

Pharmacological Characterization of AC-90179:

a Selective 5-HT_{2A} Receptor Inverse Agonist

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DMEM; Dopamine, D; Fixed Ratio, FR; Histamine, H; Krebs-bicarbonate, KBB;

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Abstract

The primary purpose of the present series of experiments was to characterize the *in vitro* and *in vivo* pharmacology profile of AC-90179, a selective 5-HT_{2A} receptor inverse agonist, in comparison to the antipsychotics haloperidol and clozapine. The secondary purpose was to characterize the pharmacokinetic profile of AC-90179. Like all atypical antipsychotics, AC-90179 shows high potency as an inverse agonist and competitive antagonist at 5HT_{2A} receptors. In addition, AC-90179 exhibits antagonism at 5HT_{2C} receptors. In contrast, AC-90179 does not have significant potency for D₂ and H₁ receptors that have been implicated in the dose limiting side effects of other antipsychotic drugs. The ability of AC-90179 to block 5-HT_{2A} receptor signaling *in vivo* was demonstrated by its blockade of the rate decreasing effects of the 5-HT_{2A} agonist, DOI, under a fixed ratio schedule of reinforcement. Similar to clozapine and haloperidol, AC-90179 attenuated phencyclidine-induced hyperactivity. Whereas haloperidol impaired acquisition of a simple autoshaped response and induced cataleptic-like effects at behaviorally efficacious doses, AC-90179 and clozapine did not. Further, unlike haloperidol and clozapine, AC-90179 did not decrease spontaneous locomotor behavior at efficacious doses. Limited oral bioavailability of AC-90179 likely reflects rapid metabolism rather than poor absorption. Taken together, a compound with a similar pharmacological profile as AC-90179 and with increased oral bioavailability may have potential for the treatment of psychosis.

Clinically effective antipsychotic drugs have diverse neurochemical actions and demonstrate significant interactions with dopamine (DA), serotonin (5-HT), noradrenaline, and histamine (H) receptors. Antipsychotic efficacy and extrapyramidal motor effects have long been correlated with affinity for D₂ dopamine receptors (Snyder, 1976). Atypical antipsychotics with lesser affinity at D₂ receptors and higher affinity at 5-HT₂ receptors compared to neuroleptics have a decreased propensity to induce these motoric side effects (Meltzer et al., 1989). Further, selected atypical antipsychotic drugs appear to improve certain aspects of cognitive function in schizophrenic patients, whereas typical neuroleptic antipsychotics do not improve and may worsen cognition (Meltzer et al., 1999; Velligan and Miller, 1999). However, many atypical antipsychotics exhibit other side effects including sedation, profound orthostasis, morbid obesity and type II diabetes as untoward side effects, likely attributable to potent interactions at multiple receptors.

Interactions of antipsychotic drugs with the 5-HT_{2A} receptor subtype is of particular interest. High affinity interactions with 5-HT_{2A} receptors have been postulated to mediate the efficacy and improved side-effect profile of atypical antipsychotics (Carlsson A et al., 1999; Kasper et al, 1999; Weiner et al., 2001). Recently, it has been recognized that most clinically effective antipsychotic drugs are, in fact, inverse agonists at 5-HT_{2A} receptors rather than simply neutral antagonists (Weiner et al., 2001). In addition, increased potency as 5-HT_{2A} inverse agonists predicts the “atypical” nature of most antipsychotic drugs. Antagonism at the other 5-HT₂ receptor subtypes, 5-HT_{2B} and 5-HT_{2C}, is shared by some atypical antipsychotic drugs (Bymaster et al., 1999; Canton et

al., 1994; Wainscott et al., 1996). However, 5-HT_{2B} receptor interactions do not appear to contribute to efficacy of antipsychotic drugs and although 5-HT_{2C} receptor inverse agonism may contribute to the effects of some antipsychotic drugs, this is still controversial (Herrick-Davis et al., 2000; Rauser et al., 2001).

Potent, selective and highly efficacious inverse agonists for 5-HT_{2A} receptors were identified, typified by AC-90179 [2-(4-methoxy-phenyl)-N-(4-methyl-benzyl)-N-(1-methyl-piperidin-4-yl)-acetamide hydrochloride; Figure 1 for structure] (Weiner et al., 2001). The purpose of the present set of experiments was to further characterize AC-90179 with respect to its *in vitro* and *in vivo* pharmacology and pharmacokinetic parameters. The *in vitro* intrinsic efficacy of AC-90179 as an inverse agonist at 5-HT_{2A} and 5-HT_{2C} receptors was determined using a cell-based functional assay. In addition, the potency and affinity of AC-90179 as an antagonist at 5-HT_{2A} and 5-HT_{2C} receptors was determined using a cell-based functional assay and a radioligand-binding assay, respectively. The interaction of AC-90179 at these 5-HT₂ receptors and lack of interaction at other receptors of particular interest, namely 5-HT_{2B}, D₂ and H₁, are compared to data for haloperidol and clozapine. Behaviorally, the efficacy and side effect profile of AC-90179 was determined in mice. Blockade of the response rate decreasing effects of the 5-HT_{2A} receptor agonist, DOI, was used to demonstrate blockade of 5-HT_{2A} receptor signaling *in vivo*. Attenuation of locomotor hyperactivity induced by phencyclidine was used to assess antipsychotic-like efficacy. In order to address potential side effects, AC-90179 was tested for its ability to induce cataleptic-like behavior with a step-down latency test and impair acquisition of a simple nose poke

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response. The behavioral pharmacological effects of AC-90179 were compared to those of haloperidol and clozapine. Finally, absorption, metabolism, and pharmacokinetics of AC-90179 were examined. Gastrointestinal absorption and metabolic stability were estimated *in vitro* using CACO-2 and liver microsomal experiments, respectively. Blood serum and brain concentrations and oral bioavailability were determined in rats.

