Original Article

Y-QA31, a novel dopamine D$_3$ receptor antagonist, exhibits antipsychotic-like properties in preclinical animal models of schizophrenia

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Aim: To investigate the potential effects of Y-QA31, a novel dopamine D$_3$ receptor antagonist, as an antipsychotic drug.

Methods: A panel of radioligand-receptor binding assays was performed to identify the affinities of Y-QA31 for different G protein-coupled receptors. [^{35}S]GTPγS-binding assays and Ca^{2+} imaging were used to assess its intrinsic activities. The antipsychotic profile of Y-QA31 was characterized in mouse models for the positive symptoms and cognitive deficits of schizophrenia and extrapyramidal side effects with haloperidol and clozapine as positive controls.

Results: *In vitro*, Y-QA31 is a dopamine D$_3$ receptor antagonist that is 186-fold more potent at the D$_3$ receptor than at the D$_2$ receptor. Y-QA31 also exhibits 5-HT$_{1A}$ receptor partial agonist and α$_{1A}$ adrenoceptor antagonist activities with medium affinity, whereas it exhibits very little affinity for other receptors (100-fold lower than for the D$_3$ receptor). *In vivo*, Y-QA31 (10–40 mg/kg, *po*) significantly inhibited MK-801-induced hyperlocomotion and methamphetamine-induced prepulse inhibition disruption in a dose-dependent manner. Y-QA31 also inhibited the avoidance response and methamphetamine-induced hyperlocomotion with potency lower than haloperidol. Y-QA31 was effective in alleviating the MK-801-induced disruption of novel object recognition at a low dose (1 mg/kg, *po*). Moreover, Y-QA31 itself did not affect spontaneous locomotion or induce cataleptic response until its dose reached 120 mg/kg.

Conclusion: Y-QA31 is a selective D$_3$R antagonist that exhibits antipsychotic effects in some animal models with positive symptoms and cognitive disorder and less extrapyramidal side effects.

Keywords: schizophrenia; D$_3$ receptor antagonist; positive symptoms; cognitive deficits; haloperidol; clozapine

Introduction

Schizophrenia is an intractable neuropsychiatric disorder characterized by positive (delusions and hallucinations) and negative (social withdrawal, blunted affect, and mutism) symptoms, as well as cognitive deficits (deficits in attention, working and verbal memory, and executive function). Dopaminergic systems play a dominant role in regulating the development of schizophrenia, and therefore, dopamine receptors have been the primary target for antipsychotic research[1]. Five types of dopamine receptor subtypes have been cloned, including D$_1$–D$_5$ receptors. Dopamine D$_1$ and D$_5$ receptors are classified as D$_1$-like receptors that activate adenylyl cyclase through coupling with the G$_s$-protein. Dopamine D$_2$, D$_3$ and D$_4$ receptors are classified as D$_2$-like receptors that inhibit adenylyl cyclase through coupling with the G$_{i/o}$-protein[2].

Blocking dopamine D$_2$ receptors (D$_2$R) with haloperidol successfully alleviates positive symptoms. However, this treatment shows limited effectiveness on negative and cognitive symptoms[3, 4]. Moreover, haloperidol causes severe side effects, such as extrapyramidal reactions and tardive dyskinesia, which lead to patients' non-compliance to these medications[3, 4].

In contrast to D$_2$R, dopamine D$_3$ receptors (D$_3$R) are found important for psychotic disorders in the brain regions such as the nucleus accumbens (NAc), the thalamus, and the cortex[5], while D$_3$R distribute less in the striatum, the brain region associated with movement function. Therefore, the antipsychotic compounds targeting D$_3$R cause less extrapyramidal

side effects than those targeting D_2R. Previous studies have suggested that the prefrontal cortex was associated with the occurrence of negative symptoms as well as the cognitive impairment of schizophrenia. In the D_3R-KO mice, cognitive performance was enhanced[6, 7], suggesting that D_3R has a regulating effect in schizophrenia, especially in its cognitive and negative symptoms. In addition, Shaikh and colleagues reported that in a study of 133 Caucasian subjects the Ser9 D_3 allele was associated with susceptibility to schizophrenia[8]. The autopsy study found that the density of D_3R in the ventral striatum increased significantly in patients with schizophrenia but remained relatively normal in patients treated with antipsychotic drugs, further suggesting the important role of D_3R in the development of schizophrenia. Therefore, D_3R has been considered as a potential target for antipsychotic agents.

Several D_3R antagonists have been developed. Among them, some selective D_3R antagonists, such as S33084[9] and SB-277011A (D_3/D_2 selectivity=112)[10], are not as effective as the D_2R antagonist L741,626[11] in reducing positive symptoms in animal models, such as amphetamine-induced hyperlocomotion and the impaired prepulse inhibition (PPI) induced by apomorphine. The preferential D_3R antagonist S33138 (D_3/D_2 selectivity=25) potently alleviates the positive, negative and cognitive symptoms of schizophrenia[12]. Because of the pharmacological properties of these compounds, however, their effects cannot be attributed solely to D_3R, and a selective D_3R antagonist is still needed to identify the effects of D_3R on the symptoms of schizophrenia.

In the current study, we evaluated Y-QA31, a novel and selective D_3R antagonist (D_3/D_2 selectivity=186) with medium affinity to the 5-HT_{1A} receptor and the α_{1A} adrenoceptor. Because the blocking of D_3 versus D_2 receptors is debated in the treatment of various symptoms of schizophrenia, we investigated the effects of Y-QA31 in animal models with positive and cognitive symptoms and analyzed its ability to induce catalepsy in mice to assess the potential of Y-QA31 as an antipsychotic drug.

Materials and methods
Materials
All radioligands used in our experiments were purchased from PerkinElmer Life Sciences (NEN, Boston, MA, USA). Other drugs used in the experiments were obtained from Sigma (St Louis, MO, USA). Y-QA31 (N-N-(4-(4-(2-methoxyphenyl) piperazin-1-yl)butyl)-2-benzothiazolinone-6-carboxamide hydrochloride), the chemical structure as shown in Figure 1, was synthesized at the Beijing Institute of Pharmacology and Toxicology and was dissolved in 25% 2-hydroxypropyl-cyclodextrin (Sigma, St Louis, MO, USA). hD_4R-, hD_5R-, $h5\text{-HT}_{1A}R$-, $h5\text{-HT}_{1B}R$-, $h5\text{-HT}_{1D}R$-, $h5\text{-HT}_{2A}R$-, $h5\text{-HT}_{2C}R$-, $h5\text{-HT}_{5A}R$-, $h5\text{-HT}_6R$-, hA_1R-, $hA_{2A}R$-, hH_1R-, hH_2R-, hH_3R-, hCB_1R-, hCB_2R-, $h\alpha_{2A}$-, and hM_2R-pcDNA3.1(+) plasmids were purchased from Missouri S&T cDNA Resource Center (Rolla, MO, USA).

Animals
Male CD1 mice (weighing 18–22 g) and SD rats (weighing 180–220 g) were obtained from the Beijing Animal Center.
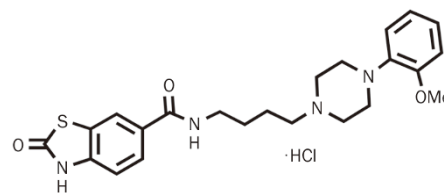

Figure 1. The chemical structure of Y-QA31.

The mice were maintained in cages with unrestricted access to food and water at 21±1 °C, 60%±5% humidity, and under a 12-h light/dark cycle. All protocols in this study were approved by the Committee of Animal Use and Protection of the Beijing Institute of Pharmacology and Toxicology.

