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Unexpected antipsychotic-like activity with the muscarinic receptor ligand (5*R*,6*R*)6-(3-propylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo[3.2.1]octane

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Abstract

(5*R*,6*R*) 6-(3-propylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo[3.2.1]octane (PTAC) is a potent muscarinic receptor ligand with high affinity for central muscarinic receptors and no or substantially less affinity for a large number of other receptors or binding sites including dopamine receptors. The ligand exhibits partial agonist effects at muscarinic M_2 and M_4 receptors and antagonist effects at muscarinic M_1, M_3 and M_5 receptors. PTAC inhibited conditioned avoidance responding, dopamine receptor agonist-induced behavior and D-amphetamine-induced FOS protein M_5 expression in the nucleus accumbens without inducing catalepsy, tremor or salivation at pharmacologically relevant doses. The effect of PTAC on conditioned avoidance responding and dopamine receptor agonist-induced behavior was antagonized by the acetylcholine receptor antagonist scopolamine. The compound selectively inhibited dopamine cell firing (acute administration) as well as the number of spontaneously active dopamine cells (chronic administration) in the limbic ventral tegmental area (A10) relative to the non-limbic substantia nigra, pars compacta (A9). The results demonstrate that PTAC exhibits functional dopamine receptor antagonism despite its lack of affinity for the dopamine receptors and indicate that muscarinic receptor partial agonists may be an important new approach in the medical treatment of schizophrenia.

Keywords: Muscarinic receptor; Antipsychotic; Schizophrenia; Fos protein immunoreactivity; Conditioned avoidance responding; Single unit recording

1. Introduction

All clinically efficacious neuroleptics block central dopamine receptors with the average antipsychotic dose of a neuroleptic correlating with its ability to inhibit binding of the dopamine D_2 receptor ligand [^{3}H]spiroperidol in brain homogenates (Seeman et al., 1976). Even though involvement of the central cholinergic system in the pathophysiology of schizophrenia has been suggested (Pfeiffer and Jenney, 1957; Chalmers and Erickson, 1964; Karson et al., 1993; Yeomans, 1995; White and Cummings, 1996) and even though muscarinic receptors are located in limbic areas associated with schizophrenia such as the nucleus accumbens and the prefrontal cortex (Zilles et al., 1989; Levey et al., 1991), most attention has been paid to the beneficial effects of anticholinergics in the treatment of extrapyramidal side effects induced by antipsychotic medication. The prominent parasympathomimetic effects of muscarinic receptor agonists however, preclude interpretation of their therapeutic potential. We report here on a novel, selective muscarinic receptor ligand (5*R*,6*R*)6-(3-propylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo[3.2.1]octane (PTAC; see Fig. 1 for chemical structure), with partial agonist effects at muscarinic m_2 and m_4 receptors, which does not produce tremor or salivation and which exhibits a preclinical profile suggestive of antipsychotic efficacy. The

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PTAC

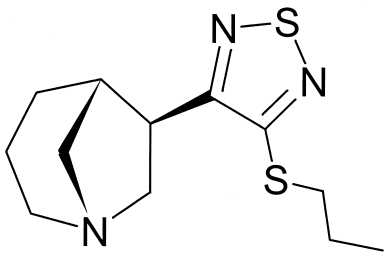


Fig. 1. Chemical structure of PTAC.

compound was selected as the best antipsychotic candidate from a large number of newly synthesized muscarinic receptor ligands.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, weighing 250–350 g, (Moellegaards Breeding Labs, Ll. Skensved, Denmark or Harlan Sprague–Dawley), male Fisher-derived F344 (Harlan Sprague–Dawley, Indianapolis, IN), weighing 250–350 g rats or NMRI mice, weighing 20 ± 2 g (Moellegaards Breeding Labs) were used. The animals were housed in temperature-controlled rooms (20–22°C) with a light/dark cycle (light from 0600–1800) and access to food and water ad libitum. The experimental protocols used were in accordance with the international accepted principles of the care and use of laboratory animals and have been approved by the Danish Committee for Animal Research or the Eli Lilly Animal Care and Use Committee. Fisher-derived F344 rats (Harlan Sprague–Dawley) were used in the conditioned avoidance experiments and the catalepsy experiments.

2.2. Drugs

PTAC was synthesized at Novo Nordisk and dissolved in 0.9% saline. D-amphetamine sulphate was purchased from Sigma (St. Louis, MO) and dissolved in sterile water. (−)-Apomorphine was purchased from Sigma and dissolved in distilled water containing 0.2 mg ml^{-1} ascorbic acid. All the solutions were prepared immediately before use. All drug doses refer to the salt.

2.3. Specific radioligand binding to muscarinic receptors

[^{3}H]Oxotremorine-M and [^{3}H]pirenzepine were used to label muscarinic receptors in radioligand binding experiments using rat brain homogenates. [^{3}H]*N*-methylscopolamine was used to label m_1–m_5 human muscarinic receptors in Chinese hamster ovary (CHO) cells transfected with human muscarinic receptors. In general, the assay conditions used have previously been described by Suzdak et al. (1992).

2.4. Second messenger measurements

cAMP formation was measured in CHO cells transfected with the human m_2 receptor or m_4 receptor. In the human m_2 receptor cell line, carbachol-induced inhibition of adenyl cyclase was determined from intracellular adenosine 3′,5′-monophosphate (cAMP) levels after stimulation with forskolin as earlier described by Harper and Brooker (1975). In the human m_4 receptor cell line cAMP accumulation was measured in pertussis toxin-treated CHO cells transfected with human muscarinic m_4 receptors. Cells attached to 96 well plates were incubated for 1 h after addition of 100 μl of serum free Dulbecco's modified Eagle medium containing 1 mM 3-isobutyl-1-methylxanthine and 1 mM forskolin plus or minus drug being tested. Incubations were terminated with serum free Dulbecco's modified Eagle medium containing 0.3% Triton X-100. After stopping incubations the plates were allowed to sit for 20 min to extract cAMP and samples were then diluted 2.5-fold and then assayed using the scintillation proximity assay of Amersham (Arlington Heights, IL). Phosphoinositol hydrolysis was measured in A9L cells transfected with human m_1 receptor and in CHO cells transfected with the human m_3 receptor as earlier described by Baumgold et al. (1995). Arachidonic acid release was measured in CHO cells transfected with the human m_5 receptor basically as described by Baumgold et al. (1995).