Methods

In Vitro Functional Assays

R-SAT® (Receptor Selection and Amplification Technology) assays were performed similarly to that previously described (Weiner et al., 2001). Briefly, NIH3T3 cells were grown in 96 well tissue culture plates to 70-80% confluence. Cells were transfected for 12-16 hours with plasmid DNAs using Superfect Reagent (Qiagen) as per manufacture's protocols. R-SAT®'s were generally performed with 1-50 ng/well of receptor and 20 ng/well of B-galactosidase plasmid DNA. For G-protein co-expression studies, 4-20 ng/well of Gq or Gqi5 was used. After overnight transfection media was replaced with serum free Delbecco's Modified Essential Media (DMEM) containing 2% cyto-sf3 (Kemp Biotechnologies), and 1% penicillin/streptomycin/glutamine and varying concentrations of drug. Cells were then grown in a humidified atmosphere with 5% ambient CO₂ for four to six days. Media was then removed from the plates and B-galactosidase activity was measured by the addition of o-nitrophenyl B-d-galactopyranoside (in phosphate buffered saline with 5% NP-40 detergent). The resulting colorimetric reaction was measured in a spectrophotometric plate reader (Titertek) at 420 nM. All data were analyzed using the computer programs Excel Fit, EC₅₀ determinations were made using least squares fit analysis with GraphPad Prism (San Diego, CA) software.

Radioligand Binding Assays

NIH-3T3 cells (mouse fibroblast) were seeded in 15 cm dishes with DMEM supplemented with 10% calf serum and 1% penicillin/streptomycin/glutamine (PSG). At

70-80% confluence, the cells were transiently transfected for 12-16 hours with 10 μ g plasmid DNA using Polyfect Reagent (Qiagen, Valencia, CA) according to manufacturer's protocols. Cells were harvested by scraping, then centrifuged at 1000 rpm for 5 min and resuspended in 20 mM HEPES buffer containing 10 mM EDTA. The cells were then homogenized and recentrifuged at 11000 rpm for 30 min at 4°C, resuspended in 20 mM HEPES/1 mM EDTA and homogenized again. After one additional centrifugation/homogenization step the membranes were resuspended in 20 mM HEPES buffer containing 0.1 mM EDTA. Protein concentration was determined by the method of Bradford (Bio-Rad Assay).

Radioligand binding assays were carried out in triplicate at room temperature for 3 hours using [3H]ketanserin (63.3 Ci / mmol, NEN) for human 5-HT_{2A} receptors and [3H]mesulergine (86 Ci/mmol, Amersham) for human 5-HT_{2C} receptors (VGV isoform) and [3H]methoxy raclopride (71.3 Ci/mmol, PerkinElmer) for human D₂ receptors as radioligands. For saturation and competition studies 12 concentrations of radioligand/ligand were used. Non-specific binding was measured with 500 nM methysergide for 5-HT_{2A} receptors and 1 μ M mianserin for 5-HT_{2C} receptors. The competition assays were conducted in 600 μ l binding buffer (20 mM HEPES/0.1 mM EDTA, pH 7.4). The incubations were terminated with rapid filtration through Whatman GF/B glass fiber filters pre-treated with 0.3% polyethylenimine, using a Packard Filtermate 96 cell harvester, followed by a 1 ml wash with ice cold buffer (50mM TRIS pH 7.4). Filters were then counted on a Packard Topcount.

The data were analyzed by nonlinear regression using the GraphPad Prism software. The pKi values were determined using the Cheng and Prusoff equation.

Behavioral Experiments

Animals and Apparatus. Male NSA mice and male C57BL/10 mice (Harlan; San Diego, CA) were used as subjects. Mice weighed 20 – 30 g. Animals were housed (One Cage; Lab Products, Inc., Seaford, DE) singly (C57BL/6 mice), 8/cage (NSA mice) or 4/cage (C57BL/10 mice) with bedding (1/8 inch Bed “O” Cob; Harlan Teklad, Madison, WI) in a room with controlled temperature $22 \pm 3^{\circ}\text{C}$ and a 12 hour light:dark cycle (lights on 6 am). Water and standard rodent chow (Harlan Teklad) were continuously available in the home cage, except as noted below for the food-restriction of C57BL/6 mice.

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). For step-down latency (catalepsy) experiments, a custom-built 8-mm rod that was raised 3.5 cm from the laboratory bench top was used. For operant schedule controlled behavior and cognitive/acquisition experiments, sound attenuating enclosures contained operant chambers (12.5 cm x 11 cm x 12.5 cm; Med Associates Inc., East Fairfield, VT), equipped with a recess (dipper well; 2.2 cm diameter, 1.3 cm deep) for dipper accessibility on one wall, two smaller holes (1.3 cm diameter, 0.9 cm deep) on the wall opposite to the dipper well, a tone and a house light. Each recess had a photocell for monitoring nose poke responses. A computer and associated interface controlled stimulus presentation and recorded nose poke responses.

Procedure. For schedule-controlled operant behavior, C57BL/6 mice were food restricted with access to ~5g chow daily post-session and the milk consumed during the session. Mice were trained to poke their nose in the lit hole on the right side in order to obtain a dipper full of evaporated milk under a fixed ratio 1 (FR1) schedule of continuous reinforcement. Over the period of several days, the fixed ratio was increased (FR1, FR2, FR3, FR5, FR10) to the final schedule of reinforcement of FR10 under which every 10th nose poke was reinforced. Nose pokes in the left hole had no consequence.

Pharmacological testing as soon as response rates under the FR10 schedule were stable (\pm 10%). Testing occurred on Tuesdays and Fridays as long as stable levels of responding were maintained during non-test sessions. Test compounds (n = 5-6) were injected i.p. 30 min before the start of the session. Sessions lasted for 1 hour or 20 reinforcers were obtained.

NSA mice were used for locomotor activity experiments. For determination of spontaneous activity, test compounds were administered alone (AC-90179 s.c. 10 min before the session; haloperidol i.p. 30 min; clozapine i.p. 30 min). For hyperactivity experiments, mice were injected with 3 mg/kg phencyclidine 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 test compound. Motor activity data was collected during a 15 min session in a lit room. Mice had no prior exposure to the motor cages. Each dose or dose combination was tested in a separate group of mice (n=8).

For step-down latency (catalepsy) experiments, NSA mice were used as subjects. Test compounds were administered s.c. or i.p. 60 min before the start of the session. A longer pretreatment time was used for step-down latency experiments than for locomotor experiments due to the shorter duration of the step-down latency test and the attempt to test at peak activity of the compounds. Each mouse was held by the tail and its forepaws were placed on the rod and hindpaws placed on the bench top. The time for forepaws to step down from the rod was measured. If the animal stepped off immediately, another attempt was made until the animal remained on the rod for more than 10 sec or 10 attempts had been made. A maximum of 2 min was allowed at which time the animal was taken away from the rod and returned to the home cage. Each dose or dose combination was tested in a separate group of animals (n=6).

