In Vitro and In Vivo Characterization of the Dopamine D₄ Receptor, Serotonin 5-HT₂A Receptor and Alpha-1 Adrenoceptor Antagonist (R)-(+)2-Amino-4-(4-Fluorophenyl)-5-[1-[4-(4-Fluorophenyl)-4-Oxobutyl]Pyrrolidin-3-yl]Thiazole (NRA0045)

SHIGERU OKUYAMA, SHIGEYUKI CHAKI, RYOKO YOSHIKAWA, YOSHIKO SUZUKI, SHIN-ICHI OGAWA, YASUO IMAGAWA, NAOYA KAWASHIMA, YOKO IKEDA, TOSHIHITO KUMAGAI, ATSURO NAKAZATO, MASASHI NAGAMINE and KAZUYUKI TOMISAWA

1st Laboratory (S.O., S.C., R.Y., Y.S., S.O., Y.I., N.K., T.K., N.A., K.T.) and Molecular Biology Laboratory (Y.I.), Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd., Ohmiya, Saitama 330, Japan and Research Center (M.N.), Nihon Nouyaku Co., Ltd., Kawachi-Nagano, Osaka 586, Japan

Accepted for publication March 6, 1997

ABSTRACT

(R)-(+)2-Amino-4-(4-fluorophenyl)-5-[1-[4-(4-fluorophenyl)-4-oxobutyl]pyrrolidin-3-yl]thiazole (NRA0045), a novel thiazole derivative, has high affinities for the human cloned dopamine D₄.2, D₄.4 and D₄.7 receptors, with Ki values of 2.54, 0.55 and 0.54 nM, respectively. NRA0045 is approximately 91-fold more potent at the dopamine D₄.2 receptor, compared with human cloned dopamine D₂L receptor. NRA0045 also has high affinities for the serotonin (5-HT)₂A receptor (Ki 1.92 nM) and alpha₁ adrenoceptor (Ki 1.40 nM) but weak affinities (IC₅₀ values are approximately 1 μM) for six other neurotransmitter receptors (adenosine₁, 5-HT₁A, 5-HT₁C, dopamine transporter, α₂A and α₂A) and negligible affinities (IC₅₀ values are over 10⁻⁵ M) for 42 other receptors, including neurotransmitters and hormones, ion channels and second messenger systems. Locomotor hyperactivity induced by methamphetamine (1 mg/kg i.p.) in mice was dose-dependently antagonized by NRA0045, whereas NRA0045 did not exceed 50% inhibition even at the highest dose given (30 mg/kg i.p.). Catalepsy was dose-dependently and significantly induced by NRA0045 in rats, whereas NRA0045 did not exceed 50% induction even at the highest dose given (30 mg/kg i.p.). Thus NRA0045 blocks behaviors associated with activation of the mesolimbic/mesocortical dopaminergic neurons more selectively than behaviors associated with nigrostriatal dopaminergic neurons. In rats, tryptamine-induced clonic seizure, a 5-HT₂ receptor-mediated behavior, was also dose-dependently inhibited by NRA0045 (ED₅₀ 1.7 mg/kg i.p.). Norepinephrine-induced lethality is regarded as being induced through the alpha₁ adrenoceptor. NRA0045 dose-dependently antagonized norepinephrine-induced lethality in rats (ED₅₀ 0.2 mg/kg i.p.). Thus NRA0045 may have a unique antipsychotic activity with regard to dopamine D₄ and 5-HT₂A receptors and alpha₁ adrenoceptor antagonistic activities, without producing the extrapyramidal side effects.

Brain dopamine synapses are considered to be overactive in schizophrenics (Seeman, 1992). This overactivity may stem from either an excess release of dopamine or overactivity of dopamine receptors. Much evidence for the hypothesis of dopamine overactivity in schizophrenics relies on findings that neuroleptics block dopamine D₂ receptors in direct relation to their clinical antipsychotic potencies (Seeman et al., 1975; Seeman, 1992; 1995). Recently, however, molecular biological approaches suggest that the cloned dopamine receptors (D₁–D₅) can be divided into two groups that correspond to the dopamine D₁ and D₂ receptor classification that had previously been identified pharmacologically (Mansour and Watson, 1995). The dopamine D₁ and D₅ receptors have a dopamine D₁-like pharmacology, whereas the dopamine D₂, D₃ and D₄ receptors have a dopamine D₂-like pharmacological profile (Mansour and Watson, 1995). Among these five

ABBREVIATIONS: ANOVA, analysis of variance; MAP, methamphetamine; NRA0045, (R)-(+)2-amino-4-(4-fluorophenyl)-5-[1-[4-(4-fluorophenyl)-4-oxobutyl]pyrrolidin-3-yl]thiazole; NE, norepinephrine; 5-HT, serotonin; PET, positron emission tomography; SPET, simple PET; mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid; RT-PCR, reverse transcription-polymerase chain reaction; NMDA, N-methyl-D-aspartate; PCP, phencyclidine; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine.
cloned dopamine receptor subtypes, the dopamine D₄ receptor had an interesting anatomical distribution and pharmacological profile.