2.5. In vitro profiling at receptors, ion channel and uptake sites

In order to evaluate the selectivity of PTAC for muscarinic receptors a large number of radioligand binding assays specific for neurotransmitters, ion channels and uptake sites were used. The assay conditions employed and the radioligands utilized in each assay, have previously been described by Andersen (1989) and Suzdak et al. (1992). The dopamine D_3 and D_4 radioligand binding assay has earlier been described by Seeman and Van Tol (1993) and the dopamine D_5 radioligand binding assay has earlier been described by Jarvie et al. (1993).

The profile included tests specific for competitive ligand binding to histamine receptors (H_1 and H_3), adrenoceptors ($\alpha_{1,2}$ and $\beta_{1,2}$), σ binding sites, dopamine receptors (D_1, D_2, D_3, D_4 and D_5), 5-HT receptors (5-HT_{1A}, 5-HT_2 and 5-HT_3), glutamate receptors (kainate, NMDA, α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid (AMPA)), glycine (A and B), adenosine (A_1 and A_2), opioid (μ and κ), benzodiazepine and GABA receptors,

Na^+ and Ca^{2+} channels and uptake sites for dopamine, GABA, noradrenaline and 5-HT.

2.6. Spontaneous locomotor activity following systemic or central injections

Sprague–Dawley male rats were placed in acrylic glass chambers (width, length, height: 29, 29 and 38 cm, respectively) situated within a frame of photocells (4 × 4) located 1 cm above the floor. Each photocell interruption was recorded on a computer. PTAC or saline were injected, animals were immediately thereafter placed individually in the glass chambers and locomotor activity was measured for the following 10 min.

In the central injection experiment, rats were anesthetized with tribromethanol (cat. no 90710, Fluka Biochemika), 400 mg kg^{-1} i.p. and placed in a stereotaxic apparatus (David Kopf) with the upper incisor bar set at 2.4 mm below the interaural line. Two 2-mm holes were drilled in the skull and the dura was opened by a hypodermic needle. Two guide cannulas were inserted by aid of a stereotaxic manipulator and cemented to the skull. The animals were allowed to recover for 6–7 days before the central injections were done, at which time the rats had regained full weight. PTAC or saline at a volume of 1 μl was injected bilaterally into the ventral striatum (0.5 mm rostral and 2.6 mm lateral to bregma, 6.0 mm below the brain surface) using an infusion pump (Carnegie Medicine) with a flow rate of 1 μl min^{-1}. Following the activity measurement, the animals were sacrificed, the brains removed and sectioned in 250 μm coronal slices. The placement of the injection needle was verified in each instance using a stereomicroscope. Results obtained from animals in which the probe had been positioned incorrectly were not used.

2.7. Apomorphine-induced climbing

In principal, the apomorphine-induced climbing was done as earlier described by Costall et al. (1978). NMRI male mice ($n = 10$ per group) were administered subcutaneously (s.c.) with saline or various doses of PTAC 25 min prior to and (−)-apomorphine (2 mg kg^{-1} s.c.) 20 min prior to trial. At the beginning of the trial, mice were placed individually in cylindrical wire cages. During the trial, the time each mouse climbed on the inside of the wire cage was automatically recorded over a 60-s period. Data are expressed as the mean ± S.E.M. number of seconds climbing during the 60-s trial. When administration of PTAC was combined with administration of the cholinergic receptor antagonist scopolamine, scopolamine was administered (s.c.) 25 min prior to trial as well as PTAC in combination with (−)-apomorphine (2 mg kg^{-1} s.c.) 20 min prior to trial as described above.

2.8. Conditioned avoidance responding

Fisher-derived F344 (Harlan Sprague–Dawley) male rats were housed individually on a 12-h light, 12-h dark cycle (light on at 0600) with free access to food and water. Conditioned avoidance responding experiments were performed, with minor modifications, as earlier described by Verhave et al. (1958). Briefly, the rats were trained to avoid or escape foot-shock in an operant conditioning chamber. At the start of each trial, the house light and a tone were presented. A response within 10 s immediately terminated the trial (avoidance response). If the rat did not respond within 10 s, foot shock (2 mA) was presented. A response during the shock immediately terminated the trial (escape response). If the rat did not respond within 10 s after the onset of shock, the trial terminated automatically (response failure). Sessions typically ended after 50 trials. Each point represents the mean ± S.E.M. number of responses for six rats.

2.9. Catalepsy

Fisher-derived F344 (Harlan Sprague–Dawley) male rats were injected with compound of interest 1 h before testing. Catalepsy was evaluated by placing one forepaw on a 30-mm rubber cork. A rat was considered cataleptic if it had not returned the paw to the floor within 30 s.

2.10. Fos protein immunoreactivity

The Fos protein immunoreactivity was essentially performed as earlier described by Fink-Jensen et al. (1995). Sprague–Dawley rats (Moellegaards Breeding Labs) were divided into five groups (six rats per group) and injected subcutaneously with saline, PTAC (0.1 mg kg^{-1}), D-amphetamine (2.5 mg kg^{-1}), PTAC (0.01 mg kg^{-1}) and D-amphetamine (2.5 mg kg^{-1}) or PTAC (0.1 mg kg^{-1}) and D-amphetamine (2.5 mg kg^{-1}). Each animal was assigned a number, the injections were done in random order and the treatment was unknown to the person who performed the image analysis. Two hours after drug administration, animals were killed, the brains removed, frozen in isopentane and stored at −80°C until cutting of sections. The brains were cut on a cryostat into 15 μm thick sections, mounted on chrome–alum gelatine coated slides. Sections were fixed with 4% paraformaldehyde in phosphate-buffered saline and washed in 0.5 M Tris–Cl pH 7.4 with 0.05 M NaCl (TBS) containing 0.5% Tween 20 and 0.2% swine serum. This buffer was used for washing during the whole staining procedure. After blocking of protein binding sites with 10% normal swine serum, sections were incubated overnight at 4°C with rabbit polyclonal antibodies against Fos (Oncogene Science (c-*fos*