For acquisition of a nose poke response, C57BL/10 mice (n = 4/group) were used. A different strain of mice was used for the acquisition experiments than for the locomotor and step-down latency because C57BL/10 mice appeared to be more sensitive to pharmacological-induced deficits than NSA mice and data were more reliable (unpublished observations). Mice were injected with a test compound s.c. (AC-90179) or i.p. (clozapine and haloperidol) and 30 min later placed into operant chambers. Mice were evaluated for their ability to acquire a simple nose poke response for a evaporated milk reinforcer under an autoshaping schedule of reinforcement. During this procedure, a tone was sounded on a variable interval schedule of presentation (mean 45 s, range 4 – 132 s) and stayed on for 6 sec or until a nose poke response in the dipper well was made, at which time the tone was turned off and a dipper with evaporated milk was presented.

If no response was made during the tone, a non-contingent dipper was presented and recorded. If a response was made during the tone, it was counted as an earned reinforcer. The number of reinforcers earned (maximum 20) during a 1 hr session was counted and the latency to earn 20 reinforcers was measured (maximum 3600 s if less than 20 reinforcers earned). Dipper well nose poke responses while the tone was off were counted but had no consequence. Nose pokes in the smaller holes on the back wall were counted for general exploratory activity but had no consequence. Each compound was tested in a separate group of mice.

Data Analysis. For schedule-controlled operant behavior, response rates (nose pokes/s) were calculated and averaged across animals in a group. For spontaneous locomotor experiments, distance traveled (cm) was calculated and averaged across animals in a group. Due to day-to-day variability in phencyclidine-induced hyperactivity, distance traveled was converted to percent of that day's vehicle + phencyclidine control value and averaged across animals in a group. Latency (s) to step down was calculated and averaged across animals in a group. For acquisition experiments, the number of reinforcers earned and rate of exploratory nose-pokes (responses/s) were calculated and averaged across animals in a group. An analysis of variance (ANOVA) and post-hoc Dunnett's t-test comparisons to vehicle control were conducted for each dose-response function, except for the schedule-controlled behavior that required an ANOVA for repeated measures.

Absorption, Metabolism, Pharmacokinetic Experiments

Animals and Apparatus. Eight male Wistar rats weighing 240 – 265 g were used as subjects. The rats were obtained from Charles River AGB (Germany) and subsequently housed in cages with food and water supply at the Panum Institute (Copenhagen) where the in vivo portion of the experiment was conducted.

Solid phase extractions were performed by Gilson Aspec robot (Gilson, France) using solid phase extraction columns (ISOLUTE SCX, IST) for serum and brain samples. Extracts were analyzed by LC-MS (HP 1100 LC/MSD) on a reversed phase C18 column (Luna, Phenomenex, 3 μ m, 75 x 4.6 mm ID). A Sorvall RC-5B centrifuge (Sorvall, US) was used for centrifugation.

The Caco-2 experiment was conducted at Cyprotex Ltd. (U.K.) and used caco-2 cells obtained from the ATCC at passage number 27. Cells (passage number 40-60) were seeded on to Falcon HTS 24-well inserts (1 μ m pore-size; area available for absorption 0.33 cm²) at 1 x 10⁵ cells/cm². They were cultured for 20 days in DMEM and media changed every two or three days. On day 20 the permeability study was performed. The trans-epithelial resistance of the monolayers was measured approximately 1 hour before use. Monolayers were used only if the resistance is between 200 and 350 ohm.cm².

The metabolic stability assay was performed using microsomes from XenoTech (US).

Procedure. For determination of serum and brain concentrations as well as oral bioavailability, AC-90179 was administered to 2 rats per dose: 2 mg/kg i.v., 4 mg/kg i.v., 4 mg/kg p.o., 8 mg/kg p.o. Blood was drawn from the rats at time points 30 min, 2, 4 and 6 hours through the eye after a brief CO₂ anesthetization. The sampling process took approximately 1-1.5 min. Blood was collected in tubes without anticoagulant, allowed to coagulate (30 min.) and centrifuged at 5000 g for 5 min. After the last time point (6 h), the animal was killed by decapitation and the brain was taken out. The brain was frozen immediately in liquid nitrogen. The obtained serum and brain samples were kept in freezer (-20 °C) until analysis.

The frozen serum samples were thawed. The 0.25-ml aliquots were diluted with ammonium acetate buffer and purified on solid phase extraction columns. The analyte was eluted off the column with acetonitrile/ammonia solution and the combined extracts were evaporated to dryness and redissolved in LC-MS mobile phase.

The frozen brains were thawed and homogenized adding water. The 0.25-g aliquots of the brain homogenates were diluted with ammonium acetate buffer and subsequently centrifuged. The supernatants were purified on solid phase extraction columns. The analyte was eluted off the column with acetonitrile/ammonia solution and the combined extracts were evaporated to dryness and redissolved in LC-MS mobile phase.

The serum and brain extracts were analyzed by LC-MS on a reversed phase C18 column (3µm, 75 x 4.6 mm ID) thermo stated at 30°C. The samples were analyzed using a

gradient run with acetonitrile/ammonium acetate mobile phase. Detection consisted of UV (DAD, 190-550 nm) and MS (Electrospray, SIM mode) detection. The concentrations in the serum and brain samples were determined using a standard curve with standards in the range 1-250 ng/ml.

Permeability was determined in caco-2 cells. Krebs-bicarbonate (KBB) pH 7.4 buffer with 10 mM glucose at 37 °C was used as the incubating medium in permeability studies. Incubations were carried out in an atmosphere of 5 % CO₂ with a relative humidity of 95%.

The monolayers were prepared by rinsing both basolateral and apical surfaces twice with KBB at 37 °C. Cells were then incubated with KBB in both apical and basolateral compartments (0.5 ml and 1 ml, respectively) for 40 minutes to stabilize physiological parameters.

For apical to basolateral permeability, KBB was then removed from the apical (donor) compartment and replaced with 0.5 ml test compound solution. The solutions were made by adding 10 mM DMSO concentrates to KBB to give a final test compound concentration of 10 µM (DMSO added to give a final concentration of 1 % v/v). The fluorescent integrity marker lucifer yellow was also included in the dosing solution at 300 µM. Solutions were filtered if drug precipitation was seen. Test compound apical to basolateral permeability was assessed in triplicate on each of two plates; one was incubated for 40 minutes and the other for 2 hours to ensure accurate permeability

assessment for high and low-permeation compounds, respectively. On each plate, propranolol was included in 3 wells as a lipophilic, passively-permeating control. Test compound was not included in these control wells.

