Full Paper

Pharmacological Characterization of FR194921, a New Potent, Selective, and Orally Active Antagonist for Central Adenosine A₁ Receptors

Takuya Maemoto¹, Miho Tada¹, Takuma Mihara¹, Noriko Ueyama¹, Hideaki Matsuoka¹, Katsuya Harada¹, Takayuki Yamaji¹, Kiyoharu Shirakawa¹, Satoru Kuroda², Atsushi Akahane², Akinori Iwashita¹, Nobuya Matsuoka^{1,*}, and Seitaro Mutoh¹

¹Medicinal Biology Research Laboratories, ²Medicinal Chemistry Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-1-6 Kashima, Yodogawa-ku, Osaka 532-8514, Japan

Received June 3, 2004; Accepted July 6, 2004

Abstract. Adenosine A₁ receptors in the brain are believed to play an important role in brain functioning. We have discovered a novel adenosine A₁ receptor antagonist, FR194921 (2-(1-methyl-4-piperidinyl)-6-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl)-3(2*H*)-pyridazinone), and characterized the pharmacological activity in the present study. FR194921 showed potent and selective affinity for the adenosine A₁ receptor without affinity for A_{2A} and A₃ receptors and did not show any species differences in binding affinity profile among human, rat, and mouse. Pharmacokinetic study in rats revealed that FR194921 was orally active and highly brain penetrable. Oral administration of FR194921 dose-dependently ameliorated the hypolocomotion induced by the A₁ receptor agonist *N*⁶-cyclopentyladenosine in rats, indicating this compound exerts A₁-antagonistic action in vivo. In the passive avoidance test, scopolamine (1 mg/kg)-induced memory deficits were significantly ameliorated by FR194921 (0.32, 1 mg/kg). In two animal models of anxiety, the social interaction test and elevated plus maze, FR194921 showed specific anxiolytic activity without significantly influencing general behavior. In contrast, FR194921 did not show antidepressant activity even at a dose of 32 mg/kg in the rat forced swimming test. These results indicate that the novel, potent, and selective adenosine A₁ receptor antagonist FR194921 exerts both cognitive-enhancing and anxiolytic activity, suggesting the therapeutic potential of this compound for dementia and anxiety disorders.

Keywords: adenosine A₁ receptor, hypolocomotion, memory deficit, anxiety, depression

Introduction

Adenosine which is metabolized from ATP is an important neuromodulator in the central nervous system and acts on seven-transmembrane spanning G-protein coupled receptors classified as A₁, A_{2A}, A_{2B}, and A₃ subtypes (1–3). The adenosine A₁ receptor couples with G_i protein, decreases cAMP by inhibiting adenylate cyclase, and shows structural homology among diverse mammalian species (4). Adenosine A₁ receptors are distributed in brain areas which are important for integrating brain functions such as the hippocampus, cerebral cortex, some thalamic nuclei, basal ganglia,

and cerebellar cortex in experimental animals (5, 6). A recent positron emission tomography (PET) study also showed that A₁ receptors are widely expressed in human brain (7). Adenosine A₁ receptors are believed to be localized in the active zone of presynaptic nerve terminals (8, 9).

A₁ receptors are known to activate several types of K⁺-channels (10) and inactivate N-, P-, and Q-type Ca²⁺ channels (11, 12). A₁ receptors located at presynaptic terminals play vital roles in controlling the release of neurotransmitters (13). Since activation of A₁ receptors inhibits glutamatergic transmission (14), cholinergic transmission (15), and dopamine D₁-dependent GABA release (16), and A₁ receptor blockade increases the release of acetylcholine and glutamate (15, 17) in higher brain areas which play important roles in integra-

*Corresponding author. FAX: +81-6-6304-5385
E-mail: nobuya_matsuoka@po.fujisawa.co.jp

tion of cognitive and emotional functions, A₁ receptor antagonists are expected to have positive effects on memory and psychological conditions. Memory deficits associated with brain cholinergic hypofunction in the rat passive avoidance paradigm can be restored by an A₁-selective antagonist (18), and CPT (8-cyclopentyl-1,3-dimethylxanthine), an A₁ receptor antagonist, can ameliorate the anxiogenic effects produced by ethanol withdrawal in the elevated plus-maze and light/dark test, two relevant animal models of anxiety (19). Thus, although the role of A₁ receptors in brain functioning is not fully understood, adenosine A₁ receptor-selective antagonists have potential for the treatment of neurological and psychiatric disorders.