In vitro studies on Y-QA31
Radioligand-receptor binding assays were performed to identify the affinity of Y-QA31 for G protein-coupled receptors, [35S] GTPγS-binding assays and intracellular calcium assays were performed to identify the intrinsic activity of Y-QA31. The protocols were performed according to previous reports[3, 13-15], with some modifications.

Radioligand-receptor binding assays
Radioligand-receptor binding assays were used to identify the affinity of Y-QA31 for G protein-coupled receptors, and the protocols were performed according to previous reports[3, 13] with some modifications. The reactions were initiated by adding diluted membranes and were incubated at 25 °C, 30 °C or 37 °C for 30–60 min, depending on the individual assay, until binding reached equilibrium (see Table 1 for details). Non-specific binding was defined in the presence of saturating concentrations of the unlabeled compounds listed in Table 1. The concentrations of Y-QA31 ranged from 10^{-12} to 10^{-5} mol/L. Radioactivity bound to filters was quantified by liquid scintillation in a Micro-Scint-20 (PerkinElmer LAS Ltd, Beaconsfield, UK). IC_{50} values were analyzed by nonlinear regression using Prism 5 (GraphPad Software, San Diego, CA, USA). K_i values were calculated according to Cheng-Pruss of: $K_i=IC_{50}/(1+L/K_d)$, where L is the radioligand concentration, and K_d is the dissociation constant.

[35S]GTPγS-binding assay
To test the intrinsic activity of Y-QA31 at the dopamine D_2 and D_3 receptors[13], cellular membrane protein (35–50 µg) was pre-incubated with unlabeled quinpirole in a reaction buffer containing 3 µmol/L GTP at 30 °C for 30 min. Then, 1.0 nmol/L [35S]GTPγS was added for incubation for another 30 min. Basal [35S]GTPγS binding was measured in the absence of quinpirole. Non-specific binding was measured in the presence of 0.2 nmol/L [35S]GTPγS and 40 µmol/L unlabeled GTPγS. To test the activity of Y-QA31 at 5-HT_{1A} receptors, the procedures for the [35S]GTPγS-binding assay were slightly modified from the reported method[16]. The intrinsic activity (agonist or antagonist) of Y-QA31 on [35S]GTPγS binding was measured in the absence or presence of quinpirole (10 µmol/L) or 8-OH-DPAT (10 µmol/L, a 5-HT_{1A} receptor agonist). The

concentrations of Y-QA31 ranged from 10^{-10} to 10^{-5} mol/L. IC_{50} values were determined as described above.

Ca^{2+} imaging

The cell line used for Ca^{2+} imaging was HEK293 cells stably expressing α_{1A} adrenoceptor (HEK293-α_{1A} cells). The cells were washed with HBSS buffer (145 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L NaH$_2$PO$_4$, 1.2 mmol/L CaCl$_2$, 1.3 mmol/L MgCl$_2$, 10 mmol/L glucose, 20 mmol/L HEPES, and 1 mmol/L probenecid; pH 7.4) and co-incubated with 10 µmol/L Fluo-3/AM in extracellular medium for 30 min at 37 °C. A laser confocal microscope was used to measure intracellular calcium. The intrinsic activity (agonist or antagonist)

of Y-QA31 (10 µmol/L) for α_{1A} adrenoceptor was tested in the absence or presence of norepinephrine (10 µmol/L).

In vivo studies of Y-QA31

Methamphetamine- or MK-801-induced hyperlocomotion and spontaneous locomotion in mice

The day before testing, mice were placed in spontaneous activity chambers (40 cm×40 cm×40 cm) for a 30 min habituation period. On the testing day, the animals were administered either the vehicle or drug 20 min prior to the injection of methamphetamine (0.5 mg/kg, ip) or MK-801 (0.25 mg/kg, ip). A video system was used to track the movement for 1 h and record the horizontal distance traveled.

Table 1. Details of radioligand-receptor binding assays.

Receptor	Species	Host cell line or tissue	Radioligand	Unlabeled ligand	Assay buffer	Reaction tempereture/time
D$_1$	Human	HEK293	^{3}H-SCH23390 (1 nmol/L)	(+)-Butaclamol (10 µmol/L)	A	25 °C/60 min
D$_2$	Human	HEK293	^{3}H-Spiperone (1 nmol/L)	Haloperidol (10 µmol/L)	A	25 °C/60 min
D$_3$	Human	CHO	^{3}H-Spiperone (1.5 nmol/L)	Haloperidol (10 µmol/L)	A	25 °C/60 min
D$_4$	Human	HEK293	^{3}H-Spiperone (1 nmol/L)	Haloperidol (10 µmol/L)	A	25 °C/60 min
D$_5$	Human	HEK293	^{3}H-SCH23390 (1 nmol/L)	(+)-Butaclamol (10 µmol/L)	A	25 °C/60 min
5-HT$_{1A}$	Human	HEK293	^{3}H-8-OH-DPAT (1 nmol/L)	Way100635 (10 µmol/L)	H	25 °C/60 min
5-HT$_{1B}$	Human	HEK293	^{3}H-LSD (6 nmol/L)	5-HT (10 µmol/L)	B	25 °C/60 min
5-HT$_{1D}$	Human	HEK293	^{3}H-LSD (10 nmol/L)	5-HT (10 µmol/L)	B	25 °C/60 min
5-HT$_{2A}$	Human	HEK293	^{3}H-Spiperone (1 nmol/L)	Clozapine (10 µmol/L)	A	25 °C/60 min
5-HT$_{2C}$	Human	HEK293	^{3}H-LSD (4 nmol/L)	5-HT (10 µmol/L)	B	25 °C/60 min
5-HT$_{5A}$	Human	HEK293	^{3}H-LSD (1 nmol/L)	5-HT (10 µmol/L)	B	25 °C/60 min
5-HT$_6$	Human	HEK293	^{3}H-LSD (1 nmol/L)	5-HT (10 µmol/L)	B	25 °C/60 min
5-HT$_7$	Rat	Rat thalamus	^{3}H-LSD (4 nmol/L)	5-HT (10 µmol/L)	B	37 °C/30 min
α_{1A}	Human	HEK293	^{3}H-Parzosin (1 nmol/L)	Chlorpromazine (10 µmol/L)	C	25 °C/60 min
α_{1B}	Human	HEK293	^{3}H-Parzosin (1 nmol/L)	Phentolamine (10 µmol/L)	C	25 °C/60 min
α_2	Rat	Brain	^{3}H-Clonidine (2 nmol/L)	Yohimbine (10 µmol/L)	C	37 °C/30 min
β_1	Human	HEK293	^{3}H-DHA (1 nmol/L)	Isopropyl noradrenalin (10 µmol/L)	B	25 °C/60 min
M$_1$	Human	CHO	^{3}H-NMS (1 nmol/L)	Atropine (10 µmol/L)	D	25 °C/60 min
M$_2$	Human	HEK293	^{3}H-NMS (1 nmol/L)	Atropine (10 µmol/L)	D	25 °C/60 min
M$_3$	Human	CHO	^{3}H-NMS (1 nmol/L)	Atropine (10 µmol/L)	D	25 °C/60 min
M$_4$	Human	CHO	^{3}H-NMS (1 nmol/L)	Atropine (10 µmol/L)	D	25 °C/60 min
M$_5$	Human	CHO	^{3}H-NMS (1 nmol/L)	Atropine (10 µmol/L)	D	25 °C/60 min
µ	Human	CHO	^{3}H-Dipnorphine (1 nmol/L)	Naloxone (10 µmol/L)	E	37 °C/30 min
δ	Human	CHO	^{3}H-Dipnorphine (1 nmol/L)	Naloxone (10 µmol/L)	E	37 °C/30 min
κ	Human	CHO	^{3}H-Dipnorphine (1 nmol/L)	Naloxone (10 µmol/L)	E	37 °C/30 min
A$_1$	Human	CHO	^{3}H-DPCPX (1 nmol/L)	R-PIA (10 µmol/L)	F	37 °C/30 min
A$_{2A}$	Human	HEK293	^{3}H-CGS21680 (10 nmol/L)	NECA (10 µmol/L)	F	25 °C/60 min
H$_1$	Human	HEK293	^{3}H-Pyrilamine (1 nmol/L)	Promethazine (10 µmol/L)	B	37 °C/30 min
H$_2$	Human	HEK293	^{3}H-Tiotidine (3 nmol/L)	Cimetidine (10 µmol/L)	B	37 °C/30 min
H$_3$	Human	HEK293	^{3}H-Histamine (10 nmol/L)	Promethazine (10 µmol/L)	B	25 °C/60 min
CB$_1$	Human	HEK293	^{3}H-CP55940 (1 nmol/L)	WIN 55,212-2 (10 µmol/L)	G	30 °C/60 min
CB$_2$	Human	HEK293	^{3}H-CP55940 (1 nmol/L)	WIN 55,212-2 (10 µmol/L)	G	30 °C/60 min