The inserts were then placed into 24-well 'receiver' plates containing 1 ml fresh KBB per well. Plates were placed on an orbital shaker set at 50 rpm throughout each permeation period. At the end of the test-period the apical (donor) compartment solution was retained for analysis to enable calculation of the initial donor concentration. Aliquots of receiver samples were transferred to fresh receiver plates to permit determination of receiver compartment binding. Samples were analyzed by LC-MS.

Metabolic stability was determined using liver microsomal preparations. A substrate solution giving a final concentration of 0.1 μ M AC-90179 in the assay was prepared, mixed with three solutions: [20 mg/ml NADP sodium salt, 20 mg/ml glucose-6-phosphate (G6P) and 13.3 mg/ml magnesium chloride hexahydrate ($\text{MgCl}_2, 6\text{H}_2\text{O}$)], [20 U glucose-6-phosphate dehydrogenase (G6PDH/ml)], and 64 mM Tris-hydroxy methyl amino methan, HCl (TRIS), and subsequently heated to 37 °C (in a water bath). The final assay conditions were 0.1 μ M substrate, 1.3 mM NADP, 3.5 mM G6P, 3.3 mM MgCl_2 , 0.4 U/ml G6PDH and 50 mM TRIS. The mixture was added to CD1 mouse liver microsomes at 20 mg protein/ml to initiate metabolism, giving a final protein concentration of 1 mg/ml in the assay. The mixture was added to 75 μ l acetonitrile 0, 5, 10 and 20 minutes to stop the metabolism and cooled on ice. The samples were centrifuged at 12,000 x g (10,000 rpm on Sorvall RC-5B centrifuge) for 10 minutes at 5

deg Celsius to precipitate the protein. A sample of 75 μ l of the supernatant was injected directly in the LC-MS system using a SIM method for analysis.

Data Analysis. The concentration data obtained from analysis of the serum samples were subjected to pharmacokinetic analysis using the program WinNonlin (Pharsight, version 3.0). Apical-basolateral permeability coefficients for each compound (P_{app}) are calculated from the following equation:

$$P_{app} = \frac{dQ/dt}{C_0 \times A}$$

dQ/dt is the rate of permeation of the drug across the cells, C_0 is the initial donor compartment concentration and A is the area of cells available to permeating drug. C_0 is determined from the end-donor sample and is thus corrected for test compound loss due to plate-binding or cellular accumulation. Apical to basolateral data were used to group test compounds into low (0 to 40%, $P_{app(A-B)}$ up to $0.6 \times 10^{-6} \text{ cms}^{-1}$), medium (40 to 70%, $P_{app(A-B)}$ 0.6 to $2 \times 10^{-6} \text{ cms}^{-1}$) or high (70 to 100%, $P_{app(A-B)}$ above $2 \times 10^{-6} \text{ cms}^{-1}$) absorption potential by comparison of human absorption data from a set of 26 validated drugs (at Cyprotex). For metabolic stability, data from two determinations were averaged. Amount of AC-90179 detected at the zero time point was defined as 100% control and subsequent amounts detected at 5, 10 and 20 min were determined as percent of control.

Test Compounds. AC-90179 was synthesized by ACADIA Pharmaceuticals Inc., whereas clozapine and haloperidol were purchased (Sigma; St. Louis, MO). For in vitro functional and radioligand binding assays, test compounds were dissolved in DMSO. For behavioral experiments, all test compounds were dissolved in 10% Tween 80 (90% water). Phencyclidine hydrochloride (PCP) (Sigma) was dissolved in 0.9% saline and (\pm)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI; Sigma) was dissolved in water. 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 in vivo phase of the pharmacokinetic experiments, AC-90179 dose was calculated based on the weight of the free base and dissolved in saline. A solution of 0.1 μ M AC-90179 in water was used for the metabolic stability study.

Results

In vitro functional assay

Table 1 shows the potency of AC-90179 as a functional competitive antagonist or inverse agonist compared to clozapine and haloperidol at human 5-HT_{2A} and 5-HT_{2C} receptors.

AC-90179 shows high potency as an inverse agonist (2.1 nM ± 0.2) and competitive antagonist (2.5 nM ± 0.8) at 5HT_{2A} receptors. AC-90179, clozapine and haloperidol all showed full efficacy as inverse agonists (≥97% of the reference standard ritanserin).

These functional data were confirmed by radioligand binding studies (Table 1).

Clozapine showed similar potency to AC-90179 at 5-HT_{2A} receptors in all three assays, whereas haloperidol was considerably less potent as a competitive antagonist and not detected as an inverse agonist at concentrations up to 100 nM.

Unlike the other drugs, AC-90179 does not have significant potency for 5-HT_{2B}, D₂ and H₁ receptors. These data indicate that the only target interaction of AC-90179, in addition to 5HT_{2A}, is an interaction with the 5HT_{2C} receptor. AC-90179 displays lower affinity (15 fold) and lower potency (26 fold) for 5-HT_{2C} receptors than 5-HT_{2A} receptors.

Behavioral Experiments

Schedule-controlled behavior. Vehicle or AC-90179 (1 – 10 mg/kg, i.p., 30 min) had little effect on response rate of schedule-controlled nose poke behavior in mice. The data for the highest dose tested of 10 mg/kg is shown in Figure 2 as a control point (AC) for AC-90179 alone. DOI (0.3 – 3 mg/kg, i.p., 15 min) produced a dose-dependent decrease

in response rate [$F(4,24) = 11.24, p = 0.0002$; Figure 2]. AC-90179 (10 mg/kg, i.p., 30 min) completely reversed the DOI-induced suppression of response rate (paired t-test of 3 mg/kg DOI versus 3 mg/kg DOI + 10 mg/kg AC-90179: $t = 24.06, df = 4, p < 0.0001$; Figure 2). Only partial reversal of DOI-induced suppression was observed at a lower dose of AC-90179 (1 mg/kg, i.p., 30 min).

Locomotor activity. The dose-response curves of AC-90179, haloperidol and clozapine are shown in Figure 3. In a fifteen minute session, vehicle treated mice traveled, on average, 666 cm to 789 cm. AC-90179 [$F(5,47) = 6.537, p < 0.001$], haloperidol [$F(3,31) = 10.166, p < 0.001$] and clozapine [$F(3,31) = 11.562, p < 0.001$] each dose-dependently decreased spontaneous locomotor activity. The rank order potency of minimum effective doses was haloperidol (0.1 mg/kg i.p., $p < 0.05$), clozapine i.p. (3 mg/kg i.p., $p < 0.05$), and AC-90179 (30 mg/kg s.c., $p < 0.01$).

