Characterization of the Potent and Highly Selective A$_{2A}$ Receptor Antagonists Preladenant and SCH 412348 [7-[2-[4-2,4-Difluorophenyl]-1-piperazinyl]ethyl]-2-(2-furanyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine] in Rodent Models of Movement Disorders and Depression


Received December 9, 2008; accepted March 27, 2009

ABSTRACT

The adenosine A$_{2A}$ receptor has been implicated in the underlying biology of various neurological and psychiatric disorders, including Parkinson's disease (PD) and depression. Preladenant and SCH 412348 [7-[2-[4-2,4-Difluorophenyl]-1-piperazinyl]ethyl]-2-(2-furanyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine] are potent competitive antagonists of the human A$_{2A}$ receptor ($K_i$ 1.1 and 0.6 nM, respectively) and have >1000-fold selectivity over all other adenosine receptors, making these compounds the most selective A$_{2A}$ receptor antagonists reported to date. Both compounds attenuate hypolocomotion induced by the A$_{2A}$ receptor agonist CGS-21680 [2-[(p-(2-carboxyethyl)phenethylamino)-5'-N-ethylcarboxamidoadenosine], suggesting that they inhibit A$_{2A}$ receptor activity in vivo. Their high degree of selectivity and robust in vivo activity make preladenant and SCH 412348 useful tools to investigate the role of the A$_{2A}$ receptor system in animal models of PD and depression. Oral administration of preladenant and SCH 412348 (0.1–1 mg/kg) to rats potentiated 3,4-dihydroxy-L-phenylalanine (L-Dopa)-induced contralateral rotations after 6-hydroxydopamine lesions in the medial forebrain bundle and potently attenuated the cataleptic effects of haloperidol. Preladenant (1 mg/kg) inhibited L-Dopa-induced behavioral sensitization after repeated daily administration, which suggests a reduced risk of the development of dyskinesias. Finally, preladenant and SCH 412348 exhibited antidepressant-like profiles in models of behavioral despair, namely the mouse tail suspension test and the mouse and rat forced swim test. These studies demonstrate that preladenant and SCH 412348 are potent and selective A$_{2A}$ receptor antagonists and provide further evidence of the potential therapeutic benefits of A$_{2A}$ receptor inhibition in PD (with reduced risk of dyskinesias) and depression (one of the primary nonmotor symptoms of PD).

Adenosine modulates neuronal function via its interaction with glutamatergic, cholinergic, GABAergic, and dopaminergic neurotransmitter systems (Kurokawa et al., 1996; Latini et al., 1996; Mori and Shindou, 2003; Popoli et al., 2003). There are four G-protein-coupled adenosine receptors (A$_{1}$, A$_{2A}$, A$_{2B}$, and A$_{3}$), so classified based on pharmacology and signal transduction mechanisms (Jacobson and Gao, 2006). Of these subtypes, the A$_{2A}$ receptor has received the most attention in the context of Parkinson's disease and depression.

ABBREVIATIONS: DA, dopamine; PD, Parkinson’s disease; Ro 201724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidone; L-Dopa, levodopa (3,4-dihydroxy-L-phenylalanine); KW-6002, istradefylline; 6-OHDA, 6-hydroxydopamine; SCH 58261, 5-amino-7(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine; FST, forced swim test; TST, tail suspension test; CGS-21680, 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine; HEK, human embryonic kidney; LMA, locomotor activity; SCH 412348, 7-[2-[4-2,4-difluorophenyl]-1-piperazinyl]ethyl]-2-(2-furanyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine; ZM-241385, (4-(2'-7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino)ethyl)phenol.
interest because it mediates part of the established interaction of adenosine and dopamine (DA) involved in the regulation of movement (Ferré et al., 1992). A$_{2A}$ receptors are distributed in areas of the basal ganglia associated with the dopaminergic nigrostriatal and mesolimbic neuronal pathways, including the striatum, globus pallidus, nucleus accumbens, and olfactory tubercle (Rosin et al., 1998). Within the striatum, A$_{2A}$ receptors are predominantly localized on GABAergic, enkephalin-expressing striatopallidal neurons, where they are colocalized with DA D$_2$ receptors (Hettinger et al., 2001). These neurons form part of the "indirect" pathway that, along with the "direct" pathway, projects to the globus pallidus and substantia nigra and is involved in the control of fine motor movement (Alexander and Crutcher, 1990).