While diverse classes of adenosine A₁ receptor antagonists have been documented since the discovery of the receptor and specific radiolabelled ligands, these compounds were derived from xanthine analogues and had poor oral bioavailability, CNS penetration, and considerable species difference in terms of receptor binding affinity (20). We have previously shown that novel pyrazolopyridine based compounds such as FK453, FK352, and FK838 are highly selective and potent A₁ receptor antagonists in radioligand binding assay (20). These compounds had similar affinity in different species (e.g., mice, rats, guinea pigs, and humans), whereas xanthine and adenosine derivative compounds have around tenfold lower affinities for human receptors than to mouse receptors (20). These compounds were also brain penetrant and inhibited A₁ receptor agonist-induced locomotor depression in vivo (21), whereas they had less potencies in vivo compared to that in vitro due to insufficient CNS permeability.

We have screened a series of pyrazolopyridine derivatives to optimize the A₁ receptor affinity and pharmacokinetic profile and recently identified a novel A₁-selective pyrazolopyridine compound, FR194921 (2-(1-methyl-4-piperidinyl)-6-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl)-3(2*H*)-pyridazinone; chemical structure shown in Fig. 1) (22). We first determined the binding

affinities of FR194921 for adenosine receptor subtypes and the antagonistic activity of the compound for adenosine A₁ receptors in vivo. Then, to investigate the involvement of adenosine A₁ receptor on memory, anxiety, and depression, FR194921 was evaluated in a variety of behavioral models: the passive avoidance test, social interaction test, elevated plus maze test, and forced swimming test in rats.

Materials and Methods

Animals

Male ddY mice (7-week-old; Japan SLC, Hamamatsu), male SD rats (7- or 8-week-old, Japan SLC), and male Wistar rats (6- to 8-week-old, Charles River, Inc., Yokohama or Japan SLC) were housed five per cage and given access to food and water ad libitum. The animals were housed for at least one week before use in breeding rooms (lighted on from 7:00 to 19:00) in which the room temperature was maintained at $22 \pm 2^\circ\text{C}$. All experiments in the present study were performed in accordance with the guidelines of the Experimental Laboratory Animal Committee of Fujisawa Pharmaceutical Co., Ltd. All efforts were made to minimize both the number of animals used and stress to the animals during experimental procedures.

Drugs

FR194921 and SCH58261(5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine) were synthesized at Fujisawa Pharmaceutical Co., Ltd. (Osaka). Scopolamine hydrobromide and dithiothreitol were purchased from Nacalai Tesque (Kyoto). Imipramine HCl was purchased from Sigma-Aldrich (St. Louis, MO, USA). α -MEM was purchased from Nikken (Tokyo) and fetal bovine serum was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). [³H]-8-Cyclopentyl-1,3-dipropylxanthine ([³H]-DPCPX) and [³H]-2-[4-(2-carboxyethyl)phenethylamino]-5'-*N*-ethyl-carboxamidoadenosine ([³H]-CGS21680) were purchased from NEN Life Science Product Inc (Boston, MA, USA), and [³H]-5'-*N*-ethyl-carboxamidoadenosine ([³H]-NECA) was purchased from Amersham Pharmacia (London, UK). Unless otherwise stated, all other materials were purchased from Sigma-Aldrich.

Radioligand binding assay for adenosine receptors

CHO cell lines permanently expressing human adenosine A₁, A_{2A}, and A₃ and brain synaptosome fractions from mice and rats brain were used in this study. The CHO cell-lines were made according to the method described by Chen et al. (23). These cells were cultured in α -MEM medium supplemented with 10% fetal

Fig. 1. Chemical structure of FR194921, 2-(1-methyl-4-piperidinyl)-6-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl)-3(2*H*)-pyridazinone.

