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[^{3}H]OSIP339391, a selective, novel, and high affinity antagonist radioligand for adenosine A_{2B} receptors

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Abstract

Until recently, the characterization of adenosine A_{2B} receptors has been hampered by the lack of high affinity radioligands. This study describes the synthesis and in vitro characterization of the radiolabeled derivative of OSIP339391, a novel, potent, and selective pyrrolopyrimidine A_{2B} antagonist. OSIP339391 had a selectivity of greater than 70-fold for A_{2B} receptors over other human adenosine receptor subtypes. The radiolabel was introduced by hydrogenation of the acetylenic precursor with tritium gas resulting in the incorporation (on average) of three tritium atoms in the molecule, yielding a ligand with specific activity of 87 Ci/mmol (3.2 TBq/mmol). Using membranes from HEK-293 cells expressing the human recombinant A_{2B} receptor, [^{3}H]OSIP339391 was characterized in kinetic, saturation, and competition binding experiments. From the association and dissociation rate studies, the affinity was 0.41 nM and in close agreement with that found in saturation binding experiments (0.17 nM). In competition, binding studies using 0.5 nM [^{3}H]OSIP339391, the affinity of a range of agonists and antagonists was consistent with previously reported data. Thus, [^{3}H]OSIP339391 is a novel, selective, and high affinity radioligand that can be a useful tool in the further exploration and characterization of recombinant and endogenous adenosine A_{2B} receptors.

Keywords: Adenosine receptors; Radioligand binding; Pharmacology; Radiolabel synthesis; Non-xanthine A_{2B} receptor antagonist; Receptor characterization

1. Introduction

Characterization of adenosine A_{2B} receptors using radioligand binding techniques has, until recently, necessitated the use of relatively low affinity ligands, for example [^{3}H]-DPCPX [19], which actually has greater affinity for A_1 receptors, and [^{3}H]-NECA, a non-selective A_2 receptor agonist [3]. By definition, the inherent low affinity (K_D values >20 nM) of these ligands for the human adenosine A_{2B} receptor translates to a rapid dissociation rate from the receptor. The filtration and washing procedures routinely employed to terminate radioligand binding assays reduce the free radioligand concentration in the assay medium, thus disturbing the equilibrium and driving dissociation of the receptor–radioligand complex. Hence, binding assays utilizing low affinity radioligands such as [^{3}H]-NECA will suffer a marked loss in specific binding during the filtration and washing process. This in turn may compromise assay reproductibility and hinder the ability to detect low receptor expression levels. The binding of high affinity radioligands (i.e. slowly dissociating) is not significantly affected by the termination process and hence tends to be more robust and reproducible [1,11]. More recently, a high affinity radioligand for A_{2B} receptors ([^{3}H]MRS 1754) has been described [12] but, this compound was not commercially available at the time of this study. In the course of our studies we have identified a series of novel pyrrolopyrimidine compounds with high affinity for the human A_{2B} receptor. One such compound is OSIP339391,

Abbreviations: TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; DMF, dimethyl formamide; DEPT, distortionless enhancement by polarization transfer

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(*N*-(2-{2-phenyl-6-[4-(3-phenylpropyl)-piperazine-1-carbonyl]-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-ylamino}-ethyl)-acetamide). The present study describes the development of a receptor binding assay for human adenosine A_{2B} receptors expressed in HEK-293 cells utilizing [^{3}H]OSIP339391 as a high affinity radioligand.