Parkinson’s disease (PD) is characterized by degeneration of nigrostriatal dopaminergic neurons, which results in an imbalance in the direct and indirect pathways. Pharmacological treatment of PD by improving dopaminergic transmission through the enhancement of DA release with L-Dopa or direct activation of DA D$_2$/D$_3$ receptors has been relatively successful but often has been accompanied by serious side effects, including dyskinesias, somnolence, and compulsive behavior (Obeso et al., 1989; Cantor and Stern, 2002; Driver-Dunkley et al., 2003). Because of the functionally opposing roles of A$_{2A}$ and D$_2$ receptors on indirect pathway neurons, A$_{2A}$ antagonists represent a potentially novel approach to the treatment of PD, either as monotherapy or as adjunctive therapy with L-Dopa and DA agonists. A$_{2A}$ antagonists facilitate intrastriatal GABA release (Ferré et al., 1997), helping to restore the indirect inhibitory output from the striatum to the globus pallidus, subthalamic nucleus, and thalamus without producing the issues associated with chronic DA receptor stimulation (Ferré et al., 1997; Richardson et al., 1997). A$_{2A}$ receptor antagonists also have been shown to attenuate catalepsy induced by D$_2$ receptor blockade in rodents (Mandhane et al., 1997; Shiozaki et al., 1999). Furthermore, pretreatment with the A$_{2A}$ receptor antagonist KW-6002, dosed either alone or in conjunction with L-Dopa, improved motor function in 6-hydroxydopamine (6-OHDA)-lesioned rats in the Rotorod test (Lundblad et al., 2003). KW-6002 also has been shown to attenuate Parkinsonian-like motor impairments in cynomolgus monkeys (Grondin et al., 1999) and common marmosets (Kanda et al., 1998) treated with the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

PD patients also suffer from nonmotor symptoms, including a 45% prevalence of depression (Lemke, 2008); current PD therapies do not treat depressive symptoms. A$_{2A}$ receptors have been shown to have a link to the pathology of depression. Rodent studies using adenosine analogs, synthetic agonists, or compounds that raise endogenous levels of adenosine have demonstrated that these approaches can produce a depression-like profile of behavioral despair (Woodson et al., 1998). Conversely, the A$_{2A}$ receptor antagonists, KW-6002, SCH 58261, and ZM-241385, produced antidepressant-like effects in the mouse forced swim test (FST) and tail suspension test (TST). Furthermore, A$_{2A}$ receptor knockout mice display reduced immobility time in these same behavioral tests (El Yacoubi et al., 2001).

Neustadt et al. (2007) recently reported the discovery of high-affinity A$_{2A}$ receptor antagonists with improved selectivity and oral activity through modification of the parent compound, SCH 58261. The aim of the present studies was to assess the profile of two of these compounds, preladenant (A$_{2A}$ $K_i = 1.1$ nM) and SCH 412348 (A$_{2A}$ $K_i = 0.6$ nM), in rodent models of movement disorders and behavioral despair.

### Materials and Methods

#### Animals

Male CD rats (Charles River Laboratories, Inc., Wilmington, MA) weighing 200 to 240 g were used for catalepsy and hypolocomotion studies. Rats were housed three or four per cage in a temperature- and humidity-controlled environment, with food and water available ad libitum. They were maintained on a 12-h light/dark cycle (lights on 7:00 AM, lights off 7:00 PM), and all studies were conducted between 9:00 AM and 5:00 PM. All studies involving animals were conducted at an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility in conformity with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and the Animal Welfare Act.

Male CD rats weighing 275 to 300 g were used for unilateral 6-OHDA lesion experiments, and male CD1 mice (Charles River Laboratories, Inc.) weighing 25 to 27 g were used for depression studies. Animals were housed under a normal 12-h light/dark cycle (lights on 7:00 AM, lights off 7:00 PM) with food and water available ad libitum. All behavioral testing was performed between 8:00 AM and 2:00 PM. These procedures involving the use of animals and their care were conducted in conformity with institutional guidelines and in compliance with the European Community Council Directive (OJ L 358, 1, December 12, 1987).

#### Drugs and Injections

Preladenant and SCH 412348 were synthesized by the Medicinal Chemistry Department of the Schering-Plough Research Institute and administered orally in 50% polyethylene glycol 400 at a dose volume of 3 to 5 ml/kg. Haloperidol (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.1 M hydrochloric acid and 0.9% saline, and CGS-21680 hydrochloride (Sigma-Aldrich) was dissolved in saline. Both compounds were administered in a volume of 1 ml/kg s.c. 6-OHDA (Sigma-Aldrich) was dissolved in saline containing 0.05% acetic acid and infused at a final concentration of 2 µg/µl (8 µg total). Desipramine and L-Dopa (3,4-dihydroxy-l-phenylalanine; Sigma-Aldrich) were dissolved in distilled water and administered at a dose volume of 3 ml/kg i.p. Benserazide hydrochloride (Sigma-Aldrich) was dissolved in saline and administered at a dose volume of 3 ml/kg i.p. All doses are expressed as free base.