bovine serum, 50 IU/mL penicillin, and 50 μ g/mL streptomycin in 5% CO₂ / 95% air atmosphere and were harvested and homogenised in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl₂, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged (36,000 $\times$ g for A₁ and A_{2A}, 30,000 $\times$ g for A₃, at 4°C for 20 min) and the pellet was collected and centrifuged again. Then the pellet was suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl₂ (assay buffer). This washing process was repeated twice and the final pellet was stored at -80°C until use. Brain synaptosome membranes were prepared from mice and rats. Male ddY mice and male SD rats were sacrificed and the cerebral cortex and basal ganglia, used for the A₁ and A_{2A} binding assay, respectively, were removed quickly and homogenized in approximately 15 volumes of 0.36 M sucrose solution. The homogenates were centrifuged (1,000 $\times$ g, 4°C, 10 min) and then the supernatants were centrifuged (17,000 $\times$ g, 4°C, 20 min). The pellets were suspended in the assay buffer and centrifuged (30,000 $\times$ g, 4°C, 10 min). This washing process was repeated twice and final pellets were stored at -80°C until use. Radioligand binding was assessed by use of a modification of the previous report (20). Radioligand binding experiments to adenosine A₁, A_{2A}, and A₃ receptors were carried out by using [³H]-DPCPX, [³H]-CGS21680, and [³H]-NECA, respectively. Membranes were incubated with 4 nM [³H]-DPCPX, 15 nM [³H]-CGS21680, or 32 nM [³H]-NECA in the absence and presence of various concentrations of FR194921 in 250 μ L of assay buffer containing 1 U/mL adenosine deaminase. DPCPX, SCH58261, and 2-chloro-N⁶-(3-iodobenzyl)-9-[5-(methylcarbamol)- β -D-ribofuranosyl]adenine (CI-IB-MECA) were also investigated for their displacement to A₁, A_{2A}, and A₃ receptors as references, respectively. The mixture was incubated for 60 min at room temperature, harvested on Whatman GF/C filters pre-soaked in 0.1% polyethyleneimine by a cell harvester, and washed eight times with 50 mM Tris-HCl buffer (pH 7.4). The radioactivity on the filter was measured by a scintillation counter. All experiments were carried out three times in duplicate using 96-well plates. The non-specific binding was defined as the binding activity in the presence of 10 μ M of the corresponding unlabeled ligand and was subtracted from the total binding activity for determination of the specific binding. K_d and B_{max} values were obtained from Scatchard analysis. K_i values in inhibition studies were calculated with the GraphPad Prism ver. 3.0 program (GraphPad Software, Inc., San Diego, CA, USA).

Pharmacokinetic study

For measurement of the concentration in plasma, FR194921 was dissolved in saline with equimolar HCl and administered orally at 3.2, 10, or 32 mg/kg in a volume of 2 mL/kg in feeding SD rats. In another set of experiments in which brain concentrations were measured, FR194921 was administered orally at 3.2 or 32 mg/kg in fasted rats. After 0.25, 0.5, 1, 2, 4, or 6 h, rats were anesthetized with ether to allow blood sampling from the abdominal aorta. Where indicated, rats were then perfused with 0.9% saline via an aortic catheter before the cerebrum was removed. The heparinized plasma and cerebrum were stored at -20°C until measurement of the concentration by HPLC. The heparinized plasma (0.1 mL) or 20% cerebrum homogenate (0.5 mL) was mixed with methanol (50 μ L) and then to the mixture was added 0.1 N NaOH (0.5 mL) and CHCl₃ (4 mL). The resulting mixture was shaken for 10 min and centrifuged at 1700 $\times$ g for 5 min. The organic layer (3 mL) was separated and evaporated in a stream of nitrogen. The residue was dissolved in 150 μ L of the HPLC mobile phase and analyzed by HPLC.

A₁ receptor agonist-induced hypolocomotion in rats

The selective adenosine A₁ receptor agonist N⁶-cyclopentyladenosine (CPA) and FR194921 were dissolved in saline with equimolar HCl. SD rats were divided into five groups (n = 7 or 8 per group): saline + saline (normal), saline + CPA (control), FR194921 (0.032 mg/kg) + CPA, FR194921 (0.1 mg/kg) + CPA, and FR194921 (0.32 mg/kg) + CPA. FR194921 or saline was administered orally 25 min prior to intraperitoneal administration of CPA (0.056 mg/kg) or saline. Each drug was given in a volume of 5 mL/kg. Rats were placed individually into plastic cages (L360 $\times$ W305 $\times$ H180 mm) of an Animex auto device (MK-110 type; Muromachi Kikai Co., Ltd., Tokyo) immediately after CPA administration. Locomotor activity was recorded for 20 min starting from 5 min after habituation.

Passive avoidance test

Wistar rats were handled for 3 days prior to the start of the behavioral experiments. The apparatus and experimental procedure used in the passive avoidance test were similar to those described previously (24). In brief, a two-compartment step-through passive avoidance apparatus made of black perspex was used. The apparatus consisted of illuminated and dark compartments attached to a grid floor and were separated by a guillotine door. The rat was placed in the illuminated compartment and the door was raised. After entering the dark compartment, the rat was returned to its home cage (habituation trial). Rats were given an i.p. injection

of scopolamine (1 mg/kg) 30 min after the habituation trial, and after further 30 min, the rat was again placed in the illuminated compartment (acquisition trial). When the rat entered the dark compartment, the guillotine door was closed. Scrambled electrical foot shocks with intensity of 4 mA were delivered for 3 s through the grid floor using a shock generator (Neuroscience Co., Tokyo; model SGS-001). FR194921 was suspended in 0.5% methylcellulose and scopolamine hydrobromide was dissolved in physiological saline. Drugs were prepared just before the tests and administered i.p. in a volume of 1 mL/kg. FR194921 was administered immediately after the acquisition trial (60 min after the habituation trial). In the test trial made 24 h after the acquisition trial, the rat was placed again in the illuminated compartment and the response latency to enter the dark compartment was measured up to a maximum of 300 s (retention trial). The results were recorded as average latency for each group of rats.