2. Methods

2.1. Synthesis

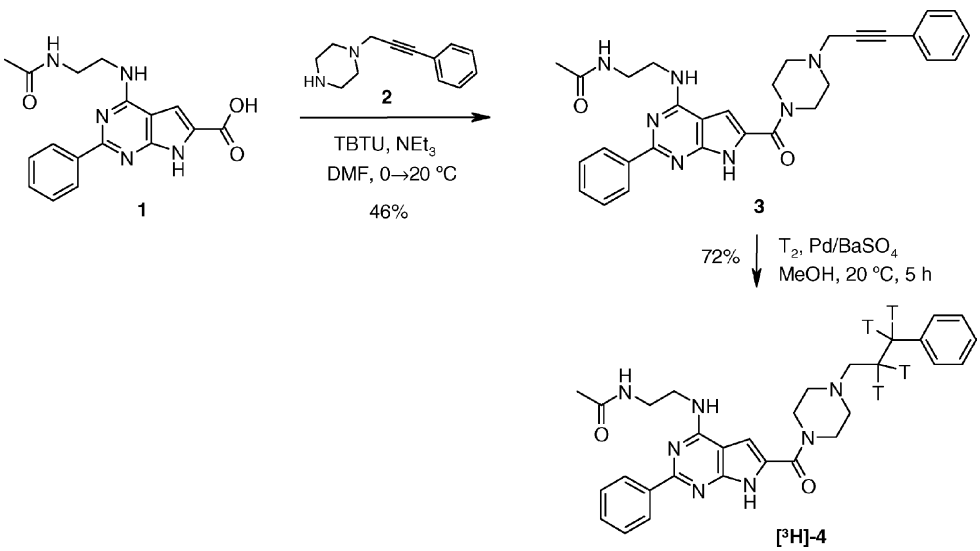
[^{3}H]OSIP339391 ([^{3}H]-**4**) was synthesized as shown below (Scheme 1).

2.1.1. N-(2-{2-Phenyl-6-[4-(3-phenylprop-2-ynyl)-piperazine-1-carbonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-ylamino}-ethyl)-acetamide (3)

TBTU (37 mg, 0.12 mmol) is added to a solution of acid **1** (30 mg, 0.088 mmol) [4,5] in dimethyl formamide (DMF) (1.3 ml), cooled by ice/water. After 30 min, amine **2** (19 mg, 0.095 mmol) [21] and triethylamine (20 μl, 0.14 mmol) are added, the cooling bath is removed, and the reaction mixture is stirred at ambient temperature for 3 h. Water (10 ml) is then added, and the mixture is extracted with EtOAc (4 × 15 ml). The combined organic layers are washed with 2N NaOH, water (3×), and brine, dried over $MgSO_4$, filtered and concentrated. The crude material is purified by chromatography on silica gel eluting with CH_2Cl_2 → 5% MeOH in CH_2Cl_2 → 7.5% MeOH in CH_2Cl_2 followed by trituration with Et_2O, yielding 21 mg (0.040 mmol, 46% yield) of **3** as an off-white solid, mp 218–219 °C. MS (ES): *m/z* 522 (100) [MH^+], 322 (100) [MH^+—(3-phenylprop-2-ynyl)piperazine]. IR (film): $\nu = 3302\ cm^{-1}$, 3061, 3022, 2924, 2850, 1654, 1589, 1573, 1531, 1455, 1429, 1386, 1367, 1327, 1298, 1270, 1171, 1135, 1027, 999, 970, 803, 776, 755, 704, 691. ^{1}H NMR ($CDCl_3$, 200 MHz): $\delta = 1.80$ (s, 3H), 2.73 (m_c, 4H), 3.50–3.61 (m, 4H), 3.80–4.00 (m, 6H), 5.93 (brs, 1H), 6.68 (s, 1H), 6.82 (brs, 1H), 7.25–7.35 (m, 3H), 7.35–7.55 (m, 5H), 8.37–8.47 (m, 2H), 9.63 (brs, 1H). ^{13}C NMR ($CDCl_3$, 50.3 MHz, additional DEPT135): $\delta = 23.03$ (+), 29.66 (−), 40.60 (−), 41.70 (−), 47.67 (−), 51.93 (−), 83.63 (C_{quart}), 85.81 (C_{quart}), 101.97 (+), 101.20 (C_{quart}), 122.70 (C_{quart}), 126.40 (C_{quart}), 128.04 (+), 128.28 (+), 129.81 (+), 131.69 (+), 138.70 (C_{quart}), 151.13 (C_{quart}), 157.67 (C_{quart}), 159.89 (C_{quart}), 161.60 (C_{quart}), 171.17 (C_{quart}). t_R (HPLC) = 7.5 min. $C_{30}H_{31}N_7O_2 \cdot 0.5H_2O$ (530.64): Calcd. C 67.91, H 6.08, N 18.48; found C 67.88, H 6.09, N 18.46.