#### In Vitro Adenosine Receptor Binding

Receptor binding was performed using membranes prepared from cells with recombinant expression of adenosine receptors as follows: human A$_{2A}$ and HEK 293 (Receptor Biology, Inc., Boston, MA), rat A$_{2A}$ and Chinese hamster ovary (PerkinElmer Life and Analytical Sciences, Waltham, MA), human and rat A$_{1}$, and Chinese hamster ovary (PerkinElmer Life and Analytical Sciences), and human A$_{3}$ and HEK 293. Radioligand competition binding assays were performed in 96-well plates in a total assay volume of 200 µl using a final test drug concentration range of between 0.1 and 3 µM. Membranes were diluted in assay buffer, pH 7.4 (A$_{1}$ and A$_{2A}$), Dulbecco’s phosphate-buffered saline with 10 mM MgCl$_2$; A$_{2A}$, 50 mM Tris-HCl, 120 mM NaCl, 10 mM MgCl$_2$). To remove endogenous adenosine from the membrane preparations, 4 U/mg adenosine deaminase (Roche Applied Science, Indianapolis, IN) was added to the membranes, which were then incubated at room temperature for 15 min. Radioligand was added to a final concentration of 0.5 ([$^3$H]SCH 58261, A$_{2A}$) and 1 ([$^3$H]DPCPX, A$_{1}$) or 0.25 ([$^{125}$I]AB-MECA, A$_{3}$) nM.
Non-specific binding was defined by adding 100 nM CGS 15923 (A2A), 100 nM NECA (A1), or 100 nM DPCPX (A3). Plates were incubated at room temperature with agitation for 1.5 h (A2A and A1) or 2 h (A3). Membranes were filtered onto Packard GF-B filter plates and washed in ice-cold assay buffer using a Brandel cell harvester (Brandel Inc., Gaithersburg, MD) to separate bound and free radioligand. The plates were dried before addition of 45 μl of Microscint 20 to each well. IC50 values were determined by fitting the displacement curves using an iterative curve-fitting program (Prism; GraphPad Software Inc., San Diego, CA). Kv values were calculated using the Cheng-Prusoff equation.

**cAMP Measurement**

HEK 293 cells stably expressing either human A2A or A3 receptors were grown to confluence, harvested using enzyme-free cell dissociation buffer (Invitrogen, Carlsbad, CA) and pelleted by centrifugation (1000g; 5 min). The cells were washed and diluted to a final density of 4 × 10^6 cells/ml in Hank’s balanced salt solution (Invitrogen) supplemented with 10 nM HPS, pH 7.4, 5 mM MgCl2, and 0.2% bovine serum albumin. Test compounds were diluted in the above buffer with inclusion of the following components to achieve the respective final assay concentrations: 0.25% dimethyl sulfoxide, 2 U/ml adenosine deaminase, and 100 μM Ro 201724. Cell suspensions (20 μl) were preincubated for 15 min at room temperature in 96-well plates containing 25 μl of vehicle or test compound. CGS 21680 (A2A) or 5-n-cyclopropylcarboxamidoadenosine (A3B) at 10-fold the desired concentration was then added, and the reactions were incubated for 15 min at 37°C. The reactions were terminated by the addition of 50 μl of assay buffer (Applied Biosystems, Foster City, CA). The concentration response curves for CGS 21680 in the presence and absence of the test compound were plotted, and the EC50 values were determined by fitting the curves using GraphPad Prism software. The Ka values for preladenant and SCH 412348 were determined by the dose-ratio method (Lazareno and Birdsell, 1993).

**Haloperidol-Induced Catalepsy in the Rat**

The dopamine D2 receptor antagonist, haloperidol (1 mg/kg s.c.) was administered to induce catalepsy. Thirty minutes after haloperidol administration, rats experience a full cataleptic response. Therefore, at that time point, each rat was placed on a wire mesh screen inclined at 60° with their heads facing up and their forelimbs and hindlimbs extended. The time taken for the rat to make a forelimb movement was measured with a cut-off time of 120 s. Only rats that remained cataleptic for the entire 120 s were used for subsequent drug studies. Test drugs (preladenant and SCH 412348) were administered orally after the 30-min baseline measure, and catalepsy was retested 1 and 4 h after administration. Haloperidol was not readministered.

**CGS-21680-Induced Hypolocomotion in the Rat**

Locomotor activity (LMA) was measured using an automated photobeam system (Digiscan; AccuScan Instruments, Inc., Columbus, OH). In the dose-response studies, rats were pretreated with an oral dose of vehicle, preladenant, or SCH 412348, followed 30 min later by an injection of a dose of the A2A receptor agonist, CGS-21680 (1 mg/kg s.c.). Ten min later, the rats were placed into one of eight Plexiglas LMA chambers. Drug doses were balanced across the eight chambers. Total distance traveled (measure of ambulation) during a 30-min test was automatically measured by a computer. In the duration of action studies, the identical apparatus and test session were used. However, a 1 mg/kg dose of preladenant was administered at various pretreatment times (1, 4, 8, and 12 h) before an injection of CGS-21680.