The distribution of dopamine D₄ mRNA was observed at a higher density in the frontal cortex and mesolimbic system than in the primary motor area—specifically, the nigrostriatal pathway (Van Tol et al., 1991; O’Malley et al., 1992). The observed higher dopamine D₄ mRNA density in the cerebral cortex (particularly in the frontal lobe) and in the mesolimbic area has been recognized as an important characteristic of this receptor, because this is a CNS area of direct interest in schizophrenia (Tamminga et al., 1992; Weinberger, 1988).

The dopamine D₄ receptor has a high affinity for the antipsychotic clozapine (Van Tol et al., 1991), which has potent antipsychotic actions and a very low incidence of extrapyramidal motor side effects (Wagstaff and Bryson, 1995). PET and SPECT studies have demonstrated that a good clinical response to clozapine occurs despite low dopamine D₂ occupancy (Brucke et al., 1992; Farde et al., 1992; Pilowsky et al., 1992), which strongly suggests that the action of clozapine is not mediated by dopamine D₂ receptor blockade. Conversely, in up to 30% of schizophrenics, there is a maximal dopamine D₂ receptor blockade (Pilowsky et al., 1993). Clozapine at 100 to 200 nM blocks dopamine D₂ receptors, in contrast with the therapeutic concentration of 10 to 20 nM found in the spinal fluid of clozapine-treated patients (Seeman, 1992). However, cloned dopamine D₄ receptors can be blocked with clozapine concentrations (10–20 nM) that are found in the spinal fluid or water phase of plasma from clozapine-treated patients (Seeman, 1995; Seeman and Van Tol, 1994). In addition, the existence of dopamine D₄-like sites and their elevation in schizophrenia have been reported (Seeman et al., 1993; Murray et al., 1995; Sumiyoshi et al., 1995). Kerwin and Collier (1996) reported that both haloperidol and clozapine mediate antipsychotic efficacy at dopamine D₄ receptors and that the additional selectivity and affinity of haloperidol at dopamine D₄ receptors are responsible for the neurological side effects. Thus clozapine’s clinical efficacy for schizophrenics was hypothesized to be associated with its dopamine D₄ preference, and a dopamine D₄ antagonist has the potential to be an effective antipsychotic agent lacking the extrapyramidal side effects.

Dopamine D₄ ligands such as 5-(4-chlorophenyl)-4-methyl-3-(1-(2-phenylethyl)piperidin-4-yl)isoxazole (Rowley et al., 1996), 3-[(4-(4-chlorophenyl)piperazin-1-yl)-methyl]-1H-pyrrolo[2,3-b]pyridine (Kulagowski et al., 1996), (S)-(−)-4-[2-(isochroman-1-yl)ethyl]-piperazin-1-yl]benzenesulfonamide (TenBrink et al., 1996), J18 (Liegeois et al., 1995), 2-naphthoate esters (Boyfield et al., 1996) and YM-43611 (Hidaka et al., 1996; Ohmori et al., 1996) have been described. The dopamine D₄ receptor antagonistic activities and potencies of these compounds, however, have not been demonstrated in in vitro and in vivo functional studies.

We report here the receptor binding and neuropharmacological activities of a novel dopamine D₄ receptor antagonist, RA0045 (fig. 1).

![Fig. 1. Chemical structure of NRA0045.](image)

**Materials and Methods**

**Animals.** Male ICR mice (20–35 g, Charles River, Atsugi, Japan) were housed 10 per cage. Male Wistar rats (150–260 g, Charles River, Atsugi, Japan) were housed three per cage and used for behavioral experiments and neurochemical studies. Male Hartley guinea pigs (150–200 g, Charles River, Atsugi, Japan) were housed three per cage and used for neurochemical studies. Animals were maintained under a 12-hr light/dark cycle (lights on 7:00 A.M.) in a temperature- and humidity-controlled holding room. Food and water were available ad libitum.

All studies reported here have been reviewed by the Taiho Pharmaceutical Co., Ltd. Animal Care Committee and have met the Japanese Experimental Animal Research Association standards as defined in the Guidelines for Animal Experiments (1987).

**Human dopamine D₄ receptor expression construct.** The human dopamine D₄ cDNA was cloned by RT-PCR. Total RNA was prepared from human neuroblastoma SK-N-MC cells by means of the acid guanidine-phenol/chloroform method described by Chomczynski and Sacchi (1987), and cDNA was synthesized using reverse transcriptase (SuperscriptII, BRL, Gaithersburg, MD, USA). The oligonucleotide primers used in the RT-PCR were 5′-CGGAAATTC-CGGGGCCGCCATGGGGAACCG-3′ (sense) and 5′-AAGTGATC-CTACAAAAAGGCGCTCTCCATCTCCTTG-3′ (antisense). The PCR conditions were 1 min at 98°C, 1 min at 63°C and 4 min at 74°C for 35 cycles. The amplified cDNA, including the entire coding region of human dopamine D₄ (an open reading frame encoding 387 amino acid residues) was then cloned into the expression vector pcDLAPE derived from pcDLpSro296 (Takebe et al., 1988). In this plasmid, the PstI site of pcDLpSro296 was converted to an EcoRI site by ligation of an EcoRI linker to its bluntling termini, and the PstI-EcoRI short segment was deleted.

**Cell culture and transfection.** COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin, in a CO₂ incubator at 37°C. Full-length cDNA clones of human dopamine D₄ ligated into pcDLpAPE were transfected into COS-7 cells, using the Lipofectin (BRL, Gaithersburg, MD) procedure (Felgner et al., 1987). The cells were harvested after 72 hr by centrifugation at 400 × g. The cell pellet was washed with phosphate-buffered saline and stored at −80°C until use.

