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ORIGINAL ARTICLE

Discovery of potential antipsychotic agents possessing pro-cognitive properties

Jelveh Lameh · Krista McFarland · Jorgen Ohlsson · Fredrik Ek · Fabrice Piu · Ethan S. Burstein · Ali Tabatabaei · Roger Olsson · Stefania Risso Bradley · Douglas W. Bonhaus

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Abstract Current antipsychotic drug therapies for schizophrenia have limited efficacy and are notably ineffective at addressing the cognitive deficits associated with this disorder. The present study was designed to develop effective antipsychotic agents that would also ameliorate the cognitive deficits associated with this disease. In vitro studies comprised of binding and functional assays were utilized to identify compounds with the receptor profile that could provide both antipsychotic and pro-cognitive features. Antipsychotic and cognitive models assessing in vivo activity of these compounds included locomotor activity assays and novel object recognition assays. We developed a series of potential antipsychotic agents with a novel receptor activity profile comprised of muscarinic M_1 receptor agonism in addition to dopamine D_2 antagonism and serotonin 5-HT_{2A} inverse agonism. Like other antipsychotic agents, these compounds reverse both amphetamine and dizocilpine-induced hyperactivity in animals. In addition, unlike other antipsychotic drugs, these compounds demonstrate pro-cognitive actions in the novel object recognition assay. The dual attributes of antipsychotic and pro-cognitive actions distinguish these compounds from other antipsychotic drugs and suggest that these compounds are prototype molecules in the development of novel pro-cognitive antipsychotic agents.

Keywords Muscarinic agonism · Dopamine antagonism · Serotonin antagonism · Schizophrenia · Antipsychotic drug (APD)

J. Lameh (✉) · K. McFarland · J. Ohlsson · F. Ek · F. Piu · E. S. Burstein · A. Tabatabaei · R. Olsson · S. R. Bradley · D. W. Bonhaus
ACADIA Pharmaceuticals, Inc, San Diego, CA 92121, USA
e-mail: jelameh@yahoo.com

Present Address:
J. Lameh
Genoptix Medical Laboratory, Carlsbad, CA, USA

Present Address:
J. Ohlsson
AstraZeneca PLC, Sodertalje, Sweden

Present Address:
F. Ek
SPAGO Imaging, Stockholm, Sweden

Present Address:
F. Piu
Otonomy, San Diego, CA, USA

Present Address:
A. Tabatabaei
DART NeuroScience, San Diego, CA, USA

Present Address:
S. R. Bradley
Merck, WestPoint, PA, USA

Present Address:
D. W. Bonhaus
Neuropore Therapies, San Diego, CA, USA

Introduction

Schizophrenia is a debilitating neuropsychiatric disorder that is characterized by hallucinations and delusions (positive symptoms), flattened affect, anhedonia, anergia (negative symptoms), and cognitive disturbances (Wong and Tol 2003). With current treatment regimens, approximately 30% of patients continue to have severe and persistent symptoms and more than 60% of patients fail to achieve full alleviation of symptoms (Kane et al. 1988; Lieberman et al. 2005; Stroup et al. 2006a, b). Moreover, for many patients, intolerable side effects, coupled with limited efficacy, result in premature discontinuation of

therapy (Kane et al. 1988; Stroup et al. 2006a). One symptom domain that is especially poorly addressed by current therapies is the cognitive deficits associated with the disease. This is a significant shortcoming because cognitive impairment is a major determinant of functional outcome in schizophrenia (Green 1996; Green et al. 2000).

Efforts to elucidate the optimal receptor profile of an antipsychotic drug (APD) to maximize overall efficacy and minimize undesired effects have pointed to the therapeutic benefits of dopamine D_2 antagonism combined with 5-HT_{2A} inverse agonism. Reduction of D_2 receptor activation (via antagonism or low-efficacy partial agonism) has been a hallmark of all clinical APDs to date and provides efficacy against the positive symptoms of schizophrenia (Meller et al. 1986). However, excessive blockade of D_2 receptors results in adverse effects on motor function, affect, and cognition. Thus, less than full D_2 receptor occupancy has proven to be advantageous. Concomitant 5-HT_{2A} receptor blockade confers several benefits to the APD therapy. Serotonin 5-HT_{2A} receptor antagonists (and inverse agonists) possess antipsychotic activity in their own right and thus may add to the efficacy conferred by D_2 receptor blockade. These compounds also reduce the level of D_2 receptor blockade required for equivalent efficacy (Abbas and Roth 2008; Gardell et al. 2007). In addition, 5-HT_{2A} antagonists may provide protection against some of the adverse effects associated with dopamine D_2 receptor blockade (Li et al. 2005; Schmidt et al. 1995; Weiner et al. 2001). Current efforts aim not only to develop APDs that contain the core benefits of D_2 and 5-HT_{2A} receptor antagonism but also carry receptor interactions which can potentially confer greater antipsychotic efficacy and specifically address the cognitive deficits in schizophrenia.

Three lines of preclinical and clinical data suggest that muscarinic acetylcholine receptor agonism may be a desired pharmacological activity to incorporate into the profile of an APD. First, there is substantial evidence to suggest that cholinergic transmission is disrupted in schizophrenia with changes in binding and expression of muscarinic M_1 and M_4 receptors (Anagnostaras et al. 2003; Crook et al. 2000; Dean 2004; Dean et al. 1996; Dean et al. 2002; Mancama et al. 2003). Second, muscarinic receptor agonists have demonstrated pro-cognitive actions in animal models and in human subjects (Fisher 2008; Friedman 2004) while disruptions of muscarinic transmission impair cognitive function in experimental animals, healthy human subjects (Anagnostaras et al. 2003; Drachman et al. 1980; Ellis et al. 2006) and potentially in schizophrenic patients (Friedman 2004; Sellin et al. 2008). Third, muscarinic cholinergic agonists have demonstrated antipsychotic activity in several animal models including those reflecting deficits in sensorimotor gating (Bymaster et al. 2002; Stanhope et al. 2001; Vanover et al. 2008). Most recently, (Shekhar et al. 2008) schizophrenia subjects treated with the M_1/M_4 agonist xanomeline showed improvements in scores on the Positive and Negative Syndrome Scale as well as in measures of learning and memory compared with placebo. Together, the findings of alterations in muscarinic receptor expression in schizophrenic patients and the pro-cognitive and antipsychotic actions of muscarinic agonists make a compelling case for the inclusion of muscarinic receptor agonism in the profile of an APD.