**Ex Vivo Receptor Occupancy**

Rats were administered vehicle or SCH 412348 (0.01–1 mg/kg) 4 h before euthanization by exposure to CO2 and decapitation. The brains were removed, and the striata and a portion of the cerebral cortex were dissected. The tissues were homogenized in 10 mM MgCl2 in Dulbecco’s phosphate-buffered saline, and the homogenates were stored at −80°C. Protein concentration was determined by the Pierce Protein BCA kit (Pierce Chemical, Rockford, IL). For the binding assay, each homogenate was preincubated with 10 U/ml adenosine deaminase for 15 min at room temperature. [3H]SCH 52681 (final concentration, 0.5 nM) was added, and the mixture was incubated for 15 min at room temperature. The membrane receptor/ligand complex was collected onto a 96-well GF/B filter plate under vacuum, washed free of unbound ligand with cold water, and allowed to dry at 57°C. The plate was counted in a Microplate after the addition of 45 μl of Microscint 20. Specific binding was defined as the counts derived from striatal tissue minus the counts from the cerebral cortex.

**Potentiation of L-Dopa-Induced Rotations in 6-OHDA-Lesioned Rats**

Animals were anesthetized by intraperitoneal administration of chloral hydrate (400 mg/kg) and treated with desipramine (10 mg/kg i.p.) 30 min before 6-OHDA injection to block the uptake of the toxin by noradrenergic terminals. Rats were subsequently placed in a stereotaxic frame, the skin over the skull was reflected, and a burr hole was drilled through the skull at the following stereotaxic coordinates: −2.2 posterior from bregma (anterior-posterior) and +1.5 lateral from bregma (medial-lateral). Subsequently, a total of 8 μg of 6-OHDA dissolved in 4 μl of saline containing 0.05% ascorbic acid was infused over 7.8 mm ventral to the dura at a constant flow rate of 1 μl/min using a 48-gauge needle attached to an infusion pump. Two weeks after the lesion, rats were administered L-Dopa (50 mg/kg i.p.) and benserazide (25 mg/kg i.p.) and selected on the basis of the number of full contralateral turns quantified by an automated rotametry system during a 2-h testing period (priming test). Rats that made fewer than 200 complete turns were not included in the subsequent studies.

To test the effect of the A2A antagonists in this assay, preladenant and SCH 412348 were delivered 40 min before the delivery of benserazide. L-Dopa was delivered 20 min later and placed in the rotametry chambers. The number of contralateral rotations was recorded during a 2-h test.

**Behavioral Sensitization Experiments**

**L-Dopa Dose Finding.** Rats were randomly assigned to two groups. One group received one of three doses of L-Dopa (4, 6, or 8 mg/kg), and the second group received the same doses but with an additional administration of 0.3 mg/kg preladenant 1 h before testing. The number of rotations was recorded in the rotametry system for 140 min after L-Dopa administration.

**Prevention of L-Dopa Sensitization.** Rats were randomly divided into two groups. One group received a combination treatment of vehicle and L-Dopa (6 mg/kg) twice a day for 23 days. The second group received a combination treatment of preladenant (0.3 mg/kg) and L-Dopa (4 mg/kg) twice a day for 23 days. Daily treatments were administered in the morning (between 8:00 AM and 10:00 AM) and 12 h later. Rotational behavior was measured on day 1 (i.e., after the first treatment) and on days 4, 8, 12, 15, 18, and 23 at a fixed time in the morning for each animal.

**Depression Model Studies**

**Mouse FST.** In the FST, mice were placed individually into glass cylinders filled to a depth of 10 cm with water (25°C) and left for 6 min. A mouse was judged to be immobile when it floated in an upright position and made only small movements to keep its head above water. The duration of immobility was recorded during the last 4 min of the 6-min testing period by an observer blind to the treatment of the animals. Animals were dosed with vehicle, preladenant, or SCH 412348 1 h before behavioral testing.
Mouse TST. In the TST, mice were suspended by the tail and displayed alternate periods of agitation and immobility. The animals were observed for a period of 6 min, and the duration of time the animals spent immobile was recorded by an observer blind to the drug treatment of the animals. Animals were dosed with vehicle, preladenant, or SCH 412348 1 h before behavioral testing.

Rat Forced Swim Test. Each rat was placed individually in a cylinder of water (25°C) and left to swim for 15 min before being removed and dried in a heated enclosure and returned its home cage. Twenty-four hours later (test day), the animal was re-exposed to the conditions described above, and the total immobility time during a 5-min period was recorded. In addition, the duration of time that the rats spent climbing the sides of the cylinder was recorded. On test day, each animal was dosed with preladenant, SCH 412348, or vehicle 1 h before behavioral testing.