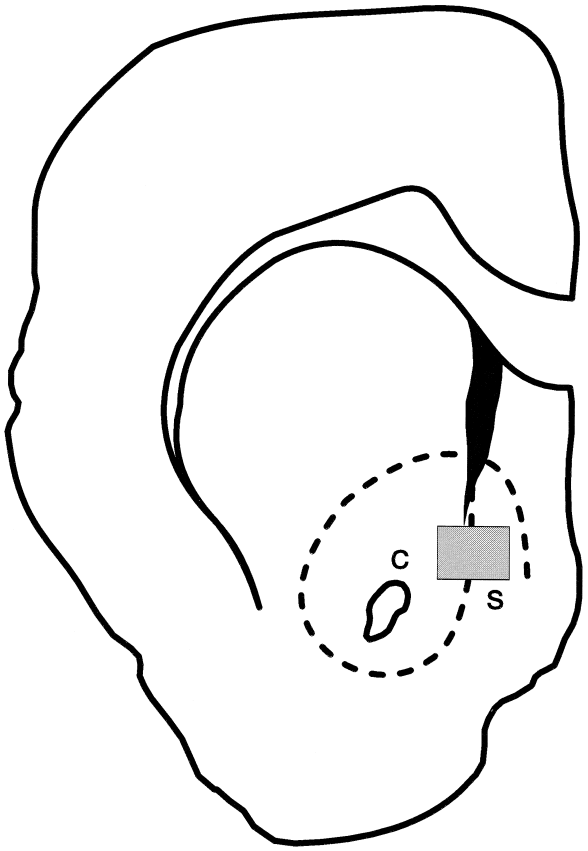


Fig. 2. Fos counting area. Camera lucida drawing of coronal rat brain section depicts the Fos counting area (grey shaded box) in the nucleus accumbens, core (C) and shell (S) region.

(ab-2))), diluted 1:200 in TBS with 0.5% Tween 20 and 4% normal swine serum. The primary antibody on the sections was detected using biotinylated swine antirabbit immunoglobulin G (IgG) (1:400, diluted in TBS) (Dakopatts) followed by avidin alkaline phosphatase (1:100, diluted in TBS) (Dakopatts). The alkaline phosphatase reactivity was demonstrated with the nitro blue tetrazolium / 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) detection system.

The Fos protein immunoreactivity was counted in an area of the nucleus accumbens that included both the shell region and the core region by use of a 10 × objective on a microscope equipped with a TV camera. The 525 μm × 875 μm counting area is depicted as a grey box in Fig. 2. Sections were discharged if more than 10% of the region under investigation was damaged. Fos positive nuclei were counted manually by one person—two sections per area—and the positive nuclei were marked on each section. An average value was calculated for the two sections.

2.11. In vivo electrophysiology

The electrophysiological effects of PTAC were basically measured as earlier described by Stockton and Rasmussen (1996). Male Sprague–Dawley rats (280–330 g; Charles River) were anesthetized with chloral hydrate (400 mg kg^{-1}, administered intraperitoneally); supplemental doses were administered through the lateral tail vein as needed. Body temperature was maintained at 35°C by a heating pad (K-module, American Pharmaseal, Valencia, CA). The anesthetized rats were mounted in a stereotaxic apparatus (Kopf Instruments), the skull exposed, and a cisternal drain performed to prevent tissue swelling. A burr hole was made in the skull over the A9 and A10 areas. To construct recording electrodes, single-barrel glass micropipettes (Radnoti, starbore glass) were pulled (Narishige PE-2 vertical puller); the resulting fine tips broken back and the barrels were backfilled with 2 M NaCl. Electrode impedances were 1.8 to 2.65 MΩ (in vivo measurements with a Dagan 2400 preamplifier utilizing a 2-Hz, 100-nA peak-to-peak square wave).

For recording neuronal activity the bandpass filter on the preamplifier was set from 0.3 to 3 kHz. The tip of the recording electrode was lowered to the dorsal border of either the substantia nigra, pars compacta, where the A9 dopaminergic cells are located, or the ventral tegmental area, where the A10 dopaminergic cells are located. Thereafter, the recording electrode was advanced, using a micropositioning device (Burleigh, Inchworm Motor Controller), in 5 mm increments through the nucleus. The electrode was passed through nine tracks (each track was separated by 0.2 mm) in a stereotaxically defined block of tissue (5.0–5.4 posterior, 2.0–2.4 mm lateral to bregma and 6.0–8.5 mm ventral to the cortical surface for the substantia nigra, pars compacta; and 5.0–5.4 mm posterior and 0.5–0.9 mm lateral to bregma and 6.0–8.5 mm ventral to the cortical surface for the ventral tegmental area), and the number of spontaneously active dopamine cells was counted. The electrode tracks were made in a preset sequence that was kept constant from animal to animal. For animals acutely treated, proper anatomical positioning of the recording electrode was ensured by scoring three control tracks prior to drug treatment. Six additional tracks were recorded 1 h following administration of drug or vehicle. Only one area, containing either the A9 cells or the A10 cells, was recorded in each acutely treated animal. For chronically treated animals, nine tracks were scored in both the substantia nigra, pars compacta and in the ventral tegmental area; the first site examined was alternated to control for order effects. Spontaneously active dopamine cells were recorded as previously reported (Chiodo and Bunney, 1983). Briefly, cells were considered dopaminergic if they possessed the following characteristics: (1) action potential duration of 2.5 to 4.5 ms; (2) triphasic waveform containing a notch in the initial rising phase of the first positive peak; and (3) slow, slightly irregular firing pattern, with a rate of 2 to 10 Hz. A digital oscilloscope (Gould 1604) was used to analyze the spike waveforms; eight waveforms were captured, averaged, and then displayed for analysis. The firing rate of each cell was

monitored for 1 to 2 min to ensure that the cells had not been mechanically excited.

For acute treatment, vehicle or PTAC was administered by intravenous injection. For chronic treatment, a constant rate of drug delivery was maintained by s.c. implantation of Alzet 2ML4 osmotic minipump (Alza, Palo Alto, CA) containing drug or vehicle into 150 g male Sprague–Dawley rats. Implant surgeries were carried out while the animals were lightly anesthetized with halothane. Electrophysiological recordings were performed 21 days post implantation; pumps were not removed prior to recording. The volume of fluid in the pump was measured before and after treatment to verify that the proper amount of drug was administered.