Buffer A (in mmol/L, pH7.4): 50 Tris-HCl, 120 NaCl, 5 KCl, 5 EDTA-Na$_2$·2H$_2$O, 5 MgCl$_2$, 1.5 CaCl$_2$;
Buffer B (in mmol/L, pH7.4): 50 Tris-HCl, 10 MgCl$_2$, 1 EDTA-Na$_2$·2H$_2$O;
Buffer C (in mmol/L, pH7.4): 20 Tris-HCl, 145 NaCl;
Buffer D (in mmol/L, pH7.4): 10 HEPES, 0.1 EDTA-Na$_2$·2H$_2$O;
Buffer E (in mmol/L, pH7.4): 50 Tris-HCl;
Buffer F (in mmol/L, pH7.4): 50 Tris-HCl, 2 MgCl$_2$, 1 ADA;
Buffer G (pH7.4): 10 mmol/L HEPES, 10 mmol/L MgCl$_2$, 0.3 g/L BSA;
Buffer H (in mmol/L, pH7.4): 130 NaCl, 4.8 KCl, 1.2 Na$_2$HPO4, 1.3 CaCl$_2$, 1.2 MgSO$_4$, 10 glucose, 25 HEPES.

Disruption of prepulse inhibition (PPI) by methamphetamine in mice
The procedures used were previously reported by Millan[12], with some modifications. Male CD1 mice were administered the drug or vehicle 20 min before the injection of methamphetamine (6 mg/kg, ip) or vehicle and then placed individually in the startle chambers connected via an interface to a computer that controlled auditory stimuli and monitored startle responses. The prepulse parameter test session consisted of 4 trial types: startle stimulus trials (pulse-alone), two different prepulse-alone trial types (prepulse-alone), and two different prepulse trial types (prepulse+pulse). The pulse-alone trial type consisted of the presentation of a 20-ms, 115-dB pulse. The two prepulse-alone trial types consisted of 4-ms, 80-dB or 85-dB prepulse stimuli that were randomly administered. The two prepulse+pulse trial types consisted of 80-dB and 85-dB prepulses, followed 30 ms later (the interstimulus interval; the time between prepulse offset and pulse onset) by the 20-ms, 115-dB startle pulse. After a 5 min acclimation period with 65-dB background noise, 10 of the 4 trial types (a total of 40 trials) were presented in a pseudo-random order with an average inter-trial interval of 15 s for a total of 40 trials. PPI was defined as the percentage of reduction in startle amplitude in the presence of prepulse versus the absence of prepulse. %PPI=100−[(startle amplitude for prepulse+pluse)/(startle amplitude for pluse alone)]×100.

Conditioned avoidance responses (CAR) in mice
Mice were trained to avoid an electric shock (0.16 mA, 5 s) by switching compartments of a shuttle box upon the appearance of a light. The mice were trained for 30 trials per day, and the intertrial interval was 15–30 s, randomly administered. Each trial consisted of a 10-s "light on" period. The mouse received a shock if it did not change compartments, or it avoided a shock by switching during the "light on" period. Testing was performed when each mouse learned to avoid the shock in over 24 trials per day. The drug or vehicle was administered 30 min before testing.

Induction of catalepsy in mice
The procedure was performed according to Reavill[10] with some modifications. The tails of the animals were lifted so that the forelimbs could touch the 3-cm-high wooden block top edge. Then, the hind limbs were placed on the desktop gently, and the duration spent in this position was determined. The animals that did not move for more than 30 s were categorized as positive animals. Three independent measurements were obtained, separated by 15-min intervals. The drug or vehicle was administered 30 min before testing.

Novel object recognition (NOR) in mice
The procedure was performed according to Karasawa[14] with some modifications. The task was performed in a 40 cm×50 cm×50 cm chamber. All animals were given a habituation session during which they were left to freely explore the environment for 10 min. No objects were placed in the chamber during the habituation trial. 24 h after habituation, training was conducted by placing individual mice into a chamber for 5 min, in which two identical objects (object A1 and A2) were positioned in two adjacent corners, 10 cm away from the walls. In a short-term memory (STM) test given 1.5 h after training, the mice explored the chamber for 5 min in the presence of one familiar and one novel object. All objects presented had similar textures, colors, and sizes, but distinctive shapes. A recognition index calculated for each animal was expressed by the ratio $T_B/(T_A+T_B)$ (T_A=time spent exploring the familiar object A; T_B= time spent exploring the novel object B). Between the trials, the objects were washed with a 10% ethanol solution. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. The mice were administered Y-QA31, haloperidol, or clozapine at the indicated doses (*po*, once daily) for four days prior to the testing day. On the testing day (5th day), the mice were injected with MK-801 (0.2 mg/kg, ip) with or without one of the abovemention drugs 30 min before the training session.

Statistical analysis
The data were expressed as the mean±SEM. All statistical analyses were performed using Prism 5 (GraphPad Software Incorporate, CA, USA). The statistical significance of the catalepsy data was calculated by Fisher's exact test. Other data were tested by one-way ANOVA or two-way ANOVA to compare the differences among multiple groups. $P<0.05$ was considered statistically significant.

Results
Y-QA31 selectively antagonized D_3R over D_2R
In radioligand-receptor binding assays, Y-QA31 bound to the recombinant dopamine D_3R with an IC_{50} value of 1.74±0.47 nmol/L and a K_i value of 0.28±0.08 nmol/L, suggesting a high affinity for D_3R (Figure 2A and Table 2). Y-QA31 also bound to the dopamine D_2R; however, the affinity was much lower (IC_{50}=220.2±48.4 nmol/L, K_i=52.1±11.1 nmol/L) (Figure 2A and Table 2). These results indicate that Y-QA31 had 186-fold selectivity for D_3R over D_2R. In contrast, the affinity of Y-QA31 for D_4R was low, with an IC_{50} value of 877.3±150.1 nmol/L. Additionally, Y-QA31 rarely bound to dopamine D_1 and D_5 receptors, with IC_{50} values>10 µmol/L. In [^{35}S]GTPγS-binding assays, Y-QA31 (0.1 nmol/L−10 µmol/L) did not activate D_3R, but it antagonized the D_3R activation stimulated by 10 µmol/L quinpirole, indicating that Y-QA31 is an antagonist for D_3R (Figure 2B). Y-QA31 also inhibited D_2R activation stimulated by 10 µmol/L quinpirole (Figure 2B). These results suggest that Y-QA31 selectively antagonized D_3 over D_1, D_2, D_4 and D_5 receptors.