Mouse Locomotor Activity
The mice were transferred from their colony and allowed to habituate to the testing room for a minimum of 30 min. Four hours after drug or vehicle treatment, the mice were individually placed in a Plexiglas chamber (24 × 24 cm) and allowed to explore for 1 h. During this time, photo beams mounted on the chamber walls (Coulbourn Instruments, Allentown, PA) measured total distance traveled by the mice.

Statistical Analysis
Data from catalepsy, LMA, ex vivo binding, 6-OHDA rotation, FST, and TST studies were analyzed by one-way analysis of variance followed by Dunnett’s post hoc test. The studies evaluating the time course of the two treatments for dyskinesia studies were evaluated by within-subject one-way analysis of variance followed by Dunnett’s post hoc test, whereas data comparing the 1st day of treatment with other treatment days were analyzed using the paired Student’s t test. In all studies, the significance level for effects was p < 0.05.

Results

Characterization of Adenosine Receptor Affinity in Recombinant Receptor Expression Systems

Preladenant and SCH 412348 are structurally novel A2A receptor antagonists described previously by Neustadt et al. (2007) (Fig. 1). The affinities of preladenant for human adenosine receptors were determined using competition binding assays. Preladenant and SCH 412348 bound A2A receptors with high affinity; Kᵢ values were 1.1 and 0.6 nM, respectively. When tested under the same conditions, KW-6002 had a Kᵢ value at the A₂A receptor of 6.6 nM. Both preladenant and SCH 412348 were greater than 1000-fold selective over the A₁ and A₃ adenosine receptor subtypes as assessed by radioligand binding, whereas KW-6002 was only 82-fold selective for the A₂A receptor over the A₁ receptor. Preladenant and SCH 412348 also completely antagonized cAMP in cells expressing the recombinant human A₂A receptor (Fig. 2). Preladenant and SCH 412348 were determined to have Kᵢ values of 1.3 and 0.3 nM, respectively at the A₂A receptor; these values are in good agreement with the Kᵢ values determined in radioligand binding assays. Neither compound demonstrated agonist activity when tested in the absence of agonist. A similar functional assay with A₂B receptor-expressing cells was used to demonstrate selectivity over A₂B receptors. In this assay, the Kᵢ values for preladenant and SCH 412348 were 1.2 μM and 273 nM, indicating that these two compounds were 923- and 910-fold selective for the A₂A receptor over the A₂B receptor. In addition to adenosine receptors, assays for over 60 additional receptors, ion channels, and transporters were conducted. At a concentration of 10 μM, no significant interaction of preladenant and SCH 412348 at any of these targets was observed (data not shown).

Attenuation of CGS-21680-Induced Hypolocomotion

In the hypolocomotion study with preladenant, there was a significant main effect of treatment [F(5,54) = 3.9, p < 0.05], with post hoc analysis indicating that CGS-21680 induced a statistically significant hypolocomotion compared with vehicle. Preladenant attenuated CGS-21680-induced hypolocomotion with significant reversal at doses of 0.1 and 1 mg/kg (Fig. 3A). In the SCH 412348 study, there was a significant main effect of treatment [F(5,89) = 4.3, p < 0.01], with post hoc analysis indicating that CGS-21680 induced a statistically significant hypolocomotion compared with vehicle. SCH 412348 produced a dose-dependent attenuation of the CGS-21680-induced hypolocomotion with significant reversal at doses from 0.3 to 1 mg/kg (Fig. 3B).

In the duration of action study, there was a significant main effect of treatment [F(5,54) = 9.8, p < 0.01], with post hoc analysis indicating that CGS-21680 induced a significant hypolocomotion compared with vehicle. Preladenant (1 mg/kg) produced a significant attenuation of the CGS-21680-induced hypolocomotion for up to 8 h (data not shown).

Attenuation of Haloperidol-Induced Catalepsy

Preladenant dose-dependently attenuated the cataleptic effects of haloperidol 1 h [F(3,20) = 5.0, p < 0.01] and 4 h [F(3,20) = 9.8, p < 0.01] after dosing, with statistically significant effects at doses of 0.3 and 1 mg/kg at both time points (Fig. 4A). Likewise, SCH 412348 (1 and 3 mg/kg) dose-dependently attenuated haloperidol-induced catalepsy 1 h [F(3,20) = 3.9, p < 0.05] and 4 h [F(3,20) = 7.5, p < 0.01] after dosing (Fig. 4B).

Potentiation of L-Dopa-Induced Rotations in 6-OHDA-Lesioned Rats

The administration of either preladenant or SCH 412348 to 6-OHDA-lesioned rats did not result in asymmetric turning behavior at doses up to 10 mg/kg (data not shown). However, both compounds induced marked contralateral turning when administered before a subthreshold dose of L-Dopa (Fig. 5). The effects were dose-dependent for preladenant (0.01–1 mg/kg) [F(5,21) = 8.6, p < 0.01] and SCH 412348 (0.1–3 mg/kg) [F(3,31) = 20.0, p < 0.01]. In the presence of a subthreshold dose of L-Dopa (4 mg/kg), preladenant induced significant contralateral turning at 1, 6, and...
12 h after drug administration (Fig. 6A) but was not active 18 h after administration $[F(4,24) = 13.2, p < 0.01]$. Likewise, the combination of a subthreshold dose of SCH 412348 evoked significantly higher contralateral turning compared with vehicle-treated animals at 1 and 6 h, returning to basal levels by 12 h $[F(3,30) = 18.1, p < 0.01]$.