Phencyclidine (3 mg/kg, i.p., 15 min) in combination with vehicle pretreatment caused a robust hyperactivity that, on average, ranged from 1200 to 2500 s (Figure 4). Due to day-to-day variability of phencyclidine hyperactivity, data were converted to that day's vehicle + phencyclidine control value and averaged across animals in the group. AC-90179 attenuated phencyclidine-induced hyperactivity [$F(5,47) = 2.745, p = 0.031$] with a minimum effective dose of 1 mg/kg s.c. ($p < 0.05$). In a separate experiment (data not shown), AC-90179 (3 mg/kg, i.p., 30 min) shifted the dose-effect curve of phencyclidine (1 – 10 mg/kg, i.p., 15 min) down and to the right, consistent with attenuation of phencyclidine-induced hyperactivity.

Haloperidol reduced phencyclidine-induced hyperactivity [$F(3,31) = 7.479$, $p = 0.001$] at 0.3 mg/kg i.p. ($p = 0.006$) and 1 mg/kg i.p. ($p = 0.007$). Clozapine attenuated phencyclidine-induced hyperactivity [$F(3,31) = 13.309$, $p < 0.001$]. Clozapine showed a significant decrease in phencyclidine activity at 1 mg/kg i.p. ($p = 0.028$) and 3 mg/kg i.p. ($p = 0.002$).

Step-down Latency (Catalepsy): The ability of the test compounds to increase step-down latencies is shown in Figure 5. Normal or vehicle treated mice typically will step down within 10 sec of having their forepaws placed on the bar. Haloperidol was the only compound that increased step-down latencies [$F(3,23) = 12.359$, $p < 0.001$] with a significant increase at 1 mg/kg i.p. ($p < 0.001$).

Acquisition of a nose poke response. The ability of test compounds to disrupt the acquisition of a simple nose poke response for milk reinforcer is shown in Figure 6. AC-90179 had no effect on acquisition of a simple nose-poke response for milk reinforcer until the highest dose tested. The 30 mg/kg (sc) dose elicited a slight, but significant decrease in the number of reinforcers earned [$F(3,15) = 4.208$, $p = 0.03$; 30 mg/kg s.c., $p < 0.05$]. Haloperidol significantly reduced number of reinforcers earned at 0.3 and 1 mg/kg, i.p. [$F(3,15) = 10.146$, $p = 0.001$; post-hoc for 0.3 and 1 mg/kg, $p < 0.05$]. Clozapine dose-dependently decreased the number of reinforcers earned, but the effect failed to reach statistical significance due to individual variability [$F(3,15) = 1.258$, $p = 0.333$].

Pharmacokinetic Experiments

Serum and brain concentrations and oral bioavailability. Values for peak concentrations (C_{\max}), time of peak effect (T_{\max}), area under the curve (AUC), elimination half-life ($t_{1/2z}$), brain/serum ratio, and oral bioavailability (%F) are shown in Table 2. The observed peak serum level occurred 30 min after i.v. administration and ranged from an average of 107.3 ng/ml after 2 mg/kg to an average of 283.9 ng/ml after 4 mg/kg. The serum levels after oral administration were low, not exceeding an average of 9 ng/ml at any time point, resulting in low oral bioavailability ($\leq 5\%$).

Permeability in CACO-2 cells. The apical to basolateral permeability at 10 μM AC-90179 after 40 min was $P_{\text{app}} = 92 \pm 1.3 \text{ cm/s} \times 10^{-6}$, indicating high absorption potential.

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***In vitro* liver microsomes.** AC-90179 was rapidly metabolized after incubation with mouse liver microsomes. With 59%, 34%, and 17% AC-90179 detected at 5, 10, and 20 min, respectively, after onset of incubation with an estimated half-life of 7 minutes and intrinsic clearance value of 94 $\mu\text{l}/\text{min}\cdot\text{mg}$.

Discussion

Consistent with a previous report (Weiner et al., 2001), AC-90179 exhibited intrinsic efficacy as an inverse agonist at 5-HT_{2A} receptors with high potency. AC-90179 also exhibited high affinity for 5-HT_{2A} receptors in a radioligand binding assay. The high potency and high efficacy inverse 5-HT_{2A} agonism is a molecular property shared by all atypical antipsychotic drugs (Weiner et al, 2001). Similar to clozapine, AC-90179 showed weaker potency as an inverse agonist and competitive antagonist at 5-HT_{2C} receptors relative to 5-HT_{2A} receptors. AC-90179 blocked DOI-induced response rate decreases in mice demonstrating the ability to block 5-HT_{2A} receptor signaling in vivo.

Unlike clozapine and haloperidol, AC-90179 exhibited a lack of effect at D₂ receptors. Also unlike clozapine, AC-90179 showed no effect at H₁ receptors. With high potency and efficacy as an inverse agonist at 5-HT_{2A} receptors without D₂ and H₁ receptor antagonism, AC-90179 was predicted to show atypical antipsychotic-like efficacy without D₂ and H₁ mediated side effects such as catalepsy, cognitive dysfunction, and sedation.

Consistent with the behavioral profile of clinically effective antipsychotic drugs, AC-90179 attenuated phencyclidine-induced hyperactivity in mice. These results extend the previous report of dizocilpine-induced hyperactivity blockade by AC-90179 (Weiner et al., 2001). Similarly, in the present study, clozapine and haloperidol attenuated phencyclidine-induced hyperactivity. These effects are consistent with previous reports that the selective 5-HT_{2A} receptor antagonist, M100907 (R(+)- α -(2,3-dimethoxyphenyl)-

1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol), less selective 5-HT₂ receptor antagonists, such as ketanserin and ritanserin, as well as atypical antipsychotics, such as clozapine and olanzapine, have been shown to reduce dizocilpine- and phencyclidine-induced behavioral effects (Carlsson ML et al., 1999; Corbett et al., 1995; Freed et al., 1984; Gleason and Shannon, 1997; Maurel-Remy et al., 1995; Millan et al., 1999; Ninan and Kulkarni, 1998, 1999; O'Neill and Shaw, 1999). Haloperidol has been reported to block dizocilpine- and phencyclidine-induced locomotion only at doses that decreased spontaneous activity (Gleason and Shannon, 1997; O'Neill and Shaw, 1999; Vanover, 1997), consistent with the present findings. Further, AC-90179 showed a wider separation, compared to clozapine, between the dose that attenuated phencyclidine hyperactivity and the dose that reduced spontaneous activity, suggesting that AC-90179 may not suppress general behavior to the same extent as clozapine.