Y-QA31 rarely bound to cholinergic muscarinic receptors (M_1, M_2, M_3, M_4 and M_5 subtypes), opioid receptors (µ, δ and κ subtypes), 5-HT$_6$ receptor, adenosine A_1 and A_{2A} receptors, cannabinoid CB_1 and CB_2 receptors, histamine H_2 and H_3 receptors, or β$_1$ adrenoceptors (IC_{50}>10 µmol/L), and it weakly bound to α$_2$ adrenergic receptors, histamine H_1 receptors and some subtypes of 5-HT receptors, including 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{2A}$, 5-HT$_{2C}$, 5-HT$_{5A}$ and 5-HT$_7$ subtypes (IC_{50}>500 nmol/L, Table 2). Y-QA31 displayed significant affinity for 5-HT$_{1A}$ receptors

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(IC$_{50}$=11.70±3.87 nmol/L, K_i=8.36±2.76 nmol/L) and α$_{1A}$ adrenoceptors (IC$_{50}$=46.3±10.4 nmol/L, K_i=14.8±3.4 nmol/L), although its affinity to 5-HT$_{1A}$ receptors and α$_{1A}$ adrenoceptors was still lower than that to D$_3$R (30-fold for D$_3$R over 5-HT$_{1A}$ receptors and 53-fold for D$_3$R over α$_{1A}$ adrenoceptors, Table 2).

Y-QA31 showed a modest affinity for α$_{1B}$ adrenoceptors (IC$_{50}$=170.8±62.5 nmol/L, K_i=56.9±21.0 nmol/L), but the affinity was much lower (203-fold for D$_3$R over α$_{1B}$ adrenoceptors). Functional assays showed weak partial agonism of 5-HT$_{1A}$ receptors and antagonism of α$_{1A}$ adrenoceptors (Figure 3).
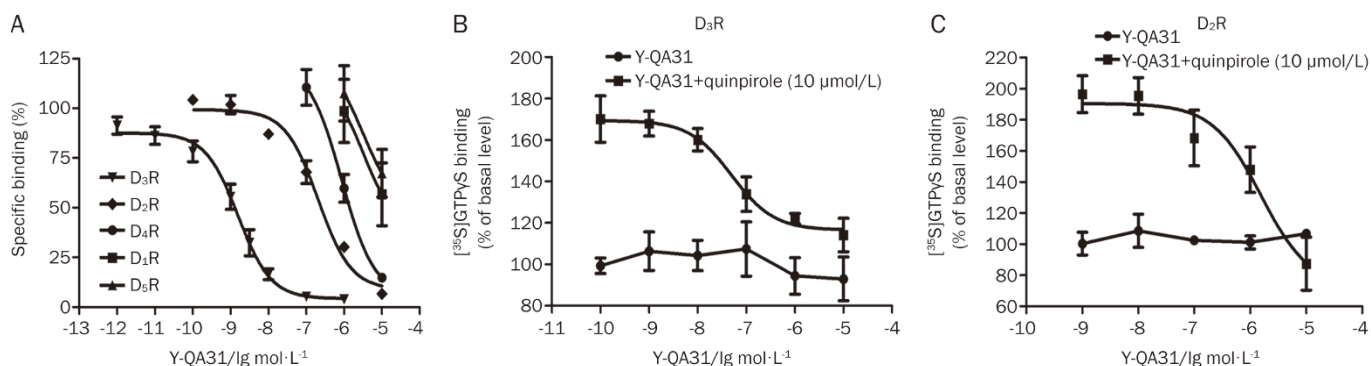

Figure 2. Affinity and intrinsic activity of Y-QA31 for dopamine receptors. (A) Competitive binding curves of Y-QA31 to dopamine receptor subtypes determined from radioligand-receptor binding assays. Data are representative of three to five independent experiments. (B and C) Antagonism of Y-QA31 for dopamine D$_3$ and D$_2$ receptors in [^{35}S]GTPγS-binding assays. The data are representative of three independent experiments.

Table 2. IC$_{50}$ values of Y-QA31 for GPCRs.

| Receptor	IC$_{50}$	Receptor	IC$_{50}$	Receptor	IC$_{50}$
D$_1$	>10.0 μmol/L	5-HT$_6$	>10.0 μmol/L	μ	>10.0 μmol/L
D$_2$	220.2±48.4 nmol/L	5-HT$_7$	1.65±1.52 μmol/L	δ	>10.0 μmol/L
D$_3$	1.74±0.47 nmol/L	α$_{1A}$	46.3±10.4 nmol/L	κ	>10.0 μmol/L
D$_4$	877.3±150.1 μmol/L	α$_{1B}$	170.8±62.5 nmol/L	A$_1$	>10.0 μmol/L
D$_5$	>10.0 μmol/L	α$_2$	2.17±2.38 μmol/L	A$_{2A}$	>10.0 μmol/L
5-HT$_{1A}$	11.70±3.87 nmol/L	β$_1$	>10.0 μmol/L	H$_1$	568.5±95.7 nmol/L
5-HT$_{1B}$	7.52±6.16 μmol/L	M$_1$	>10.0 μmol/L	H$_2$	>10.0 μmol/L
5-HT$_{1D}$	677.9±254.1 nmol/L	M$_2$	>10.0 μmol/L	H$_3$	>10.0 μmol/L
5-HT$_{2A}$	2.40±4.77 μmol/L	M$_3$	>10.0 μmol/L	CB$_1$	>10.0 μmol/L
5-HT$_{2C}$	839.0±472.2 nmol/L	M$_4$	>10.0 μmol/L	CB$_2$	>10.0 μmol/L
5-HT$_{5A}$	1.68±0.32 μmol/L	M$_5$	>10.0 μmol/L		
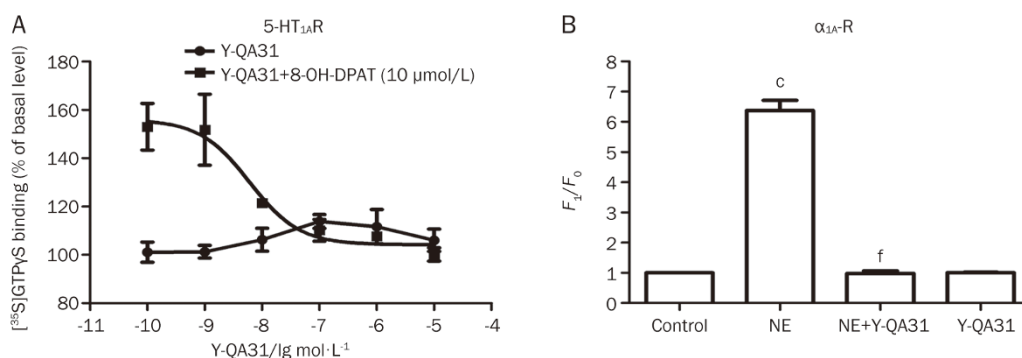
Figure 3. Antagonism of Y-QA31 for α$_{1A}$ adrenoceptor and partial agonism for 5-HT$_{1A}$ receptor. (A) [^{35}S]GTPγS-binding assay was used for the 5-HT$_{1A}$ receptor. Data are representative of three independent experiments. (B) An intracellular Ca^{2+} release assay was used for the α$_{1A}$ adrenoceptor. F_0: the basis fluorescence intensity that did not receive drug treatment, F_1: the peak fluorescence intensity after drug treatment. F_1/F_0: the ratio of the fluorescence intensity after drug treatment compared to that before drug treatment. The data are representative of five to six independent experiments. ^{c}$P<0.01$ *vs* control, ^{f}$P<0.01$ *vs* NE; one way ANOVA followed by Bonferroni's multiple comparisons test. NE, norepinephrine, 10 μmol/L; Y-QA31, 10 μmol/L.