**Membrane preparation.** The cell pellet was homogenized with 50 mM Tris-HCl buffer containing 5 mM EDTA (pH 7.4) using an ultra-turrax T25 homogenizer (IKA-Labortechnik, Staufen, Germany), and centrifuged in a Hitachi 55P-72 centrifuge at 48,000 × g for 20 min at 4°C. The supernatant was discarded, and the pellet obtained was rehomogenized with 50 mM Tris-HCl buffer containing 5 mM EDTA (pH 7.4) and re-centrifuged at 48,000 × g for 20 min at 4°C. The final pellet was suspended in 50 mM Tris-HCl buffer containing 5 mM EDTA, 1.5 mM CaCl₂, 5 mM KCl and 120 mM NaCl (pH 7.4) at a protein concentration of 0.3 mg/ml and was used as the membrane preparation. Protein concentration was determined by the method of Lowry et al., (1951) using the Folin phenol reagent.

**Receptor binding assays.** The binding assay for dopamine D₄ receptor was performed according to Van Tol et al. (1991). The membranes (0.5 ml) were incubated with [³H]spiperone (0.5 nM) for 120 min at 27°C. RA0045 or dopamine receptor-related compounds were included in the reaction mixture, simultaneously. The reaction was terminated by rapid filtration through Whatman GF/B glass-fiber filters presoaked with 0.3% polyethyleneimine, after which the
filters were washed with 3 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) three times. Nonspecific binding was determined in the presence of 10 μM haloperidol. Specific binding was defined by subtracting the nonspecific binding from the binding in the absence of haloperidol. For these steps, we used a multiple cell harvester M-24R (Brandel Biomedical Research and Development Laboratories, Inc., Gaithersburg, MD). Aquazol-2 scintillator (New England Nuclear, Wilmington, DE) (7 ml) was added, and filter-bound radioactivity was quantified in a liquid scintillation spectrometer (Beckman LS6000TA).

Dopamine D<sub>1</sub> receptor binding assay was performed using [3H]spiperone (0.2 nM) and human cloned dopamine D<sub>1</sub>, in 50 mM Tris-HCl buffer containing 10 mM MgCl<sub>2</sub> and 1 mM EDTA (pH 7.4) for 60 min at 27°C according to Lahti et al. (1993). Nonspecific binding was determined in the presence of 10 μM haloperidol. Selective bindings for several receptors, except for both dopamine D<sub>1</sub> and D<sub>2</sub>, were carried out according to established protocols, and the methods are summarized in table 1.

**Spontaneous locomotor activity in mice.** The animals were housed individually in transparent acrylic cages (47 x 28.5 x 29.5 cm), and spontaneous locomotor activity was recorded every 5 min for 60 min, using a SCANET apparatus (Neuroscience Inc., Tokyo, Japan) placed in a sound-proof box. Animals were given i.p. NRA0045 (0.3–3 mg/kg), clozapine (1–10 mg/kg), haloperidol (0.1–1 mg/kg), chlorpromazine (0.3–3 mg/kg) or an appropriate vehicle (10 ml/kg) and placed in individual cages 30 min later. Six groups of five mice for the vehicle and each of three dosages of drugs were used to generate dose-response reactions. The total count of vehicle-treated control group was defined as 100%, and the percent inhibition of each treatment group was calculated and ED<sub>50</sub> values determined.

MAP-induced locomotor hyperactivity in mice.** The animals were housed individually in transparent acrylic cages (47 x 28.5 x 30 cm) and acclimatized for 90 min with a SCANET apparatus placed in a sound-proof box. Animals were given i.p. and p.o. NRA0045 (0.3–3 mg/kg i.p. and 1–10 mg/kg p.o.), clozapine (0.3–3 mg/kg i.p. and 1–10 mg/kg p.o.), haloperidol (0.1–1 mg/kg i.p. and 0.1–1 mg/kg p.o.), chlorpromazine (0.3–3 mg/kg i.p. and 1–10 mg/kg p.o.) or an appropriate vehicle (10 ml/kg) 15 min before the i.v. administration of MAP (1 mg/kg). Fifteen minutes later, locomotor activity was recorded every 5 min for 30 min using a SCANET apparatus placed in a sound-proof box. Six group of five mice for the vehicle and each of three dosages of drugs, were used to generate dose-response reactions. The total count for the vehicle-treated control group was defined as 100%, and the percent inhibition of each group was calculated and ED<sub>50</sub> values determined.

**MAP-induced stereotyped behavior in mice.** The animals were placed individually in transparent acrylic cages (24 x 17.6 x 12 cm) and allowed a minimum of 60 min to acclimatize to the new environment. The mice were given i.p. NRA0045 (3–30 mg/kg), clozapine (3–30 mg/kg), haloperidol (0.3–3 mg/kg), chlorpromazine (1–10 mg/kg) or an appropriate vehicle (10 ml/kg) 30 min before the i.v. administration of MAP (10 mg/kg). Ten minutes later, stereotyped behavior was scored every 10 min for 80 min, using the following scoring system: 0, normal behavior; 1, exploratory behavior and discontinuous sniffing; 2, continuous sniffing; 3, continuous sniffing, discontinuous licking, biting or gnawing; 4, continuous licking, biting and gnawing (Okuyama et al., 1993). Eight mice for the vehicle and each of three dosages of drugs were used to generate dose-response reactions. The total score for the vehicle-treated control group was defined as 100%, and the percent inhibition of each group was calculated and ED<sub>50</sub> values determined.