Haloperidol caused catalepsy in mice in the present experiment, an effect that has been reported previously in rodents (Hoffman and Donovan, 1995). Catalepsy is associated with high dopamine D₂ receptor occupancy (Crocker and Hemsley, 2001) and is predictive of extrapyramidal side effects observed in humans (Kurz et al., 1995). One of the hallmarks of atypical antipsychotics is the reduced liability for extrapyramidal side effects (Barnes and McPhillips, 1998; Kurz et al., 1995; Worrel et al., 2000). This is modeled in rodents by the lack of ability to produce catalepsy (Hoffman and Donovan, 1995). The present data with clozapine are consistent with those reports. The present study is the first to demonstrate the lack of cataleptic-like behavior with the novel 5-HT_{2A} receptor inverse agonist, AC-90179.

Haloperidol has been shown to cause cognitive impairment in humans (Goldstone et al., 1979; Weiser et al., 2000). The acquisition of a nose poke response using an autoshaping procedure in mice has been demonstrated previously to be sensitive to pharmacologically induced deficits (Vanover and Barrett, 1998). The present experiment shows that haloperidol induces a deficit in acquisition using this autoshaping procedure at the same dose that caused a decrease in phencyclidine-induced hyperactivity. Clozapine caused a dose-related, but not statistically significant, decrease in acquisition. The lack of a significant cognitive impairment at efficacious doses is consistent with reduced liability for cognitive dysfunction with atypical antipsychotic treatment (Meltzer and McGurk, 1999; Weiser et al., 2000). AC-90179 caused a slight but statistically significant impairment of performance in this task at a dose that was 30 times higher than the dose required to attenuate phencyclidine-induced hyperactivity. This suggests that AC-90179 and related compounds would not cause cognitive impairment in humans at therapeutically efficacious doses.

Cognitive impairment in schizophrenia significantly contributes to unemployment (McGurk and Meltzer, 2000). Extrapyramidal side effects cause subjective discomfort and distress and likely contribute to poor compliance with prescribed medication regimens (Barnes and McPhillips, 1998). The reduced liability of such side effects along with equal or greater efficacy for the treatment of psychotic symptoms have led to the success of atypical antipsychotic treatment. However, many atypical antipsychotics have other side effects that are likely due to interaction with other receptors. There are

multiple hypotheses about what mechanisms contribute to an atypical antipsychotic drug being “atypical”. The relative potency at 5-HT_{2A} receptors over D₂ receptors is one such hypothesis. Selective 5-HT₂ receptor inverse agonists may offer an advantage over other less selective atypical antipsychotic drugs. AC-90179 has shown selectivity for 5-HT_{2A} receptors with no interaction as a competitive antagonist or inverse agonist at 32 monoaminergic G-protein coupled receptors (Weiner et al., 2001). Addressing this potential selectivity in the present study, AC-90179 appeared to impart a greater therapeutic window between antipsychotic-like efficacy (i.e., attenuation of phencyclidine-induced hyperactivity) and psychomotor impairment (i.e., reduction of spontaneous locomotor behavior) than the atypical antipsychotic, clozapine.

Taken together with previous data showing blockade of DOI-induced head twitch behavior or pre-pulse inhibition deficits and blockade of dizocilpine-induced hyperactivity in rodents (Weiner et al., 2001), the present blockade of phencyclidine-induced hyperactivity in mice by AC-90179 is consistent with the behavioral profiles of clinically active antipsychotic drugs. That AC-90179 did not increase step-down latency, even at relatively high doses, and did not cause cognitive impairment or spontaneous locomotion decreases at efficacious doses, suggests that AC-90179 has a favorable side effect profile. Unfortunately, AC-90179 exhibited poor oral bioavailability. This is likely due to extensive metabolism as predicted in liver microsomes, rather than poor intestinal absorption. In fact, AC-90179 showed high permeability in CACO-2 cells and is likely to show good intestinal absorption *in vivo*. Therefore, a compound structurally related to AC-90179 with protection against liver metabolism while maintaining *in vitro*

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and behavioral profiles may be an excellent candidate for the treatment of psychotic disorders.

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Figure Legends

Figure 1. The chemical structure for AC-90179 [2-(4-methoxy-phenyl)-N-(4-methyl-benzyl)-N-(1-methyl-piperidin-4-yl)-acetamide hydrochloride].

Figure 2. The effects of AC-90179 and DOI alone and in combination on schedule-controlled operant behavior in mice. Dose is shown as a function of nose poke response rate (resp/min). Vehicle alone is represented by the open triangle. AC-90179 (1 mg/kg, i.p., 30 min) alone or in combination with DOI (3 mg/kg) is represented by the filled triangle. AC-90179 (10 mg/kg, i.p., 30 min) alone or in combination with DOI (3 mg/kg) is represented by the filled circle. The DOI (0.3 – 3 mg/kg, i.p., 15 min) dose-effect curve is represented by the open circles. Each point represents the mean of 5-6 mice and vertical lines show the standard errors of the mean.

Figure 3. The effects of test compounds on spontaneous locomotor activity in mice. Dose is shown as a function of distance traveled (cm). Panel A shows AC-90179 (filled squares). Panel B shows haloperidol (filled squares) and clozapine (filled circles). Vehicle treatment (V) for each experiment is shown for comparison. Each point represents the mean of 8 mice and vertical lines show the standard errors of the mean. Asterisks designate statistical significance ($p < 0.05$) compared to vehicle control.

Figure 4. The effects of test compounds on phencyclidine-induced hyperactivity in mice. Dose is shown as a function of percent of control phencyclidine hyperactivity. Panel A shows AC-90179 (filled squares) in combination with 3 mg/kg phencyclidine (i.p., 15 min). Panel B shows haloperidol (filled squares) and clozapine (filled circles) in combination with 3 mg/kg phencyclidine (i.p., 15 min). Vehicle treatment (V) plus phencyclidine for each experiment is shown for comparison (open squares). Each point

represents the mean of 8 mice and vertical lines show the standard errors of the mean.

Asterisks designate statistical significance ($p < 0.05$) compared to vehicle control.

Figure 5. The ability of test compounds to induce cataleptic-like behavior in mice. Dose is shown as a function of latency to step down, measured in seconds. Dose-response functions are shown for AC-90179 (panel A), haloperidol (panel B) and clozapine (panel C). Vehicle (veh) is shown for comparison. Each bar represents the mean of 6 mice and vertical lines show the standard errors of the mean. Asterisks designate statistical significance ($p < 0.05$) compared to vehicle control.