Effects of Y-QA31 on methamphetamine- or MK-801-induced locomotion activity in mice

Antagonism of hyperlocomotion induced by dopamine system agonists has traditionally been used to predict antipsychotic efficacy. Methamphetamine (0.5 mg/kg, ip) induced hyperlocomotion activity was used to test the effect of Y-QA31. Y-QA31 inhibited methamphetamine-induced hyperlocomotion at 20 mg/kg and 40 mg/kg [$F_{(5,80)}$=14.79; $P<0.0001$] (Figure 4C). Y-QA31 alone did not influence spontaneous locomotion until its concentration was higher than 80 mg/kg [$F_{(4,38)}$=28.86; $P<0.0001$] (Figure 4A). In comparison, haloperidol (0.16 and 0.64 mg/kg, *po*) significantly decreased methamphetamine-induced hyperlocomotion [$F_{(4,44)}$=36.45; $P<0.0001$] (Figure 4D), and, by itself, also inhibited spontaneous locomotor activity [$F_{(3,29)}$=28.07; $P<0.0001$] at these doses (Figure 4B).

Hyperactivity induced by MK-801 was then used to assess the effect of Y-QA31. Figure 4E shows that Y-QA31 (10–40 mg/kg, *po*) significantly inhibited MK-801-induced hyperlocomotion (0.25 mg/kg, ip) in a dose-dependent manner [$F_{(5,73)}$=30.98; $P<0.0001$].

Disruption by Y-QA31 of performance of CAR in mice

Y-QA31 (20 mg/kg and 40 mg/kg, *po*) inhibited the avoidance response in a dose-dependent manner [$F_{(4,86)}$=4.233; $P<0.01$] without affecting the escape response (Figure 5A). Haloperi-dol (0.01−0.08 mg/kg, *po*) significantly inhibited the avoidance response [$F_{(5,72)}$=10.24; $P<0.0001$] (Figure 5C) and did not affect the escape response. Clozapine also suppressed the avoidance response at 2.5 and 5 mg/kg [$F_{(4,44)}$=6.05; $P<0.001$] (Figure 5B) but had the trend to produce the escape response failure at a high dose (5 mg/kg), probably because of its motor side effect. In this experiment, the effect of haloperidol was more potent, which was consistent with a previous report[12].

Effects of Y-QA31 on PPI in mice

Y-QA31 alone had no influence on the basal %PPI at low doses; Y-QA31 at dosage of 40 mg/kg reduced the %PPI by 85-dB prepulse [dB: $F_{(1,35)}$=10.666, $P<0.01$; treatment: $F_{(4,35)}$=1.5, $P<0.05$; interaction: $F_{(4,35)}$=0.333, $P>0.05$] (Figure 6C). Methamphetamine (6 mg/kg, ip) disrupted PPI, as expected, and Y-QA31 (5–40 mg/kg, *po*) significantly reversed this disruption of PPI in a dose-dependent manner [dB: $F_{(1,45)}$=24.418, $P<0.01$; treatment: $F_{(5,45)}$=5.121, $P<0.01$; interaction: $F_{(5,45)}$=0.101, $P>0.05$] (Figure 6A). This perturbation of PPI was also abolished by clozapine (1.25–10 mg/kg, *po*) [dB: $F_{(1,45)}$=1.741, $P>0.05$; treatment: $F_{(5,45)}$=4.497, $P<0.01$; interaction: $F_{(5,45)}$=1.416, $P>0.05$] (Figure 6B), which was consistent with a previous report[12].

Effects of Y-QA31 on MK-801-induced NOR disruption in mice

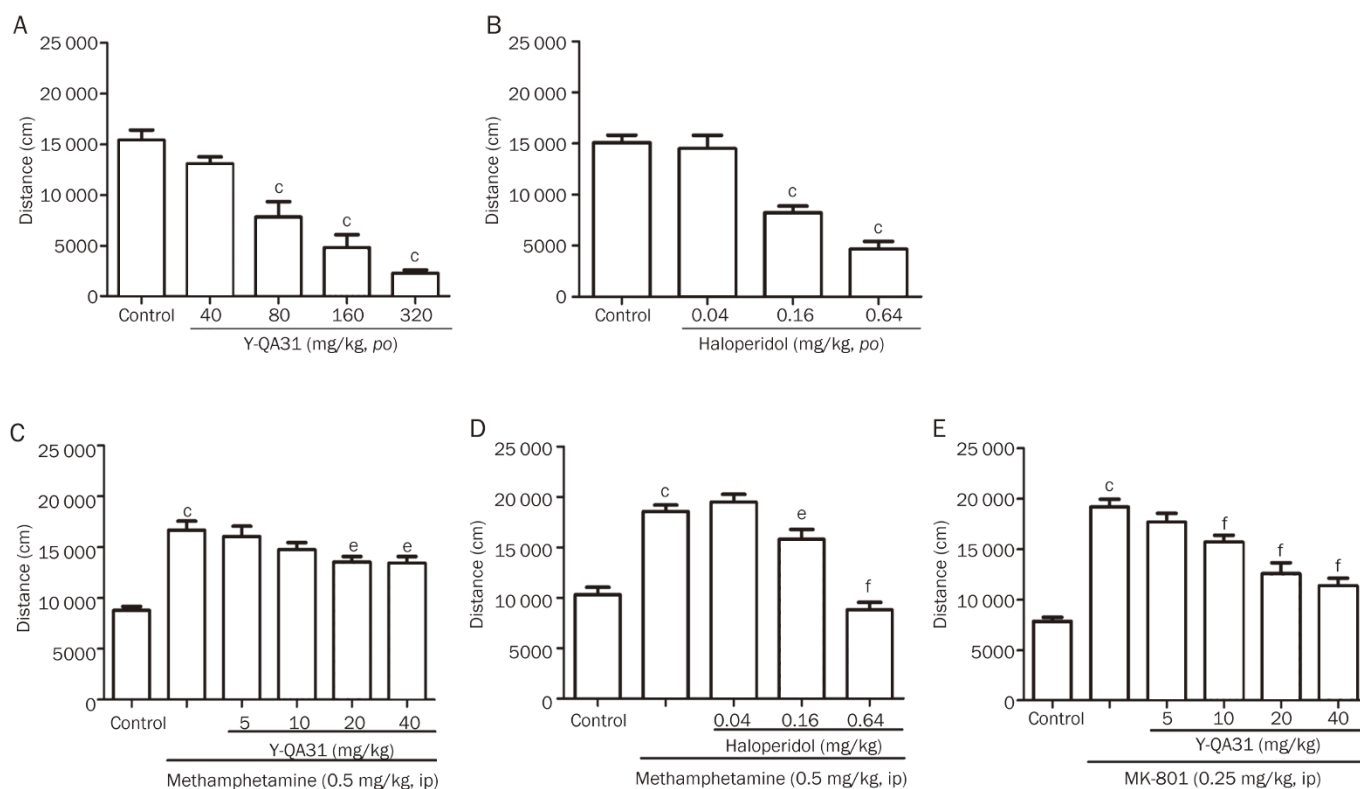During the training trial, no difference was found among the

