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Pharmacological characterization of a novel, potent adenosine A_1 and A_{2A} receptor dual antagonist, ASP5854, in models of Parkinson's disease and cognition

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Abbreviations:

PD, Parkinson's disease; ASP5854, 5-[5-amino-3-(4fluorophenyl) pyrazin-2-yl]-1-isopropylpyridine-2(1*H*)-one; KW-6002, 7-methyl-3,7-dihydro-1*H*-purine-2,6-dione; CNS, central nervous system; CGS21680, (2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethyl-carboxamidoadenosine); NECA, N-ethylcarboxamidoadenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; CPA, N^6-cyclopentyladenosine; CHO, Chinese hamster ovary; MEM, Minimum essential medium; MPTP, 1-metyl-4-phenyl-1,2,3,6-tetrahydropyridine; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; 6-OHDA, 6-hydroxydopamine; L-DOPA, l-dihydroxyphenylalanine

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Abstract

Central adenosine A_{2A} receptor is a promising target for drugs to treat Parkinson's disease (PD), and the central blockade of adenosine A_1 receptor improves cognitive function. In the present study, we investigated the effect of a novel adenosine A_1 and A_{2A} dual antagonist, 5-[5-amino-3-(4fluorophenyl) pyrazin-2-yl]-1-isopropylpyridine-2(1*H*)-one (ASP5854), in animal models of PD and cognition. The binding affinities of ASP5854 for human A_1 and A_{2A} receptors were 9.03 and 1.76 nM, respectively, with higher specificity and no species differences. ASP5854 also showed antagonistic action on A_1 and A_{2A} agonist-induced increases of intracellular Ca^{2+} concentration. ASP5854 ameliorated A_{2A} agonist (CGS21680)- and haloperidol-induced catalepsy in mice, with the minimum effective doses of 0.32 and 0.1 mg/kg, respectively, and also improved haloperidol-induced catalepsy in rats at doses higher than 0.1 mg/kg. In unilateral 6-hydroxydopamine-lesioned rats, ASP5854 significantly potentiated l-dihydroxyphenylalanine (L-DOPA)-induced rotational behavior at doses higher than 0.032 mg/kg. ASP5854 also significantly restored the striatal dopamine content reduced by 1-metyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment in mice at doses higher than 0.1 mg/kg. Furthermore, in the rat passive avoidance test, ASP5854 significantly reversed the scopolamine-induced memory deficits, whereas the specific adenosine A_{2A} antagonist, KW-6002 (istradefylline), did not. Scopolamine- or MK-801-induced impairment of spontaneous alternation in the mouse Y-maze test were ameliorated by ASP5854, whereas KW-6002 did not exert improvement at therapeutically relevant dosages. These results demonstrate that the novel, selective, and orally active dual adenosine A_1 and A_{2A} receptors antagonist, ASP5854, improves motor impairments, is neuroprotective via A_{2A} antagonism, and

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also enhances cognitive function through A_1 antagonism.

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Introduction

Adenosine is a ubiquitous neuromodulator in the central nervous system (CNS). Its major role in the CNS is to modulate neurotransmitter release, the postsynaptic components, and also the non-synaptic components such as glial cell signaling. Adenosine exerts these diverse physiological actions through activation of specific G-protein-coupled receptors termed A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 1994).

Adenosine A_{2A} receptors are specifically localized in the striatum (Svenningsson et al., 1999), where they are coexpressed with dopamine D_2 receptors in the GABAergic striatopallidal neurons; in contrast, there are no A_{2A} receptors in the neurons projecting from the striatum to the substantia nigra that express D_1 receptors (Ferre et al., 1997). Stimulation of adenosine A_{2A} receptors decreases the binding affinity of D_2 receptors (Ferre et al., 1991) and elicits effects opposite to D_2 receptor activation at the level of second-messenger systems and early gene expression (Svenningsson et al., 1999). These data suggest that antagonistic adenosine-dopamine interactions may regulate basal ganglia activity, and could explain the depressant and stimulating effects of adenosine A_{2A} receptor agonists and antagonists on motor behavior (Ferre et al., 1997). Parenteral administration and local striatal infusion of adenosine A_{2A} receptor agonists, such as CGS21680, induces sedation and catalepsy, like dopamine antagonists, and also inhibits the motor-activating effects of dopamine receptor agonists (Svenningsson et al., 1999).

In contrast, adenosine receptor antagonists, including caffeine and related methylxanthines, produce motor stimulant effects, which appear to be related to an action on A_{2A}, rather than A_1, receptors (Fredholm et al., 1999). The A_{2A} selective

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antagonist, KW-6002 (istradefylline) exhibits anti-parkinsonian activities in experimental models of the Parkinson's disease (PD) (Kanda et al., 1998; Shiozaki et al., 1999; Koga et al., 2000), and is developing in clinical stage. More resently, other adenosine A_{2A} antagonists, SCH-420814 and BII014/V2006, also have been developed in clinical stage as anti-parkinsonian drugs. These findings support a role for A_{2A} receptors as neuromodulators of dopaminergic function and suggest that they may play an important role in movement disorders such as PD. In addition, antagonism of adenosine A_{2A} receptors exerts dual actions on motor dysfunction and neurodegenerative processes in animal models of PD (Ikeda et al., 2002). Consistent with its pharmacology, A_{2A} receptor knockout mice are resistant to both motor impairment and neurochemical changes relevant to neurodegenerative disorders such as PD (Ledent et al., 1997; Chen et al., 2001). Hence, adenosine A_{2A} receptor antagonists may represent a novel therapeutic approach to pathologies characterized by neurodegenerative events, since they both reverse motor impairment and are neuroprotective.

Adenosine A_1 receptors are expressed throughout the brain, including the hippocampus and prefrontal cortex, which are important areas for cognitive function, and A_1 receptors in the hippocampus are densely concentrated in the CA1 and CA3 regions (Onodera and Kogure, 1988). Adenosine A_1 receptors play an important role in memory formation (Normile and Barraco, 1991; Costenla et al., 1999). In fact, administration of an A_1 agonist prior to passive avoidance training impaired memory (Normile and Barraco, 1991), and selective adenosine A_1 antagonists enhance cognition in rodents (Maemoto et al., 2004). Therefore, the blockade of both adenosine A_1 and A_{2A} receptors might have therapeutic implications for neurodegenerative disease such as PD, Alzheimer's disease, and other neurological

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diseases. In particular, blockade of both adenosine A_1 and A_{2A} receptors have potential for the treatment of PD, which presents with both motor disability and cognitive impairment. In fact, theophylline improved both motor and mental impairment scores in a short, open-label study in PD patients (Mally and Stone, 1994).

We have recently identified a novel, non-xanthine, adenosine A_1 and A_{2A} receptor antagonist, ASP5854 (shown in Fig. 1). Here, we characterize the mechanism of action and the pharmacological features of ASP5854 using in vitro studies and various animal models of PD and cognition, and compare its effects to the specific A_{2A} receptor antagonist, KW-6002.

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Materials and Methods

Materials

5-[5-Amino-3-(4fluorophenyl)pyrazin-2-yl]-1-isopropylpyridine-2(1*H*)-one (ASP5854; chemical structure shown in Fig. 1) and 8-((*E*)-2-(3,4-Dimethoxyphenyl)ethenyl)-1,3-diethyl-7-methyl-3,7-dihydro-1*H*-purine-2,6-dione (KW-6002) were synthesized at Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharmaceutical Inc., Tsukuba, Japan). Chinese hamster ovary (CHO) cells were purchased from Biogen (Cambridge, MA USA). FuGENE 6 Transfection Reagent was purchased from Roche Applied Science (Mannheim, Germany). α-Minimum essential medium (α-MEM) and opti-minimum essential medium I (opti-MEM I) were purchased from GIBCO, Invitrogen Corporation (Tokyo, Japan). F-127 was purchased from Molecular Probes, Invitrogen Corporation (Tokyo, Japan). Fluo3-AM was purchased from DOJINDO (Kumamoto, Japan). N^6-cyclopentyladenosine (CPA), 2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethyl-carboxamidoadenosine (CGS21680), (-)-scopolamine hydrobromide trihydrate, (+)-MK-801 hydrogen maleate were purchased from SIGMA ALDRICH Co. (Steinhein Germany). [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-DPCPX) and [^{3}H]-CGS21680 were purchased from PerkinElmer Life Science (Boston, MA USA). [^{3}H]-N-ethylcarboxamidoadenosine ([^{3}H]-NECA) was purchased from Amersham Bioscience UK Ltd. (Buckinghamshire, UK). Haloperidol (Serenace® Injection, 5 mg/mL) was purchased from Dainippon Pharmaceutical (Osaka, Japan). Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

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Binding affinities for adenosine receptors in various species

Preparation of membranes from CHO cells expressing the human adenosine receptors: CHO cell lines permanently expressing human adenosine A_1, A_{2A} and A_3 were used in this study. CHO cell-lines were made according to the method described previously (Chen and Okayama, 1987). These cells were cultured in α-MEM supplemented with 10% fetal bovine serum, 50 IU/mL penicillin, and 50 μg/ml streptomycin in a 5% CO_2/95% atmosphere, and were harvested and homogenised in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged (30,000 g) 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 $MgCl_2$ (assay buffer). This washing process was repeated twice, and the final pellet was stored at -80°C until use.

Preparation of membranes from mouse and rat brain: Brain synaptosome membranes were prepared from mice and rats. Animals were sacrificed, and the cerebral cortex and basal ganglia, used for A_1 and A_{2A} binding assays, respectively, were removed quickly and homogenised in approximately 15 volumes of 0.36 M sucrose solution. The homogenates were centrifuged (1,000 g) at 4 °C for 10 min, and then the supernatants were centrifuged (17,000 g) at 4 °C for 20 min. The pellets were suspended in the assay buffer and further centrifuged (30,000 g) at 4 °C for 10 min. This washing process was repeated twice and final pellets were stored at -80 °C until use.

