo[a,d]cyclohepten-5,10-imine (dizocilpine maleate; 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.

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 (Snyder, 1976; Carlsson et al., 1999a). Unfortunately, antagonism of D$_2$ receptors also causes profound motor, endocrine, and cognitive side effects, which can

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; AC-90179, 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)acetamide, hydrochloride; ACP-103, N-(4-fluorophenylmethyl)-N-(1-methylpiperidin-4-yl)-N′-(4-(2-methylpropyloxy)phenylmethyl) carbamide (2R,3R)-dihydroxybutanedioate (2:1); DOI, (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride; PPI, prepulse inhibition; NMDA, N-methyl-D-aspartate; MK-801, 5H-dibenzo[a,d]cyclohepten-5,10-imine (dizocilpine maleate); R-SAT, receptor selection and amplification technology; DMEM, Dulbecco's modified Eagle's medium; M100907, R-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol; SST, somatostatin; MDL-100,151, (±)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol.

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 (Leysen et al., 1978; Meltzer et al., 1989; Carlsson et al., 1999a; 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, was shown to have efficacy in animal models for antipsychotic-like activity and lacked the side effects associated with other antipsychotic drugs, but it demonstrated poor oral bioavailability (Weiner et al., 2001; Vanover et al., 2004). Additional synthetic efforts designed to improve metabolic stability resulted in the discovery of ACP-103 (Fig. 1).

The pharmacological properties of the novel 5-HT$_{2A}$ receptor inverse agonist ACP-103 were characterized and compared with 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 ($\pm$)-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 maleate). 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.

Materials and 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 subcloned into a mammalian expression vector as described previously (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 used to create the various 5-HT$_{2C}$ receptor isoforms as described previously (Marion et al., 2004). All 5-HT$_{2C}$ receptor selection and amplification technology (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 fmol/mg protein ($n = 18$) for 5-HT$_{2A}$, 3251 ± 1457 fmol/mg protein ($n = 20$) for 5-HT$_{2C}$, and 277 ± 139 fmol/mg protein ($n = 4$) for 5-HT$_{2B}$.

Receptor Selection and Amplification Technology. R-SAT assays were performed as described previously (Weiner et al., 2001), with the following modifications. In brief, NIH-3T3 cells were grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (Hyclone Laboratories, 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 contained 0.4% calf serum (Hyclone Laboratories), 30% UltraCulture (BioWhittaker, Rockland, ME), and 1% penicillin/streptomycin/glutamine, and then they were added to half-area 96-well microtiter plates containing either test compounds or reference ligands. After 5 days in culture, media were removed from the wells, and the cells were incubated at room temperature in 200 μl of phosphate-buffered saline, pH 7.4, with 3.5 mM o-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich, St. Louis, MO) and 0.5% Nonidet P-40 (Sigma-Aldrich). After 2 to 4 h, the plates were read at 420 nm on a plate-reader (Bio-Tek Instruments, 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 11,000g at 4°C for 30 min. The supernatant was discarded, and the pellet was 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 using 1 nM [³H]ketanserin (PerkinElmer Life and Analytical Sciences, Boston, MA) for the 5-HT$_{2A}$ receptor and 3 nM [³H]mesulergine (Amersham Biosciences, Piscataway, NJ) for the 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors. Membranes
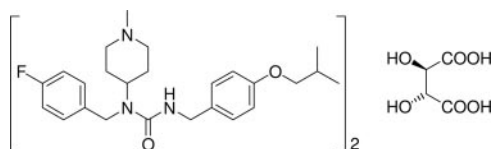

Fig. 1. Molecular structure of ACP-103.

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, and dried, and radioactivity was determined using TopCount (Packard Bioscience, Shelton, CT).