2.12. Salivation and tremor

Salivation and tremor were assessed in NMRI male mice 30 min following s.c. injection of PTAC with 10 mice per group. The salivation and tremor scores were assigned to each animal corresponding to the severity of the effect with 0 = no effect and 3 = maximal effect. For salivation, a score of 0 indicates no observed signs; 1: wet around the mouth; 2: wet around mouth, throat and/or neck; 3: wet around mouth, throat and/or neck as well as on chest and/or belly. For the validation of tremor a score of 0 indicates no observed signs; 1: weak tremor upon handling; 2: tremor and flexing of neck upon handling; 3: spontaneously occurring pronounced clonic tremor. The scores were multiplied by their frequencies, summated and calculated as a percentage out of the maximal possible total scores.

2.13. Tissue levels of DOPAC

Striatal DOPAC concentrations were determined by high performance liquid chromatography with electrochemical detection as earlier described by Fuller and Perry (1989). In brief, striatum of the rat was dissected and frozen on dry ice before analysis. Tissues were weighed and sonicated in 0.1 M trichloroacetic acid containing 0.2 mg ml^{-1} of cysteine as a stabilizing agent and an internal standard of 40 ng ml^{-1} of 5-hydroxyindolecarboxylic acid. The resulting homogenates were centrifuged at $12\,000 \times g$ for 10 min and DOPAC was assayed by injecting 20 μl of supernatant onto the Econosphere C18 analytical column (4.6 mm × 150 mm, Alltech Associates).

2.14. Data analysis

The results from drug-treated and control group animals were compared by use of a one-way analysis of variance followed by Student Newman–Keuls post-hoc test. Log probit analysis was used to calculate ED_{50} values.

3. Results

3.1. Specific radioligand binding to muscarinic receptor subtypes

PTAC showed high affinity for the [^{3}H]oxotremorine-M labeled muscarinic receptors in radioligand binding experiments using rat brain homogenates (see Table 1), and for [^{3}H]*N*-methylscopolamine labeled m_1, m_2, m_3, m_4 and m_5 human muscarinic receptors in CHO cells with K_i values of 0.6 ± 0.2, 2.8 ± 1.4, 0.2 ± 0.02, 0.2 ± 0.05 and 0.8 ± 0.09 nM, respectively.

3.2. Second messenger measurements

In order to evaluate agonist/antagonist properties of PTAC at the muscarinic receptor subtypes, cell lines expressing human muscarinic receptor subtypes coupled to second messenger systems were produced. cAMP formation was measured in CHO cells transfected with the human m_2 or m_4 receptors. Phosphoinositol hydrolysis was measured in A9L and CHO cells transfected with

Table 1
Affinity of PTAC for neurotransmitter receptors, uptake sites and ion channels in rat brain

Receptor uptake site ion channel	Ligand	IC_{50} value (nM)
Muscarinic	[^{3}H]oxotremorine-M	0.7
Nicotine	[^{3}H]methylcarbachol	6300
5-HT_{1A}	[^{3}H]8-OH-DPAT	47 000
5-HT_2	[^{3}H]ketanserin	140 000
5-HT_3	[^{3}H]GR65630	235
Adrenoceptor α_1	[^{3}H]prazosin	318
Adrenoceptor α_2	[^{3}H]idazoxan	39 000
Adrenoceptor β_1 and β_2	[^{3}H]dihydroalprenolol	44 000
Histamine H_1	[^{3}H]pyrilamine	887
Histamine H_3	[^{3}H]methylhistamine	4801
Dopamine D_1	[^{3}H]SCH23390	100 000
Dopamine D_2	[^{3}H]spiperone	630 000
Glutamate, AMPA	[^{3}H]CNQX	> 450 000
Glutamate, kainate	[^{3}H]kainate	> 1 000 000
Glutamate, NMDA	[^{3}H]CPP	420 000
Glycine A	[^{3}H]strychnine	290 000
Glycine B	[^{3}H]glycine	> 100 000
σ binding site	[^{3}H]-3-PPP	79
Adenosine A_1	[^{3}H]PIA	> 70 000
Adenosine A_2	[^{3}H]CGS21680	> 70 000
Opioid μ	[^{3}H]naloxone	72 000
Opioid κ	[^{3}H]EKC	13 000
Benzodiazepine	[^{3}H]flunitrazepam	> 100 000
GABA	[^{3}H]GABA	51 000
Na^+ channels	[^{3}H]BTX-T	> 100 000
Ca^{2+} channels	[^{3}H]nitrendipine	> 100 000
Dopamine uptake	[^{3}H]dopamine	108 000
GABA uptake	[^{3}H]GABA	108 000
Noradrenaline uptake	[^{3}H]noradrenaline	4400
5-HT uptake	[^{3}H]5-HT	4100

human m_1 and m_3 receptors, respectively, whereas arachidonic acid release was measured in CHO cells transfected with the human m_5 receptor. PTAC exhibited partial agonist properties at the human m_2 and m_4 receptors (57% of maximal carbachol effect on cAMP with an EC_{50} value of 0.6 nM and 50% of maximal oxotremorine-M effect on cAMP with an EC_{50} value of 5 nM, respectively) and antagonist properties at the human m_1, m_3 and m_5 receptors (K_i values of 0.4 ± 0.1 nM (m_1), 3.4 ± 0.2 nM (m_3) and 0.3 ± 0.1 nM (m_5) for inhibition of oxotremorine-M stimulated phosphoinositol hydrolysis (m_1 and m_3) and arachidonic acid release (m_5).

3.3. In vitro profiling at receptors, ion channel and uptake sites

In comparison to the binding at muscarinic receptors, PTAC binds with considerable lower affinity (more than 100 times) at the following binding sites: [^{3}H]pyrilamine and [^{3}H]methylhistamine (histamine H_1 and H_3 receptors, respectively), [^{3}H]GR65630 (5-HT_3 receptor), [^{3}H]prazosin (adrenoceptor α_1) and [^{3}H]-3-PPP (σ binding site) (Table 1). In addition, PTAC was inactive ($IC_{50} > 50\,000$ nM) at all other receptor and channel binding sites, as shown in Table 1. PTAC had very low affinity for dopamine D_3 and D_4 receptors labeled with [^{3}H]spiperone and dopamine D_5 receptors labeled with [^{3}H]SCH23390 (IC_{50} values > 1000 nM). Moreover, PTAC showed no affinity ($IC_{50} > 50\,000$ nM) for the dopamine- and GABA-uptake sites and very low affinity for the noradrenaline- and 5-HT uptake sites (IC_{50} values > 4000 nM) as shown in Table 1.