**Tryptamine-induced clonic seizure in rats.** The animals were placed individually in clear acrylic cages (36 x 30 x 17 cm) and allowed a minimum of 45 min to acclimatize to the new environment. The rats were given i.p. NRA0045 (0.1–3 mg/kg), clozapine (1–10 mg/kg), haloperidol (0.3–3 mg/kg), chlorpromazine (1–10 mg/kg) or an appropriate vehicle (10 ml/kg) 30 min before the i.v. administration of tryptamine (20 mg/kg). The duration of tryptamine-induced clonic seizure was monitored. Six rats for the vehicle and each of 3 to 4 dosages of drugs were used to generate dose-response reactions. The duration for the vehicle-treated control group was defined as 100%, and the percent inhibition of each group was calculated and ED<sub>50</sub> values determined.

**NE-induced lethality in rats.** The animals were placed individually in a clear acrylic cage (36 x 30 x 17 cm) and allowed a minimum of 60 min to acclimatize to the new environment. The rats were given i.p. NRA0045 (0.03–1 mg/kg), clozapine (0.3–10 mg/kg), haloperidol (0.3–10 mg/kg), chlorpromazine (0.1–3 mg/kg) or an appropriate vehicle (10 ml/kg). Thirty minutes later, NE (1.25 mg/kg) was administered i.v. Inhibition of NE-induced lethality was judged to be positive unless death had occurred 30 min after NE administration. Ten rats for the vehicle and each of four dosages of drugs were used to generate dose-response reactions. The vehicle-treated control group was defined as 100%, and the percent inhibition of each group was calculated and ED<sub>50</sub> values determined.

**Statistical analysis.** For determination of the equilibrium dissociation constant (K<sub>d</sub>), saturation binding data were analyzed by Scatchard plot analysis, and the K<sub>d</sub> values were calculated using a computer program, sp123, developed by Dr. H. Ono of the University of Tokyo for PC-9801 (NEC, Tokyo, Japan) personal computer.

In the competition binding assay, the concentration of test compound that caused 50% inhibition of specific radiolabeled ligand binding (IC<sub>50</sub> values) was determined from each concentration-response curve. After determination of IC<sub>50</sub> values using the Marquardt-Levenberg nonlinear least-squares curve-fitting procedure of the MicroCal ORIGIN program (MicroCal, Northampton, MA) running on a Microsoft Windows 3.1, and K<sub>d</sub> values for each test com-

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Membrane</th>
<th>Radiolabeled Ligand</th>
<th>Reaction Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine D&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Human cloned D&lt;sub&gt;1&lt;/sub&gt;</td>
<td>[3H]TSCH23390 (2 nM)</td>
<td>27°C, 60 min</td>
<td>Jarvie et al. (1993)</td>
</tr>
<tr>
<td>Dopamine D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Human cloned D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>[3H]YM-09151-2 (0.1 nM)</td>
<td>37°C, 15 min</td>
<td>Sokoloff et al. (1990)</td>
</tr>
<tr>
<td>Dopamine D&lt;sub&gt;5&lt;/sub&gt;</td>
<td>Human cloned D&lt;sub&gt;5&lt;/sub&gt;</td>
<td>[3H]TSCH23391 (1 nM)</td>
<td>25°C, 30 min</td>
<td>Jarvie et al. (1993)</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;1A&lt;/sub&gt;</td>
<td>Rat cerebral cortex</td>
<td>[3H]R16203 (0.2 nM)</td>
<td>25°C, 60 min</td>
<td>Doble et al. (1992)</td>
</tr>
<tr>
<td>α&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Rat whole brain</td>
<td>[3H]Prazosin (0.2 nM)</td>
<td>25°C, 60 min</td>
<td>Timmermans et al. (1981)</td>
</tr>
<tr>
<td>α&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Guinea pig whole brain</td>
<td>[3H]N(+)Pentazocine (2 nM)</td>
<td>25°C, 120 min</td>
<td>Chaki et al. (1994)</td>
</tr>
</tbody>
</table>
bound were calculated according to the equation of Cheng and Prusoff (1973), using \( K_d \) values obtained by Scatchard analysis.

Data regarding spontaneous locomotion, MAP-induced hyperactivity and tryptamine-induced clonic seizure were analyzed by ANOVA, and significant differences between groups were determined using Dunnett’s test. Data from MAP-stereotyped behavior and induction of catalepsy were analyzed by Kruskal-Wallis test, with significant differences between groups determined using non-parametric Dunnett’s test. Data from NE-induced lethality was analyzed by one-way cumulative chi-square (\( \chi^2 \)) test, with significant differences between groups determined using Fisher exact test. The ED50 values were calculated by Allfit analysis using percent inhibition.