For the whole-cell binding, 6 million human embryonic kidney 293T cells were plated in 10-cm dishes and transfected with 5 μg of plasmid DNA using Polyfect according to manufacturer's instructions. Two days after transfection, cells were harvested with 10 mM EDTA, washed, and resuspended in binding buffer ($1\times$ DMEM with 0.1% bovine serum albumin). Then, 60,000 cells transfected with the 5-HT$_{2A}$ receptor or 20,000 cells transfected with the 5-HT$_{2C}$-INI receptor were incubated at 37°C for 3 h in the presence of 5 nM radioligand ([^{3}H]ketanserin for 5-HT$_{2A}$ receptors and [^{3}H]mesulergine for 5-HT$_{2C}$-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 filter plate (Packard Bioscience) and washed with 300 ml of wash buffer (25 mM HEPES, 1 mM CaCl$_2$, 5 mM MgCl$_2$, and 0.25 M 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 Bioscience). Plates were counted on a TopCount (Packard Bioscience). Separately, the hydrochloride salt form of ACP-103 (10 μM) 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 mice (Harlan, San Diego, CA) were used as subjects. Rats weighed 200 to 300 g, and mice weighed 20 to 30 g. Animals were housed two per cage (rats) or eight per 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 reported previously (Mansbach et al., 1988) in commercially available startle chambers (San Diego Instruments, San Diego, CA). Plastic locomotor activity cages (20 × 20 × 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 before locomotor testing, mice were evaluated for myorelaxation/ataxia using a custom-built apparatus consisting of a metal wire (2 mm in diameter) suspended 25 cm above the benchtop.

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 eight to 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 24 40-ms presentations of a 120-dB broadband pulse, 10 20-ms presentations of each prepulse intensity (68, 71, and 77 dB) 100 ms before a 40-ms presentation of a 120-dB broadband pulse, and eight no-stimulus trials in which no acoustic pulse was delivered 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 five 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 pseudorandom order with an average intertrial interval of 15 s (range 7–23 s), and air puffs were presented every 10 s.

Thirty minutes 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, or 10.0 mg/kg s.c.). Five minutes 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 37 min. After 1 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).

Non-Swiss albino 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 (i.p.) 15 min presession (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 before placing the mice in the locomotor chambers, effects on myorelaxation/ataxia were determined by placing each of the 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 Software Inc., San Diego, CA) and are reported as averaged values derived from multiple experimental replicates (n) $\pm$ S.D. 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 formula $K_i = (IC_{50})/(1 + [L]/K_d)$, in which [L] is the concentration of the test compound, and K_d is the dissociation constant of the radioligand.

For DOI head-twitch experiments, latency (seconds) 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 (centimeters) was calculated and averaged across animals in a group. Means $\pm$ S.E.M. 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. Percentage of 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)] × 100}. A three-way repeated measures analysis of variance with pretreatment as a between-subject factor and treatment and prepulse intensity as within-subject 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. Because there was 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 α level was adjusted to 0.025 for the two-way pairwise 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-subject factor and treatment and time as within-subject 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 intravenous administration, 0 (predose), 2, 5, 30, 60, 120, 240, 360, and 480 min; and for oral administration, 0 (predose), 5, 30, 60, 120, 240, 360, and 480 min. 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. After centrifugation, the plasma was placed on ice until frozen at $-70°C$ before 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 (Pipersville, PA) (lot number 062631954) to contain 1000, 300, 100, 30, 10, 3, 1, and 0.3 ng/ml 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 min at 10,000 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 10,000 rpm for 10 min, the sample was injected into the liquid chromatograph/tandem mass spectrometer. The concentration of the internal standard solution was 2 μg/ml AC-90179. Pharmacokinetic analysis was performed on the average plasma concentration for each time point. The data were subjected to noncompartmental analysis using the pharmacokinetic program WinNonlin version 3.1 (Pharsight, Mountain View, CA).

Drugs. ACP-103, eplivanserin, and M100907 and its racemate MDL-100,151 were synthesized by ACADIA Pharmaceuticals Inc. Ritanserin, haloperidol, MK-801, and DOI were obtained from Sigma-Aldrich, and clozapine was obtained from Tocris Cookson Inc. (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% Tween 80 (90% water). All compounds were administered in a volume of 0.1 ml/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 polyethylene glycol-400 [4:1 (v/v)].