Figure 4. Effect of Y-QA31 on methamphetamine- or MK-801-induced hyperlocomotion in mice. (A) Effects of Y-QA31 alone on spontaneous locomotion. (B) Effects of haloperidol alone on spontaneous locomotion. (C) Inhibitory effect of Y-QA31 on methamphetamine-induced hyperlocomotion. (D) Inhibitory effect of haloperidol on methamphetamine-induced hyperlocomotion. (E) Inhibitory effect of Y-QA31 on MK-801-induced hyperlocomotion. *n*=10−15. [c]$P<0.01$ *vs* the control; [e]$P<0.05$, [f]$P<0.01$ *vs* methamphetamine treated group or MK-801 treated group; one-way ANOVA followed by Newman-Keuls test.

vehicle, MK-801, or MK-801+drug groups in the total object exploration time (Figure 7A). In the testing session, vehicle-treated mice spent more time exploring the novel object than the familiar object. In contrast, throughout the experiment, MK-801 (0.2 mg/kg, ip)-treated mice spent less time exploring the novel object, indicating that MK-801 treatment resulted in cognitive deficit. The deficit in NOR induced by MK-801 was alleviated by Y-QA31 (1 mg/kg, *po*) [trial: $F_{(1,54)}=0.126$, *P*>0.05; treatment: $F_{(5,54)}=5,565$, *P*<0.01; interaction: $F_{(5,54)}=4.831$, *P*<0.05] (Figure 7A). In comparison, the atypical antipsychotic clozapine (1.25 mg/kg, *po*), but not

the classical agent haloperidol, reversed the NOR disruption [trial: $F_{(1,28)}=19.281$, *P*<0.01; treatment: $F_{(5,28)}=1.377$, *P*<0.05; interaction: $F_{(5,28)}=0.784$, *P*>0.05] (Figure 7B), which was consistent with a previous report[14].

Y-QA31 induced catalepsy in mice

Within the effective doses of 5–40 mg/kg (*po*), Y-QA31 did not elicit catalepsy. Induction of catalepsy by Y-QA31 was observed at 120 mg/kg (Table 3). Clozapine did not induce catalepsy until its dose was over 7.5 mg/kg (Table 3). Haloperidol elicited potent catalepsy at doses up to 0.64 mg/kg (Table 3).
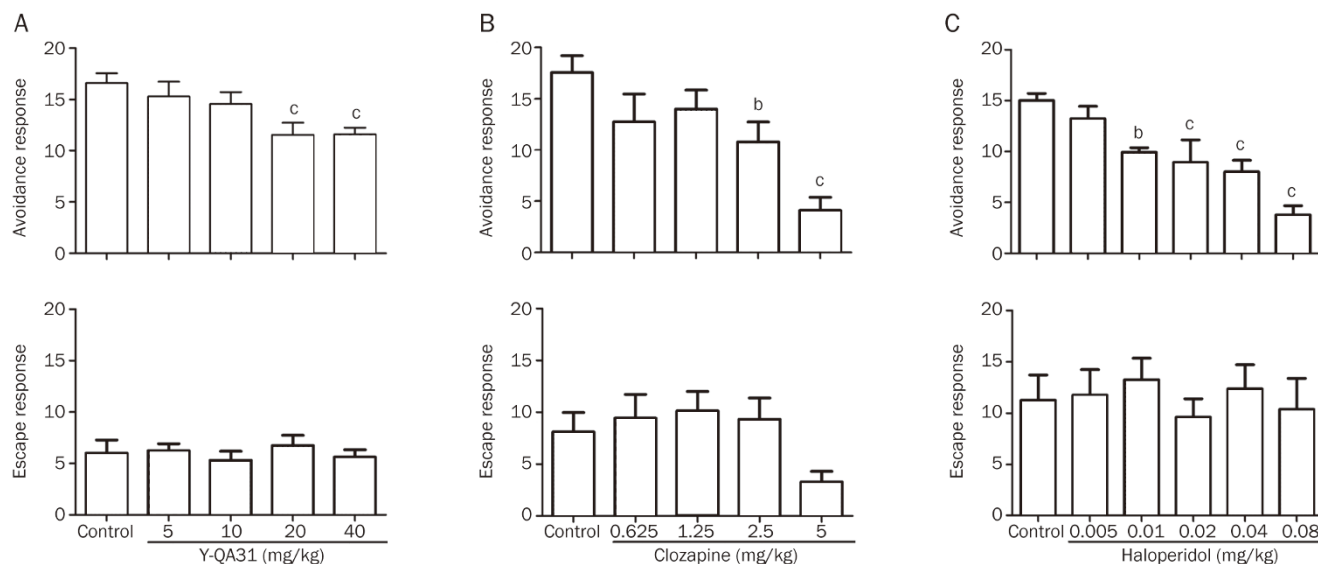

Figure 5. Inhibition of the conditioned avoidance response in mice by Y-QA31 (A), clozapine (B), and haloperidol (C). *n*=15–20; [b]*P*<0.05, [c]*P*<0.01 *vs* control; one-way ANOVA followed by Newman-Keuls test.
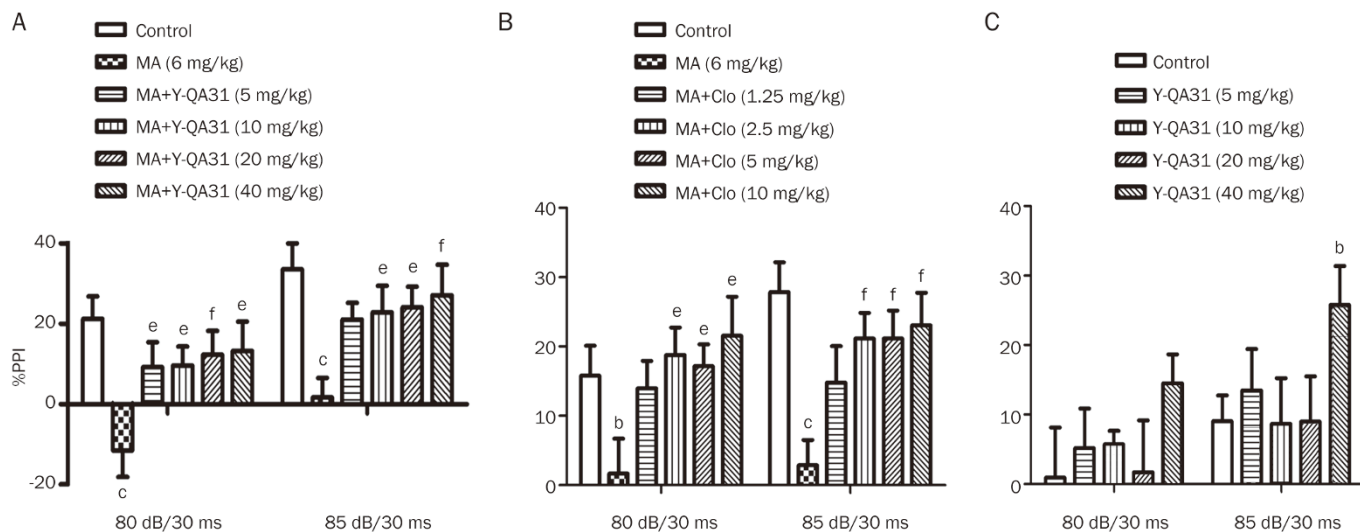