Social interaction test

SD rats were handled a day before the behavioral experiments. Animals were dosed orally with either compound or vehicle in weight-matched (± 10 g) pairs, which had not previously been housed together and were naive to the test arena, and returned to home cage. FR194921 was suspended in 0.5% methylcellulose and administered orally in a volume of 2 mL/kg 1 h before the test. Sixty minutes later, they were placed in a clear plastic test box (60 $\times$ 60 $\times$ 35 cm) set in a locomotor analyzer SCANET MV-10MT (Toyo Sangyo, Toyama) under white light (110 lux) in a darkened sound-proof room. The behavior of rats was recorded for 10 min with a video camera suspended obliquely from above. Time spent in active social interaction (sniffing, following, genital investigation, grooming, kicking, boxing, biting, wrestling, crawling under or over the partner, and mounting) was scored by an observer by monitoring records using the computerized score pad CIF-E12 (Toyo Sangyo). Locomotor activity of each pair of rats during the observation period was also measured. Time spent in active social interaction (social interaction time) was regarded as an index of the anxiolytic effect.

Elevated plus maze test

Wistar rats were handled a day before the behavioral experiments. The elevated plus-maze test apparatus (NS-M506, Neuroscience) consisted of a plus-shaped gray-colored maze elevated 50 cm from the floor. It comprised two open arms (50 $\times$ 10 cm) and two enclosed arms (50 $\times$ 10 $\times$ 45 cm), arranged so that the arms of the same type were opposite each other, connected by an open central area (platform dimensions;

10 $\times$ 10 cm). A video camera for monitoring rats was suspended above the center of the maze. Behavioral testing was conducted in a darkened sound-proof room and the maze was lit obliquely from above, giving 10 lux at the ends of the open arms. Testing was conducted during the light part of the light-dark cycle. The test was started by placing the rat onto the central platform facing an open arm 1 h after oral administration of the vehicle or FR194921. The observation started immediately thereafter and lasted 5 min. The number of entries of the whole animal (four paws) and the time spent in each arm were recorded from a video monitor in real time. The number of total arm entries (open + closed) and the percentage of open arm entries ($100 \times \text{open} / \text{total}$) were calculated. The time spent in the open arm, the percentage of entries into the open arm, and the number of entries into the open arm was regarded as indices of the anxiolytic effect. The number of entries into the closed arm and the number of total arm entries were regarded as indices of the anxiogenic effect and spontaneous locomotor activity, respectively. FR194921 was dissolved in saline with equimolar HCl and administered orally in a volume of 2 mL/kg 1 h before the test.

Forced swimming test

The procedure used was similar to that described previously (25). Swim sessions were conducted by placing Wistar rats in individual Plexiglas cylinders (46-cm-tall $\times$ 20-cm diameter) containing 20 cm water at $24 \pm 1^\circ\text{C}$. Two swim sessions were conducted: an initial 15-min pretest followed 24 h later by a 5-min test. FR194921 was administered 30 min after initial 15-min pretest, 5 h and 1 h before the 5-min test. In contrast, imipramine was administered 30 min after the initial 15-min pretest, 5 and 0.5 h before 5-min test. FR194921 was suspended in 0.5% methylcellulose and administered orally in a volume of 5 mL/kg. Imipramine was dissolved in saline and administered intraperitoneally in a volume of 2 mL/kg. Following swim sessions, the rats were removed from cylinders, dried with a paper towel, placed in heated cages, and then returned to their home cage. Test sessions were videotaped from above the cylinder for measuring immobility time later. A rat was judged to be immobile when it remained floating in the water without struggling and was making only those movements necessary to keep its head above water. Grooming behaviors (head shaking and face washing with paws) were not considered struggling.

Statistical analyses

The results are expressed as the mean $\pm$ S.E.M. For the statistical analysis of non-parametric data from the passive avoidance test, the Mann-Whitney U-test was

used for two group comparison (normal versus scopolamine treatment), and the Kruskal-Wallis test followed by the Mann-Whitney U-test was used for comparison of FR194921-treated multiple groups. Parametric data in other behavioral tests were analyzed by Student's *t*-test/Aspin-Welch test for two group comparison and by Dunnett's multiple comparisons test for comparison of FR194921-treated multiple groups.