Results

Pharmacology Experiments

Functional Antagonism and Inverse Agonism. Transient expression of the three human 5-HT$_2$ receptors in NIH-3T3 cells as part of the R-SAT assay generates robust and pharmacologically appropriate receptor-mediated responses (Fig. 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 nonselective 5-HT$_2$ receptor
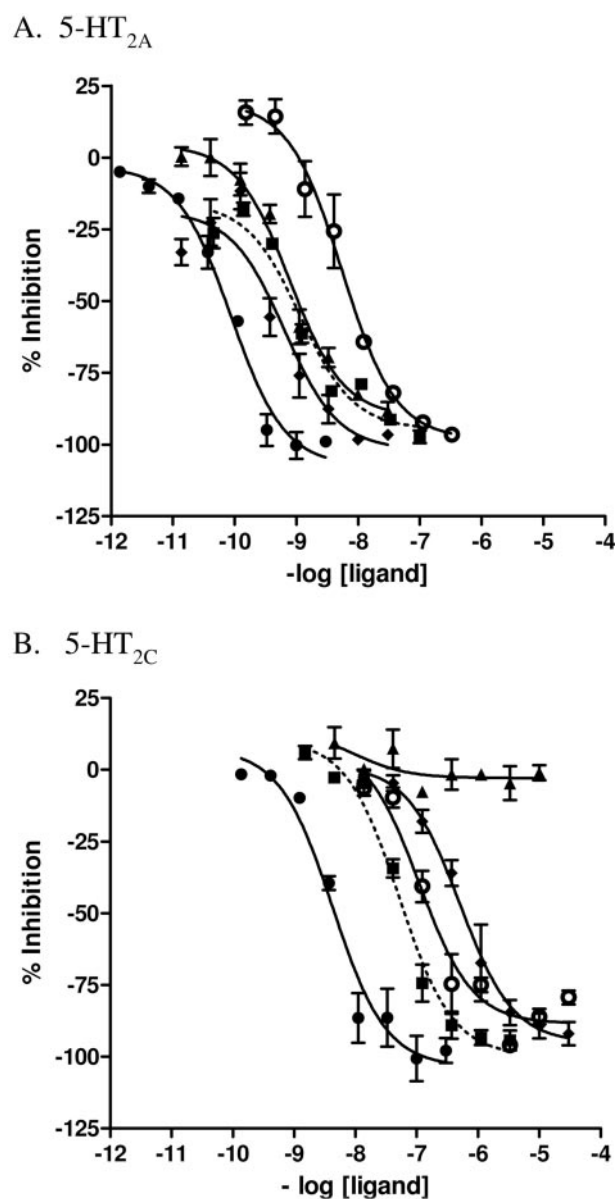

Fig. 2. Inverse agonism of ACP-103 at human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. Representative concentration-response curves for ACP-103 and various 5-HT$_2$ compounds as inverse agonists at the human 5-HT$_{2A}$ (A) and 5-HT$_{2C}$ (B) receptors, as determined by R-SAT assays, are reported. Compounds were tested at eight different concentrations where each point was assayed in duplicate, with error bars depicting the mean $\pm$ S.E. Data are reported as percentage of 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$_{50}$ values) in the reported experiments (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$_{50}$ values) in the reported experiments (B) were ritanserin (8.4), ACP-103 (7.3), M100907 (6.3), eplivanserin (no activity), and clozapine (7.0).

inverse agonist ritanserin, as well as those observed for ACP-103 (Fig. 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$_{2A}$ (Fig. 2A) and 5-HT$_{2C}$ (Fig. 2B) receptors. Each of these compounds was found to be potent (pIC$_{50}$ values

TABLE 1
Pharmacological activity of ACP-103 at human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors

This table reports the potencies and inverse agonist efficacies (relative to ritanserin) of ACP-103, eplivanserin, M100907, ritanserin, and clozapine at human 5-HT$_2$ receptors (5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$) 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 ± S.D. Inverse agonist efficacies are reported as percentage of inhibition relative to that observed for ritanserin (100%). Affinity determinations are reported as average pK_i values ± S.D.

Compound	R-SAT Functional Data								
	5-HT$_{2A}$			5-HT$_{2B}$			5-HT$_{2C}$		
	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	N.A.	N.A.	4	6.27 ± 0.15	76 ± 5
Eplivanserin	2	8.98 ± 0.38	87 ± 7	2	N.D.	35 ± 12	6	N.A.	N.A.
ACP-103	19	8.73 ± 0.3	93 ± 6	3	N.D.	N.A.	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							
	Membrane Binding pK_i				Whole Cell Binding pK_i			
	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

n, number of experiments performed; N.A., no activity (< 20% inhibition); and N.D., not determined.