**Drugs.** The following drugs and chemicals were used in this study: human cloned dopamine receptors such as dopamine D1, D2L, D2, D4, D4, and D6 (Research Biochemicals International, Natick, MA), human neuroblastoma SK-N-MC cells and COS-7 cells (American Type Culture Collection, Rockville, MD), [\(^{3}H\)]pireperone (spec. act. 3290 GBq/mmol) and [\(^{3}H\)]RIP62203 (spec. act. 2480 GBq/mmol) (Amer sham International PLC. Buckinghamshire, England), [\(^{3}H\)]YOM-91512-1 (spec. act. 3182.0 GBq/mmol), [\(^{3}H\)]SCH23390 (spec. act. 2638.1, 3011.8 GBq/mmol), [\(^{3}H\)]pirazin (spec. act. 2752.8 GBq/ mmol) and [\(^{3}H\)](+)-pentazocine (spec. act. 1417.1 GBq/mmol) (New England Nuclear, Wilmington, DE). Apomorphine HCl (Sigma Chemical Co., St. Louis, MO), methamphetamine HCl (Dainippon Pharmaceutical, Osaka, Japan), tryptamine HCl (Research Biochemicals International, Osaka, Japan), haloperidol (Serenase injections, Dainippon Pharmaceutical, Osaka, Japan) were dissolved in 0.9% saline with the addition of 0.1% ascorbic acid for apomorphine. Clozapine (Gee Lawson Chemicals, London, UK) was dissolved in a minimal amount of 0.5 N HCl and saline and then titrated with 0.5 N NaOH to a final pH of 7.5.

NRA0045 was synthesized in the laboratories of Taisho Pharmaceutical, Osaka, Japan, and was suspended in 5% arabic gum. Cell culture reagents were obtained from GIBCO (Grand Island, NY). All other chemicals were obtained commercially and were of analytical grade.

**Results**

**Receptor binding studies.** NRA0045 bound potently to dopamine D4.2 with a \( K_i \) value of 2.54 nM. Among the dopamine D2 receptor family, NRA0045 showed high selectivity for the dopamine D4 receptor subtype, whereas clozapine, an atypical antipsychotic, showed moderate selectivity for the dopamine D4 receptor (table 2, fig. 2). NRA0045 bound to human cloned dopamine D4.2 with an affinity 91 and 42 times higher than to human cloned dopamine D2L and to human cloned dopamine D3, respectively. Human dopamine D4 has differences between groups determined using Fisher exact test. The ED50 values were calculated by Allfit analysis using percent inhibition.

**Effect on spontaneous locomotor activity in mice.** A reduction of spontaneous locomotor activity was recorded after i.p. administration of either NRA0045 [\( \chi^2(3,20) = 6.64, P < 0.01 \), ED50 = 0.9 mg/kg], clozapine [\( \chi^2(3,20) = 42.15, P < 0.01 \), ED50 = 3.0 mg/kg], haloperidol [\( \chi^2(3,20) = 15.45, P < 0.01 \), ED50 = 0.3 mg/kg] or chlorpromazine [\( \chi^2(3,20) = 28.27, P < 0.01 \), ED50 = 1.5 mg/kg] in mice (table 5).

**Effect on MAP-induced hyperactivity in mice.** MAP-induced hyperactivity was dose-dependently and significantly attenuated after i.p. \( \chi^2(3,20) = 22.84, P < 0.01 \) or p.o. \( \chi^2(3,20) = 32.50, P < 0.01 \) administration of NRA0045 (ED50 = 0.5 mg/kg i.p. and 1.9 mg/kg p.o., respectively) (fig. 3; table 5). A dose-dependent blockade of MAP-induced hyperactivity was also observed in mice pretreated with clozapine \( \chi^2(3,20) = 19.98, P < 0.01 \), ED50 = 1.0 mg/kg i.p. and 1.9 mg/kg p.o., respectively) with haloperidol [\( \chi^2(3,20) = 24.16, P < 0.01 \), ED50 = 0.2 mg/kg i.p. and F(3,20) = 9.82, P < 0.01, ED50 = 0.5 mg/kg p.o., respectively] and with chlorpromazine [\( \chi^2(3,20) = 37.88, P < 0.01 \), ED50 = 0.6 mg/kg i.p. and F(3,20) = 15.37, P < 0.01, ED50 = 2.2 mg/kg p.o., respectively] (fig. 3; table 5). With i.p. administration of all five drugs, the potency of inhibition of MAP-induced hyperactivity was much stronger than that of the spontaneous locomotor activity (table 5).

**Effects on MAP-induced stereotyped behavior in mice.** MAP-induced stereotyped behavior was dose-dependently and significantly attenuated after i.p. administration of either haloperidol [\( H(3) = 25.92, P < 0.01 \), ED50 = 0.1 mg/kg] or chlorpromazine [\( H(3) = 27.94, P < 0.01 \), ED50 = 3.5 mg/kg] (fig. 3; table 5). In contrast, i.p. administration of NRA0045 [\( H(3) = 17.91, P < 0.01 \) or clozapine [\( H(3) = 12.86, P < 0.01 \)] dose-dependently and significantly inhibited MAP-induced stereotyped behavior, whereas these compounds did not exceed 50% inhibition even at the highest dose given (30 mg/kg).