Results

Adenosine receptors binding affinities and selectivity of FR194921

Radioligand binding to adenosine A_1 , A_{2A} , and A_3 receptors were carried out by using [3 H]-DPCPX, [3 H]-CGS21680, and [3 H]-NECA, respectively. The binding of each radioactive ligand to membranes prepared from CHO cells expressing human A_1 , A_{2A} , or A_3 receptors was best-fitted to a one-site model of binding using Scatchard analysis, with estimated K_d (dissociation constant) values of 2.58, 51.0, and 2.88 nM, respectively, and B_{max} values of 5040, 4300, and 681 fmol/mg protein, respectively. The binding of [3 H]-DPCPX and [3 H]-CGS21680 in the membrane prepared from the cerebral cortex (for A_1) and basal ganglia (for A_{2A}) of mice and rats was also best fitted to a one-site model of binding in the Scatchard analysis. The K_d values of mice A_1 and A_{2A} receptors were 0.50 and 16.0 nM, respectively, and B_{max} values were 410 and 192 fmol/mg protein, respectively. The K_d values of rat A_1 and A_{2A} receptors were 0.62 and 15.7 nM, respectively, and B_{max} values were 368 and 214 fmol/mg protein, respectively.

The affinity of FR194921 was compared with representative adenosine receptors antagonists DPCPX, SCH58261, and CI-IB-MECA as reference for A_1 , A_{2A} , and A_3 , respectively (Fig. 2). Each reference compound showed potent affinity for the receptors confirming the validity of our assay; K_i values of DPCPX, SCH58261, and CI-IB-MECA for each subtype receptors were 1.15 ± 0.28 , 0.96 ± 0.23 , and 1.00 ± 0.16 (nM, mean $\pm$ S.E.M.), respectively. The K_i value of FR194921 for the human adenosine A_1 receptors was 2.91 ± 0.67 , but it did not show any binding affinity for A_{2A} and A_3 receptors up to 1μ M. FR194921 also showed high affinity for A_1 receptors and low affinity for A_{2A} receptors in mice and rats brain preparations (Table 1), thus indicating a lack of species difference between humans and rodents.

Pharmacokinetic study

The plasma concentrations of FR194921 in fed rats were determined at 0.25, 0.5, 1, 2, 4, and 6 h following p.o. administration. At doses of 0.32, 3.2, and 32 mg/kg,

Fig. 2. Effects of adenosine A_1 receptor antagonist FR194921 and reference compounds on specific binding of [3 H]-DPCPX (A), [3 H]-CGS21680 (B), and [3 H]-NECA (C) in membranes prepared from CHO cells expressing human adenosine A_1 , A_{2A} , and A_3 receptors, respectively. The K_i values of DPCPX, SCH58261, and CI-IB-MECA for each subtype receptors were 1.15 ± 0.28 , 0.96 ± 0.23 , and 1.00 ± 0.16 , respectively. Values are the means $\pm$ S.E.M. of three separate experiments.

Table 1. Adenosine receptors binding affinities of FR194921 in human, rat, and mouse

	K _i value (nM)		
	Human	Rat	Mouse
A ₁	2.91 ± 0.67	4.96 ± 1.65	6.49 ± 0.47
A _{2A}	>100	>100	>100
A ₃	>100	NT	NT

K_i values for adenosine receptor subtypes of human, mouse, and rat were measured. In the binding assay for human receptors, membrane prepared from CHO cells expressing human A₁, A_{2A}, or A₃ receptors were used. Brain synaptosome preparations were used in the binding assay for rat and mouse receptors. Data are presented as the mean ± S.E.M. from three independent experiments. NT indicates "not tested".

Fig. 3. The plasma concentrations of FR194921 were determined at 0.25, 0.5, 1, 2, 4, and 6 h following p.o. administration of FR194921 at doses of 3.2, 10, or 32 mg/kg in rats. Data are presented as the mean ± S.E.M. values (n = 3 per each time point).

mean C_{max} plasma concentrations were 87, 220, and 690 ng/mL at 1 h following drug administration, respectively. Even 6 h after dosing, the plasma concentration at 32 mg/kg dosing was relatively highly sustained (501 ng/mL in the plasma) as shown in Fig. 3. Brain concentration of FR194921 was evaluated at 30 min after the administration of 3.2 or 32 mg/kg doses of FR194921 in fasted rats. Mean plasma and brain concentration were 150 ng/mL and 139 ng/g tissue at a dose of 3.2 mg/kg, and 1310 ng/mL and 1420 ng/g tissue at a dose of 32 mg/kg, respectively. Thus, the concentration of FR194921 in the brain was equivalent to that in the blood, and the brain/plasma concentration ratio was approximately 1.0 at 30 min after the dosing.