**Ex Vivo Receptor Occupancy**

Occupancy of striatal $A_{2A}$ receptors by preladenant (1 mg/kg) was also tested 3, 6, 12, and 18 h after administration. There was a time-dependent receptor occupancy that mirrored the time-dependent effect of the compound on L-Dopa-induced contralateral rotations (Fig. 6B). SCH 412348 (0.01, 0.03, 1 mg/kg) produced a dose-dependent occupancy of striatal $A_{2A}$ receptors as measured by an ex vivo binding assay with $^3$H$\text{SCH 58261}$. All doses significantly displaced ex vivo $^3$H$\text{SCH 58261}$ binding compared with the vehicle control group $[F(5,30) = 152, p < 0.01]$, with the highest doses of 0.3 and 1 mg/kg SCH 412348 occupying approximately 73 and 83% of the striatal $A_{2A}$ receptors, respectively (data not shown).

**Dyskinesia Experiments**

### L-Dopa Dose Finding

Preladenant (0.3 mg/kg), dosed 60 min before a subthreshold dose of L-Dopa (4 mg/kg), elicited a marked contralateral turning behavior in 6-OHDA rats (Table 1). To establish the dose of L-Dopa that produced the same turning behavior intensity as the L-Dopa-preladenant combination, dose-response studies were performed by administering L-Dopa at 4, 6, and 8 mg/kg. A dose of 6 mg/kg L-Dopa alone induced a similar number of contralateral turns to that induced by L-Dopa (4 mg/kg) in combination with preladenant (0.3 mg/kg). Thus, a dose of 6 mg/kg L-Dopa was chosen for the following experiments.

### Prevention of L-Dopa-Induced Sensitization

6-OHDA-lesioned rats were chronically treated twice a day for 23 days with preladenant (0.3 mg/kg) and L-Dopa (4 mg/kg) or with vehicle (orally) and L-Dopa (6 mg/kg). The rotational response to
L-Dopa alone gradually increased, reaching a plateau on days 12 to 18 before decreasing slightly by day 23 (Fig. 7). The L-Dopa-induced turning behavior in this group was significantly different between day 1 and days 4, 8, 12, 15, 18, and 23 (p < 0.05, df = 10; paired Student’s t test). In contrast, rats receiving preladenant in combination with L-Dopa displayed a rotational response on all subsequent days of the study that was not significantly different from day 1 (Fig. 7).

Depression Model Studies

Mouse FST. Preladenant [F(3,34) = 9.7, p < 0.01] and SCH 412348 [F(2,43) = 16.5, p < 0.01], at 1 mg/kg, reduced immobility time in the mouse FST (Fig. 8). Again, the effects were similar to those of desipramine, which reduced immobility with a minimal effective dose of 3 mg/kg (Fig. 8) [F(3,56) = 8.1, p < 0.01].

Rat Forced Swim Test. Preladenant (0.1–1 mg/kg) produced a dose-dependent reduction in immobility time in the rat FST [F(3,64) = 16.9, p < 0.01], with effects observed as low as 0.3 mg/kg (Fig. 9). Preladenant also increased the time spent climbing [F(3,64) = 4.1, p < 0.01] at 0.1 to 1 mg/kg. Desipramine (40 mg/kg) also decreased the immobility time and induced a marked activation of climbing behavior (Fig. 9) [p < 0.01, df = 2].

Mouse Locomotor Activity. SCH 412348 significantly increased activity levels in the mouse [F(4,27) = 2.9, p <...
establishes the balance between the inhibitory and excitatory (direct) pathways required for the optimal control of fine motor movement. As such, A2A receptor antagonists are an attractive putative target for the treatment of PD. Here, we assessed the effect of two novel, high-affinity, highly selective A2A receptor antagonists, preladenant and SCH 412348, in animal models of PD and depression. The profiles of these compounds represent substantial improvements on both the selectivity and affinity (5–10 fold) of the well characterized A2A antagonist KW-6002, which makes them excellent tools for investigating the role of the A2A receptor in movement disorders.

Preladenant and SCH 412348 both reversed the hypolocomotion induced by treatment with the A2A agonist CGS-21680 and achieved substantial striatal A2A receptor occupancy after oral doses > 0.1 mg/kg. Furthermore, both compounds maintain substantial receptor occupancy and reduced CGS-21680-induced hypolocomotion for at least 6 h after a dose of 1 mg/kg. These data confirm that preladenant and SCH 412348 achieve substantial occupancy of brain A2A receptors at relatively low doses that also cause effects in vivo. Neither compound displays affinity for any of the DA receptors, monoamine transporters, or monoamine oxidase, suggesting that the mechanism does not involve direct manipulation of the DA system.