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Radioligand binding assay: Radioligand binding experiments for adenosine A_1, A_{2A} and A_3 receptors were performed with [^{3}H]-DPCPX, [^{3}H]-CGS21680 and [^{3}H]-NECA, respectively. Membranes were incubated with 4 nM [^{3}H]-DPCPX, 15 nM [^{3}H]-CGS21680 or 3.2 nM [^{3}H]-NECA (32 nM [^{3}H]-NECA for KW-6002) in the absence and presence of various concentrations of ASP5854 or KW-6002 in 250 μL of assay buffer containing 1 U/mL adenosine deaminase. 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 (PerkinElmer Inc., Waltham, MA), 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 performed three times in duplicate using 96 well plates. The non-specific binding was defined as the binding activity in the presence of 10 μM of the corresponding unlabeled ligand and was subtracted from the total binding activity for determination of specific binding. Ki values in inhibition studies were calculated with the Prism 3.0 computer program (GraphPad Software, San Diego, CA). To compare the affinity of compounds to A_1 and A_{2A} receptors, the ratio of Ki values between A_1 and A_{2A} receptors was calculated.

Functional assay for adenosine A_1 and A_{2A} receptors in vitro

Preparation of CHO cells expressing the human adenosine A_1 and A_{2A} receptors: A CHO cell line permanently expressing the human adenosine A_1 or A_{2A} receptor was cultured in α-MEM supplemented with 10% (v/v) fetal bovine serum, 50 U/ml penicillin, and 50 μg/mL streptomycin, and grown at 37 °C in an environment of 5% CO_2. These cells were seeded on black 96-well assay plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) at a density of 15,000 cells/well and then cultured for

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24 h.

Transfection of G16 to human adenosine A_1 and A_{2A} CHO cells: Human-A_1 and -A_{2A} CHO cells were transfected with G16, a human Gα protein, at 50 ng/well by means of FuGENE 6 Transfection Reagent and Opti-MEM, and these cells were cultured at 37 °C in 5% CO_2 for 24 h.

Measurement of intracellular Ca^{2+} concentration using FLIPR: Hanks' balanced salt solution, without phenol red, containing 20 mM HEPES and 2.5 mM probenecid was prepared fresh on the day of assay and used as the assay buffer. The dye-loading buffer contained 5 μM Fluo3-AM (Ca^{2+}-sensitive dye) and 0.05% pluronic F-127 (Molecular Probes). The cells were dye-loaded at 37°C by removing the existing maintenance media and adding 100 μL of dye-loading buffer to each well. The cells were washed using plate washer (ELx405 Select, Bio-Tek Instruments) with the assay buffer and 100 μL/well was left in each well. Antagonists at 50 μL/well were added 10 seconds into the fluorescent measurements. After adding drugs, 10 nM CPA (adenosine A1 receptor agonist) or 10 nM CGS21680 (adenosine A_{2A} receptor agonist) was added at 50 μL/well. A 96-well plate contained three wells dedicated to the positive control and three wells as a negative control (assay buffer alone). Each experiment was repeated 3 times, in triplicate at each concentrations. All data were normalized to the positive and negative control wells, which were expressed as 100% and 0% signal. The inhibition % at each concentration was averaged for three IC_{50} values determined by the logistic regression method.

Specificity of ASP5854

The affinity of ASP5854 for 70 receptors, 8 ion channels, 5 transporters, and 3 enzymes was evaluated using radioligand binding assays (performed by Daiichi

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Pure Chemical Co., Ltd., ibaraki, Japan). ASP5854 and each positive substances were dissolved in DMSO, and diluted serially with the same solvent to prepare the solutions at 100-fold concentrations of the final concentrations (ASP5854: 1×10^{-5} mol/L, positive substances: 1×10^{-5} mol/L). These stock solutions were 10-fold diluted with Milli-Q water just prior to the use to prepare test substance and positive substance solutions. Duplicate samples of each concentration of ASP5854 and positive substance solutions were assayed once. These assays utilized standard radioligand binding techniques with tissue membrane preparations. Each inhibition rate was calculated from "100 – binding ratio". Binding ratio was determined by "[(B – N)/(B_0 – N)] × 100 (%)", B: Radioactivity or fluorescence intensity in the tube for calculation of inhibition rate (individual value)

B_0: Radioactivity or fluorescence intensity in the tube for calculation of total reaction (mean value)

N: Radioactivity or fluorescence intensity in the tube for calculation of non-specific reaction (mean value).

CGS21680- or haloperidol-induced catalepsy in mice

Adult male ddY mice were purchased from Japan SLC Co. Ltd. (Hamamatsu, Japan) and were used at seven weeks old. The number of mice in one group was fourteen. The mouse forepaws were placed on a horizontal steel bar (ϕ 0.3 cm), elevated 3 cm above the tabletop. The duration during which each mouse maintained position with both forepaws on the bar and both hindpaws on the tabletop was recorded, up to 30 seconds, as the cataleptic time. Catalepsy was measured 30 min after intracerebroventricular injection of CGS21680 (10 μg/20 μl/head) or intraperitoneal injection of haloperidol (0.32 mg/kg). ASP5854 (0.01-3.2 mg/kg) or

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KW-6002 (0.01-3.2 mg/kg) was orally administered 1 hr before catalepsy measurement. If the catalepsy time reached 30 sec in at least in one trial out of two, catalepsy was scored as positive, otherwise it was scored as negative. The incidence of catalepsy was expressed as the % of the mice that were scored as positive versus the total mice in a group. ASP5854 and KW-6002 were suspended in 0.5% methylcellulose and were administered in a volume of 10 mL/kg. As the vehicle treated group, 0.5% methylcellulose was administered instead of test compounds. CGS21680 was dissolved and diluted in saline, and haloperidol was diluted with saline prior to the experiments.

Haloperidol-induced catalepsy in rats

Adult male SD rats were purchased from Japan SLC Co. Ltd. (Hamamatsu, Japan) and were used at seven weeks old. The number of rats in one group was ten to twelve. Forepaws of a rat were placed on a horizontal plastic bar (ϕ 0.8 cm) which was wrapped with gauze and was 11 cm above the tabletop. The duration during which each rat maintained this position was recorded up to 120 seconds as the cataleptic time. All rats were given two trials at intervals of one minute. Catalepsy was measured 90 minutes after the intraperitoneal (i.p.) injection of haloperidol (1 mg/kg). ASP5854 (0.01-1 mg/kg) or KW-6002 (0.032-1 mg/kg) was administered orally 60 min before the catalepsy test.

Rotational behavior in 6-OHDA lesion rats

Animals: 13-week-old male Fisher rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and were subjected to the surgery at 14 weeks of age.

Unilateral 6-hydroxydopamine lesion: Rats were anaesthetized with sodium

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pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic frame (Narishige, Tokyo, Japan). 6-OHDA (10 μg/2 μL in saline containing 0.2% ascorbic acid) was stereotaxically injected into the left substantia nigra at the following coordinates (in mm) relative to the bregma and the skull surface: A = -4.3, L = 1.7, V = 7.5. Thirty minutes before 6-OHDA injection, rats received an injection of desipramine hydrochloride (25 mg /kg, i.p.) to protect the noradrenergic neurons.

Animal selection: To select successfully denervated animals, all rats were challenged with apomorphine (0.5 mg/kg, s.c.) at more than 7 days post-surgery. The 24 animals showing the highest number of contralateral rotations were selected for subsequent experiments. Rotational behaviors were recorded using the Roto-Rat system (MED Associates, Inc., East Fairfield, NJ).

Evaluation of turning behavior: Selection was performed more than 2 weeks after the 6-OHDA lesion. The 24 apomorphine-challenged rats were challenged with 32 mg/kg L-DOPA plus 8 mg/kg benserazide to induce intense turning behavior, and animals with the top 8 rotational responses were tested in subsequent experiments with drugs. L-DOPA at 10 mg/kg plus benserazide at 2.5 mg/kg, which induces modest contralateral turning behavior, was administered at the same time as ASP5854 (0.01-1 mg/kg), or 30 min after the treatment with KW-6002 (0.1-10 mg/kg). Turning was recorded in 10 min intervals over a 180 min period after L-DOPA plus benserazide administration. At least 5 days were allowed to elapse between experiments. ASP5854 and KW-6002 were suspended in 0.5% methylcellulose and orally administered in a volume of 5 mL/kg. L-DOPA (administered with benserazide at the ratio of 4:1) was suspended in 0.5% methylcellulose containing 0.2% ascorbic acid and administered p.o. in a volume of 5 mL/kg.

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Contents of striatal DA and its metabolites in MPTP-treated mice

Animals: Male C57BL/6 mice (7 or 8 weeks old) were purchased from Charles River Laboratories Japan Co. Ltd. (Yokohama, Japan).

MPTP treatment: ASP5854 (0.01-0.32 mg/kg) or KW-6002 (0.1-3.2 mg/kg) suspended in 0.5% methylcellulose was administrated twice by p.o., 30min or one hour prior to each MPTP injection, in volumes of 10 mL/kg. As the vehicle group, 0.5% methylcellulose was administered instead of the test compounds. Mice received two i.p. injections of MPTP-HCl (20 mg/kg free base) dissolved in saline or saline alone at a 2-hr interval (10 mL/kg).

Measurement of striatal DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels: The contents of DA, DOPAC, and HVA in the striatum were quantified using HPLC with electrochemical detection. Four days after MPTP treatment, brains were quickly removed and the striatum were dissected out. Samples were immediately frozen and stored at -80 °C until analysis. On the day of the assay, tissue samples were homogenized with 0.1 M perchloric acid and 0.1 mM EDTA-2Na containing 50 ng/ml isoproterenol as an internal standard. After centrifugation, the supernatant, with added sodium acetate, was injected onto a reversed phase catecholamine column (SC-5ODS, Eicom Co., Ltd., Kyoto, Japan). The mobile phase contained sodium acetate, methanol, and sodium octane sulphate. DA, DOPAC, and HVA contents were normalized to the normal group (100%). Percent recovery was calculated relative to control animals (control=0%; normal=100%).

Passive avoidance test

Six to seven weeks-old male Wistar rats were obtained from Charles River Inc.