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 Fig. 2B, ritanserin, ACP-103, M100907, and clozapine displayed 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$_{50}$ or pK_i values less than 6.0) at the 31 monoaminergic receptors and additional five nonmonoaminergic (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 (Fig. 2B; 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 < 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, 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 ± 0.9 (S.E.M.) head twitches with the latency to head-twitch onset of 183 ± 25.3 s. ACP-103 (n = 8–16/group) attenuated DOI-induced head twitches at a dose of 3 mg/kg p.o. after a 2-h pretreatment (Fig. 3). The analysis of variance for number of head twitches [$F(5,71) = 2.795$; $p = 0.02$] was statistically significant, and for latency [$F(5,71) = 1.970$; $p = 0.09$], it 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 three-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

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 percentage of inhibition is listed in the table. The IC$_{50}$ and K_i values were only determined at those receptors for which 10 μM ACP-103 demonstrated 100% inhibition. ACP-103 (10 μM) demonstrated less than 50% inhibition in receptor binding assays at the following receptors: adenosine A$_1$, adenosine 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$_1$, and testosterone.

Receptor	% Inhibition at 10 μM	IC$_{50}$	K_i
Adrenergic norepinephrine transporter	64	N.D.	N.D.
Calcium channel type L, benzothiazepine	101	0.34 μM	0.31 μM
Calcium channel type L, dihydropyridine	88	N.D.	N.D.
Dopamine D$_3$*	60	N.D.	N.D.
Muscarinic M$_1$*	87	N.D.	N.D.
Muscarinic M$_2$*	69	N.D.	N.D.
Muscarinic M$_3$*	68	N.D.	N.D.
Serotonin 5-HT$_{2A}$	100	0.46 nM	0.13 nM
Sigma σ1	67	N.D.	N.D.
Sigma σ2	75	N.D.	N.D.
Sodium channel site 2	103	1.41 μM	1.29 μM

N.D., not determined.

* 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).
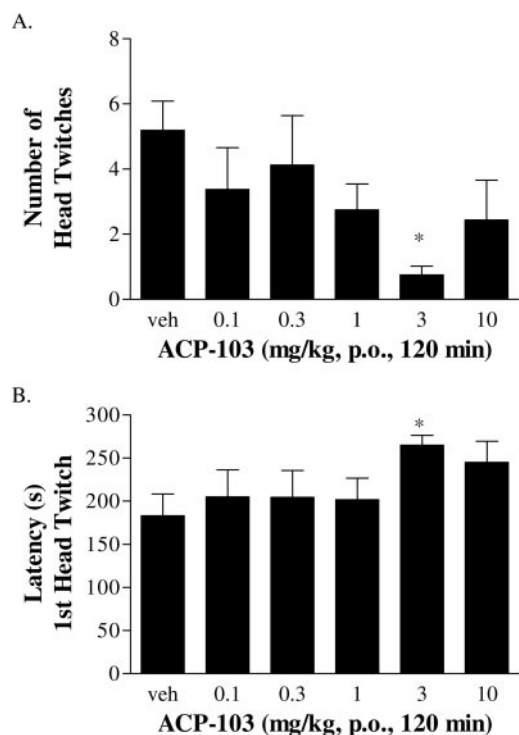

Fig. 3. Attenuation of DOI-induced head twitches by ACP-103. DOI (2.5 mg/kg i.p.) was administered in combination with vehicle (veh) or various doses of ACP-103. A, number of head twitches produced by DOI as a function of ACP-103 dose. B, latency in seconds to the first head twitch as a function of ACP-103 dose. Each bar represents the mean + S.E.M. from a separate group of animals (n = 6/dose). Asterisks indicate statistical significance ($p < 0.05$) compared with DOI + vehicle.
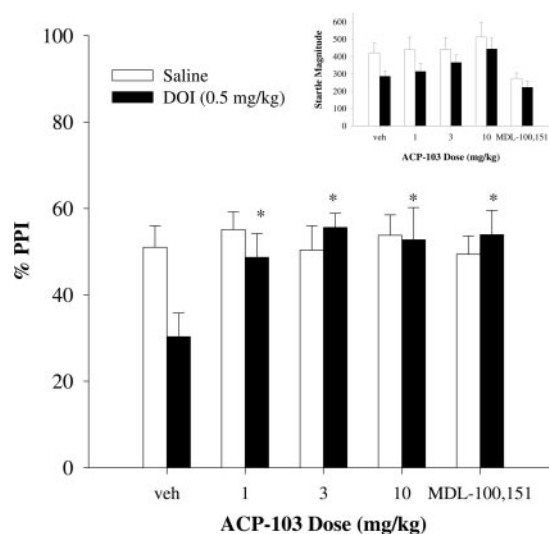