3.4. Spontaneous locomotor activity following systemic or central injections

Behaviorally, the compound significantly and dose dependently inhibited spontaneous locomotor activity in male Sprague–Dawley rats following systemic administration (Fig. 3A). The effect of PTAC was antagonized by the muscarinic receptor antagonist scopolamine at doses of 0.01 and 0.05 mg kg^{-1} s.c. (Fig. 3C). The inhibition of locomotor activity by PTAC observed after systemic administration could be mimicked by bilateral injection of PTAC (5–25 μg) directly into the ventral striatum (Fig. 3B). The activity counts in the control group receiving

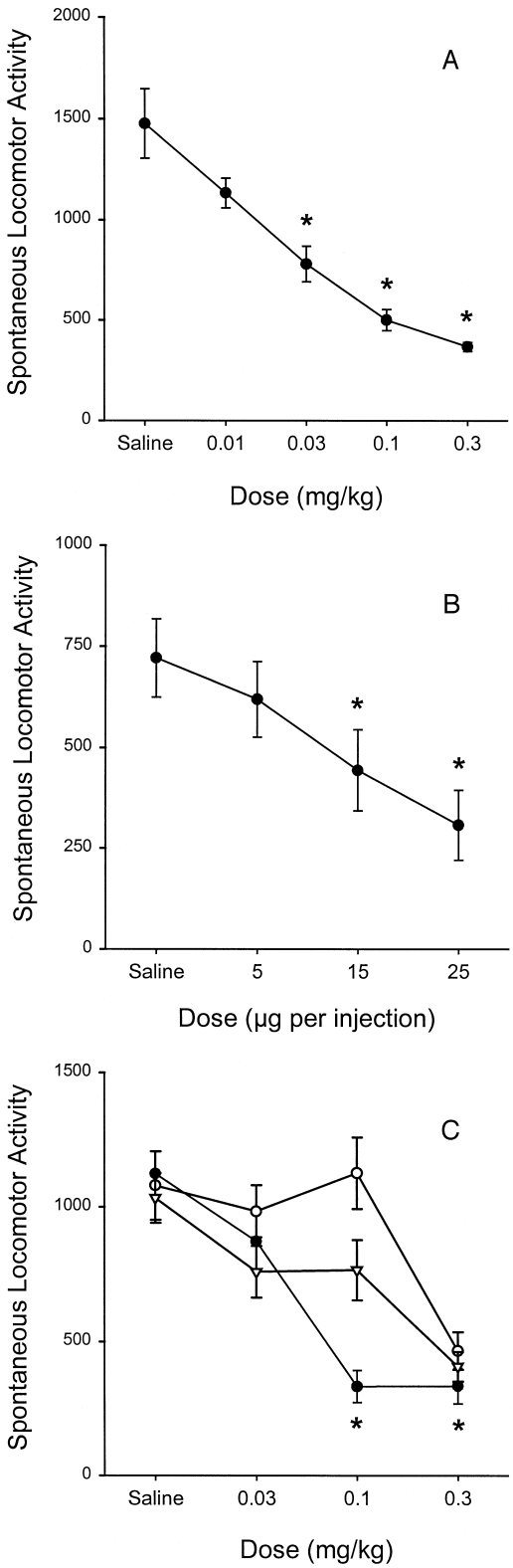


Fig. 3. (A) Effects of PTAC, 0.01, 0.03, 0.1 and 0.3 mg kg^{-1}, on spontaneous locomotor in rats immediately following s.c. administration. Results are expressed as the mean ± S.E.M. ($n = 8$ rats per dose). $* P < 0.05$, compared to the control (one-way analysis of variance followed by Student Newman–Keuls post-hoc test). (B) Effects of PTAC on spontaneous locomotor activity in rats immediately following intracerebral injection of 5, 15 and 25 μg bilaterally into the ventral striatum. Results are expressed as the mean ± S.E.M. ($n = 8$ rats per dose). $* P < 0.05$, compared to the control (one-way analysis of variance followed by Student Newman–Keuls post-hoc test). (C) Effects of PTAC, 0.03, 0.1 and 0.3 mg kg^{-1} s.c., alone (black circle) or in combination with scopolamine, 0.01 mg kg^{-1} s.c. (open triangle) and 0.05 mg kg^{-1} s.c. (open circle) on spontaneous locomotor activity in rats. PTAC and scopolamine were administered 20 and 25 min before start of the measurement, respectively. Results are expressed as the mean ± S.E.M. ($n = 8$ rats per dose). $* P < 0.05$, compared to the control (one-way analysis of variance followed by Student Newman–Keuls post-hoc test).

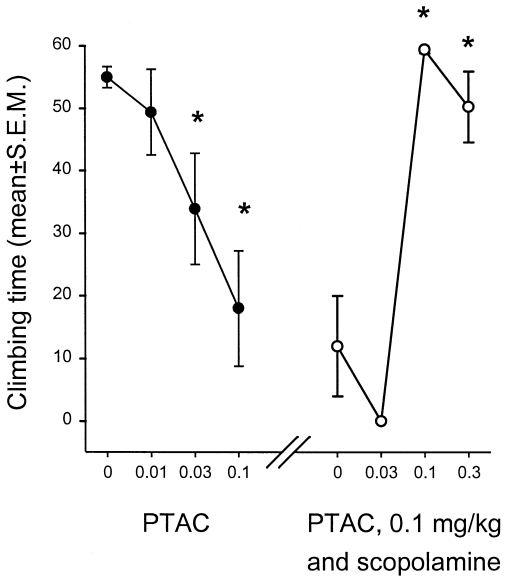


Fig. 4. Apomorphine-induced climbing in mice. Effects of PTAC, 0.03, 0.1 and 0.3 mg kg^{-1} s.c., alone (black circle) or PTAC, 0.1 mg kg^{-1} in combination with increasing doses (0.03, 0.1 and 0.3 mg kg^{-1} s.c.) of scopolamine (open circle). PTAC and scopolamine were administered 20 and 25 min before start of the measurement, respectively. Results are expressed as the mean ± S.E.M. ($n = 10$ mice per dose). $* P < 0.05$, compared to the control (one-way analysis of variance followed by Student Newman–Keuls post-hoc test).