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(Tsukuba, Japan). The minimum quarantine period was at least one week before the experiments, and they were used at eight weeks old. They were handled 3 days prior to the start of the passive avoidance test. The apparatus and experimental procedure used in the passive avoidance task were similar to those described previously (Matsuoka et al., 1992). 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). In the scopolamine-treated model, rats were given an i.p. injection of scopolamine (1 mg/kg) 30 min after the habituation trial, and after a further 30 minutes, 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 0.4 mA was delivered for 4 sec through the grid floor using a shock generator (Neuroscience Co., Tokyo, Japan; model NS-SG01). ASP5854 (0.032-3.2 mg/kg) or KW-6002 (0.1-10 mg/kg) was administered by i.p. injection 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 sec (retention trial). The results were recorded as average latency for each group of rats. The percentage of rats reaching criterion (300 sec) was also calculated. All drugs were prepared just before the tests and administered in a volume of 1 mL/kg.

Y-maze test

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Male ddY mice (Japan SLC Inc., Shizuoka, Japan) aged 5 weeks, were used for the Y-maze test. Spontaneous alternation behavior was assessed in the Y-maze test (Ukai et al., 1998). The maze was made of gray vinyl chloride. Each arm was 40 cm long, 13 cm high, 3 cm wide at the bottom, 10 cm wide at the top, and converged at an equal angle. Mice were acclimated in the experiment room for 1h. Each mouse was placed at the end of one arm and the number of arm entries and alternations were counted for 8 min. Alternation was defined as entries into all three arms on consecutive occasions. The percent alternation was calculated as follows; Alternation rate (%) = (Alternations / Total entry - 2) × 100. ASP5854 (0.032-3.2 or 0.032-1 mg/kg) or KW-6002 (0.1-10 or 0.32-10 mg/kg) was administered orally in a volume of 10 mL/kg 50 minutes before the Y-maze test. Scopolamine (0.5 mg/10 mL/kg, i.p.) or MK-801 (0.15 mg/10 mL/kg, i.p.) was injected 30 minutes after administration of the drug.

All animal procedures were carried out as approved by the Animal Care and Use Committee at Fujisawa Pharmaceutical Co., Ltd. or Astellas Pharma Inc.

Statistical Analysis

In catalepsy test in mice, statistical analysis between vehicle-treated group and drug-treated groups were calculated with the appearance ratio Dunnett's test. The ED_{50} values were calculated using the probit method. In the rat catalepsy test and rat passive avoidance test, statistical analysis between vehicle-treated group and drug-treated groups were made using the Kruskal-Wallis test followed by Dunnett's multiple comparisons. In unilateral 6-OHDA-lesioned rat model, analysis of variance, based on randomized block design, followed by Dunnett's multiple comparisons test was used for comparison between the L-DOPA+benserazide (10+2.5 mg/kg)-treated

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group and multiple drug-treated groups. For striatal DA and metabolite content in MPTP-treated mice and the mice Y-maze test, Dunnett's multiple comparison test was used for comparison between the vehicle-treated control group and multiple drug-treated groups.

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Results

Adenosine receptor binding affinities and selectivity of ASP5854

ASP5854 and KW-6002 were evaluated for their potencies in radioligand binding to the human adenosine receptor subtypes. Ki values for inhibiting specific radioligand binding to the three receptor-subtypes are summarized in Table 1. These compounds were also tested for their binding affinities to adenosine A_1 and A_{2A} receptors obtained from mouse and rat brains (Table 1). ASP5854 showed high affinity for human A_1 and A_{2A} receptors, with Ki values of 9.03 and 1.76 nM, respectively, but lacked affinity for human A_3 receptors. In contrast, KW-6002 showed lower affinity for human A_{2A} receptors compared with ASP5854, and no significant affinity for either human A_1 or A_3 receptors. ASP5854 showed similar binding affinity for A_1 and A_{2A} receptors in human, rats and mice.

Functional assay for adenosine A_1 and A_{2A} receptors in vitro

In CHO cells stably expressing cloned human adenosine A_1 and A_{2A} receptors transfected with Gα16 protein, CPA (10nM) or CGS21680 (10nM) induced increase in the intracelluler Ca^{2+} concentration ($[Ca^{2+}]i$), but ASP5854 alone did not increase (data not shown). In contrast, CPA-induced $[Ca^{2+}]i$ was concentration-dependently blocked by ASP5854 treatment (Fig. 2A). ASP5854 also concentration-dependently inhibited increased $[Ca^{2+}]i$ by 10 nM CGS21680 (Fig. 2B). The IC_{50} values of ASP5854 for increased $[Ca^{2+}]i$ by CPA and CGS21680 were 59.81 ± 17.27 nM and 4.21 ± 0.30 nM, respectively.

Specificity of ASP5854

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ASP5854 was screened for interaction against an array of 70 receptors, 8 ion channels, 5 transporters, and 3 enzymes, including molecular sites which are relevant to anti-parkinsonian activity (Table 2). No significant binding to other proteins was found.

CGS21680- or haloperidol-induced catalepsy in mice

Intracerebroventricular injection of CGS21680 (10 μg/head), an A_{2A} agonist, induced cataleptic behavior in mice. Oral administration of ASP5854 dose-dependently ameliorated CGS21680-induced catalepsy, with an ED_{50} value of 0.147 mg/kg and statistical significance at the doses higher than 0.32 mg/kg (Fig. 3A).

ASP5854 also dose-dependently ameliorated haloperidol (0.32 mg/kg, i.p.)-induced catalepsy in mice, with an ED_{50} value of 0.066 mg/kg, and statistical significance at doses higher than 0.1 mg/kg (Fig. 3B). KW-6002 similarly reversed the haloperidol-induced catalepsy in mice, with an ED_{50} value of 0.092 mg/kg (Fig. 3B).

Haloperidol-induced catalepsy in rats

Haloperidol (1 mg/kg, i.p.) induced catalepsy in rats. ASP5854 reduced the cataleptic duration, with a minimum effective dose of 0.1 mg/kg (Fig. 4A). KW-6002 also inhibited haloperidol-induced catalepsy, though the minimum effective dose was 0.32 mg/kg (Fig 4B).

Turning behavior in 6-OHDA-lesioned rats

Administration of 10 mg/kg L-DOPA plus 2.5 mg/kg benserazide produced

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modest turning behavior, in contrast to the intense contralateral turning behavior elicited by 32 mg/kg L-DOPA plus 8 mg/kg benserazide in unilateral 6-OHDA-lesioned rats (Fig. 5). Thus, 10 mg/kg L-DOPA plus 2.5 mg/kg benserazide was used in this study. ASP5854 significantly potentiated L-DOPA-induced contralateral turning behavior at doses of 0.032 to 1 mg/kg (Fig. 5A). ASP5854 alone minimally induced turning behavior in all groups (data not shown). KW-6002 significantly potentiated L-DOPA-induced contralateral turning behavior at doses of 0.32 to 10 mg/kg (Fig. 5B). ASP5854 was more potent than KW-6002.

Contents of striatal DA and its metabolites in MPTP-treated mice

The striatal DA contents of normal and MPTP-treated control mice were 120.76 ± 4.36 and 43.40 ± 2.88 ng/mg protein, respectively. MPTP treatment significantly reduced the striatal DA content to 35.94% compared with normal mice. ASP5854 dose-dependently improved the reduction of striatal DA content by MPTP administration. The % recovery of DA content from the vehicle-treated control level were 7.09%, 21.11%, 38.78% and 60.41% at doses of 0.01, 0.032, 0.1 and 0.32 mg/kg, respectively, with statistical significance at doses of 0.1 mg/kg and higher (Fig. 6A). The effects of ASP5854 on DOPAC and HVA contents paralleled that on DA. KW-6002 similarly restored the striatal DA content reduced by MPTP treatment in a dose-dependent manner. The % recovery in DA contents at doses of 0.1, 0.32, 1 and 3.2 mg/kg were 22.91%, 28.37%, 38.79% and 42.45%, respectively. Significant effects were obtained at the doses of 1 and 3.2 mg/kg (Fig. 6B).

Scopolamine-induced memory deficits in the rat passive avoidance test

Scopolamine (1 mg/kg) given 30 min before the acquisition trial significantly

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reduced the latency in the retention trial determined 24 hr thereafter (Fig. 7A & B). Dosing with ASP5854 (0.032-3.2 mg/kg) immediately after the acquisition trial prolonged the latency in the scopolamine-treated rats, and the dose-response curve was bell-shaped, with a statistically significant effect at 0.1 to 3.2 mg/kg (Fig. 7A), and the percentage of rats reaching criteria tended to increase (data not shown). On the other hand, KW-6002 at doses of 0.1 to 10 mg/kg had no effect in this model (Fig. 7B).

Scopolamine-induced memory deficit in the mouse Y-maze test

In the Y-maze test, administration of scopolamine (0.5 mg/kg ip) induced a marked decrease in alternation rate compared with normal animals (Fig. 8A & B). This scopolamine-induced decrease of alternation rate was significantly ameliorated by treatment of ASP5854 at 0.32 and 1 mg/kg (Fig. 8A). KW-6002 significantly improved the impairment of spontaneous alternation induced by scopolamine at only the highest dose of 10 mg/kg, and the lower doses (0.1-3.2 mg/kg) did not show significant effects (Fig. 8B).

MK-801-induced memory deficits in the mouse Y-maze test

Administration of MK-801 (0.15 mg/kg, ip) induced a marked decrease in alternation rate compared with normal animals (Fig. 9A & B). ASP5854 (0.032-1 mg/kg, p.o.) improved the spontaneous alteration impaired by MK-801, with significance at 0.32 and 1 mg/kg (Fig. 9A). KW-6002 significantly improved the impairment of spontaneous alternation induced by MK-801 only at 10 mg/kg (Fig. 9B).