To test this hypothesis, we embarked on a drug discovery effort aimed at developing molecules that possessed muscarinic M_1 agonism in addition to D_2 and 5-HT_{2A} antagonism or inverse agonism. A series of small molecules with this pharmacological profile were elaborated and two molecules (pro-cognitive antipsychotic (PCAP)-1 and PCAP-2; Fig. 1) were selected for further in vitro and in vivo characterization.

Materials and methods

Materials

D-Amphetamine sulfate and dizocilpine ((+)-MK-801 hydrogen maleate) were purchased from Sigma (St. Louis, MO). Cell culture media and reagents were purchased from Life Technologies (Carlsbad, CA). 8-Chloro-4-methyl-11-piperizin-1-yl-5*H*-dibenzo[*b,e*][1,4]diazepine (PCAP-1) and 4,8-dichloro-11-piperazin-1-yl-5*H*-dibenzo[*b,e*][1,4]diazepine (PCAP-2) were synthesized at ACADIA Pharmaceuticals Inc. (Malmö, Sweden). All other reagents were purchased from standard vendors. ^{3}H-raclopride (D_2 receptor ligand) and ^{3}H-ketanserin (5-HT_{2A} receptor ligand) were purchased from Perkin Elmer Life Sciences (Boston, MA), and ^{3}H-*N*-methylscopolamine (M_1 receptor ligand) was purchased from GE Healthcare (Piscataway, NJ).

Synthetic procedures

A mixture of an anthranilic acid (1 equivalent), a 2-fluoronitrobenzene (1 equivalent), K_2CO_3 (1 equivalent),

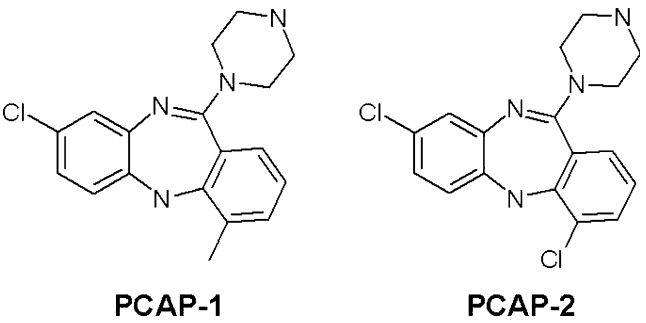

Fig. 1 Chemical structures of PCAP-1 and PCAP-2

Cu_2O (0.05 equivalent), and Cu (0.1 equivalent) was refluxed in 2-ethoxyethanol for 16 h and then cooled to ambient temperature where after water was added, the solution was made acidic and then extracted with EtOAc. Activated carbon was added to the combined organic phases which were then filtered through celite, dried (Na_2SO_4), and concentrated under reduced pressure. The residue was dissolved in EtOH and a mixture of $Na_2S_2O_4$ (5 equivalents) and K_2CO_3 (5 equivalents) in water was added slowly to the solution which thereafter was stirred for 1 h at room temperature. Aq HCl (2 M) was added to the mixture and the aqueous phase was extracted three times with EtOAc, where after the combined organic phases were concentrated under reduced pressure. The residue was taken up in CH_2Cl_2 and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (3 equivalents) was added, and the resulting mixture was stirred at room temperature for 1 h, and then concentrated under reduced pressure. The residue was diluted with EtOAc, washed with aqueous NaOH (2 M), and concentrated under reduced pressure. The resulting oil was taken up into toluene and $POCl_3$ (3 equivalents) and *N,N*-dimethylaniline (4 equivalents) were added, and the subsequent reaction mixture was heated at 100°C for 4 h, and then allowed to reach ambient temperature and concentrated. The residue was taken up in EtOAc and sequentially washed with saturated aqueous $NaHCO_3$, water, and brine, followed by drying (Na_2SO_4) and removal of solvent under reduced pressure. To the resulting yellow oil dissolved in dioxane, triethylamine (2 equivalents) and *N-Boc*-piperazine were added and the reaction mixture was refluxed over night. After cooling to ambient temperature, the reaction mixture was poured into aqueous NH_4Cl and extracted with EtOAc. The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 where after TFA was added and the reaction mixture was stirred for 30 min at room temperature and concentrated. The residue was dissolved in EtOAc, and the resulting solution sequentially washed with 2 M NaOH, water and brine and then dried (Na_2SO_4) and concentrated under reduced pressure.

The HCl salt was formed adding 4 M HCl (1.1 equivalent) in dioxane to the free base in CH_2Cl_2.

8-Chloro-4-methyl-11-(piperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepine. HCl (PCAP-1) 2-Amino-3-methylbenzoic acid (1.0 g, 6.6 mmol) and 5-chloro-2-fluoronitrobenzene (1.6 g, 6.6 mmol) were reacted according to the synthetic procedure to give 776 mg of the title compound. MS (electrospray ionization (ESI)) 327 (MH^+).

4,8-Dichloro-11-(piperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepine. HCl (PCAP-2) 2-Amino-3-chlorobenzoic acid (1.5 g, 8.8 mmol) and 5-chloro-2-fluoronitrobenzene (2.2 g, 9.0 mmol) were reacted according to the synthetic procedure to give 725 mg of the title compound. MS (ESI) 347 (MH^+).

Receptor selection and amplification assays

Receptor selection and amplification (RSAT) functional assays were carried out essentially as described previously (Spalding et al. 2002). NIH-3 T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and 1% penicillin/streptomycin/L-glutamine (PSG) in roller flasks or multilayer flasks and transfected with the plasmid DNA for the human receptor of interest and the plasmid DNA for the beta-galactosidase gene using 0.5% (*v*/*v*) Polyfect reagent (QIAGEN, Valencia, CA). Cells transfected with human M_2 and M_4 receptors were co-transfected with the gene for the chimeric G-protein $G_{qi\text{-}5}$. After 18–20 h, transfected cells were harvested and frozen in aliquots at −80°C until use. On the day of the assay, cells were resuspended in DMEM containing 1% PSG, 0.5% calf serum, and 25% Ultraculture synthetic supplement (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) and plated onto 96-well tissue-culture plates containing varying concentrations of ligand being tested. Cells were grown in a humidified atmosphere with 5% ambient CO_2 for 5 days. Medium was removed from the plates, and beta-galactosidase activity was measured by the addition of *O*-nitrophenyl-D-galactopyranoside in phosphate-buffered saline with 5% Nonidet P40. The resulting colorimetric reaction was measured in a spectrophotometric plate reader (Titertek, Huntsville, AL) at 420 nm. Concentration response curves were generated using nonlinear regression to fit the data to appropriate logistic equations using Prism™ software (Graph-Pad Software, Inc., San Diego, CA).