Figure 6. Inhibition of the disruption of prepulse inhibition (PPI) elicited by methamphetamine in mice by Y-QA31 (A), clozapine (B), and the effect of Y-QA31 alone on prepulse inhibition (C). *n*=8–10. [b]*P*<0.05, [c]*P*<0.01 *vs* control; [e]*P*<0.05, [f]*P*<0.01 *vs* the methamphetamine treated group; two-way ANOVA with repeated measurement followed by Bonferroni post-tests. MA, methamphetamine. Clo, clozapine.
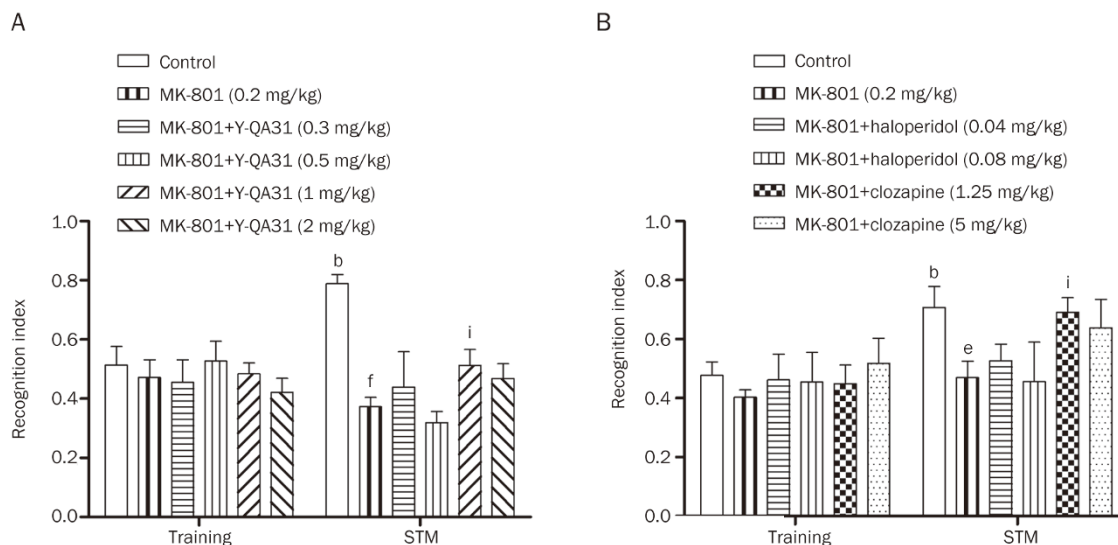
A

B

Figure 7. Effects of 4-d pretreatment with Y-QA31, haloperidol, or clozapine on MK-801-induced object recognition disruption in mice. n=8. $^{b}P<0.05$ *vs* saline control in training. $^{e}P<0.05$, $^{f}P<0.01$, the MK-801 group *vs* the saline control in short-term memory (STM) testing; $^{i}P<0.05$ *vs* MK-801; two-way ANOVA with repeated measurement followed by Bonferroni post-tests.

Table 3. Induction of catalepsy in mice by Y-QA31, clozapine, or haloperidol.

Drug	Dose (mg/kg)	n	Catalepsy (n)
Control		10	0
Y-QA31	40	10	0
	80	10	3
	120	15	7^{b}
	160	10	7^{c}
	240	10	10^{c}
	320	10	10^{c}

Drug	Dose (mg/kg)	n	Catalepsy (n)
Control		5	0
Clozapine	2.5	5	0
	5	5	0
	7.5	10	7^{c}
	10	10	8^{c}
	20	10	7^{c}
	40	5	5^{c}
	80	5	5^{c}

Drug	Dose (mg/kg)	n	Catalepsy (n)
Control		10	0
Haloperidol	0.16	10	0
	0.32	10	3
	0.64	10	8^{c}
	1.28	10	10^{c}
	2.56	10	10^{c}

n=5–15 per value. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control; Fisher's exact test.

Discussion

This study demonstrates that Y-QA31 binds with a high affinity to human D_3R with 186-fold selectivity for D_3R over D_2R and over 1000-fold selectivity over D_1, D_4 and D_5 receptor subtypes. Y-QA31 also exhibited 5-HT$_{1A}$ receptor partial agonism (D_3/5-HT$_{1A}$ receptor selectivity=30) and α_{1A} adrenoceptor antagonism (D_3/α_{1A}-R selectivity=53) with medium affinity, and had very little affinity for other G protein-coupled receptors, such as other subtypes of 5-HT receptors, cholinergic muscarinic receptors (M_1–M_5), opioid receptors (μ, δ and κ subtypes) and adenosine A_1 and A_{2A} receptors. Therefore, the *in vitro* results showed that Y-QA31 is a highly selective antagonist for D_3R and a fairly selective partial agonist for 5-HT$_{1A}$ receptor.

Excess dopamine in the brain caused by dysfunction in the dopamine system is considered to be an important mechanism for positive symptoms of schizophrenia. Methamphetamine induces hyperactivity by facilitating the release of dopamine from presynaptic nerve terminals and inhibiting reuptake, which increases extracellular dopamine levels in the brain. For this reason, methamphetamine-induced hyperlocomotion is used as a classic dopaminergic model for the identification of antipsychotics[17]. The current data showed that Y-QA31 inhibited methamphetamine-induced hyperlocomotion at doses lower than those used to inhibit spontaneous locomotion. Thus far, most of the evidence indicates that blocking D_3R may not inhibit methamphetamine-induced hyperactivity. For example, the selective D_3R antagonist S33084 did not alter methamphetamine-induced hyperactivity[10, 11]. Furthermore, knocking down D_3R with mRNA antisense oligodeoxynucleotides[18] or knocking out the receptor entirely results in increased locomotion[19]. By comparison, D_2R knockout mice

do not exhibit the hypolocomotor effects of 7-OH-DPAT, a D_2/D_3R agonist, which produced decreased locomotion, while D_3R knockout and wild-type mice showed similar decreases of locomotor activity response to 7-OH-DPAT[20]. Y-QA31 exerted low potency in inhibiting hyperlocomotion induced by methamphetamine, whereas the D_2R-selective antagonist haloperidol markedly inhibited hyperlocomotion. One possible explanation for the weak effect of Y-QA31 against methamphetamine might be the low D_2R occupancy present even at high doses of Y-QA31 (20 mg/kg and 40 mg/kg). In addition, because Y-QA31 binds the α_{1A} adrenoceptor with medium affinity and the α_{1A} adrenoceptor may play a role in hyperactivity[21], another explanation for Y-QA31's weak inhibitory action may due to its α_{1A} adrenoceptor antagonism.

In comparison with methamphetamine-induced hyperlocomotion, MK-801-induced hyperlocomotion was significantly inhibited by Y-QA31. Previous studies have also found that MK-801-induced hyperactivity is blocked by inhibiting D_3R[22], such as using the D_3R selective antagonist F17141[23]. The NMDA receptor antagonists PCP, ketamine, and MK-801 produce similar hyperlocomotion as methamphetamine does[24]. However, the mechanism underlying MK-801-induced hyperlocomotion is different from methamphetamine-induced hyperlocomotion, possibly because of an increased release of dopamine and associated activation of the dopamine system in the mesolimbic zone caused by the influence of glutamate[25]. Nakajima *et al* proposed that D_3R may control NMDA receptor signaling by acting on pyramidal cells, either directly at postsynaptic levels in the NAc or indirectly at presynaptic levels in the PFC[26].