Figure 6. The ability of test compounds to impair acquisition of a nose poke response in mice. Dose is shown as a function of the numbers of reinforcers earned. Dose-response functions are shown for AC-90179 (panel A), haloperidol (panel B) and clozapine (panel C). Vehicle (veh) is shown for comparison. Each bar represents the mean of 4 mice and vertical lines show the standard errors of the mean. Asterisks designate statistical significance ($p < 0.05$) compared to vehicle control.

Table 1: Pharmacological characterization of AC-90179. AC90179, and the known antipsychotics haloperidol and clozapine, were assayed in both the cell based functional assay R-SAT (as competitive antagonists and inverse agonists), and by radioligand binding assays. Competitive antagonist experiments were performed in the presence of 1-1.2 micromolar concentration of 5-carboxytryptamine. Binding affinities were determined by displacement of [3H]ketanserin for 5-HT_{2A} and [3H]mesulergine for 5-HT_{2C} receptors. Average Ki values (nM) ± standard deviation are reported based on 2-15 replicate experiments. nr = no response at concentrations up to 100 nM.

Competitive Antagonist – Functional Assay

Receptor	AC-90179	Haloperidol	Clozapine
5-HT_{2A}	2.5 ± 0.8	85 ± 30	6.3 ± 2.2
5-HT_{2B}	nr	nr	17.0 ± 7.9
5-HT_{2C}	64.6 ± 16.8	nr	57.5 ± 40.7
D₂	nr	0.61 ± 1.03	61.1 ± 43.6
H₁	nr	54.2 ± 68.2	0.5 ± 0.6

Inverse Agonist – Functional Assay

5-HT_{2A}	2.1 ± 0.2	nr	13.5 ± 10
5-HT_{2C}	nr	nr	nr

Competitive Antagonist – Receptor Binding Assay

5-HT_{2A}	0.2 ± 0.1	70.2 ± 42.4	1.0 ± 1.3
5-HT_{2C}	2.9 ± 1.2	nr	1.8 ± 1.8
D₂	not tested	0.7 ± 0.2	49.9 ± 4.7

Table 2: Pharmacokinetic values, AC-90179 (Average values, 2 rats per dose)

	i.v. (2 mg/kg)	i.v. (4 mg/kg)	p.o. (4 mg/kg)	p.o. (8 mg/kg)
C _{max} , observed	107.3	283.9	5.54	8.98
T _{max} , observed	30 min	30 min	2 h	30 min
AUC (h·ng/mL)	374	912	38.1	42.7
t _{1/2z} (h)	1.7	1.4	3.7	5.0
Brain (ng/g)/ serum (ng/mL) ratio: 6 h post dose	1.6	1.3	2.1	2.3
Bioavailability, F (%)	-	-	4-5	2-3

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Figure 1.

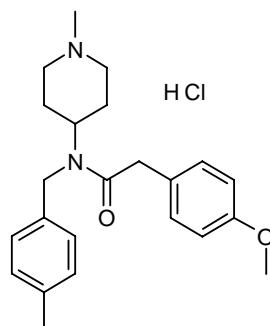


Figure 2.

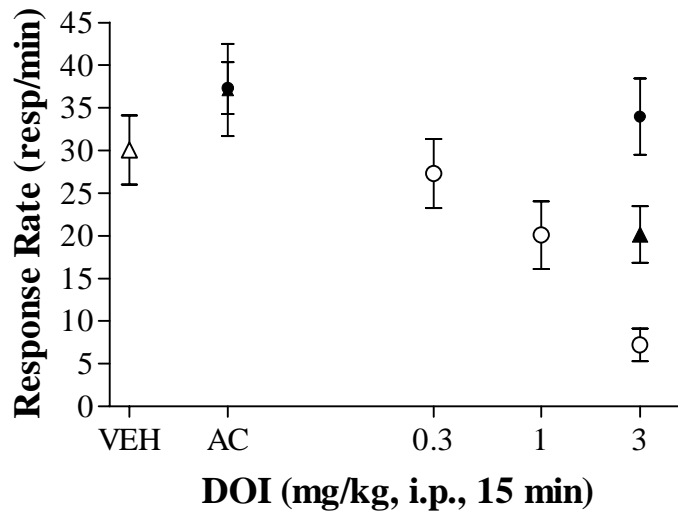
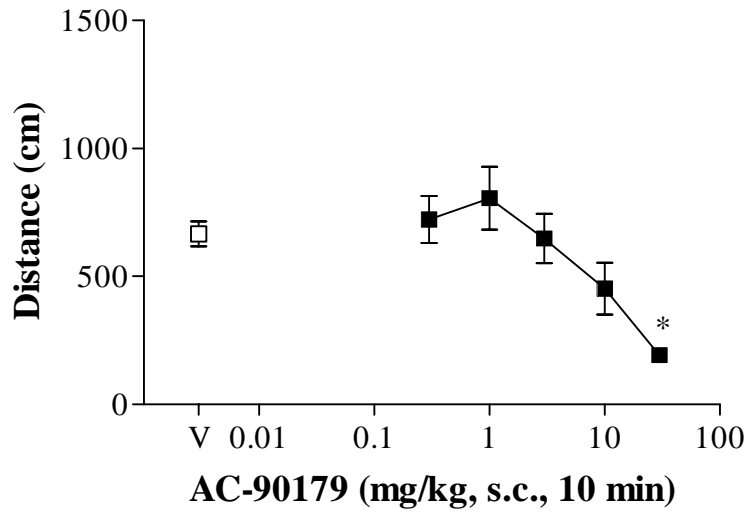


Figure 3.

A.