For agonist and inverse agonist assays, the cells were only treated with varying concentrations of the ligand under study. For antagonist assays, the cells were exposed to a fixed concentration of the appropriate agonist following addition of the antagonist.

Receptor binding assays

Exponentially growing HEK-293 T cells were plated onto 15-cm tissue-culture plates. Cells were transiently transfected with cDNA for the human receptor under study using polyfect transfection reagent (QIAGEN, Valencia, CA). After 48 h, cells were harvested and homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.4. Cell lysates were centrifuged, and the pellets washed three times with ice-cold buffer followed by centrifugation. The final pellet was

resuspended in buffer, frozen in aliquots and stored at −80°C until use. For binding assays, the membranes prepared from cells transfected with appropriate human receptor was added to 96-well plates containing the radioligand of choice for that receptor and varying concentration of the ligand under study and incubated for 3 h at room temperature. At the end of incubation, samples were harvested by filtration onto 24-well GF/B plates (Perkin Elmer) presoaked in 0.3% polyethyleneimine, and washed three times with ice-cold PBS. The radioactivity on the filters was quantitated by scintillation counting using a Top Count (Perkin Elmer).

Locomotor activity assays

All in vivo experiments were conducted in accordance with NIH *Guidelines for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at ACADIA Pharmaceuticals Inc.

Male CF1 mice (15–25 g; Charles River Laboratories) were housed in groups of 8 and received free access to food and water prior to the experiment. Experiments were conducted a minimum of 48 h after arrival, in order to allow acclimation to vivarium conditions. The vivarium was maintained on a 12-h light/dark cycle (lights on, 7 a.m.).

All locomotor activity studies were conducted in mice placed in acrylic chambers (42×42×30 cm) equipped with 16 infrared photo beams along each horizontal axis (front-to-back and side-to-side), from Accuscan Instruments, Inc. (Columbus, OH). Vehicle or test compound was injected 45 min prior to vehicle, D-amphetamine (3 mg/kg, ip) or dizocilpine (0.3 mg/kg, ip) and 15 min later, subjects were placed into the locomotor activity chambers and activity was recorded during a 15 min test session. Thus, animals received two injections and were randomly assigned to one of the following treatment groups (N=8/group): vehicle/vehicle, vehicle/D-amphetamine or vehicle/dizocilpine, PCAP/D-amphetamine, or PCAP/dizocilpine. In order to generate dose–response curves, raw distance traveled (DT) data for the PCAP treatment groups (DT PCAP) were converted to percent inhibition of the augmented locomotor response induced by D-amphetamine or dizocilpine where % inhibition=100−(100*(DT PCAP−mean DT vehicle)/(mean DT D-amphetamine or dizocilpine−mean DT vehicle)). Linear regression analyses were conducted and the ID_{50} values and the corresponding 95% confidence intervals were determined. Mice had no prior exposure to the chambers and each dose combination was tested in separate groups of mice.

To determine the effect of PCAP-1 and PCAP-2 on spontaneous locomotor activity, vehicle or test compound was injected subcutaneously into mice and locomotor activity measured as described above. To determine what doses (if any) produced a statistically significant reduction in spontaneous locomotor activity, one-way analyses of variance (ANOVAs) were conducted on the data for distance traveled followed by post hoc Dunnett's multiple comparisons tests.

Novel object recognition assay

Testing was conducted in CL57BL/6 mice (15–25 g, Charles Rivers Laboratories) in three phases: acclimation, sample, and test. During the acclimation phase, each group of six mice was placed collectively into a novel chamber and allowed to explore freely for 30 min. In the sample phase, mice were treated with vehicle, PCAP-1 or PCAP-2, and 30 min later were placed individually into the NOR chamber containing two identical objects. Each mouse was allowed to explore the chamber and the objects for 3 min, and the time spent exploring at each position was recorded. During the test phase, one familiar object (seen during sample) and one novel object were placed into the chamber, and each mouse was allowed 3 min to explore. The test phase was conducted either 1 or 2 h after the sample phase. These time points were chosen based on pilot experiments demonstrating that 1 h following exposure, untreated mice retained memory for the familiar object and thus showed a preference for the novel object. In contrast, 2 h after exposure untreated mice had no preference for either the novel or familiar object. Thus, there were two test conditions, the 1-h condition where animals demonstrated recognition memory and compounds could be tested to see if they impaired memory, and the 2-h condition where untreated animals did not display recognition memory and compounds could be tested to see if they improved memory. The test sessions were recorded on video and scored by an observer blind to each subject's treatment regimen. The time an animal explored the novel object was calculated as a percent of the total time spent in exploration and a one-way (treatment) ANOVA was conducted followed by Dunnett's multiple comparisons post hoc analyses to determine the dose(s) where performance differed significantly from vehicle control.

Pharmacokinetics

PCAP-1 and PCAP-2 were prepared at 1 mg/ml in 5% EtOH, 45% propylene glycol, and 50% H_2O. Sprague Dawley rats (n=3; Charles River Laboratories, Indianapolis, IN) were dosed per compound either orally at 10 mg/kg or intravenously at 1 mg/kg. Plasma samples were collected at 0, 0.083, 0.176, 0.25, 0.5, 1, 2, 4, 6, and 24 h post-dosing and analyzed by LC/MS/MS. The LC/MS/MS analysis was performed using a Micromass Quattro Ultima (Copenhagen, Denmark) tandem quadropole mass spectrometer equipped with ESI, in positive ion mode and operated in multiple reaction monitoring mode. PCAP-1 and PCAP-2 ion pairs were 326.9/284 and 347.0/304, respectively. The mass

spectrometer was coupled to a Waters 1525 μ HPLC system (Copenhagen, Denmark) and a CTC HTC PAL autosampler (Vaerlose, Denmark). Separation was performed using a 50× 1-mm Phenomenex Synergi Polar RP (Genetec, Lund, Sweden). LC solvent A was water/acetonitrile (95:5) and B was acetonitrile/water (95:5), each containing 0.5% acetic acid. Data collection and processing were performed using Masslynx software v 4.0. The pharmacokinetic parameters were calculated with WinNonlin 5.0 (Pharsight, Mountain View, CA).

Microsomal stability assay

Compounds were incubated at final concentration of 1 μM in the presence of rat or human liver microsomes (0.5 mg protein/mL) in Tris buffer (100 mM, pH 7.4). Following a 5-min pre-incubation at 37°C, reactions were commenced with the addition of NADPH to a final concentration of 2 mM. Samples were collected at 0, 5, 10, and 30 min post-incubation. Reaction was terminated by addition of samples to wells containing acetonitrile. Following centrifugation, the supernatant was subject to analysis by LC/MS/MS.