**Effect on induction of catalepsy in rats.** Induction of catalepsy was dose-dependently and significantly attenuated after i.p. administration of haloperidol [\( H(3) = 18.31, P < 0.01 \),

<table>
<thead>
<tr>
<th>Compound</th>
<th>hD2L</th>
<th>hD3</th>
<th>hD4.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRA0045</td>
<td>231.90 ± 51.58</td>
<td>107.49 ± 2.07</td>
<td>2.54 ± 0.78</td>
</tr>
<tr>
<td>Clozapine</td>
<td>546.60 ± 216.6</td>
<td>84.07 ± 21.21</td>
<td>72.06 ± 9.30</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>9.90 ± 0.87</td>
<td>1.50 ± 0.23</td>
<td>8.63 ± 0.99</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>13.61 ± 4.12</td>
<td>1.56 ± 0.57</td>
<td>66.80 ± 10.29</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. of three to nine separate experiments, each done in duplicate.

**TABLE 2**

**Affinities of NRA0045 and dopamine receptor antagonists for the human cloned dopamine D4 family**

\( K_i \) (nM)
Discussion

The present study is apparently the first to show in vitro and in vivo pharmacological properties of a potent dopamine D₄ receptor antagonist, NRA0045, in laboratory animals.

NRA0045 showed a high affinity for dopamine D₄, 5-HT₂A receptors and alpha-1 adrenoceptor with weak or negligible affinities for 48 other receptors, including neurotransmitters and hormones, ion channels and second messenger systems. Among the dopamine D₄ receptor family, NRA0045 was more potent at dopamine D₄ than at dopamine D₂ and D₃ receptors, which indicates that this compound is relatively selective for the dopamine D₄ receptor subtype. By contrast, clozapine, which is selective for the dopamine D₄ receptor over the D₂ receptor, had practically the same affinity for dopamine D₃ receptor as for dopamine D₄ receptor in binding studies, and both haloperidol and chlorpromazine, which cause extrapyramidal symptoms, showed low selectivity for dopamine D₄ receptor over dopamine D₂ and D₃ receptors. Thus NRA0045 is most selective and potent for the dopamine D₄ receptor, among the dopamine D₂ receptor family.

In vivo studies indicate that NRA0045 and clozapine exhibit a behavioral profile distinct from that of haloperidol and chlorpromazine in rodent models commonly used to predict antipsychotic potential and side effects in humans. NRA0045, clozapine, haloperidol and chlorpromazine produced dose-related decreases in MAP-induced hyperactivity in mice. These data support the idea that antagonism of MAP-induced locomotor activity in rodents is predictive of therapeutic efficacy in schizophrenic patients (Evenden and Ryan, 1990). NRA0045 showed low potency on stereotyped behaviors induced by MAP in mice and a low incidence of catalepsy in rats. In contrast, haloperidol and chlorpromazine blocked MAP-induced stereotyped behavior in mice, and they induced catalepsy in rats. Dopamine agonist-induced locomotor hyperactivity (an animal model of antipsychotic activity) and stereotyped behavior (an animal model of extrapyramidal symptoms) are mediated through stimulation of the mesolimbic/mesocortical and nigrostriatal dopaminergic systems, respectively (Evenden and Ryan, 1990; Hoffman and Donovan, 1995). Thus NRA0045 selectively blocked behaviors associated with activation of the mesolimbic/mesocortical dopaminergic system and showed some similarities to the atypical antipsychotic clozapine, which also failed to block MAP-induced stereotyped behavior at doses at least 30-fold over those that blocked MAP-induced hyperactivity. Clozapine seems to be the only antipsychotic that produced a very low incidence of extrapyramidal side effects and virtually no tardive dyskinesia (Casey, 1989).

In situ hybridization studies have shown that mRNA for dopamine D₂ receptor is heavily localized in the striatum, nucleus accumbens and olfactory tubercles and has lower levels in various cortical areas (Bouthenet et al., 1991; Giros et al., 1991). In contrast, the dopamine D₄ receptor is most highly concentrated in the dopamine cortical and limbic areas, and lesser amounts occur in the striatum (Van Tol et al., 1991). It seems likely that haloperidol and chlorpromazine blocked both MAP-induced hyperactivity and MAP-in-
duced stereotyped behavior, with a resulting blockade of the dopamine D2 receptors in the nucleus accumbens and striatum, respectively. In contrast, the selective dopamine D4 receptor blocking action in the cortical and limbic areas may be attributed to the behavioral effects of NRA0045 and clozapine.

NRA0045 has a high affinity for the 5-HT2A receptor and dose-dependently antagonized tryptamine-induced chronic seizures (Janssen et al., 1988). There is some evidence for the relationship between the 5-HT2A receptor and schizophrenia. First, typical antipsychotics, such as haloperidol, bind to the 5-HT2A receptor with a high affinity. Most significantly, clozapine and risperidone, atypical antipsychotics with dopamine D2 and/or D4 and 5-HT2A receptor antagonistic properties, were found to alleviate psychosis in previously refractory schizophrenics—and to do so with a greatly reduced incidence of extrapyramidal side effects (Megens et al., 1994; Wagstaff and Bryson, 1995). 5-HT2 receptor antagonists such