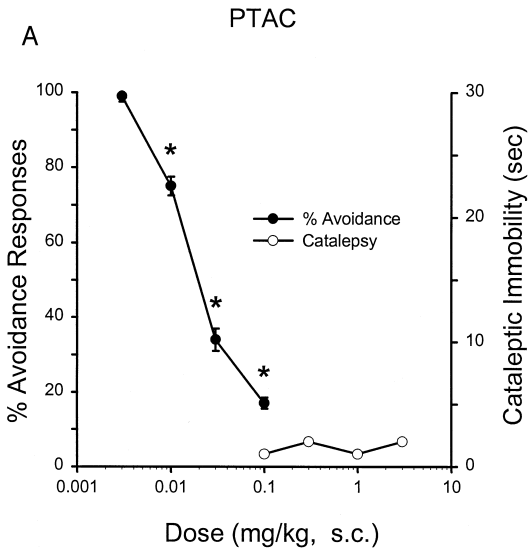
central injections were about half of the activity counts in the control group receiving peripheral injections.

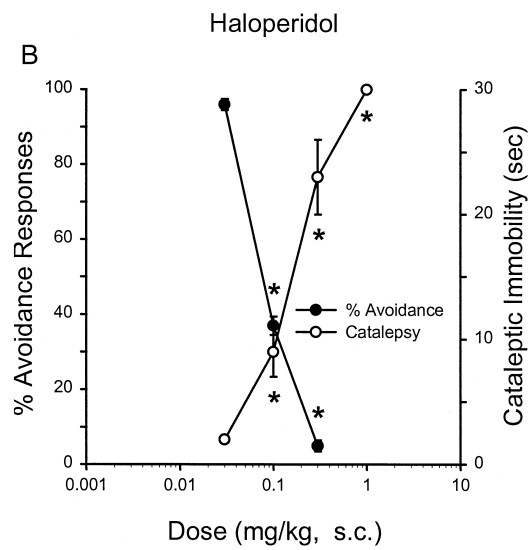
3.5. Apomorphine-induced climbing

PTAC significantly ($P < 0.05$) and dose dependently inhibited apomorphine-induced climbing in male NMRI mice with an ED_{50} value of 0.04 mg kg^{-1}, an effect that could be antagonized by the muscarinic receptor antagonist scopolamine at doses of 0.1 and 0.3 mg kg^{-1} s.c. (Fig. 4).

3.6. Conditioned avoidance responding and catalepsy

PTAC dose-dependently and significantly ($P < 0.05$) inhibited conditioned avoidance responding, a classical test





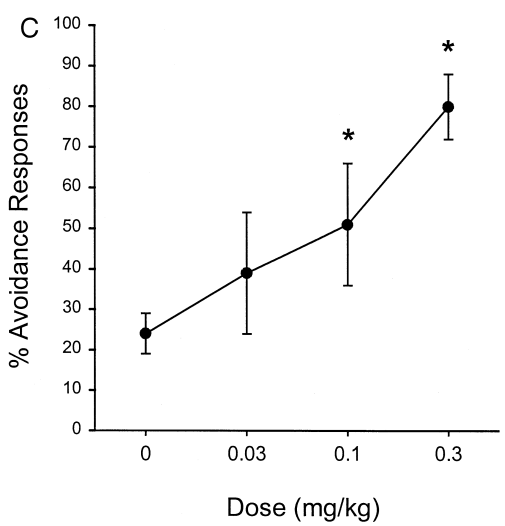


Fig. 5. (A) Effects of PTAC on conditioned avoidance responding and catalepsy in rats. Results are expressed as the mean ± S.E.M. ($n = 6$ rats per dose). $* P < 0.05$, compared to the control (one-way analysis of variance followed by Student Newman–Keuls post-hoc test). (B) Effects of haloperidol on conditioned avoidance responding and catalepsy in rats. Results are expressed as the mean ± S.E.M. ($n = 6$ rats per dose). $* P < 0.05$, compared to the control (one-way analysis of variance followed by Student Newman–Keuls post-hoc test). (C) Effect of scopolamine on PTAC-induced inhibition of conditioned avoidance responding in rats. PTAC, 0.1 mg kg^{-1} is combined with increasing doses (0.03, 0.1 and 0.3 mg kg^{-1} s.c.) of scopolamine. PTAC and scopolamine were administered 20 and 25 min before start of the measurement, respectively. Results are expressed as the mean ± S.E.M. ($n = 6$ rats per dose). $* P < 0.05$, compared to the control (one-way analysis of variance followed by Student Newman–Keuls post-hoc test).

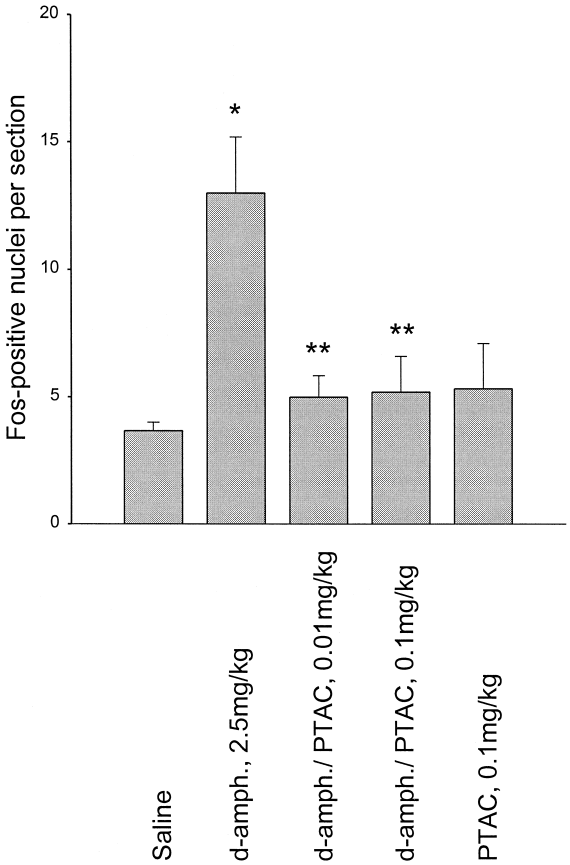


Fig. 6. Effect of PTAC on D-amphetamine-induced Fos protein immunoreactivity in the rat nucleus accumbens. Results are expressed as the mean ± S.E.M. ($n = 6$ per dose). $* P < 0.05$, compared to the saline control and $* * P < 0.05$, compared to the D-amphetamine group (one-way analysis of variance followed by Student Newman–Keuls post-hoc test).

for antipsychotic activity (Arnt, 1982) in male rats without inducing catalepsy (Fig. 5A) in contrast to the effect of the prototypical neuroleptic haloperidol which induces catalepsy at the doses which inhibit conditioned avoidance responding (Fig. 5B). The effect of PTAC on conditioned avoidance responding was antagonized by scopolamine at doses of 0.1 and 0.3 mg kg^{-1} (Fig. 5C).