Results

Receptor activity profiles of PCAP-1 and PCAP-2 were determined and compared with the activities of other antipsychotic agents using RSAT assays.

PCAP-1 and PCAP-2 were found to have agonist activity at muscarinic M_1, M_2, M_4, and M_5 receptors (Table 1) while most other APDs did not show agonist activity at these receptors. Clozapine exhibited weak agonist activity at M_1, M_2, and M_4 but not M_3 nor M_5 receptors. While most APDs did not have any agonist activity at M_1 receptors, all of the compounds bound to M_1 receptors (Table 3), and olanzapine, quetiapine, and clozapine were found to be functional antagonists at these receptors (data not shown). None of the compounds tested had agonist activity at the muscarinic M_3 receptor subtype.

Both PCAP-1 and PCAP-2 were found to be antagonists with potencies similar to olanzapine and clozapine at 5-HT_{2A} receptors (Table 2). These compounds also had antagonist activity at 5-HT_{2C} receptors. Since these compounds also inhibited constitutive activity at 5-HT_{2A} and 5-HT_{2C} receptors (data not shown), they can be classified as inverse agonists.

Binding assays confirmed the direct interaction of these compounds with 5-HT_{2A} receptors yielding estimates of affinity that were similar to the potencies observed in the functional assays (Table 3). PCAP-1 and PCAP-2 bound to dopamine D_2 receptors with an affinity lower than that of most other APDs but comparable to that of quetiapine (Table 3). Like other APDs, excepting aripiprazole, PCAP-1 and PCAP-2 had no detectable agonist activity at the D_2 receptor (data not shown).

Activity of PCAP-1 and PCAP-2 was also assessed at a number of other related receptors. These compounds had no agonist activity at alpha adrenoceptors (alpha1 or alpha2), H_4 histamine, $5HT_{1A}$ serotonin, or D_1 dopamine receptors. These compounds showed antagonist activity at alpha adrenoceptors (alpha1 and alpha2) and H_1 histamine receptors, but with 100-fold lower potency compared with their activity at serotonin $5HT_2$ and dopamine D_2 receptors.

Table 1 Agonist activity at muscarinic receptors

Target	M_1 pEC_{50} (% E_{max})	M_2 pEC_{50} (% E_{max})	M_3 pEC_{50} (% E_{max})	M_4 pEC_{50} (% E_{max})	M_5 pEC_{50} (% E_{max})
Compound					
Aripirazole (Abilify)	NA (n=2)	NA (n=2)	NA (n=1)	NA (n=1)	NA (n=1)
Olanzapine (Zyprexa)	NA (n=13)	NA (n=18)	NA (n=10)	NA (n=10)	NA (n=9)
Risperidone (Risperdal)	NA (n=5)	NA (n=2)	NA (n=2)	NA (n=5)	NA (n=5)
Quetiapine (Seroquel)	NA (n=5)	NA (n=5)	NA (n=3)	NA (n=3)	NA (n=2)
Ziprasidone (Geodone)	NA (n=3)	NA (n=2)	NA (n=4)	NA (n=4)	NA (n=4)
Clozapine (Clozaril)	8±0.6 21±9 (n=17)	6.2±0.5 59±30 (n=20)	NA (n=13)	7.4±0.3 52±14 (n=10)	NA(n=11)
PCAP-1	7.1±0.4 103±13 (n=4)	6.3±0.4 153±26 (n=4)	NA (n=3)	6.6±0.2 100±7 (n=3)	6.8±0.1 62±8 (n=2)
PCAP-2	7.1±0.2 96±22 (n=4)	6.1±0.3 124±24 (n=4)	NA (n=3)	7.3 50	7.3 68

Percent efficacy is relative activity of each compound compared with the muscarinic agonist carbachol. Olanzapine, quetiapine, and clozapine are antagonists at M_1 with pK_i values of 7.2±0.5 (n=9), 6.4±0.3 (n=2), and 7.7±0.3 (n=6), respectively

NA no activity, *ND* not determined

Table 2 Antagonist activity at serotonin receptors

Target	5-HT_{2A} (pK_i)	5-HT_{2C} (pK_i)
Compound		
Aripirazole (Abilify)	NA (n=7)	6.6±0.8 (n=3)
Olanzapine (Zyprexa)	7.9±0.6 (n=15)	6.8±0.4 (n=12)
Risperidone (Risperdal)	9.7±0.5 (n=14)	7.0±0.3 (n=10)
Quetiapine (Seroquel)	6.6±0 (n=2)	NA (n=3)
Ziprasidone (Geodone)	8.8±0.8 (n=10)	7.5±0.3 (n=6)
Clozapine (Clozaril)	8.0±0.5 (n=17)	7.3±0.3 (n=14)
PCAP-1	7.9±0.3 (n=5)	7.7±0.4 (n=4)
PCAP-2	8.3±0.1 (n=7)	9.3±0.3 (n=2)

Antagonist activity of each compound was assessed in the presence of a fixed concentration of the serotonin receptor agonist 5-CT and expressed as pK_i (the negative of the logarithm of K_i). K_i values were calculated from IC_{50} (concentration in molars that produces 50% maximal repression in the antagonist assay) using Cheng–Prusoff equation; $K_i=IC_{50}/\{1+[agonist]/EC_{50}agonist\}$

Finally, PCAP-1 and PCAP-2 inhibited hERG channels with IC_{50} values of 1.1 and 0.6 μM, respectively (study conducted by Aviva Biosciences San Diego California).

The activity of these compounds was assessed in two different animal models of antipsychotic activity. Both PCAP-1 and PCAP-2 inhibited D-amphetamine-induced hyperactivity with ID_{50} values of 3.4 and 0.25 mg/kg, respectively (Fig. 2). In a similar manner, these compounds potently inhibited dizocilpine-induced hyperactivity with ID_{50} values of 0.17 and 0.21 mg/kg, respectively (Fig. 3). During the course of these studies, no overt adverse effects on behavior were noted for these compounds. Moreover, neither salivation nor diarrhea was observed after treatment with these compounds.