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Discussion

The purpose of the present study was to characterize the in vitro and in vivo profiles of the novel adenosine A_1 and A_{2A} receptor dual antagonist, ASP5854, which was recently identified from our optimization screening of pyrazoropyrimidin derivatives. ASP5854 showed potent and selective binding affinity for A_1 and A_{2A} receptors, with no significant affinity for A_3 receptors, and did not show species differences. Furthermore, ASP5854 showed no apparent affinity for many other receptors, channels, monoamine transporters, or enzymes, including the adenosine A_{2B} receptor and phosphodiesterase, which are common targets for other adenosine antagonists that are xanthine derivatives. ASP5854 concentration-dependently inhibited the increase in $[Ca^{2+}]i$ by CPA and CGS21680, with IC_{50} values of 59.81 nM and 4.21 nM, respectively. The Ki value of binding affinity for human adenosine A_1 and A_{2A} receptors are 9.03 nM and 1.76 nM, respectively, and ratios of the IC_{50}/Ki value were between 2.4 and 6.6. These results clearly demonstrated that ASP5854 is a potent and selective antagonist of the adenosine A_1 and A_{2A} receptors.

Many behavioral, biochemical, and functional studies have indicated an antagonistic interaction between adenosine A_{2A} receptors and dopamine D_2 receptors in the striatum (Svenningsson et al., 1999). Modulation of adenosine A_{2A} receptors profoundly influence motor function (Ferre et al., 1997). In fact, the i.c.v. injection of CGS 21680, an A_{2A} agonist, induced catalepsy in mice, as do dopamine D_2 antagonists such as haloperidol (Shiozaki et al., 1999). We also found that i.c.v. injection of CGS21680 and i.p. injection of haloperidol induced catalepsy in mice and rats, and ASP5854 dose-dependently reversed the catalepsy in this study. Interestingly, ED_{50} values obtained from CGS21680- and haloperidol-induced

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catalepsy in mice were comparable, suggesting that A_{2A} antagonism of ASP5854 mediated the anti-cataleptic actions in both models.

Contralateral turning behavior in rats subjected to unilateral 6-hydroxydopamine lesions of the dopaminergic nigrostriatal pathway is another well-established animal model of PD (Ungerstedt, 1971). In this model, a selective A_{2A} antagonist reportedly potentiated the contralateral turning behavior induced by direct dopamine agonists (Koga et al., 2000), implying that adenosine A_{2A} antagonists might be beneficial for the treatment of PD. In fact, KW-6002 shortened the wearing-off time in PD patients in clinical trials (Hauser et al., 2003). In the present study, ASP5854 potentiated the rotational behavior induced by L-DOPA without inducing stereotyped behavior on its own. Drugs capable of selectively reversing the reduction in the duration of L-DOPA anti-parkinsonian action without augmenting its response intensity have significant therapeutic potential. Therefore, while further studies in non-human primates are warranted, the present findings suggest that ASP5854 may be useful as adjunct therapy in the treatment of advanced PD.

The pathogenesis of PD involves degeneration of striatal neurons that project to the external pallidum (indirect pathway) leading to overactivity, while the activity of striatal neurons projecting directly to the basal ganglia output nuclei (direct pathway) is reduced (Kase, 2001). Since adenosine A_{2A} receptors are located on striatal projection neurons (Svenningsson et al., 1999), especially within the indirect pathway, ASP5854 may work by blocking adenosine A_{2A} receptors on striatal neurons projecting to the external pallidum. This assumption implies a selective suppressive action of ASP5854 on the indirect pathway. The density of A_1 receptors is moderately higher in the motor regions of the brain, such as the cerebellum and

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motor cortex, compared to A_{2A} receptors (Rivkees et al., 1995). In the striatum, A_1 receptors are weakly expressed in approximately 40 % of striatal neurons (Rivkees et al., 1995), and are present presynaptically on glutamate, dopamine and acetylcholine afferents to medium-sized neurons (Fredholm and Dunwiddie, 1988; Flagmeyer et al., 1997). A_1 receptors antagonistically and specifically modulate the binding and functional characteristics of dopamine D_1 receptors, much like the A_{2A}-D_2 antagonistic interaction (Ferre et al., 1997), suggesting that adenosine A_1 antagonism could improve motor impairment. In fact, adenosine A_1 antagonist potentiated the motor effects induced by D_1 agonist in rats (Popoli et al., 1996). However, our specific adenosine A_1 antagonist, FR194921, did not improve motor impairment in the same PD models employed here (data not shown). Another A_1 antagonist, DPCPX, alone did not improve motor impairment in a non-human primate PD model (Kanda et al., 1998). Therefore, the anti-parkinsonian effect of ASP5854 is primarily mediated by the adenosine A_{2A} antagonism. Nevertheless, it is tempting to speculate that the A_1 antagonism of ASP5854 could potentiate the effects of dopamine D_1 or non-selective dopamine agonists, especially since ASP5854 was more potent in the 6-OHDA-lesion model than other PD models, although it warrants the further investigation to address this hypothesis.

While the pathological hallmark in PD patients is progressive dopaminergic neurodegeneration in the basal ganglia, none of the currently existing dopaminergic-based drugs affect the neurodegeneration that occurs in the basal ganglia in PD. There is an urgent need for a neuroprotective approach that may delay disease progression. MPTP-induced dopaminergic neuronal cell death model provides a useful test for neuroprotective drugs (Iwashita et al., 2004). ASP5854 improved the striatal dopamine depletion produced by MPTP administration.

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ASP5854 did not bind MAO-B which oxidizes MPTP to the active toxin, MPP^+, or the dopamine transporter, which transports MPP^+ into the cell body of dopaminergic neurons (Table 2). ASP5854 also did not significantly affect MPP^+ concentrations in plasma and brain following the injection of MPTP (data not shown). Chen *et al*. demonstrated that MPTP toxicity was reduced by administration of adenosine A_{2A} receptor antagonists, and in genetically A_{2A} knock-out mice (Chen et al., 2001). Ikeda *et al*. showed that A_{2A} receptor antagonists, but not adenosine A_1 antagonists, protected against dopaminergic neurodegeneration induced by MPTP and 6-OHDA (Ikeda et al., 2002). Our study also suggests that blockade of the A_{2A} receptor could result in neuroprotection: however, further immunohistochemical and mechanistic studies will be required.

Scopolamine, a centrally active anti-cholinergic agent, and MK-801, a non-competitive NMDA antagonist, are commonly used in the study of learning and memory. Pre-treatment of scopolamine 30 min before training impaired retention performance in the rat passive avoidance test, in which animals acquire memory related to aversive stimulus, consistent with earlier observations (Matsuoka et al., 1992). In the Y-maze test, scopolamine and MK-801 induced spontaneous alternation memory impairments in mice, which is consistent with earlier findings (Fraser et al., 1997; Ukai et al., 1998). ASP5854 ameliorated the scopolamine-induced impairment of memory in the rat passive avoidance test and the scopolamine- and MK-801-induced impairment of memory in the mouse Y-maze test, with comparable potency to PD models. While these animal models employed here do not perfectly mirror the cognitive deficits associated with PD and further studies will be required, the present findings suggest that ASP5854 could improve cognition in patients with memory disturbances.

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A_1 activation depresses cholinergic, noradrenergic and GABAergic transmission (Phillis and Kostopoulos, 1975; Hollins and Stone, 1980). The cholinergic system is important in learning and memory processes, and cholinesterase inhibitors are useful treatments for Alzheimer's disease (Seltzer, 2006). Thus, blockade of adenosine A_1 receptors may indirectly stimulate cholinergic neurotransmission and thereby enhance cognition. The selective adenosine A_1 antagonist, KFM-19, enhanced acetylcholine release and synaptic transmission in rat hippocampal slices and improved learning performance in aged and nucleus basalis magnocellularis-lesioned rats (Schingnitz, 1991). FR194921, a selective and potent A_1 antagonist, also improved scopolamine-induced memory deficits in the rat passive avoidance test (Maemoto et al., 2004). These findings suggest that the adenosine A_1 antagonist activity of ASP5854 mediates its cognitive function improvements in the context of cholinergic dysfunction. Endogenous acetylcholine regulates the induction of long-term potentiation (Ovsepian et al., 2004), an underlying event learning and memory (Malenka et al., 1989). The activation of NMDA receptors also has key role in the induction of LTP, and a competitive NMDA receptor antagonist interfered with spatial learning in the adult rat by blockade of LTP (Morris et al., 1986). Adenosine A_1 receptor activations play a role in the development of LTP, particularly in the CA1 region (Costenla et al., 1999). Thus, the preset study demonstrates, for the first time, that adenosine A_1 antagonism leads to improvement of the memory deficits associated with hypo-glutamatergic state.

Role for adenosine A_{2A} receptors in memory has been controversial. While there is a report showing that genetic deletion of the receptor exhibits improvement in mnemonic process (Wang et al., 2006), the effect of selective adenosine A_{2A} antagonists on memory disruption is not clear. Although both A_{2A} agonist and

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antagonist improve MK-801-induced memory disruption in the mice Y-maze (Fraser et al., 1997), improvement in this model in our hands did not require A_{2A} antagonism. Specifically, KW-6002 did not show any effect in the rat passive avoidance test. Furthermore, KW-6002 required a 10-fold higher dose in the Y-maze study than in the PD models including catalepsy in mice and rats and the turning behavior in 6-OHDA lesioned rats. KW-6002 has no binding affinity for human adenosine A_1 receptors but moderate affinity for A_1 receptors in rodents (Table 1), being consistent with previous results by other (Shimada J., 1997). Thus, central adenosine A_1 receptors may be more useful targets for treatment of cognitive impairment than adenosine A_{2A} receptors. It was suggested that ASP5854 might improve both cholinergic and glutamatergic memory disruption via potent adenosine A_1 antagonistic activity. Although adenosine A1 antagonism can influence the cardiovascular system (Hayes, 2003), ASP5854 with higher doses did not affect cardiovascular function in rodent and dog toxicity studies (data not shown).

In conclusion, the novel adenosine A_1 and A_{2A} dual antagonist ASP5854 might improve both motor impairment and neuronal degeneration via A_{2A} receptor blockade, and cognitive impairments via A_1 receptor blockade, and could represent a novel class of agents for PD and dementia.

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Legends for Figures

Figure 1

Chemical structure of ASP5854.