PPI of the startle reflex is a currently accepted sensorimotor gating model in mammals. Clinical studies have confirmed the phenomenon of disrupted PPI in schizophrenia patients and found that this disruption is related to cognitive deficits such as disordered thought and poor attention[27]. In animal models, PPI disruption can be induced by dopamine agonists, NMDA receptor blockers and 5-HT_{2A} receptor agonists; pharmacological reversal of PPI disruption indicates antipsychotic potential. Similar to other selective D_3R antagonists, SB-277011A and A-437203[28], Y-QA31 alone did not affect the basal PPI at low doses but increased the PPI at high doses. In the model of methamphetamine-induced PPI disruption, the inhibitory effect of Y-QA31 (5–40 mg/kg) on PPI deficit indicated its antipsychotic effect and also suggested a role for D_3R in PPI dysfunction. Previous findings showed that PPI dysfunction induced by PD128907, a D_3R agonist, was reversed by two selective D_3R antagonists, SB-277011A and A-691990, but not by raclopride or haloperidol, both of which are D_2R antagonists[29, 30]. SB-277011A also reversed PPI disruption induced by social isolation[10, 30]. However, research in genetically modified mice was not consistent with the pharmacological findings. Amphetamine-induced PPI disruption was unaffected in D_3R-KO mice but absent in D_2R-KO mice[31]. Therefore, more research is needed to address the involvement of D_3R in PPI. Many other receptors are involved in the development of PPI, such as 5-HT_{1A} receptors and α_{1A} adrenoceptor[21]. Previous studies showed that 5-HT_{1A} receptor antagonists attenuate or abolish the PPI disruption induced by MK-801[32], 8-OH-DPAT, or apomorphine[33]. Considering Y-QA31's modest affinity for the 5-HT_{1A} receptor and α_{1A} adrenoceptor, these receptors may mediate the effects of Y-QA31 on PPI disruption[21]. Additionally, D_2R antagonism may also contribute to the effect of Y-QA31 on PPI at high doses.

Y-QA31 produced weak suppression of CAR compared with the D_2R antagonist haloperidol. The behavioral mechanisms of antipsychotic-induced suppression of CAR are still not fully understood, but some evidence suggests that CAR is mediated primarily by D_2R in the ventral striatum[34]. Generally, 50% D_2R occupancy is required for CAR suppression[35]. Considering the low D_2R occupancy of Y-QA31, we speculate that this weak effect may occur because the D_2R antagonism of Y-QA31 is not strong enough for potent inhibition of CAR. Some evidence suggests that CAR may be at least partially dissociated from D_2R blockade. The 5-HT_{1A} and 5-HT_{2A} receptors, the α_{1A} adrenoceptor and NMDA receptor may participate in this process[36]. For example, the 5-HT_{1A} receptor agonist 8-OH-DPAT produced a moderate suppression of CAR[37]. We propose that the moderate 5-HT_{1A} receptor partial agonism and α_{1A} adrenoceptor antagonism of Y-QA31 may also contribute to CAR. Thus far, the role of D_3R in CAR remains unclear.

Cognitive deficit is considered as a core feature of schizophrenia, and it lacks responsiveness to many current antipsychotic drugs. NOR is a form of visual-recognition memory that is dependent on animals' innate preference to investigate novel objects, which is decreased in schizophrenia patients because of their visual-recognition memory impairments[38]. The current study found that NOR was disrupted by MK-801, consistent with previous reports[39, 40]. Y-QA31 (1 mg/kg) significantly alleviated the NOR disruption induced by MK-801, suggesting that blocking D_3R improved the cognitive and learning dysfunction. It has recently been confirmed that D_3R plays an important role in cognitive dysfunctions[41, 42]. D_3R knockout mice exhibit increased cognitive flexibility in the attentional set-shifting task and improved retention in a passive-avoidance test[43]. Moreover, the pharmacological findings also support the potential role of D_3R in treating cognitive dysfunctions and negative symptoms. For example, D_3R antagonist S33084 enhances cognitive performance in wild-type mice but not in D_3R knockout mice[44]. Other D_3 antagonists, such as the highly selective SB-277011A and RGH-1756 and the moderately selective U-99194A, significantly attenuated the learning deficit caused by FG-7142 and scopolamine-induced amnesia[41]. D_2R antagonists, however, produced deleterious effects or no effect on cognition. For example, L741,626 had no pro-cognitive effect in models on social isolation rearing and the social recognition paradigm[45, 46]. Notably, Y-QA31 was effective in improving cognitive symptoms in the NOR model at 10-fold lower doses than the doses used to attenuate positive symptoms. This phenomenon was also observed in the dual D_2R/D_3R antagonist, amisulpride, which controls both cognitive deficit and positive symptoms in the clinic. The effective dose of amisulpride is 50–100 mg per day

when cognitive deficit predominate but 400–800 mg per day when positive symptoms predominate[47, 48]. The mechanism of amisulpride in treating cognitive and positive symptoms with different doses possibly involves its preferential blockade effects of presynaptic or postsynaptic mechanisms and limbic structures. Blocking presynaptic dopamine D_2/D_3 autoreceptors with low doses of amisulpride induces increased dopaminergic neurotransmission, whereas high doses may block postsynaptic dopaminergic activity. The reason that Y-QA31 treats cognitive deficit and positive symptoms at different doses remains unknown, but preferential binding to presynaptic or postsynaptic receptors is a possible mechanism.

Blockade of D_2R in the striatum can induce extrapyramidal motor side effects such as catalepsy, whereas D_3R blockade is unlikely to provoke extrapyramidal side effects. Consistent with this idea, Y-QA31 did not induce catalepsy until dosage reached 120 mg/kg, whereas haloperidol (0.64 mg/kg) and clozapine (7.5 mg/kg) significantly induced catalepsy in therapeutic doses. These results further support the observation that D_3R blockade is associated with very low incidence of catalepsy. In addition, D_3R antagonists such as SB-277011A and S33084 were found to inhibit haloperidol-induced catalepsy[49, 50], which may predict the therapeutic value of D_3R blockade in the treatment of schizophrenia.

Taken together, Y-QA31 exhibits anti-psychotic effects in some animal models of positive symptoms without inducing the extrapyramidal side effects and the inhibition of spontaneous locomotion compared with haloperidol and clozapine. Moreover, similar to clozapine, Y-QA31 alleviates cognitive dysfunction in the NOR model. Given the high affinity of Y-QA31 to D_3R, the mechanism of its antipsychotic effect might be associated with D_3R blockade, but D_2R, as well as α_{1A} adrenoceptor antagonism and 5-HT$_{1A}$ receptor partial agonism, should also be considered. Further pharmacological profiling studies with Y-QA31 could contribute to a more precise understanding of the function of D_3R or the 5-HT$_{1A}$ receptor, as well as the α_{1A} adrenoceptor relevant to schizophrenia.

Conclusions

Y-QA31 is a dopamine D_3 receptor antagonist that also exhibits partial agonism of the 5-HT$_{1A}$ receptor and antagonism to the α_{1A} adrenoceptor with medium affinity. Y-QA31 produced antipsychotic effects in some animal models of positive symptoms at high doses and pro-cognitive effects at low doses, with low extrapyramidal syndrome. The results of this study further suggest that D_3 receptor antagonism is still of therapeutic value for treating symptoms of schizophrenia.

Author contribution

Jin LI, Rui-bin SU and Fei LI designed the research; Xue SUN, Hong-yan GOU, Guan-yi LU and Rui SONG performed the experiments; Ri-fang YANG synthesized the compound; Xue SUN, Fei LI and Ning WU analyzed the data and wrote the draft; Jin LI, Rui-bin SU and Bin CONG revised and edited the manuscript. All authors read and approved the manuscript.

Abbreviations

D_3R, dopamine D_3 receptor; D_2R, dopamine D_2 receptor; PPI, prepulse inhibition; CAR, conditioned avoidance responses; NOR, novel object recognition; NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; STM, short-term memory.

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