Table 3 Binding to various receptors

	M_1 (pK_i)	5-HT_{2A} (pK_i)	D_2 (pK_i)
Aripirazole (Abilify)	5.8±0.2 (n=2)	7.8±0.2 (n=5)	8.9±0.3 (n=3)
Olanzapine (Zyprexa)	8.0±0.1 (n=2)	9.6±0.2 (n=6)	8.5±0.3 (n=5)
Risperidone (Risperdal)	5.4±0.1 (n=2)	10.4±0.2 (n=6)	8.8±0.8 (n=6)
Quetiapine (Seroquel)	6.6±0.1 (n=2)	7.5±0.2 (n=3)	6.6±0.4
Ziprasidone (Geodone)	5.8±0.2 (n=2)	9.9±0.8 (n=2)	8.0±0.4 (n=8)
Clozapine (Clozaril)	7.8±0.6 (n=9)	8.7±0.3 (n=5)	7.0±0.4 (n=5)
PCAP-1	7.4±0.3 (n=3)	7.9±0.4 (n=5)	6.0±0.5 (n=5)
PCAP-2	7.5±0.4 (n=3)	8.0±0.2 (n=2)	6.3±0.5 (n=6)

Binding affinity of PCAP-1 and PCAP-2 as compared with other APDs were assessed at various receptors. Data presented are mean± SD. Binding affinity of each compound was assessed in the presence of a fixed concentration of the radioligand for each receptor as outlined in "Materials and methods." pK_i (the negative of the logarithm of K_i) and K_i values were calculated from IC_{50} (concentration in molars that produces 50% inhibition of binding) using Cheng–Prusoff equation; $K_i=IC_{50}/\{1+[ligand]/K_d ligand\}$

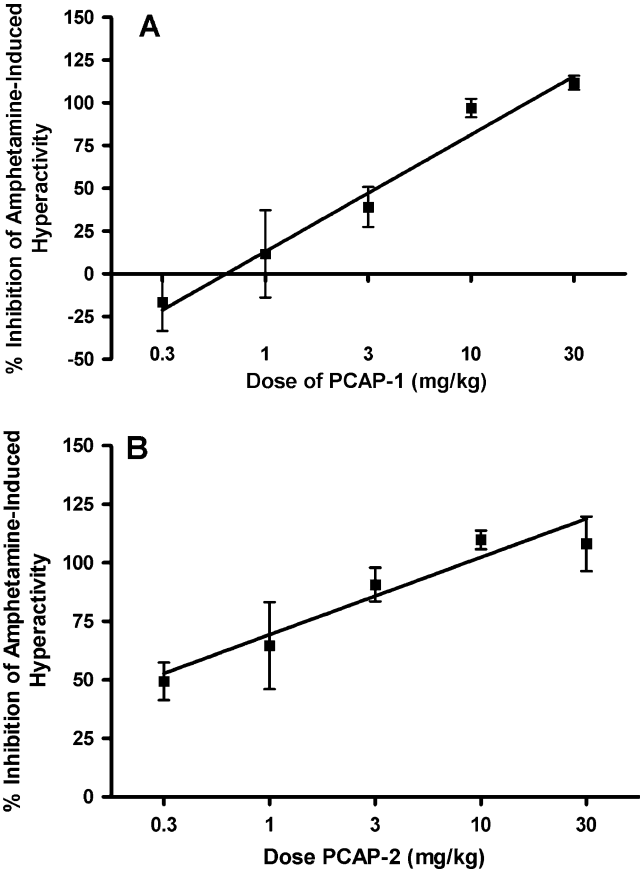

Fig. 2 Effect of PCAP-1 and PCAP-2 on D-amphetamine-induced locomotor activity. PCAP-1 and PCAP-2 were assessed for their ability to inhibit hyperactivity induced by amphetamine, a behavioral assay indicative of D_2 antagonism and predictive of antipsychotic activity in humans. Both compounds inhibited amphetamine-induced hyperactivity in mice with ID_{50} of 3.4 and 0.25 mg/kg, respectively

The effect of these compounds on spontaneous locomotor activity was also measured. A one-way (treatment) ANOVA on the data in Fig. 4a demonstrated that PCAP-1 did not have any effect on spontaneous locomotion at doses as high as 30 mg/kg ($F(4, 34)=0.57$, $p>.05$). A one-way (treatment) ANOVA on the data in Fig. 4b showed a significant effect of treatment ($F(4, 34)=11.83$, $p<.05$). A Dunnett's multiple comparisons test, showed that PCAP-2 did not have an effect on spontaneous locomotor activity at doses below 10 mg/kg.

These data show that 100-fold or higher doses of these compounds are required to cause any reduction in spontaneous locomotor activity compared with the doses required to reduce D-amphetamine- or dizocilpine-induced hyperactivity.

A key aim of the present study was to identify novel antipsychotic agents with beneficial pro-cognitive effects. First, PCAP-1, PCAP-2, and selected APDs (risperidone, olanzapine, quetiapine, clozapine, and aripiprazole) were tested to see if they interfered with performance in the

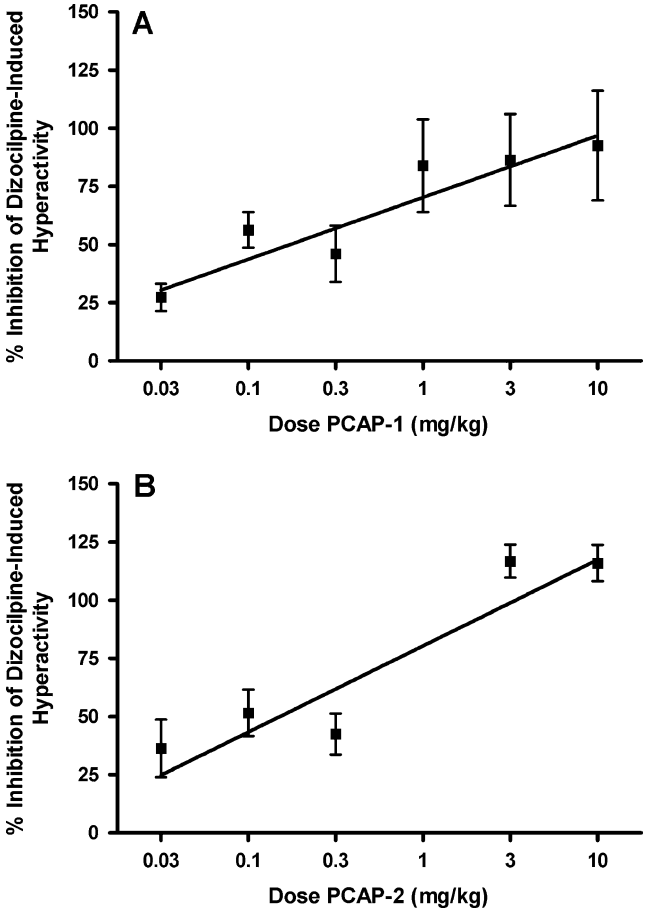

Fig. 3 Effect of PCAP-1 and PCAP-2 on dizocilpine-induced locomotor activity. PCAP-1 and PCAP-2 were assessed for their ability to inhibit hyperactivity induced by dizocilpine, a behavioral assay indicative of 5-HT_{2A} antagonism and predictive of antipsychotic activity in humans. Both compounds inhibited dizocilpine-induced hyperactivity in mice with ID_{50} of 0.17 and 0.21 mg/kg, respectively