Figure 2

Antagonistic effects of ASP5854 for human adenosine A_1 and A_{2A} receptors on $[Ca^{2+}]i$ elevation by CPA or CGS216980 in CHO cells expressing the human adenosine A_1 receptor or adenosine A_{2A} receptor. $[Ca^{2+}]i$ was measured using FLIPR. (A) 10nM CPA or (B) 10nM CGS21680 was added immediately after ASP5854 addition. ASP5854 dose-dependently inhibited increased $[Ca^{2+}]i$ by CPA and CGS21680 with IC_{50} values of 59.81 ± 17.27 and 4.21 ± 0.30 nM, respectively. Values are the mean ± SE of three separate experiments. IC_{50} values determined by the logistic regression method.

Figure 3

Effects of ASP5854 on CGS21680- or haloperidol-induced catalepsy in mice. In CGS21680-induced catalepsy, catalepsy was measured 30 minutes after intracerebroventricular injection of CGS21680. (A) ASP5854 was orally administered 30 minutes before CGS21680 injection. The column represent the % of the mice with catalepsy within the group (n=14). ASP5854 suppressed CGS21680-induced catalepsy dose-dependently, with an ED_{50} value of 0.147 mg/kg. In haloperidol-induced catalepsy, catalepsy was measured 30 minutes after intraperitoneal injection of haloperidol. ASP5854 or KW-6002 was orally administered 30 minutes before haloperidol injection. Each column represents the %

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of the mice with catalepsy within the group (n=14). ASP5854 (B) and KW-6002 (C) suppressed haloperidol-induced catalepsy dose-dependently, with ED_{50} values of 0.066 and 0.092 mg/kg, respectively. *: $P<0.05$, **: $P<0.01$; statistically significant compared with the vehicle-treated controls (opened columns) (by the appearance ratio Dunnett's test). ED_{50} values were calculated by probit method.

Figure 4

Effects of ASP5854 and KW-6002 on haloperidol-induced catalepsy in rats. Catalepsy was measured 90 minutes after intraperitoneal injection of haloperidol. ASP5854 (A) or KW-6002 (B) was orally administered 30 minutes following haloperidol injection. Each column represents the mean ± SE of ten to eleven rats. *: $P<0.05$, **: $P<0.01$; statistically significant compared with the vehicle-treated controls (opened columns) (by Kruskal-Wallis followed by Dunnett's multiple comparison test).

Figure 5

Effects of ASP5854 and KW-6002 on L-DOPA-induced turning behavior in 6-OHDA lesioned, hemi-parkinsonian rats. ASP5854 (A) was administered orally at same time with the oral administration of L-DOPA (administered with benserazide at the ratio of 4:1). KW-6002 (B) was administered 30 min before L-DOPA+benserazide administration. Each column represents the mean ± SEM of total turning counts for 180 min after L-DOPA administration. *: $P<0.05$, **: $P<0.01$; statistically significant compared with L-DOPA+benserazide (10+2.5 mg/kg) alone controls (opened columns) (by analysis of variance based on randomized block design, followed by Dunnett's multiple comparisons test). ##: $P<0.01$; statistically significant compared

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with L-DOPA+benserazide (10+2.5 mg/kg) alone controls (opened columns) (by paired t-test).

Figure 6

Effects of ASP5854 and KW-6002 on MPTP-induced depletion of striatal DA, DOPAC, and HVA content in mice. ASP5854 (A) or KW-6002 (B) was administered twice by p.o. at 2 hr intervals. MPTP was intraperitoneally injected 30 minutes or one hour after administration of each drug. The striatum was dissected 4 days after drug administration. DA, DOPAC, and HVA were measured by HPLC with electrochemical detection. Data are presented as mean ± SE of six to eight mice. *: $P<0.05$, **: $P<0.01$; statistically significant compared with the vehicle-treated controls (closed columns) (by Dunnett's multiple comparison test). ##: $P<0.01$; statistically significant compared with vehicle-treated controls (closed columns) (by Student's t-test).

Figure 7

Effects of ASP5854 and KW-6002 on scopolamine-induced memory deficits in the passive avoidance task in rats. Scopolamine (1 mg/kg) was administered by i.p. 30 min before the acquisition trial. ASP5854 (A) or KW-6002 (B) was administered i.p. immediately after the acquisition trial. Ordinate represents the median retention latencies in a 24-hr retention test. *: $P<0.05$, **: $P<0.01$; statistically significant compared with scopolamine-alone controls (closed columns) (by Kruskal-Wallis followed by Dunnett's multiple comparisons). ##: $P<0.01$, ###: $P<0.001$; statistically significant compared with scopolamine-alone controls (closed columns) (by Wilcoxon's rank sum test). Each column and bar represents the mean ± SE of

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twelve to thirteen rats.

Figure 8

Effects of ASP5854 and KW-6002 on scopolamine-induced impairment of spontaneous alternation in the Y-maze task in mice. ASP5854 (A) or KW-6002 (B) were administered 50 minutes before the Y-maze test, and scopolamine was administered 30 min after drug administration. Each value represents the mean ± SEM of ten mice. *: $P<0.05$, **: $P<0.01$; statistically significant compared with scopolamine alone controls (closed columns) (by Dunnett's multiple comparison test). ###: $P<0.001$; statisticallyy significant compared with scopolamine alone controls (closed columns) (by Student's t-test).

Figure 9

Effects of ASP5854 and KW-6002 on MK-801-induced impairment of spontaneous alternation in Y-maze in mice. ASP5854 (A) or KW-6002 (B) were administered 50 minutes before the Y-maze test, and MK-801 was administered 30 min after drug administration. Each value represents the mean ± SEM of ten mice. *: $P<0.05$, **: $P<0.01$; statistically significant compared with MK-801 alone controls (closed columns) (by Dunnett's multiple comparison test). ##: $P<0.01$; statistically significant compared with MK-801 alone controls (closed columns) (by Student's t-test).

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TABLE 1

Binding affinities of ASP5854 and KW-6002 for adenosine receptor subtypes in human, rat, and mouse.

Ki values of ASP5854 and KW-6002 for adenosine receptor subtypes of human, rat and mouse were measured. In the binding assay for human receptors, membrane prepared from CHO cells expressing human A_1, A_{2A}, or A_3 receptors were used. Brain synaptosome preparations were used in the binding assay for rat and mouse receptors. Data are presented as the mean ± SE from three independent experience. NT indicates "not tested".

Agent	Species	Ki (nM)			A_1/ A_{2A} ratio
		A_1 receptor	A_{2A} receptor	A_3 receptor	
ASP5854	Human	9.03 ± 1.63	1.76 ± 0.24	>557	5.13
	Rat	12.48 ± 2.80	1.24 ± 0.34	NT	10.06
	Mouse	7.89 ± 3.03	1.62 ± 0.41	NT	4.87
KW-6002	Human	>287	9.12 ± 0.99	>681	>31.47
	Rat	50.90 ± 8.67	1.57 ± 0.21	NT	32.42
	Mouse	105.02 ± 5.66	1.87 ± 0.17	NT	56.16

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TABLE 2

Specificity of ASP5854

The affinity of ASP5854 for 70 receptors, 8 ion channels, 5 transporters, and 3 enzymes was evaluated using radioligand binding assays. Each inhibition rate was calculated from "100 – binding ratio". Binding ratio: $[(B - N)/(B_0 - N)] \times 100$ (%), B: Radioactivity or fluorescence intensity in the tube for calculation of inhibition rate (individual value), B_0: Radioactivity or fluorescence intensity in the tube for calculation of total reaction (mean value), N: Radioactivity or fluorescence intensity in the tube for calculation of non-specific reaction (mean value). The data are expressed as the mean values of duplicate samples.

Assay name (Original source)	Inhibition(%)				
	1×10^{-7} mol/L	1×10^{-6} mol/L	1×10^{-5} mol/L	Positive substance	
Adenosine A2b (Human)	14.77	25.06	33.03	98.23	(DPCPX)
Adenosine transporter (Human)	0.00	0.00	0.60	96.74	(NBTI)
α1A-Adrenergic (Rat)	6.60	0.85	3.07	96.22	(Prazosin)
α 1B-Adrenergic (Rat)	4.78	5.40	6.52	99.42	(Prazosin)
α 2A-Adrenergic (Human)	5.04	0.00	1.21	99.01	(Rauwolscine)
α 2B-Adrenergic (Human)	0.14	0.00	0.00	100	(Rauwolscine)
α 2C-Adrenergic (Human)	0.00	0.00	0.00	100	(Rauwolscine)
β1-Adrenergic (Human)	0.85	5.88	2.73	99.45	(Propranolol)
β 2-Adrenergic (Human)	2.33	0.62	2.68	100	(Propranolol)
β 3-Adrenergic (Human)	0.00	0.00	8.64	100	(Propranolol)
Angiotensin AT1 (Human)	0.00	0.22	0.00	95.94	(Angiotensin II)
Angiotensin AT2 (Human)	0.69	11.65	24.00	97.76	(Angiotensin II)
Bradykinin B2 (Human)	5.95	19.49	12.26	97.16	(HOE140)
Ca channel (Type L, Rat)	0.00	1.40	0.27	97.52	(Nitrendipine)
Ca channel (Type N, Rat)	1.99	0.00	7.66	100	(w-Conotoxin)
Cannabinoid CB1 (Human)	0.00	6.50	0.00	97.37	(WIN55212-2)
Cannabinoid CB2 (Human)	5.46	0.44	1.56	100	(WIN55212-2)
CCK A (Human)	0.00	2.05	0.00	100	(CCK-8 sulfated)
CRF1 (Human)	0.00	0.00	15.83	93.68	(human Urocortin)
Cl channel (Rat)	0.00	0.00	0.00	92.17	(Picrotoxin)