Both preladenant and SCH 412348 potentiated l-Dopa-induced contralateral rotations in unilaterally 6-OHDA-lesioned rats, which is consistent with previous findings using less selective A2A receptor antagonists in this model (e.g., Koga et al., 2000). The time course of the effect on contralateral rotation mimics the time course of occupancy of striatal A2A receptors. In addition, both preladenant and SCH 412348 were effective in reducing catalepsy induced by the D2 receptor antagonist, haloperidol, which is consistent with a previous report of SCH 412348 blocking haloperidol-induced catalepsy in squirrel monkeys (Varty et al., 2008). These findings suggest that the compounds are acting, as hypothesized, to provide counterbalance to the loss of D2 receptor-mediated effects on the indirect pathway.

Anticataleptic properties have been predictive of clinical efficacy for other antiparkinsonian agents. For example, compounds with potent D2 receptor agonist activity, such as pramipexole have, also been shown to block catalepsy in this

### Table 1

<table>
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<tr>
<th>Treatment Group</th>
<th>Number of Rotations</th>
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<tr>
<td>Preladenant (0.3 mg/kg) + L-Dopa (4 mg/kg)</td>
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<tr>
<td>Vehicle + L-Dopa (4 mg/kg)</td>
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<td>Vehicle + L-Dopa (6 mg/kg)</td>
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<td>Vehicle + L-Dopa (8 mg/kg)</td>
<td>647 ± 176</td>
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Fig. 6. Duration of the effect of preladenant (1 mg/kg) on l-Dopa-induced contralateral rotations in 6-OHDA-lesioned rats correlates with receptor occupancy. The data represent mean ± S.E.M. after various pretreatment times (1–18 h) (n = 4–8 per group). A, number of contralateral rotations in a 2-h test in 6-OHDA-lesioned rats after treatment with preladenant. B, in a separate group of animals, ex vivo receptor occupancy was measured using competitive binding procedure with a radiolabeled A2A antagonist, [3H]SCH 58261. *p < 0.05; **p < 0.01 versus vehicle (Veh).

Fig. 7. Effect of repeated treatments with preladenant + l-Dopa or l-Dopa in 6-OHDA-lesioned rats. The data represent the numbers of contralateral turnings evaluated during a 2-h test on days 1, 4, 8, 12, 15, 18, and 23. ■, l-Dopa treatment (6 mg/kg p.o.); ▴, l-Dopa (4 mg/kg) + preladenant (0.3 mg/kg) (n = 11 per group). *p < 0.05; **p < 0.01 versus day 1.

0.05) (Fig. 10). Both the 0.3 and 3 mg/kg treatment groups were significantly more active than vehicle-treated mice. The 1 mg/kg group approached significance (p = 0.052).

### Discussion

The progressive loss of the inhibitory influence of DA through activation of D2 receptors on the striatopallidal indirect pathway leads to the paucity of movement characteristic of PD. Adenosine A2A receptors are colocalized with D2 receptors on the GABA-releasing striatopallidal neurons of the indirect pathway. Blockade of A2A receptors in the indirect pathway reduces hyperactive striatopallidal GABAergic activity, which then restores the inhibitory function and re-
Moreover, these data are consistent with previous findings that administration of KW-6002 blocks catalepsy induced with either haloperidol or reserpine treatment (Shiozaki et al., 1999) and that A2A receptor knockout mice are resistant to D2 antagonist-induced catalepsy (El Yacoubi et al., 2001). These findings collectively support the potential therapeutic potential of A2A receptor antagonists for the treatment of PD.

One of the drawbacks of current dopamine replacement therapies for PD is the development of dyskinesias after chronic activation of D2 receptors. PD patients have an increased number of A2A receptors, which correlates with the onset of dyskinesias (Calon et al., 2004). Therefore, treatment with an A2A receptor antagonist, by blocking the up-regulated A2A receptors while avoiding direct activation of D2 receptors, may treat PD symptoms while reducing the risk of the development of dyskinesias. Recent clinical studies have also demonstrated that combining A2A antagonists with L-Dopa provides symptomatic benefits and reduced risk of dyskinesias in PD patients (for review, see Schwarzschild et al., 2006).