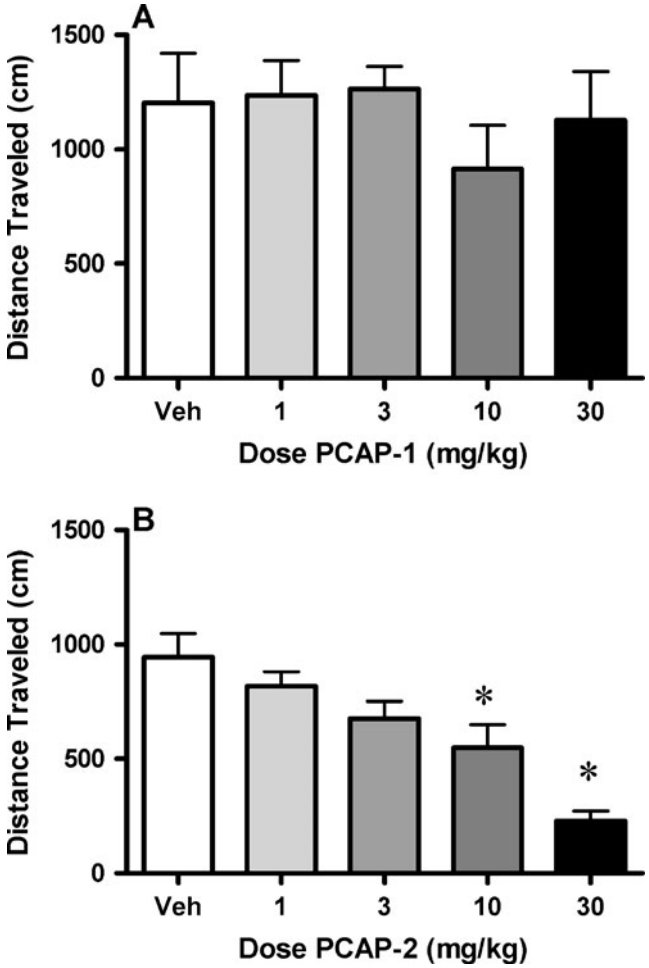

Fig. 4 Effect of PCAP-1 and PCAP-2 on spontaneous locomotor activity. PCAP-1 and PCAP-2 were assessed for their ability to inhibit spontaneous locomotor activity. PCAP-1 did not have any effect on spontaneous locomotor activity at doses up to 30 mg/kg (one-way (treatment) ANOVA ($F(4, 34)=0.57$, $p>.05$)). PCAP-2 inhibited locomotor activity at doses 10 mg/kg and higher. A one-way (treatment) ANOVA on this data showed a significant effect of treatment ($F(4, 34)=11.83$). However, subsequent Dunnett's multiple comparisons test showed that PCAP-2 did not have a statistically significant effect on locomotor activity at doses below 10 mg/kg

novel object recognition assay at the 1-h time point where recognition memory for the familiar object is retained by vehicle-treated subjects. While PCAP-1 and PCAP-2 did not adversely affect novel object recognition, the APDs tested, with the exception of aripiprazole, adversely affected performance in this assay. A one-way ANOVA on the data in Table 4 revealed a significant effect of treatment condition, $F(7,141)=7.44$, $p<.05$ (Table 4). A Dunnett's multiple comparisons test showed that animals pretreated with risperidone (0.01 mg/kg, sc), olanzapine (0.1 mg/kg, sc), quetiapine (1.0 mg/kg, sc), or clozapine (1.0 mg/kg, sc) showed impaired novel object recognition compared with vehicle-treated subjects. Since PCAP-1 and PCAP-2 did not impair novel object recognition performance, we were able to test them for the ability to improve performance in the 2-h condition where untreated animals did not display recognition memory. Notably, both these compounds demonstrated robust pro-cognitive activity, at doses of 10 and 3 mg/kg, respectively (Fig. 5). A one-way ANOVA on the data shown in Fig. 5a revealed a statistically significant effect of treatment condition ($F(3, 52)=3.41$, $p<.05$). Subsequent post hoc analyses using Dunnett's multiple comparisons test revealed that a dose of 10 mg/kg of PCAP-1 produced a statistically significant improvement in novel object recognition performance. Similarly, a one-way ANOVA on the data shown in Fig. 5b revealed a significant effect of treatment condition ($F(3, 50)=4.68$, $p<.01$). Subsequent post hoc analyses using Dunnett's multiple comparisons test revealed that a dose of 3 mg/kg of PCAP-2 produced a significant improvement in novel object recognition performance. Thus, both PCAP-1 and PCAP-2 were able to improve recognition memory performance.

Table 4 Activity of various antipsychotic drugs in novel object recognition assay

	% novel±SEM
Vehicle	63.5±1.7
Aripirazole (Abilify)	53.7±4.2 at 0.1 mg/kg
Olanzepine (Zyprexa)	49.5±3.6* at 0.1 mg/kg
Risperidone (Risperdal)	46.8±3.7* at 0.01 mg/kg
Quetiapine (Seroquel)	47.9±3.8* at 1 mg/kg
Clozapine (Clozaril)	48.5±3.3* at 1 mg/kg
PCAP-1	64.8±3.5 at 10 mg/kg
PCAP-2	71.1±3.9 at 3 mg/kg

Novel object recognition was assessed 1 h after exposure to a familiar object. With the exception of aripiprazole-treated subjects that showed no reliable difference from either chance performance (50%) or vehicle-treated subjects, subjects pretreated with antipsychotic drugs showed impaired recognition relative to vehicle-treated subjects. *$p<.05$ in a one-way ANOVA followed by Dunnett's multiple comparisons test