A₁ receptor agonist-induced hypolocomotion

FR194921 at doses from 0.32 to 32 mg/kg did not affect spontaneous locomotor activity (Fig. 4A). Loco-

Fig. 4. Adenosine A₁ receptor antagonistic activity of FR194921 in rats in vivo. FR194921 did not affect the spontaneous locomotor activity following oral dosing from 0.32 to 32 mg/kg (A). FR194921 dose-dependently attenuated hypolocomotion induced by the adenosine A₁ specific agonist CPA in rats (B). Data are represented as the mean ± S.E.M. from n = 7 or 8 rats. **P < 0.01, statistically significant compared with the control group (by Dunnett's multiple comparisons test). ##P < 0.01, statistically significant compared with the CPA-treated control group (by Student's *t*-test).

motor activity was significantly reduced in CPA (0.056 mg/kg, i.p.)-treated control group compared to the normal group (P < 0.01, by Student's *t*-test). FR194921 dose-dependently attenuated the hypolocomotion induced by CPA with an ED₅₀ value of 0.08 mg/kg and statistical significance at 0.32 mg/kg (P < 0.01, by Dunnett's multiple comparisons test), as shown in Fig. 4B.

Fig. 5. Effects of FR194921 on memory deficits induced by scopolamine in rats in the passive avoidance test. Scopolamine (1 mg/kg, i.p.) was administered 30 min before the acquisition trial. FR194921 was administered i.p. immediately after the acquisition trial. Ordinate represents the median retention latencies in a 24-h retention test. * $P < 0.05$, statistically significant compared with scopolamine-alone control (closed column) (by Kruskal-Wallis test followed by Mann-Whitney U-test). ### $P < 0.001$, statistically significant compared with scopolamine-alone control (closed column) (by Mann-Whitney U-test). Each column and bar represents the mean $\pm$ S.E.M. from 8 or 9 animals per group.

Passive avoidance test

Figure 5 shows the effect of FR194921 on the memory deficits in scopolamine-treated rats in a passive avoidance test. Scopolamine (1 mg/kg, i.p.) given 30 min before the acquisition trial significantly ($P < 0.001$ by Wilcoxon ranking test) reduced the latency in the retention trial determined 24 h thereafter. Dosing with FR194921 (0.1–10 mg/kg, i.p.) immediately after the acquisition trial prolonged the latency in the scopolamine-treated rats. While FR194921 at the lowest dosage (0.1 mg/kg) did not affect the latency, statistically significant improvements were observed in the doses of 0.32 and 1 mg/kg ($P < 0.05$, by Kruskal-Wallis test followed by Mann-Whitney U-test).

Social interaction test

We next evaluated the activity of FR194921 in the social interaction test, the model which is predictive for the anxiolytic actions of drugs. FR194921 increased social interaction time at all doses, with statistical significance at 3.2 mg/kg ($P < 0.05$, by Dunnett's multiple comparisons test), as shown in Fig. 6. FR194921 did not significantly alter the total locomotor activity of the pair of rats. Therefore, FR194921 exhibited a behaviorally specific anxiolytic effect in this model.

A

B

Fig. 6. Effect of FR194921 on the behavior of rats in the social interaction test. FR194921 was orally administered 1 h before the test, and then the time spent in active social interaction (social interaction time) and locomotor activity of each pair of rats were measured for the subsequent 10 min. Social interaction time was regarded as an index of the anxiolytic effect. Data represent the mean $\pm$ S.E.M. from 9 to 10 pairs in each group. * $P < 0.05$, statistically significant compared with the vehicle-treatment group (by Dunnett's multiple comparisons test).

Elevated plus maze test

To expand the evaluation of anxiolytic activity of FR194921, the compound was also investigated for its effect in rat exploratory behavior on the elevated plus-maze. FR194921 dose-dependently increased the time spent in open arms and the percent of entries into the open arm with statistical significance at a dose of 10 mg/kg (Table 2); however, the number of open arm entries was not significantly increased by FR194921 treatment. In addition to these changes, FR194921 did not significantly alter the number of entries into the closed arms and total arms (Table 2).