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Dopamine D1 (Human)	0.00	0.00	0.00	96.98	(SCH23390)
Dopamine D2 short (Human)	11.01	0.00	0.00	100	((+)-Butaclamol)
Dopamine D2 long (Human)	3.54	5.02	2.84	99.68	((+)-Butaclamol)
Dopamine D3 (Human)	10.11	15.51	8.43	100	((+)-Butaclamol)
Dopamine D4.2 (Human)	1.09	0.00	0.00	99.61	(Haloperidol)
Dopamine D4.4 (Human)	5.52	13.22	23.83	100	(Haloperidol)
Dopamine D4.7 (Human)	13.16	14.28	8.05	96.73	(Haloperidol)
Dopamine D5 (Human)	6.72	1.40	7.02	100	(SCH23390)
Dopamine transporter (Human)	7.27	3.78	14.82	100	(GBR12909)
Estrogen (Rat)	0.11	0.00	0.08	98.88	(b-Estradiol)
Endothelin ETA (Human)	0.00	0.00	6.68	98.63	(Endothelin)
GABA A (Agonist site, Rat)	2.84	5.19	5.18	98.81	(Muscimol)
GABA A (BZ central, Rat)	0.95	2.77	21.56	99.81	(Diazepam)
GABA A (Chloride channel, Rat)	0.00	0.00	0.00	95.25	(Picrotoxin)
GABA B (Rat)	0.00	0.00	22.07	84.22	(GABA)
GABA transporter (Rat)	0.00	0.00	0.60	95.93	(GABA)
Glucocorticoid (Human)	0.00	1.08	0.00	98.53	(Dexamethasone)
Glutamate (AMPA, Rat)	0.00	0.00	12.15	99.75	(AMPA)
Glutamate (Kainate, Rat)	0.00	0.00	12.88	100	(Kainic acid)
Glutamate (NMDA agonist site, Rat)	0.00	0.00	10.50	100	(Glutamic acid)
Glutamate (NMDA glycine site, Rat)	0.00	0.00	3.47	100	(MDL105519)
Glutamate (NMDA phencyclidine site, Rat)	4.45	9.98	3.48	98.09	(MK-801)
Glutamate (NMDA polyamine site, Rat)	2.78	0.00	14.71	95.15	(Ifenprodil)
Glycine (Strychnine sensitive, Rat)	0.00	0.00	5.98	100	(Strychnine)
Histamine H1 (Human)	0.11	0.00	7.14	91.83	(Pyrilamine)
Histamine H2 (Human)	0.00	4.87	6.87	100	(Cimetidine)
Histamine H3 (Human)	0.00	6.53	23.64	93.52	(a-methyl histamine)
K channel KA (Rat)	0.00	7.66	0.72	97.12	(Dendrotoxin)
K channel KATP (Rat)	1.03	1.03	4.44	100	(Glibenclamide)
K channel Kv (Rat)	0.42	2.38	4.61	100	(Charybdotoxin)
K channel SkCa (Rat)	0.00	0.00	4.37	100	(Apamin)
Melatonin MT1 (Human)	0.00	10.04	9.57	100	(Melatonin)
Muscarinic M1 (Human)	0.22	0.50	0.00	100	(Atropine)
Muscarinic M2 (Human)	2.02	2.31	0.00	99.68	(Atropine)
Muscarinic M3 (Human)	0.00	0.00	0.00	99.40	(Atropine)
Muscarinic M4 (Human)	8.53	23.34	11.99	100	(Atropine)
Muscarinic M5 (Human)	0.00	0.00	0.01	100	(Atropine)
Mineralcorticoid (Rat)	2.98	0.00	0.08	97.31	(Aldosterone)

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Na channel Site2 (Rat)	0.00	0.00	14.96	99.20	(Dibucaine)
Neurokinin NK1 (Human)	3.03	16.58	32.28	100	(L-703,606)
Neurokinin NK2 (Human)	0.00	5.16	3.90	99.68	(Neurokinin A)
Neurokinin NK3 (Human)	2.37	7.39	2.09	99.72	(Senktide)
Neuropeptide Y1 (Human)	0.00	0.21	14.78	99.14	(Neuropeptide Y)
Norepinephrine transporter (Human)	0.00	0.00	0.00	100	(Desipramine)
Nicotinic Ni (Human)	6.09	5.86	4.32	100	(Epibatidine)
Opiate d (Human)	0.87	2.52	2.52	99.91	(Naltriben)
Opiate k (Human)	0.00	3.05	11.31	100	(U-69593)
Opiate m (Human)	3.23	3.51	2.84	100	(DAMGO)
Opiate ORL1 (Human)	0.00	0.00	0.92	100	(Orphanin FQ)
Oxytocin (Rat)	1.67	0.00	0.00	100	(Oxytocin)
Serotonin 5HT1A (Human)	0.00	0.00	7.47	84.89	(Serotonin)
Serotonin 5HT2A (Human)	9.54	9.90	3.38	99.77	(Ketanserin)
Serotonin 5HT2B (Human)	0.00	0.18	0.00	100	(Serotonin)
Serotonin 5HT2C (Human)	1.12	0.00	6.94	96.71	(Mianserin)
Serotonin 5HT3 (Human)	3.07	0.00	0.00	94.61	(MDL72222)
Serotonin 5HT4 (Guinea pig)	13.82	8.36	37.46	89.47	(Serotonin)
Serotonin 5HT5A (Human)	4.90	7.80	10.91	99.60	(Methiothepin)
Serotonin 5HT6 (Human)	2.70	10.92	0.00	100	(Methiothepin)
Serotonin 5HT7 (Human)	1.94	4.22	10.12	100	(Methiothepin)
Serotonin transporter (Human)	0.00	6.37	21.37	100	(Imipramine)
Sigma (Non-selective, Guinea pig)	2.01	3.73	0.44	100	(Haloperidol)
Testosterone (Human)	0.16	0.21	5.24	100	(Testosterone)
Vasopressin V1 (Rat)	1.03	0.00	0.90	100	([Arg^8]-Vasopressin)
Acetylcholin esterase (Electric eel)	7.34	5.52	4.31	99.12	(Eserine)
MAO-A (Rat)	8.62	14.95	12.15	97.09	(Clorgyline)
MAO-B (Rat)	5.77	8.10	0.00	73.65	(Ro 16-6491)

Fig. 1

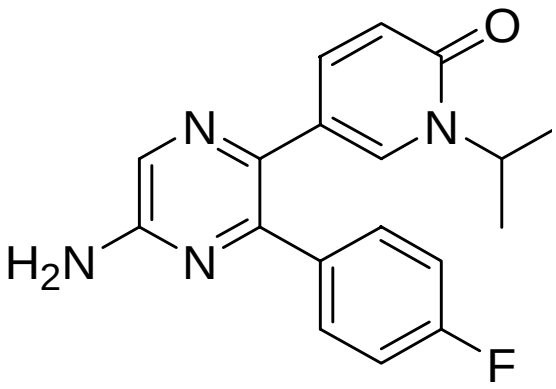
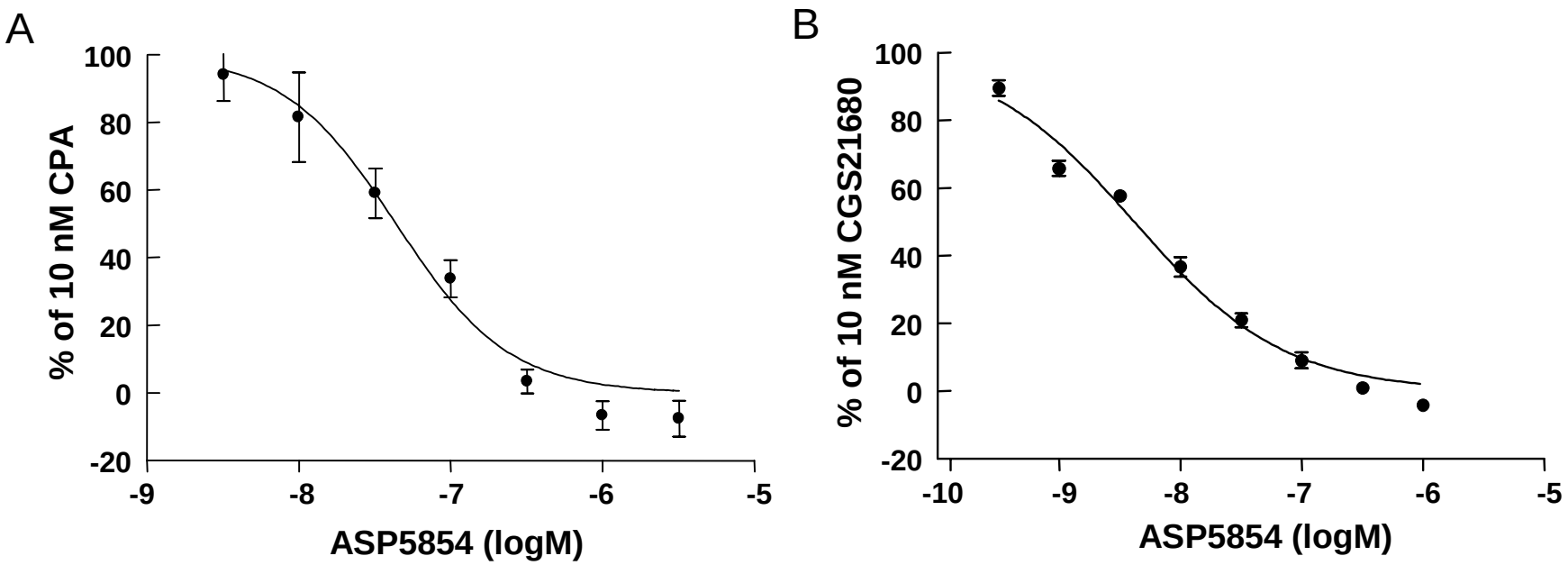