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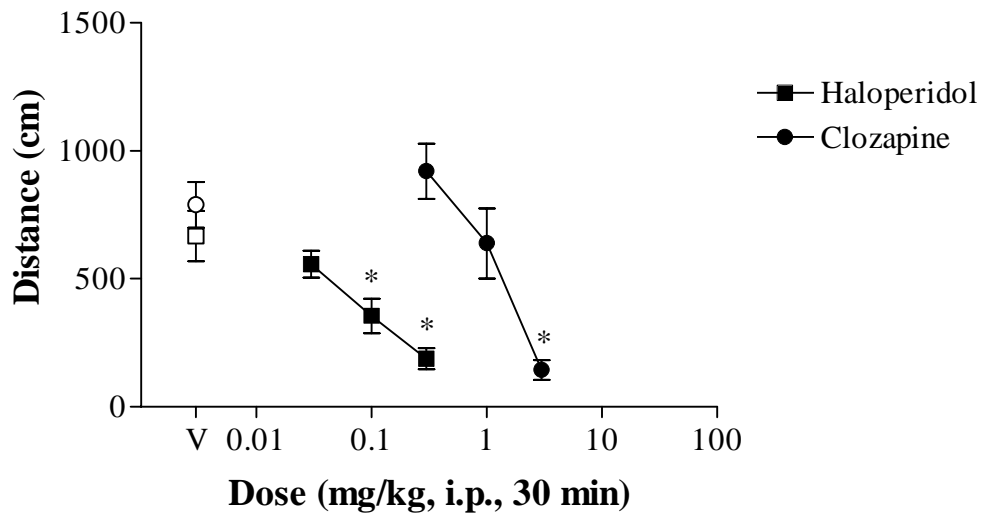
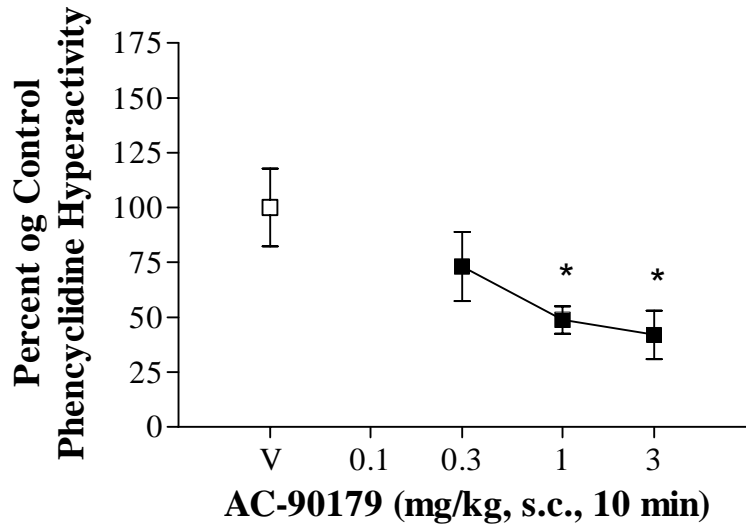


Figure 4.

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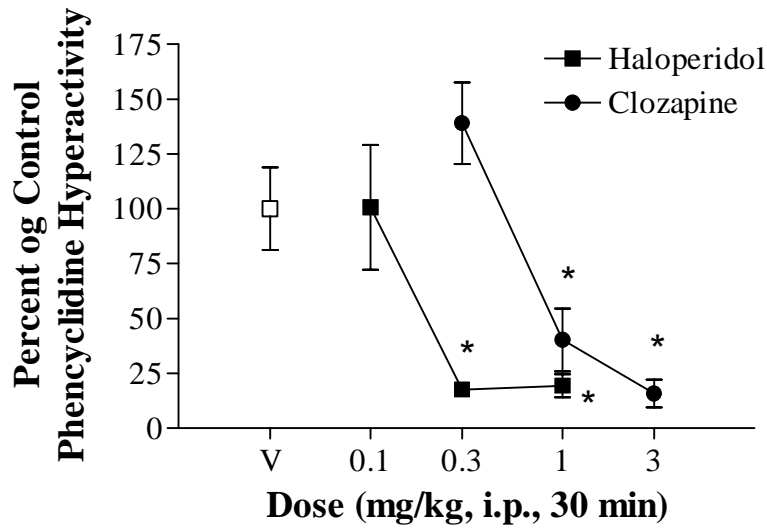
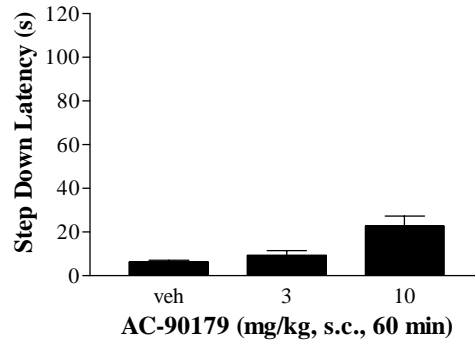
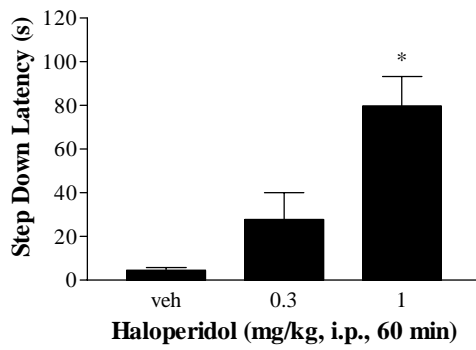


Figure 5.

A.



B.



C.

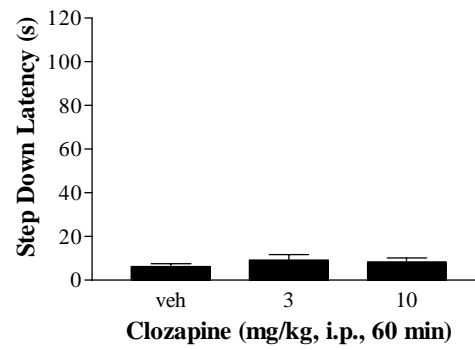
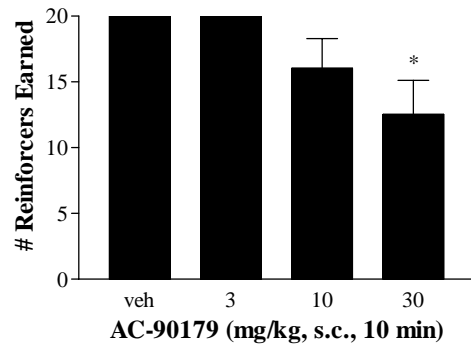
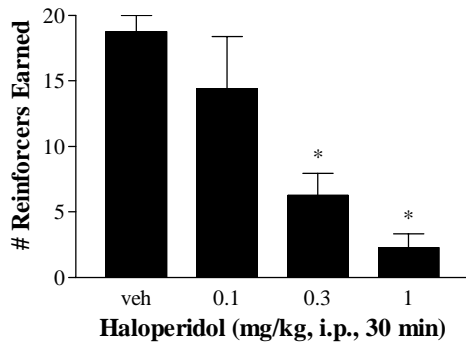


Figure 6.

A.



B.



C.