Table 2. Effect of FR194921 on rat exploratory behavior in the elevated plus-maze test

Dose (mg/kg)	n	Time spent in open arm (s)	% of open arm entries	Open arm entries	Closed arm entries	Total arm entries
Vehicle	11	44.7 ± 7.0	20.5 ± 3.6	2.5 ± 0.5	8.5 ± 0.9	10.9 ± 1.3
1	9	72.9 ± 14.5	29.2 ± 4.7	4.4 ± 1.0	9.9 ± 0.6	14.3 ± 1.3
3.2	9	73.6 ± 10.5	26.3 ± 2.9	3.6 ± 0.4	10.1 ± 1.1	13.7 ± 1.2
10	11	91.7 ± 15.3*	35.8 ± 5.6*	5.1 ± 1.0	8.3 ± 0.7	13.4 ± 1.2

The behavioral test in the elevated plus maze was started by placing the rat onto the central platform of the maze facing an open arm 1 h after oral administration of FR194921. The observation started immediately thereafter and lasted 5 min. The number of entries and the time spent in each arm were recorded from a video monitor in real-time. Data are represented as the mean ± S.E.M. from 9–11 animals in each group. * $P < 0.05$, statistically significant compared with the vehicle-treated group (by Dunnett's multiple comparisons test).

Fig. 7. Effects of FR194921 and imipramine on forced swimming test in rats. Two swim sessions were conducted: an initial 15-min pretest followed 24-h later by a 5-min test. FR194921 (0.32–32 mg/kg) was administered 30 min after the initial 15-min pretest, 5 and 1 h before the 5-min test. Imipramine (10 mg/kg, i.p.) was administered 30 min after the initial 15-min pretest, 5 and 0.5 h before the 5-min test. The immobility time was recorded for 5 min in the test swim. Data are the mean ± S.E.M. from 8 animals per group. *** $P < 0.01$, statistically significant compared with the control group (by Student's *t*-test).

Forced swimming test

To evaluate the antidepressant effects of FR194921, the forced swimming test, which is an animal model of depression, was performed. As shown in Fig. 7, the tricyclic antidepressant agent imipramine (10 mg/kg, i.p.) significantly reduced immobility time in this model; however, FR194921 (0.32–32 mg/kg) had no effect on immobility time even at the highest dose (32 mg/kg).

Discussion

The aim of the present study was to characterize the pharmacological properties of the novel adenosine A₁ antagonist FR194921, which was recently discovered from the screening of a series of pyrazolopyridine derivatives. FR194921 showed potent and selective binding affinity for A₁ receptors, without significant affinities for A_{2A} or A₃ receptors and did not show species differences in binding affinity, being consistent with the nature of non-xanthine derivatives and our previous reports that pyrazolopyridine derivatives (e.g., FK453, FK838, FK352) exhibit similar affinity for [³H]-DPCPX binding sites in the brain membranes from four species (20). The adenosine A₁ receptor is more widely distributed in the brain than the adenosine A_{2A} receptor; however, its physiological and pathophysiological roles still remain unclear in spite of extensive pharmacological studies. In this study, relevant behavioral tests were designed and FR194921 was evaluated not only to validate the potential of this compound as a therapeutic candidate, but also to clarify the functional role of central adenosine A₁ receptors in memory, anxiety, and depression.

FR194921 had high blood-brain barrier permeability, and the brain concentration of FR194921 following oral administration of the compound at 3.2 mg/kg was higher than 10^{-7} M at 0.5 h post treatment. Furthermore, even at 6 h, the plasma concentration was sustained at the level of more than half maximum concentration (C_{max}). Thus the single oral administration should yield high brain levels of FR194921 to sufficiently antagonize A₁ receptors in the brain. As expected from the good pharmacokinetic profile, oral administration of FR194921 antagonized selective A₁ receptor agonist CPA-induced hypolocomotion, indicating that FR194921 is able to block central A₁ receptors in vivo. Assuming linear pharmacokinetics, the brain concentration of FR194921 following the dosage of ED₅₀ value

inhibiting CPA-induced hypolocomotion is approximately 10 nM, which is similar to the K_i value for A_1 receptors. These results indicate that FR194921 behaves like a potent brain adenosine A_1 -receptor antagonist by systemic oral administration and is useful for subsequent studies in animal models.