3.7. Fos protein immunoreactivity

The Fos protein is an immediate-early gene product which is involved in the regulation of gene transcription (Morgan and Curran, 1989) and regarded as a marker of neuronal activation (Sagar et al., 1989). In the present experiment, D-amphetamine (2.5 mg kg^{-1} s.c.) caused a 3-fold increase in the Fos protein immunoreactivity level in the nucleus accumbens. The elevation in the Fos protein immunoreactivity was totally inhibited by PTAC at 0.01 and 0.1 mg kg^{-1} s.c. (Fig. 6) whereas the compound itself did not change the Fos protein immunoreactivity in the nucleus accumbens compared to control levels (Fig. 6).

3.8. In vivo electrophysiology

Electrophysiological in vivo experiments have shown that chronic (i.e., 3 weeks) administration of traditional neuroleptics, e.g., haloperidol, decreases the number of spontaneously active dopamine cells in the ventral tegmental area (A10) and in the substantia nigra, pars compacta (A9) whereas the 'atypical' neuroleptic clozapine selectively decreases the number of active cells in the ventral tegmental area (Chiodo and Bunney, 1983; White and Wang, 1983).

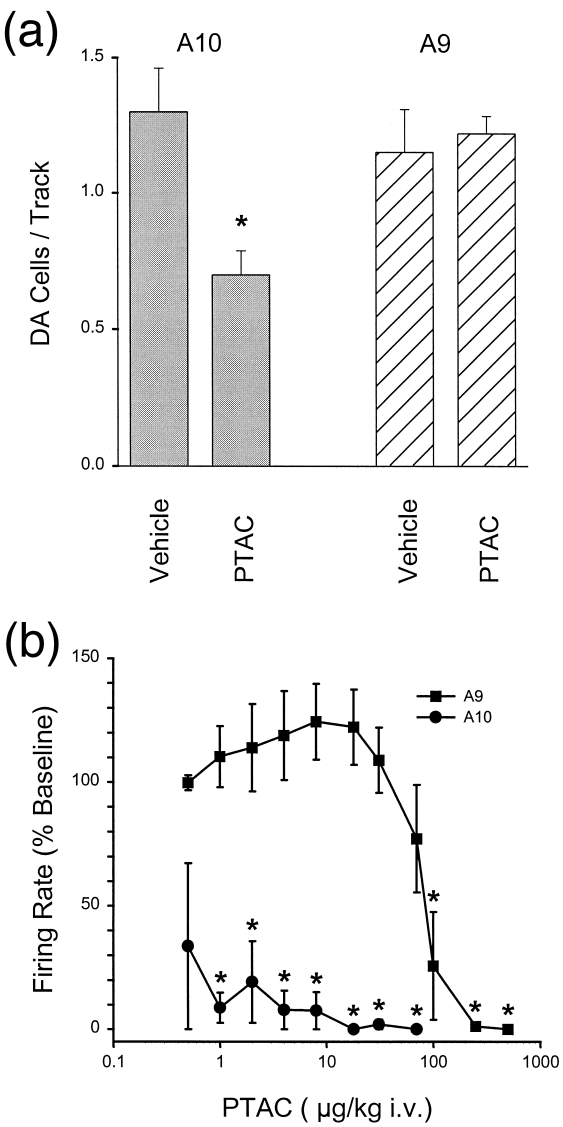


Fig. 7. (A) Effects of chronic PTAC-administration on the number of actively firing A9 and A10 dopaminergic neurons in the rat. Results are expressed as the mean ± S.E.M. ($n = 4–8$ rats per dose). $* P < 0.05$, compared to the control (one-way analysis of variance followed by Student Newman–Keuls post-hoc test). (B) Acute effects of PTAC on the firing rate of A9 ($n = 5$) and A10 ($n = 12$) dopaminergic neurons in the rat. Results are expressed as the mean ± S.E.M. $* P < 0.05$, compared to the control (one-way analysis of variance followed by Student Newman–Keuls post-hoc test).

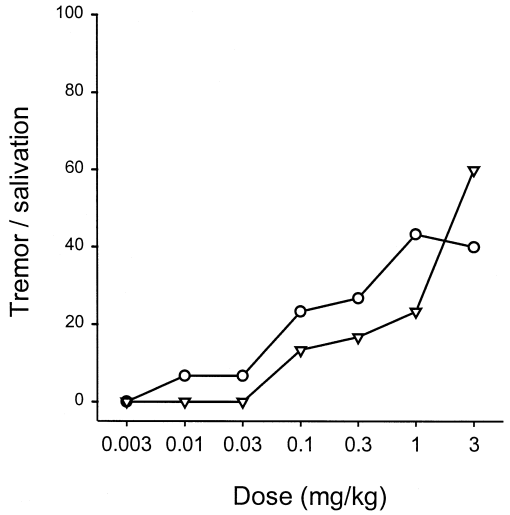


Fig. 8. Effect of PTAC on tremor (open circle) and salivation (open triangle) in mice ($n = 10$ mice per dose). Salivation and tremor were assessed 30 min following s.c. injection of PTAC and are expressed as % maximal effect.

In the present study PTAC exhibits a clozapine-like profile following chronic administration, i.e., 0.5 mg kg^{-1} day^{-1} for 21 days (Fig. 7A) by selectively decreasing the number of spontaneously active dopamine cells in the ventral tegmental area. Interestingly, in contrast to the effect of the known antipsychotics, PTAC also selectively reduced dopamine cell firing rate in the ventral tegmental area after acute administration (Fig. 7B).

3.9. Salivation and tremor

In vivo, PTAC does not induce tremor and salivation in mice at pharmacologically relevant doses (ED_{50}-values for induction of tremor and salivation are 2.2 and > 3 mg kg^{-1}, respectively) (Fig. 8) and actually blocks oxotremorine-induced salivation and tremor (data not shown).