Fig. 2



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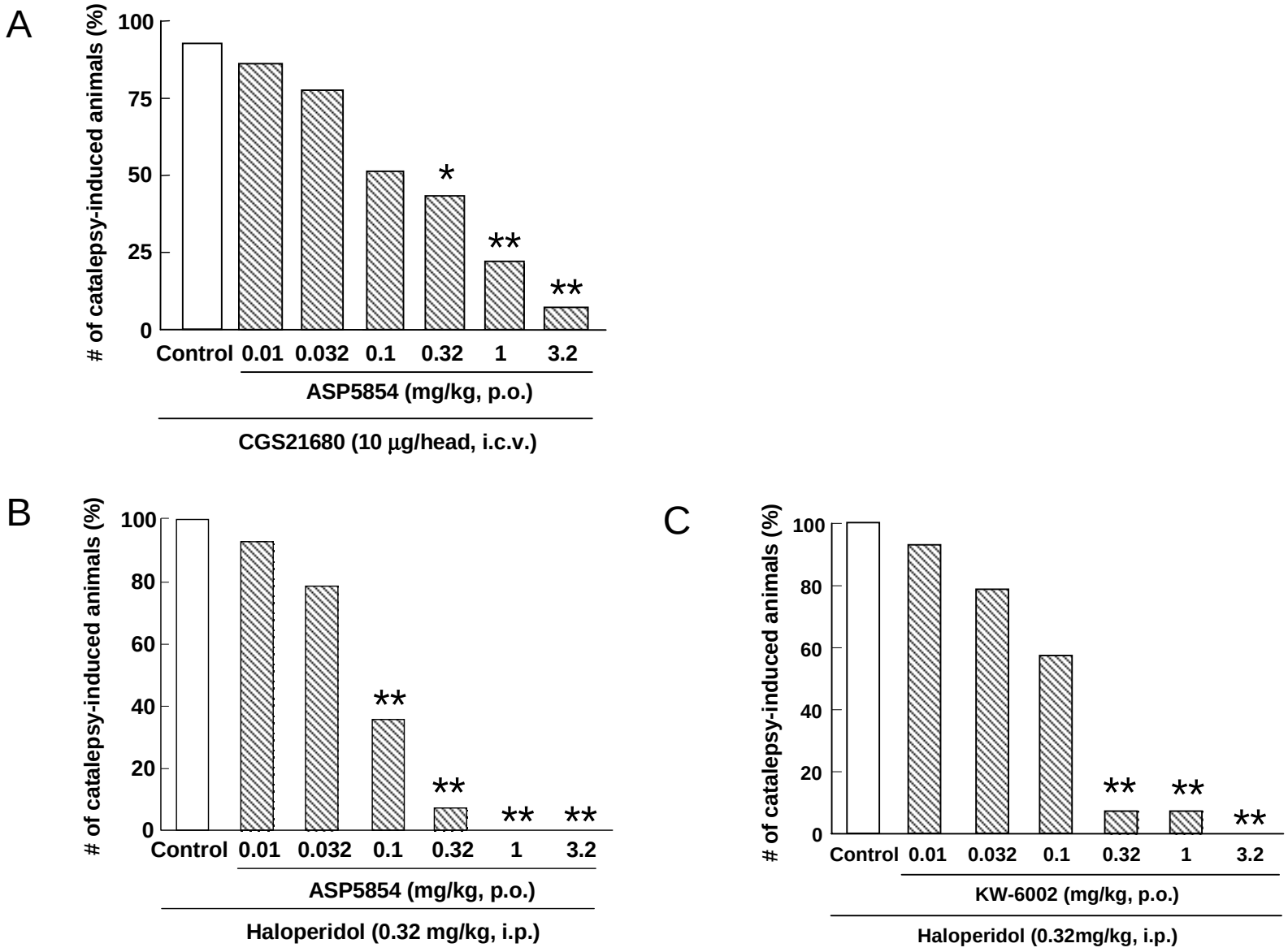

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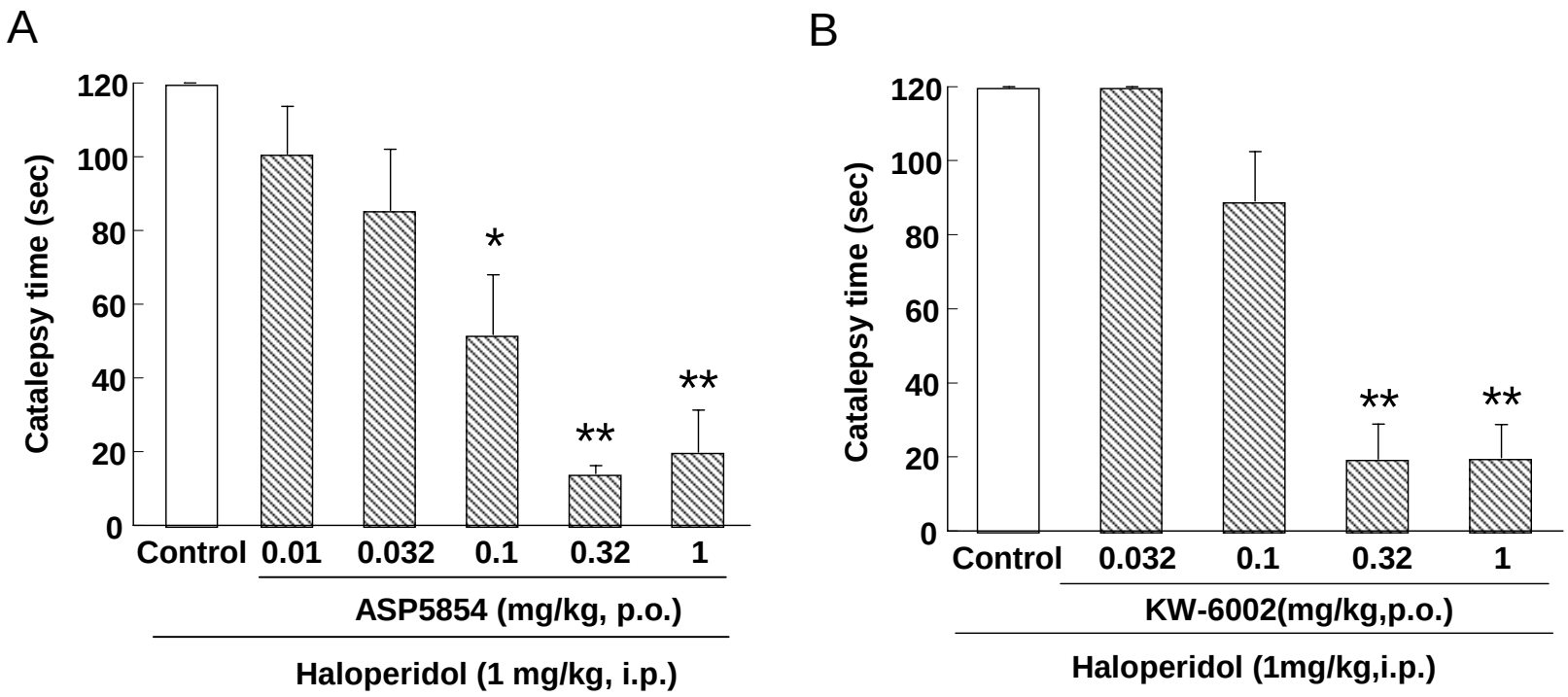

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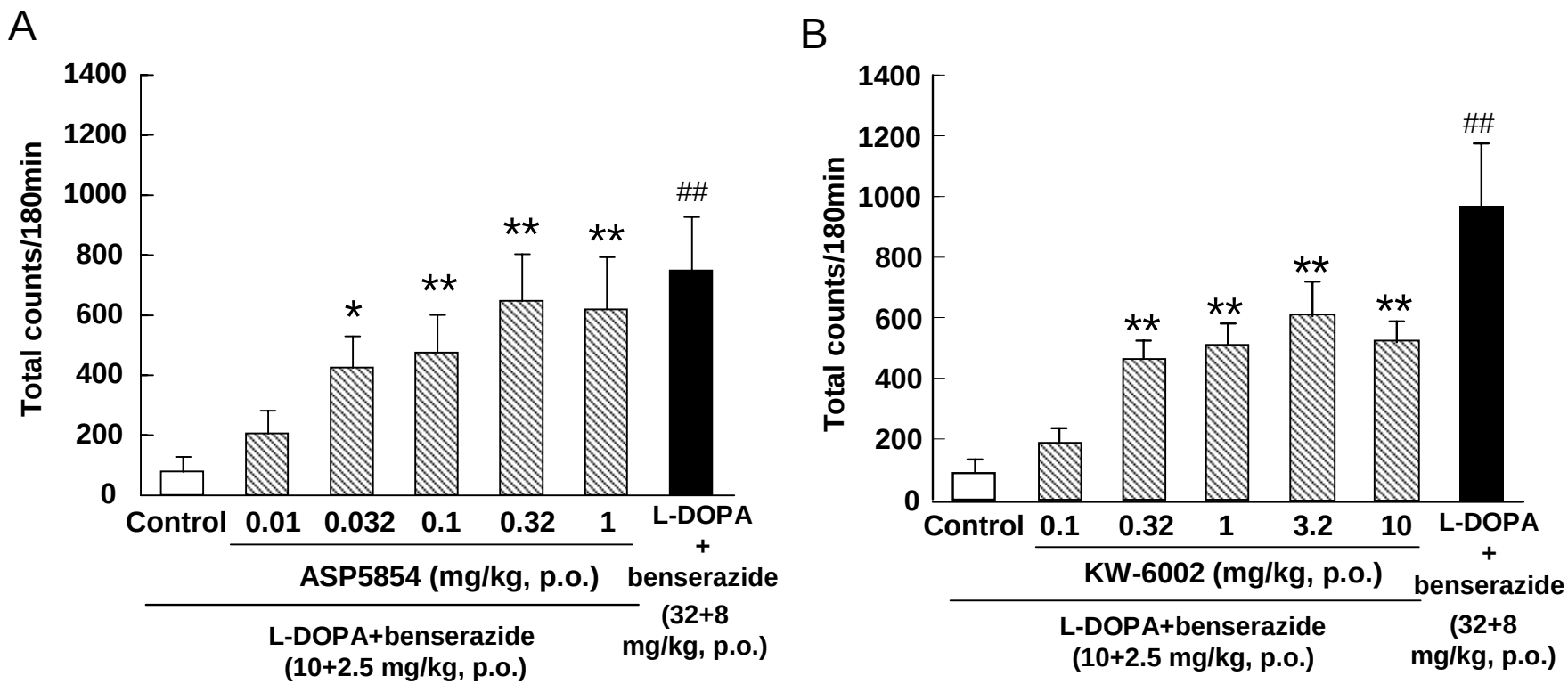