Fig. 4. Attenuation of DOI-induced PPI by ACP-103. DOI (0.5 mg/kg s.c.) was administered in combination with vehicle (veh), various doses of ACP-103, or 1.0 mg/kg MDL-100,151 (racemic M100907) (s.c.). %PPI is shown as a function of ACP-103 dose + S.E.M. The insert shows that ACP-103 had no effect on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

interaction [$F(3,37) = 3.72$; $p < 0.05$; Fig. 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.]. Because there was a significant pretreatment by treatment interaction, pairwise two-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 two-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 three-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-100,151 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 two-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. [$F_{(7,63)} = 6.010$; $p < 0.0001$; Fig. 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 hindpaws up to the horizontal wire after administration of ACP-103, which was tested just before placing the mice in the locomotor chamber. In a separate experiment, seven of eight mice were able to pull their hindpaws 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. [$F_{(4,39)} = 4.681$; $p = 0.0039$; Fig. 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 of the doses after oral administration of ACP-103 alone, indicating that none of the mice exhibited ataxia.
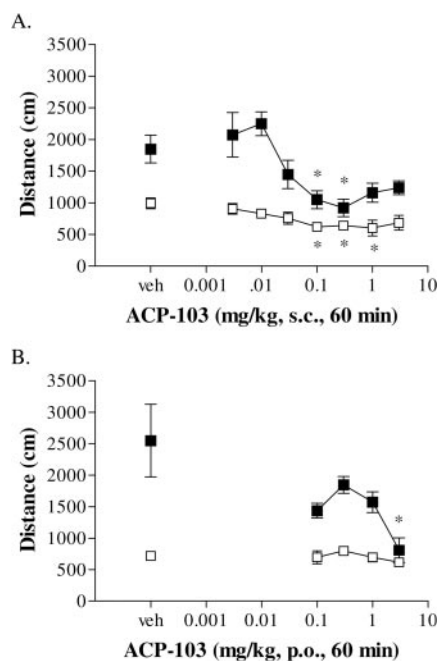

Fig. 5. Effects of ACP-103 on spontaneous locomotion and MK-801-induced hyperactivity in mice. Distance traveled is shown as a function of ACP-103 dose. A, effects of ACP-103 after s.c. administration. B, effects of ACP-103 after p.o. administration. Each point represents the mean and S.E. of a separate 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 with respective vehicle control.

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-min half-life observed. Considering that the area under the curve 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 maximal plasma concentrations occurring 60 to 120 min postdosing.

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 were similar to those reported previously by our group and others (Leysen et al., 1985; Rinaldi-Carmona et al., 1992; Kehne et al., 1996; Bonhaus et al., 1999; 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 et al. (1999). The whole-cell binding affinity for clozapine at the 5-HT$_{2C}$ receptor was similar to that reported previously (Canton et al., 1990; Roth et al., 1992; Herrick-Davis et al., 2000), whereas our observed 5-HT$_{2C}$ receptor affinity for clozapine in membranes was approximately 100-fold lower. The affinity observed for M100907 was slightly higher than described previously (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 among 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$_{2B}$ re-

TABLE 3

Bioavailability and pharmacokinetic parameters of ACP-103 in rats

Route	AUC	$t_{1/2}$	Bioavailability	Volume of Distribution[a]	CLs[a]
	min ng/ml	*min*	*%*	*l kg^{-1}*	*ml min^{-1} kg^{-1}*
Intravenous, 1 mg/kg	2106	58.8	N.A.	29.91	473.1
Oral, 10 mg/kg	8978	ND	42.6	N.A.	N.D.

AUC, area under the curve; N.A., not applicable; N.D., not determined; CLs, clearance.
[a] Normalized to the average weight of the rats.

ceptors 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 tested compounds 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. Furthermore, all antipsychotic 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 central nervous system (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 they 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 minimal effective oral dose of 3.0 mg/kg. Interestingly, this effect was not seen at a higher dose. Whether this was due to other activity, perhaps 5-HT$_{2C}$, or variability at the higher dose is unclear. ACP-103 also reversed DOI-induced PPI deficits in rats at doses between 1.0 and 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, con-

sistent 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 minimal 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 (Freed et al., 1984; Carlsson et al., 1999b; Weiner et al., 2001; Vanover et al., 2004).

Because of 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 with 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. Indeed, 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 (Nordstrom et al., 1993; Meltzer et al., 1995). 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. seems 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 were 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). Because 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, that exacerbate motor symptoms (Breier et al., 2002). 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.

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