Adenosine acts by depressing cholinergic transmission via adenosine A_1 receptors in the cortex and hippocampus, and it is well documented that the cholinergic system in these brain areas is involved in learning and memory processes (26–28). Systemic administrations of adenosine A_1 receptor agonists indeed cause learning and memory deficits (29–31), while antagonists improve impairment of learning and memory performance caused by adenosine A_1 receptor agonists (31) and central cholinergic dysfunction (18, 32). We therefore tested whether FR194921 was effective in a passive avoidance test, one model of learning and memory. FR194921 (0.32 to 1 mg/kg) significantly ameliorated scopolamine-induced memory deficits, suggesting that FR194921 exerts cognitive-enhancing actions. Considering a range of pharmacologically relevant doses of FR194921 caused significant anti-dementia effects, the putative mechanism of cognitive enhancement by FR194921 could be primarily A_1 receptor blockade. It is tempting to speculate that FR194921 reversed the memory deficits via enhancing acetylcholine or glutamate release in the hippocampus and cerebral cortex, since presynaptic A_1 -receptors play important roles in regulating release of these neurotransmitters in the brain areas (12). Supporting this view, adenosine A_1 antagonists are capable of improving memory deficits caused by adenosine A_1 receptor stimulation and attenuating adenosine-induced suppression of long-term potentiation induction in the hippocampus (33, 34). While further studies are necessary to understand the role of A_1 receptors in memory formation and we are currently conducting further studies with FR194921 using other memory tasks such as the Morris water maze or 8-arm radial maze, the present finding demonstrates that brain A_1 receptors positively modulate memory processes and further suggest that A_1 receptors may be an interesting target for the development of effective cognitive enhancers.

The involvement of adenosine in regulating complex central functions such as anxiety has also been investigated. Several findings available to date indicate that the existence of interactions between adenosine and anxiolytic agents. It has been reported that benzodiazepine, the most widely used anxiolytic agent, blocks adenosine uptake (35, 36) and decrease adenosine A_1 receptor binding capacity in vivo at doses of clinical relevance (37). In addition, a direct involvement of

adenosine in regulating affective state has been suggested by the finding that papaverine, an inhibitor of adenosine uptake, causes anxiolytic effects (38). In the present studies, to detect the anxiolytic effect of FR194921, the social interaction test and elevated plus-maze test were used as relevant animal models of anxiety. In the social interaction test, FR194921 significantly increased social interaction time without increasing locomotor activity. In addition, in the elevated plus-maze test, FR194921 increased the time spent in the open arms and the number of entries into open arm. While the effective dosages in anxiety models were bit higher than the dosages counteracting brain A_1 receptors, the finding was not surprising considering effective dosages of many of anxiolytic drugs in rats models vary depending on the rats strains and they often require higher dosages (39). Thus, the present findings in two different measures of anxiety in rats strongly support the hypothesis that blockade of adenosine A_1 receptor results in anxiolytic actions, and thus a role of adenosine A_1 antagonistic activity of FR194921 may be important to induce the anxiolytic effect. On the other hand, there are other reports demonstrating that the A_1 antagonist DPCPX did not affect the anxiety state in the elevated plus maze in mice (40), and also mice lacking genetically the adenosine A_1 receptor showed signs of increased anxiety in the light-dark test (41). As variety of factors such as differences in behavioral tests (social interaction test and elevated plus maze tests in our studies versus the light-dark test (41)), animal species /strains (rats in our study versus mice (40) and (41), nature of manipulations blocking receptors (pyrazolopyridine-type specific blocker in our study versus less selective xanthine-type antagonist (40) and genetic deletion (41)) could potentially underlie the discrepancy, further intensive studies performed with A_1 receptor-specific agonist and other ligands for adenosine receptor subtypes would be required to clarify the reason for the controversial results.

Adenosine and its analogue have been shown to induce “behavioral despair” in animal models believed to be relevant to depression. Several recent studies showed that selective adenosine A_{2A} receptor antagonists such as SCH58261, ZM241385, and KW-6002, as well as genetic inactivation of the receptor, was effective in reversing signs of behavioral despair in the forced swim test (42, 43). Although a role of adenosine has been suggested in models of depression, there are no reports demonstrating the influences of pharmacological blockade of adenosine A_1 receptors or by genetic disruption on despair behavior of animals. Therefore, the present finding is the first one to our knowledge suggesting that adenosine A_1 receptor might not be

involved in depression, at least in the animal behavior assessed by the forced swimming test, and may further support the view by others that depressant activity of adenosine could involve adenosine A_{2A} receptors.

In conclusion, the newly synthesized adenosine A₁ antagonist FR194921 exhibited potent A₁ antagonistic activity both in vitro and in vivo, with significant cognitive enhancement following scopolamine-induced memory deficits in rats, and anxiolytic activity in two animal models of anxiety, suggesting that FR194921 and/or other derivatives could be not only an important tool for investigation of the physiological role of central adenosine A₁ receptor in regulating cognitive and anxiety functions, but also an attractive therapeutic candidate for dementia and anxiety disorders.

Acknowledgments

We thank Mrs. Fujiko Takamura for her technical assistance in the pharmacokinetic study and Dr. Raymond D. Price for his helpful comments in preparing the manuscript.

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