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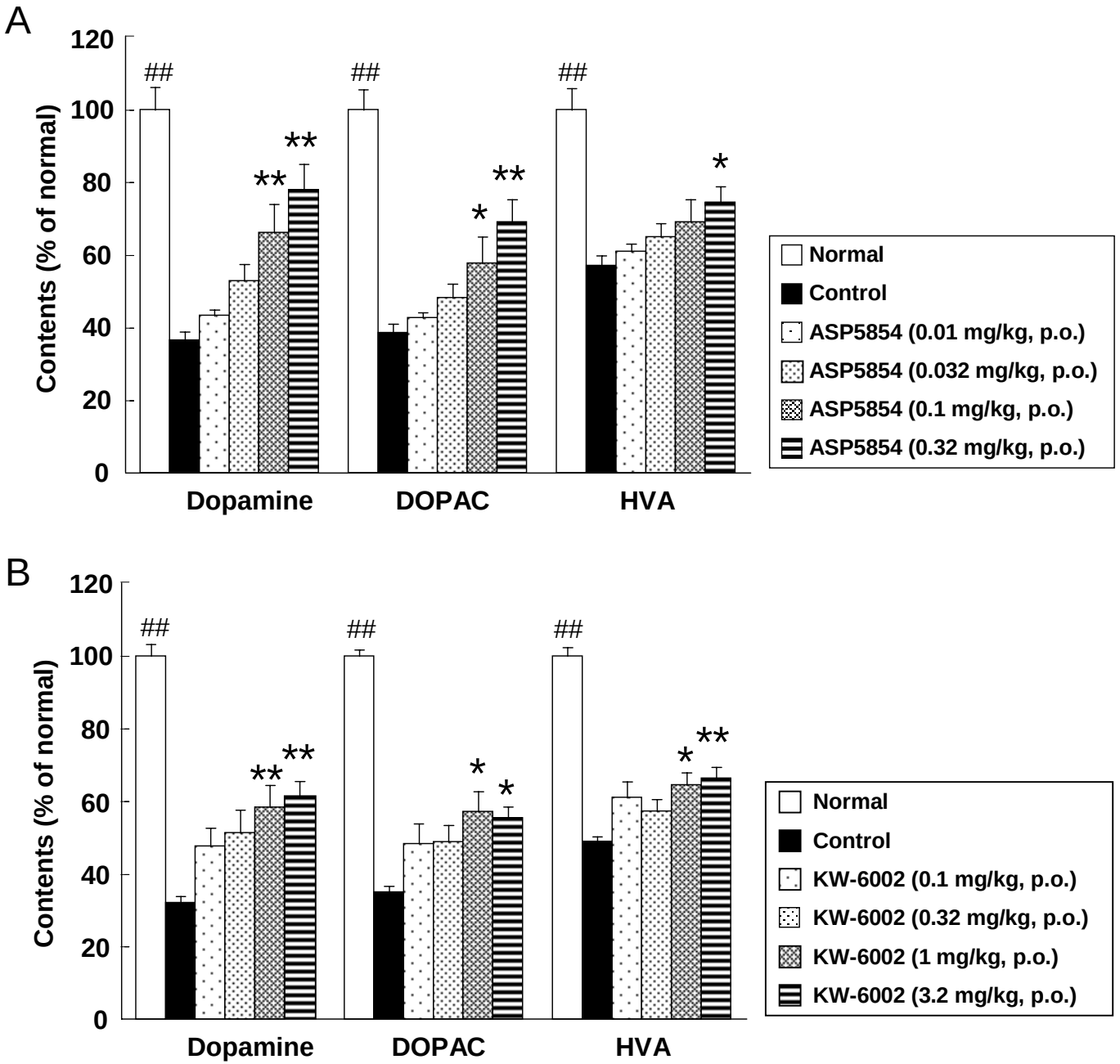

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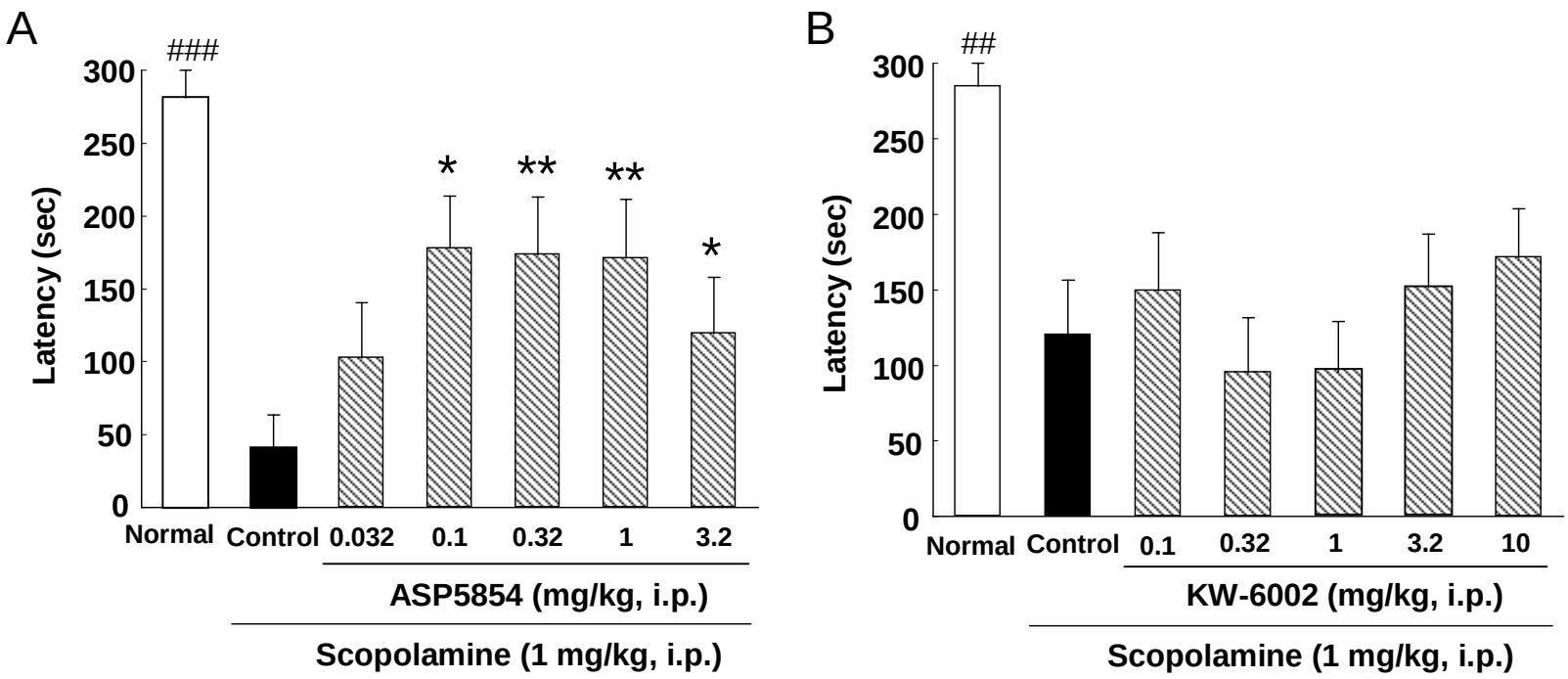

Fig. 8

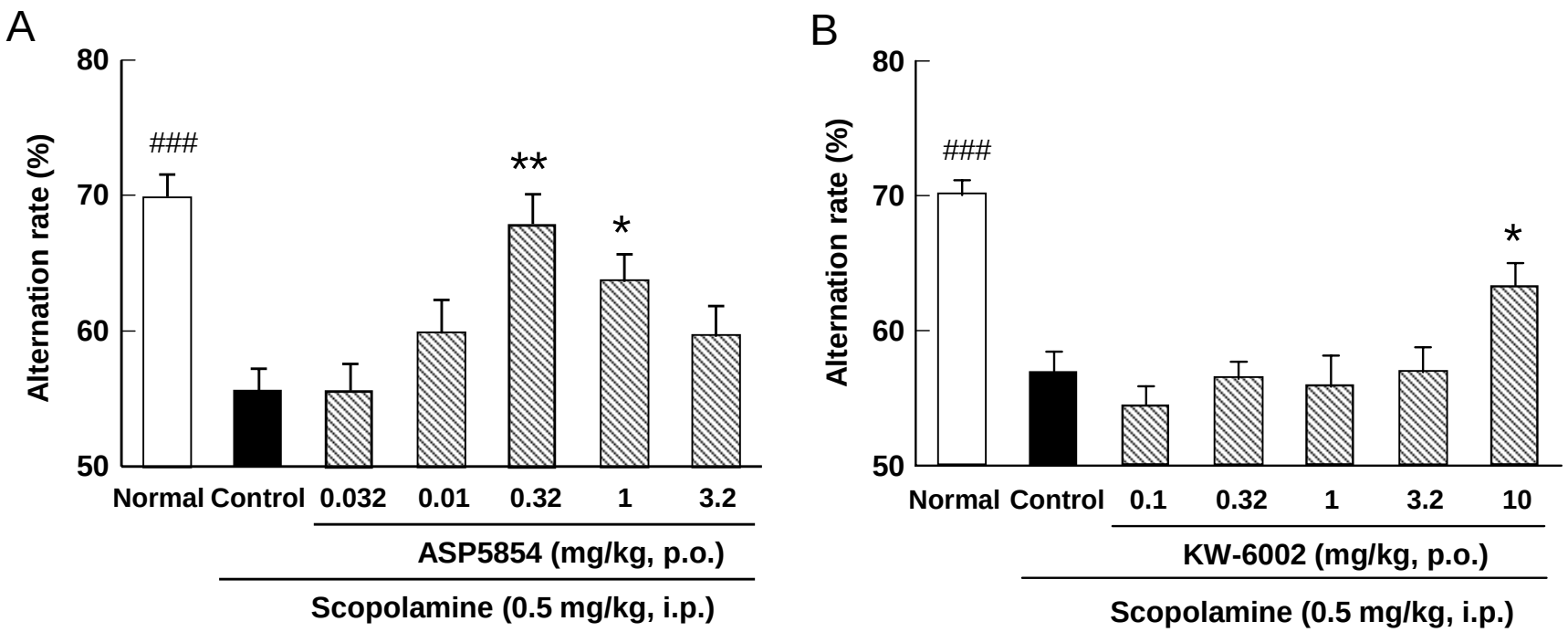

Fig. 9