Despite the promise of reduced dyskinesias with A2A treatment, results from both clinical and preclinical studies with the A2A receptor antagonist KW-6002 have not demonstrated reduced dyskinesias. In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated marmosets, for example, KW-6002 was not effective in reversing L-Dopa-induced dyskinesias (Kanda et al., 2000). Evidence suggests that KW-6002 may even exacerbate dyskinesias during “on” time in PD patients when given as combination treatment with L-Dopa (Hauser et al., 2003). This may be related to the relative lack of selectivity of KW-6002, which also has a substantial affinity for the A1

Fig. 8. Effect of preladenant (0.1–1 mg/kg), SCH 412348 (0.1–1 mg/kg), and desipramine (3–30 mg/kg) in the forced swim and tail suspension tests in mice. The data represent mean duration of immobility ± S.E.M. (n = 9–10 per group). *, p < 0.05; **, p < 0.01 versus vehicle (Veh).

Fig. 9. Effect of preladenant (0.1–1 mg/kg) and desipramine (40 mg/kg) in the rat forced swim test. Rats were injected with vehicle, preladenant, or desipramine 24, 5, and 1 h before testing. The data represent mean duration of immobility ± S.E.M. (n = 10–17 per group). *, p < 0.05; **, p < 0.01 versus vehicle (Veh).
receptor. There is some evidence that antagonizing A1 receptors may disrupt the activity of antiparkinsonian agents (Ismayilova et al., 2004). Tolerance also has been shown to develop to nonselective A1/A2A receptor antagonists because of their effects on A1 but not A2A receptors (Karcz-Kubicha et al., 2003).

In 6-OHDA-lesioned rats, repeated administration produces behavioral sensitization, manifested as a marked increase in L-Dopa-induced contralateral rotations across days of treatment (Henry et al., 1998). Behavioral sensitization has been suggested to predict the development of dyskinesias after chronic treatment with L-Dopa (Tronci et al., 2007). Here, we found that L-Dopa produced behavioral sensitization after only 4 days of treatment in 6-OHDA-lesioned rats. However, when L-Dopa was delivered concurrently with preladenant, the rate displayed no behavioral sensitization for as long as 23 days of treatment. The blockade of behavioral sensitization by preladenant in this model suggests that this agent may not only have antiparkinsonian effects on its own but also may reduce dyskinesias when used in combination with L-Dopa.

Aside from the motor symptoms that characterize PD, there are a collection of nonmotor symptoms that are not treated by current pharmacotherapies. One of the most severe is depression, which afflicts roughly 45% of PD sufferers (Allain et al., 2000). This may be due to a reduction in DA in the frontal cortex. Increases in DA levels in the frontal cortex are thought to play a role in the efficacy of antidepressants (Tanda et al., 1994). Ishiwata et al. (2000) has demonstrated a high density of A2A receptors in the frontal cortex that may modulate D3 receptor activity. Previous findings have suggested that pharmacological inhibition or genetic manipulation (El Yacoubi et al., 2001) of the A2A receptor produces antidepressant-like behaviors in animal models. In mice, preladenant and SCH 412348 reduced immobility time in the FST and TST with a degree of efficacy comparable with a high dose of tricyclic antidepressant desipramine. In the rat, preladenant dose-dependently reduced immobility and had a modest effect on climbing. Both preladenant and SCH 412348 increase locomotor activity in mice and rats, which complicates the interpretation of the FST and TST results. However, the doses that increase activity levels are consistently lower than those that are active in behavioral despair models. For example, SCH 412348 significantly increased activity in the mouse at 0.3 mg/kg and, when dosed at 0.1 mg/kg, produced a 36% increase in activity over vehicle, albeit not statistically significant. Neither of these doses was active in the mouse behavioral despair models. This suggests that the efficacy of the compounds in the FST and TST are not solely due to their effects on locomotor activity. More work needs to be done to determine the potential utility of A2A antagonists in the treatment of depression, preferably in animal models that do not rely on activity levels (e.g., chronic mild stress or hippocampal neurogenesis) and especially in the context of depression as manifested in PD sufferers.

In summary, the A2A receptor antagonists, preladenant and SCH 412348, represent a significant improvement over previously reported compounds in terms of A2A receptor affinity and selectivity. Preladenant and SCH 412348 are the most optimized compounds to date to be utilized to assess the potential of A2AReceptor antagonists for the treatment of PD without the complications of interpreting results in the context of activity at other adenosine receptors. Our data demonstrate that these highly selective A2A antagonists are efficacious in rodent models of PD with a potential for a lack of dyskinesias both as monotherapy or coupled with L-Dopa. Furthermore, preladenant and SCH 412348 may treat PD symptoms without inducing dyskinesias and may even reduce dyskinesias when used in combination with L-Dopa. This would represent a substantial improvement upon existing therapies. Given the high prevalence of depression in PD patients, the positive results in the FST and TST models of behavioral despair suggest the potential of A2A antagonists to dramatically improve over existing PD agents in the treatment of nonmotor PD symptoms. Collectively, these results suggest that preladenant and SCH 412348 are excellent candidates for clinical proof of concept of A2A receptor antagonists for the treatment of both the motor and nonmotor symptoms of PD.

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