| Affinities of NRA0045 and dopamine receptor antagonists for neurotransmitter receptors |
|---------------------------------|--------|--------|-----------|--------|--------|
| Compound                        | hD1    | hD2    | 5HT2A    | a1     | a1     |
|                                 | (nM)   | (nM)   | (nM)     | (nM)   | (nM)   |
| NRA0045                         | 21.68 ± 1.35 | 2217.0 ± 498.1 | 1.92 ± 0.29 | 1.40 ± 0.31 | 106.10 ± 5.43 |
| Clozapine                       | 22.92 ± 3.62 | 376.30 ± 124.90 | 13.45 ± 3.74 | 3.72 ± 0.48 | >10000 |
| Haloperidol                     | 4.75 ± 0.40 | 147.80 ± 28.71 | 46.37 ± 6.63 | 5.2 ± 0.42 | 1.40 ± 0.20 |
| Chlorpromazine                  | 8.01 ± 1.03 | 118.50 ± 27.11 | 13.00 ± 4.62 | 1.35 ± 0.53 | 282.40 ± 34.04 |

Values are mean ± S.E.M. of three to five separate experiments, each done in duplicate.

| Behavioral profiles of NRA0045: comparison with clozapine, haloperidol and chlorpromazine |
|---------------------------------|--------|--------|--------|--------|
| Route                           | ED50 (mg/kg) |
| N/A045                          | Clozapine | Haloperidol | Chlorpromazine |
| Spontaneous locomotor activity (mouse) i.p. | 0.9 | 3.0 | 0.3 | 1.5 |
| MAP-induced locomotor hyperactivity (mouse) i.p. | 0.5 | 1.0 | 0.2 | 0.6 |
| MAP-induced locomotor hyperactivity (mouse) p.o. | 1.9 | 2.7 | 0.5 | 2.2 |
| MAP-induced stereotyped behavior (mouse) i.p. | >30 | >30 | 0.1 | 3.5 |
| Induction of catalepsy (rat) i.p. | >30 | >30 | 0.2 | 1.4 |
| Tryptamine-induced seizure (rat) i.p. | 1.5 | 2.4 | 1.1 | 2.1 |
| NE-induced lethality (rat) i.p. | 0.2 | 2.6 | 3.2 | 0.6 |

Fig. 3. Effects of NRA0045 (A), clozapine (B), haloperidol (C) and chlorpromazine (D) on MAP-induced hyperactivity (●) and MAP-induced stereotyped behavior (○) in mice. In MAP-induced hyperactivity, the total count for the vehicle-treated control group was defined as 100%, and the percent inhibition of each group was calculated. A dose-dependent blockade of MAP-induced hyperactivity was observed in mice pretreated with i.p. administration of NRA0045 [F(3,20) = 22.8, P < .01], clozapine [F(3,20) = 19.98, P < .01], haloperidol [F(3,20) = 24.16, P < .01] and chlorpromazine [F(3,20) = 37.88, P < .05] and ***P < .05 vs. vehicle-treated group (Dunnett’s test). In MAP-induced stereotyped behavior, the total score for the vehicle-treated group was defined as 100%, and the percent inhibition of each group was calculated. A dose-dependent blockade of MAP-induced stereotyped behavior was observed in mice pretreated with i.p. administration of NRA0045 [F(3) = 17.91, P < .01], clozapine [F(3) = 12.80, P < .01], haloperidol [F(3) = 25.92, P < .01] and chlorpromazine [F(3) = 27.94, P < .01]; #P < .05 and **P < .01 vs. vehicle-treated group (nonparametric Dunnett’s test). However, inhibition percents of the highest doses of NRA0045 (30 mg/kg i.p.) and clozapine (30 mg/kg i.p.) were 33.6 and 44.2, respectively. Thus the ED50 values of NRA0045 and clozapine were not calculated.
as ritanserin have been shown to produce improvements of the negative symptoms of schizophrenia (Leysen et al., 1985; Reyntjens et al., 1986) as well as reduction of the extrapyramidal symptoms resulting from chronic treatment with haloperidol (Bersani et al., 1986). Thus NRA0045 with combined dopamine D4 and 5-HT2A receptor antagonistic activities may be useful as a novel antipsychotic drug for the treatment of both positive and negative symptoms of schizophrenia and may reduce the extrapyramidal symptoms resulting from chronic treatment with dopamine D2 receptor antagonists.

NE-induced lethality is regarded as being mediated by alpha-1 adrenoceptor (Peroutka et al., 1977). NRA0045 has a high affinity for the alpha-1 adrenoceptor, and NE-induced lethality was antagonized by NRA0045. Risperidone, a dopamine D2 and 5-HT2 receptor antagonist, also had a high affinity for alpha-1 adrenoceptor (Megens et al., 1994) and antagonized NE-induced lethality (Janssen et al., 1988). The alpha-1 adrenoceptor antagonist of drugs is thought to be related to clinical sedative and hypotensive side effects. The alpha-1 adrenoceptor antagonism of NRA0045 may contribute to the inhibition of spontaneous locomotor activity. In preliminary studies, the potency of hypotensive effects of NRA0045 (30 mg/kg p.o.) was roughly equivalent to that of chlorpromazine (30 mg/kg p.o.) in freely moving spontaneously hypertensive rats (data not shown).