3.10. Tissue levels of DOPAC

Blockade of dopamine receptors in the accumbens and the striatum of rats results in increased levels of the dopamine metabolite, DOPAC in these areas (Westerink and Korf, 1976). PTAC (0.3 mg kg^{-1} s.c.) did not appreciably increase DOPAC levels in the accumbens ($126 \pm 10\%$ relative to control) in contrast to the effect of the dopamine antagonist haloperidol, 0.3 mg kg^{-1} s.c. ($279 \pm 22\%$).

4. Discussion

In radioligand binding experiments using CHO cells expressing the human muscarinic m_1, m_2, m_3, m_4 and m_5 receptors, respectively, PTAC showed high affinity for all five muscarinic receptor subtypes with K_i values of about 1 nM using [^{3}H]*N*-methylscopolamine as a radioligand. Even though PTAC binds with equipotent affinity to the five muscarinic receptors, the effect at the receptor subtypes varies, since PTAC exhibits partial agonist properties at the human m_2 and m_4 muscarinic receptors and antagonist properties at the human m_1, m_3 and m_5 muscarinic receptors. Moreover, radioligand binding experiments show that PTAC was ineffective or binds with considerable lower affinity at a large number of other receptor binding sites and transmitter uptake sites. These experiments establish PTAC as a compound with high affinity and selectivity for the muscarinic receptors in vitro.

In vivo, non-selective muscarinic receptor agonists are endowed with serious side effects such as tremor and salivation which has hampered elucidation of the muscarinic pharmacology. PTAC does not induce tremor and salivation in mice at pharmacologically relevant doses and the compound actually blocks oxotremorine-induced salivation and tremor (data not shown).

Behaviorally, PTAC has a profile suggestive of antipsychotic activity. It inhibits apomorphine-induced climbing and locomotor activity in male NMRI mice, an effect that could be antagonized by the muscarinic receptor antagonist scopolamine, demonstrating in vivo agonist mode of action of PTAC. The inhibition of spontaneous locomotor activity observed after systemic administration of PTAC to rats could be mimicked by bilateral injection of PTAC (5–25 μg) directly into the ventral rat striatum, suggesting that striatal areas are involved in the pharmacological response of PTAC. The activity counts in the control group receiving central injections was only about half of the activity level measured in the control group which received peripheral injections. This difference in activity levels may well be due to the fact that rats receiving central injections have been handled more than rats not undergoing operations. In addition, PTAC inhibits conditioned avoidance responding, a classical test for antipsychotic activity (Arnt, 1982), in male rats without inducing catalepsy, suggesting that the observed effect was not due to non-specific motor side effects. This is in contrast to the effect of the prototypical neuroleptic haloperidol which induces catalepsy at the same dose level where conditioned avoidance responding is blocked. The effect of PTAC on conditioned avoidance responding was also antagonized by scopolamine, again suggesting that the pharmacological effects of PTAC are caused by a muscarinic receptor agonist mode of action.

Due to the lack of selective ligands for all the muscarinic receptor subtypes we could not directly determine which receptor subtype(s) mediates the antipsychotic-like effects of PTAC. However, since the above mentioned pharmacological effects of PTAC can be reversed by the cholinergic antagonist scopolamine and since PTAC exhibits (partial) agonist effects at the muscarinic m_2 and m_4 receptor subtypes, these receptor subtypes are the most likely candidates.

The traditional neuroleptics are believed to produce their antipsychotic effects through influences on the mesocorticolimbic dopamine pathway. The reinforcing and psychotomimetic properties of the indirect dopamine receptor agonist D-amphetamine are probably mediated through the same system. To establish whether the anatomical substrate for the functional dopamine antagonism elicited by PTAC involves this pathway, we investigated the effect of PTAC on D-amphetamine-induced Fos protein expression in the nucleus accumbens, which is one of the terminal areas of the mesolimbic dopamine pathway (Fig. 6). The Fos protein is an immediate-early gene product which is involved in the regulation of gene transcription (Morgan and Curran, 1989) and regarded as a marker of neuronal activation (Sagar et al., 1989). The present data demonstrates that the mesocorticolimbic dopamine pathway is involved in the effects of PTAC and further substantiates its antipsychotic potential.

Electrophysiological in vivo experiments have shown that chronic (i.e., 3 weeks) administration of PTAC selectively decreases the number of spontaneously active dopamine cells in the ventral tegmental area relative to the substantia nigra. This is similar to the effect of the atypical neuroleptic clozapine and in contrast to the effects of the traditional neuroleptics, e.g., haloperidol, which decrease the number of spontaneously active dopamine cells in the ventral tegmental area and in the substantia nigra (Chiodo and Bunney, 1983; White and Wang, 1983).

Moreover, the fact that PTAC *acutely* inhibits dopaminergic firing rate—in contrast to the effect of all known typical and atypical neuroleptics—indicates that medical use of muscarinic receptor partial agonists may offer a more rapid onset of the therapeutic action than seen with traditional neuroleptics (Beckmann et al., 1979). PTAC acutely inhibits dopaminergic firing rate in the limbic ventral tegmental area at doses 200 times lower than needed to inhibit dopaminergic firing rate in the non-limbic substantia nigra demonstrating a limbic selectivity also after acute administration.

Even though PTAC by itself does not bind to dopamine receptors, as shown in the radioligand binding experiments, the functional dopamine antagonism elicited by PTAC in vivo could theoretically be caused by one or more metabolites of PTAC blocking dopamine receptors. However, blockade of dopamine receptors in the accumbens and the striatum of rats results in increased levels of the dopamine metabolite DOPAC in these areas (Westerink and Korf, 1976). PTAC did not appreciably increase DOPAC levels in the accumbens in contrast to the effect of the dopamine receptor antagonist haloperidol, showing that the observed pharmacological effects are not simply caused by one or several metabolites of PTAC with dopamine receptor blocking properties.

In conclusion, PTAC is a potent and highly selective muscarinic receptor ligand which exhibits potent functional dopamine antagonism without binding to dopamine receptors. The results are in line with work of Carlsson et al. (1997) suggesting that several transmitter systems, including the central cholinergic system, may affect the overall striatal output system back to the cortex (the striato-thalamo-cortical loop). The data demonstrate that potent muscarinic receptor partial agonists (possibly m_2 or m_4), not endowed with parasympathomimetic side effects, may serve as a new approach to the treatment of schizophrenia and hopefully give new insight into the etiology of this devastating illness.

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