To further assess the potential effects of the PCAP compounds on general motoric capacity, an analysis of time (in seconds) spent exploring objects was conducted. For PCAP-1 (Fig. 6a), a two-way dose (vehicle, 1, 3, and 10 mg/kg) *X* test condition (sample or test) ANOVA revealed a significant main effect of dose ($F(3, 52)=6.61$, $p<.01$), a significant main effect of test condition ($F(1, 52)=76.09$, $p<.01$), and a significant dose *X* test condition interaction ($F(3, 52)=9.85$, $p<.01$). Bonferroni repeated measures post hoc analyses showed that there were no significant differences in exploration time between mice treated with vehicle or PCAP-1 during test. There was a statistically significant reduction in exploration time during sample in mice treated with 10 mg/kg of PCAP-1. Although it seems unlikely that a reduction in sampling time would underlie improved memory for the sampled object, it is possible that it could change a subject's ability to recognize the novel object. Therefore, a correlation analysis was conducted on sample times and percent of exploration time at novel object (% novel). This analysis revealed no significant correlation between sample times and novel object recognition formed, Pearson $r=-0.36$ ($r^2=0.13$), $p<.05$, suggesting that the decrease in sample time did not lead to a concomitant change in novel object recognition. For PCAP-2 (Fig. 6b), a two-way dose (vehicle, 0.3, 1, and 3 mg/kg) *X* test condition (sample or test) ANOVA revealed only a significant main effect of test condition ($F(1, 53)=89.95$, $p<.01$), but no main effect of dose ($F(3, 53)=0.81$, $p>05$), and no dose *X* test condition interaction ($F(3, 53)=1.91$, $p>05$). The main effect of treatment condition for both PCAP-1 and PCAP-2 demonstrates that mice spent less time exploring during the test session than during the sample session, presumably because

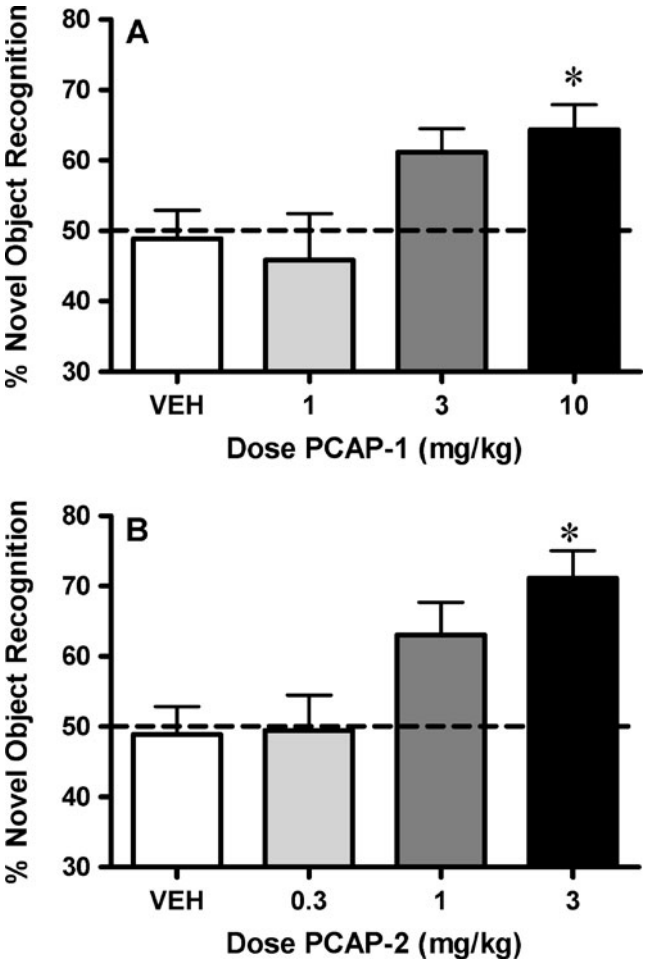

Fig. 5 Effect of PCAP-1 and PCAP-2 on novel object recognition. Pro-cognitive activities of PCAP-1 and PCAP-2 were assessed in the novel object recognition assay. Both compounds increased the percent of time spent exploring the novel object, indicative of improved memory for the familiar object (at 10 and 3 mg/kg, respectively). Statistical analysis was performed using one-way ANOVA test followed by Dunnett's multiple comparisons post hoc analyses; *$p<0.05$ relative to vehicle controls

the chamber and familiar object were no longer novel. Since exploration times during sample were decreased only at the highest dose of PCAP-1 tested and not by any dose of PCAP-2 tested and since mice still showed augmented attention to the novel object as compared with vehicle controls, interpretation of the novel object recognition data is not confounded by the motoric effect of PCAP-1.

Pharmacokinetic profiles of these compounds were assessed in rats (Table 5). Both PCAP-1 and PCAP-2 were orally bioavailable in the rat (65% and 90% respectively). Plasma clearance were moderate (3.5 and 7 mL/min*kg) with terminal half lives of elimination of 2.2 and 3.1 h, respectively. The compounds were stable in human microsomes suggesting acceptable human bioavailability and half-life.

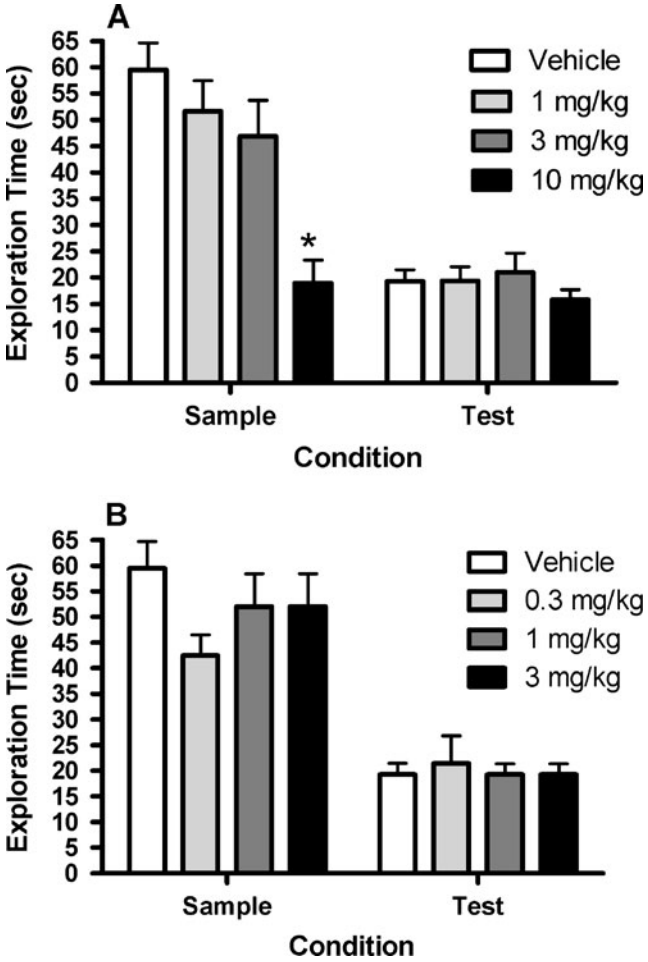

Fig. 6 Effect of PCAP-1 (**a**) and PCAP-2 (**b**) on exploration time. Locomotor effects of PCAP-1 and PCAP-2 were assessed by measuring exploration times during novel object recognition. PCAP-1 decreased exploration time during sample at 10 mg/kg but had no effect on exploration time during test. PCAP-2 had no significant effect on exploration times during sample or test. Statistical analysis was performed using two-way ANOVA, followed by Bonferroni repeated measures post hoc analyses; *$p<0.05$ relative to vehicle controls