With the exception of the affinity for dopamine (D1, D2 and D4) and 5-HT receptors, clozapine also binds the alpha-1 adrenoceptor, muscarinic ACh, histamine receptors that could generate the well-known side effects of orthostatic hypotension, tremor, seizures and hypersalivation (Wagstaff and Bryson, 1995). NRA0045 has a high affinity for dopamine D4, 5-HT2A and alpha-1 adrenoceptor but has weak or negligible affinities for 48 other receptors in vitro. Thus NRA0045 is more dopamine D4-selective than clozapine.

In conclusion, NRA0045 is a potent D4 and 5-HT2 receptor antagonist with anti-NE activity. It blocks hyperactivity but not stereotyped behavior, as induced by MAP. Given that MAP-induced hyperactivity reflects activation of the mesolimbic/mesocortical dopamine system and that an overactivity in mesolimbic/mesocortical function has been implicated in the pathophysiology of schizophrenia (Tammenga et al., 1992), these results suggest that dopamine D4 and 5-HT2A receptor antagonists may have antipsychotic potential. Whether these data on rodents can be extrapolated to humans remains to be determined.
PILOWSKY, L. S., COSTA, D. C., ELL, P. J., MURRAY, R. M., VERHOEFF, N. P. L. G.
AND KERWIN, R. W.: Clozapine, single photon emission tomography and the
D₂ dopamine receptor blockade hypothesis of schizophrenia. Lancet 340:
PILOWSKY, L. S., COSTA, D. C., ELL, P. J., MURRAY, R. M., VERHOEFF, N. P. L. G.
AND KERWIN, R. W.: Antipsychotic medication D₂ dopamine receptor blockade
and clinical response: A ¹²³I-IBZM SPET (single photon emission tomogra-
REYNTJENS, A., GEELDERS, Y. G., HOPPENBROUWERS, N. -L. J. A. AND VAN DEN
BUSSCHE, G.: Thymostenic effects of ritanserin (R 55 667), a centrally acting
ROWLEY, M., BROCCOLI, H. B., COLLINS, J., BAKER, R., EMMS, F., MARWOOD, R.,
PATEL, S., PATIEL, S., IAN RAGAN, C., FREEDMAN, S. B. AND LEESON, P. D. :
5-(4-Chlorophenyl)-4-methyl-3-(1-(2-phenylethyl)piperidin-4-yl)isoxazole,
SEEMAN, P.: Dopamine receptor sequences. Therapeutic levels of neuroleptics
occupy D₂ receptors, clozapine occupies D₄. Neuropsychopharmacology
SEEMAN, P.: Dopamine receptors: Clinical correlates. In Psychopharmacology:
The Fourth Generation of Progress, ed. by F. E. Bloom and D. V. Kupfer, pp.
SEEMAN, P., CHAU-WONG, M., TEDESCO, J. AND WONG, K.: Brain receptors for
SEEMAN, P., GUAN, H.-C. AND VAN TOL, H. H. M.: Dopamine D₄ receptors
SEEMAN, P. AND VAN TOL, H. H. M.: Dopamine receptor pharmacology. Trends
SOLOLOFF, P., GHOS, B., MARTHS, M.-P., BOUTINNET, M.-L. AND SCHWARTZ,
J.-C.: Molecular cloning and characterization of a novel dopamine receptor
SUMIYOSHI, T., STOCKMEIER, C. A., OVERHOLSER, J. C., THOMPSON, P. A.
AND MELTZER, H. Y.: Dopamine D₄ receptors and effects of guanidine nucleotides
on ['H]raclopride binding in postmortem caudate nucleus of subjects with
TAKKE, Y., SEIKI, M., FUJISAWA, J., HOY, P., YOKOTA, K., ARAI, K., YOSHIDA, M.
AND ARAI, N.: SR₃ promoter: An efficient and versatile mammalian cDNA
expression system composed of the simian virus 40 early promoter and the
R-U5 segment of human T-cell leukemia virus Type 1 long terminal repeat.
TAMMINGA, C. A., TRAVER, G. K., BUCHANAN, R., KIRKPATRICK, B., ALPES, D. L.,
CHASE, T. N. AND CARPENTER, W. T.: Limbic system abnormalities identified in
schizophrenia using positron emission tomography with fluorodeoxyglucose
and neocortical alteration with deficit syndrome. Arch. Gen. Psychiatry 49:
TIMMERMANS, P. B. M. W. H., ALI, F. K., KWAK, H. Y., SCHOOP, A. M. C., SLO-
THORST-GREDLICK, F. P. AND VAN ZWIETEN, P. A.: Identical antagonist selec-
tively of central and peripheral alph₃-adrenoceptors. Mol. Pharmacol. 20:
295–301, 1981.
VAN TOL, H. H. M., BUNZLOW, J. R., GUAN, H.-C., SUNAHARA, R. K., SEEMAN, P.,
NIZNIK, H. B. AND CIEVILLI, O.: Cloning of the gene for a human dopamine D₄
receptor with high affinity for antipsychotic clozapine. Nature (Lond.) 356:
properties and therapeutic use in patients with schizophrenia who are
unresponsive to or intolerant of classical antipsychotic agents. CNS Drugs 4:
WEINBERGER, D. R.: Schizophrenia and the frontal lobe. Trends Neurosci. 11:
Send reprint requests to: Shigeru Okuyama, Ph.D., 1st Laboratory, Medic-
inal Research Laboratories, Taisho Pharmaceutical Co., Ltd., 1-403, Yoshino-
cho, Ohmiya, Saitama 330, Japan.