Discussion

Drug discovery efforts yielded compounds with a pharmacological profile, comprised of muscarinic M_1 agonism, dopamine D_2 antagonism, and serotonin 5-HT_{2A} antagonism/inverse agonism.

With respect to their interaction with the dopamine D_2 receptor the two selected compounds acted as relatively low micromolar affinity antagonists with no agonist activity. This relatively low affinity interaction may be beneficial in reducing the risk of motoric side effects (Seeman 1992). In this context, the affinity of PCAP-2 for dopamine D_2 receptors is similar to that of the APD quetiapine, which is known to confer little risk for motoric side effects (Kapur and Remington 2001).

With respect to their interaction with serotonin receptors, both compounds demonstrated high potency as antagonists and inverse agonists at the 5-HT_{2A} receptor and thus have, what has been proposed to be, an ideal 5-HT_{2A} to D_2 potency ratio (Meltzer et al. 1989). As noted, a high ratio of 5-HT_{2A} to D_2 receptor affinities may confer increased efficacy with lower risk for liabilities associated with D_2 receptor blockade. Both compounds were also antagonists at 5-HT_{2C} receptors. The possible consequences of 5-HT_{2C} receptor blockade are not certain. However, 5-HT_{2C} blockade may contribute a beneficial effect in reducing the risk for adverse motoric effects and contributing to antipsychotic efficacy (Wood et al. 2001; Millan et al. 1998) but could also confer a risk for undesired metabolic effects (Miller 2005).

The pharmacological feature of these ligands that distinguishes them from other APDs is their agonist properties at muscarinic M_1, M_2, M_4, and M_5 (but not M_3) receptors. This profile of activity across muscarinic receptor subtypes may be nearly ideal since substantial evidence points to the M_1 receptor being the mediator of the pro-cognitive actions by muscarinic agonists (Anagnostaras et al. 2003; Christie et al. 1981; Sitaram et al. 1978) while both the M_1 and M_4 receptor may contribute to an antipsychotic action (Shekhar et al. 2008; Stanhope et al. 2001). The absence of M_3 agonist activity is important since M_3 receptors mediate most of the dose-limiting adverse effects associated with nonselective muscarinic agonists (Bymaster et al. 2003). Relative to other APDs, these compounds have lower potency at H_1 receptors raising the possibility that they would have a lower propensity to cause sedation and weight gain (Kim et al. 2007; Kroeze et al. 2003). These ligands also have relatively low potency at α_{1A} adrenoceptors suggesting lower propensity for sedation or orthostatic hypotension (Lepor et al. 2000; Takata et al. 1999).

Table 5 Pharmacokinetic profiles of PCAP-1 and PCAP-2

	Human liver microsome intrinsic clearance (μl/min*mg)	Rat liver microsome intrinsic clearance (μl/min*mg)	Bioavailability in rat (%)	Plasma clearance in rat (mL/min*kg)	Half-life in rat (h)
PCAP-1	0	15	65	3.5	2.2
PCAP-2	5	23	90	7	3.1

Consistent with their receptor pharmacology profiles at dopamine D_2 and serotonin 5-HT_{2A} receptors, PCAP-1 and PCAP-2 were active in the amphetamine and dizocilpine hyperactivity models of antipsychotic activity. The absence of any effect on spontaneous locomotor activity at relevant doses demonstrates that this activity was specific to the psychostimulant-induced hyperactivity. These findings are not in and of themselves surprising, given the extensive precedence of activity of atypical APDs in these models. They do, however, confirm that the compounds are biologically active and that they do not posses any undiscovered activity which could counteract their antipsychotic actions.

The activity of PCAP-1 and PCAP-2 in the novel object recognition assay is a remarkable finding since this activity distinguishes these molecules from all of the commonly prescribed APDs. And, as in the case of the assays of antipsychotic activity, the observed effects were specific to object recognition and not a nonspecific artifact of altered locomotor activity. The pro-cognitive action of these compounds is almost certainly attributable to their agonist activity at muscarinic receptors since this pharmacological feature is the only clearly distinguishing feature of these molecules as compared with other APDs. Other APDs tested in this study (olanzapine, quetiapine, risperidone, and clozapine) all impair novel object recognition. This effect of these compounds is likely due to their antagonist activity at M1 receptors. Thus, these findings, in addition to highlighting the potential utility of PCAP-1 and PCAP-2 as novel APD drugs with beneficial effects on cognitive function, lend further support to clinical findings suggesting the benefits of M_1 agonism in the receptor activity profile of antipsychotic agents (McArthur et al. 2010).

It is worth noting that, in contrast to the present study, clozapine has shown pro-cognitive activity in some previous novel object recognition studies (Grayson et al. 2007; Karasawa et al. 2008; Mizoguchi et al. 2008; Snigdha and Neill 2008). In this previous work, clozapine was examined for the ability to reverse a deficit in novel object recognition induced by phencyclidine, dizocilpine or methamphetamine. Thus, while clozapine impaired NOR similar to delay-induced decrements, it may have the ability to rectify decrements in performance caused by phencyclidine, dizocilpine and methamphetamine. Clearly however, the current results in the time-delay NOR task distinguish PCAP-1 and PCAP-2, with their robust muscarinic agonist activity, from all commonly used APDs.

In summary, a drug discovery effort aimed at incorporating muscarinic receptor agonism into the D_2/5-HT_{2A} receptor antagonist profile common to other APDs has yielded two molecules, PCAP-1 and PCAP-2, with the unique attribute of being active in both cognition and psychosis models. These molecules were orally bioavailable and well tolerated in animals at doses well above those associated with antipsychotic activity. Further studies will determine whether inclusion of muscarinic receptor agonism into the D_2/5-HT_{2A} profile, common to most atypical APDs, in fact confers additional, clinically meaningful, benefits.

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