2.1.2. N-(2-{2-Phenyl-6-[4-(2,2,3,3-tetratritio-3-phenylpropyl)-piperazine-1-carbonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-ylamino}-ethyl)-acetamide ([^{3}H]-4)

A solution of the alkyne **3** (2.6 mg, 4.95 μmol) in methanol (2 ml) was reacted for 5 h at room temperature in an atmosphere of tritium gas using 10% Pd/$BaSO_4$ (18 mg). The catalyst was removed by filtration, and labile tritium was removed by repeated evaporations to dryness from methanol. The crude product was purified using an ultrasphere ODS column (10 mm × 250 mm) and a MeOH:H_2O:NEt_3 gradient system (solvent A: water with 0.1% NEt_3, solvent B: methanol with 0.1% NEt_3; elution: 0–18 min, 60% B isocratic; 18–37 min, 65% B isocratic; 37 min, 70% B isocratic). The purified material (72% yield) had a radiochemical purity of 98% and co-chromatographed with the non-labeled reference. The triple-bond reduction gives a mixture of species with zero to four tritium ions incorporated, presumably, as a consequence of scrambling. Mass spectrometric analysis showed an



Scheme 1.

average of 3.02 tritiums, MW = 531.7 with a specific activity of 87 Ci/mmol (3.2 TBq/mmol).

2.1.3. N-(2-{2-Phenyl-6-[4-(3-phenylpropyl)-piperazine-1-carbonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-ylamino}-ethyl)-acetamide (**4**)

White solid, mp 198–200 °C. MS (ES): *m/z* 526 (51) [MH^+]. IR (film): $\nu = 3295\ cm^{-1}$, 3062, 3024, 2929, 2857, 1654, 1589, 1573, 1530, 1454, 1432, 1387, 1328, 1297, 1170, 1132, 1027, 1001, 776, 749, 703. ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 1.79$ (s, 3H), 1.83 (quint, $J = 7.4$ Hz, 2H), 2.39 (t, $J = 7.6$ Hz, 2H), 2.47 (m_c, 4H), 2.65 (t, $J = 7.6$ Hz, 2H), 3.52–3.56 (m, 2H), 3.80–3.90 (m, 6H), 6.19 (brs, 1H), 6.67 (s, 1H), 7.01 (brs, 1H), 7.16–7.42 (m, 8H), 8.37–8.40 (m, 2H), 10.12 (brs, 1H). ^{13}C NMR ($CDCl_3/CD_3OD$, 50.3 MHz, additional DEPT135): $\delta = 22.97$ (+), 28.31 (−), 33.50 (−), 40.85 (−), 41.19 (−), 41.35 (−), 53.01 (−), 57.66 (−), 101.95 (2C, +, C_{quart}), 125.85 (+), 126.35 (C_{quart}), 127.96 (+), 128.33 (+), 129.91 (+), 138.64 (C_{quart}), 141.84 (C_{quart}), 150.95 (C_{quart}), 157.59 (C_{quart}), 160.07 (C_{quart}), 161.49 (C_{quart}), 171.42 (C_{quart}). t_R (HPLC) = 4.4 min. $C_{30}H_{35}N_7O_2$ (525.66): Calcd. C 68.55, H 6.71, N 18.65; found C 68.93, H 6.78, N 18.26.

2.2. Analytical methods

LC–MS analysis was performed using a Gilson 215 autosampler and Gilson 819 autoinjector, attached to a Hewlett Packard HP110. Mass spectra were obtained on a Micromass Platform II mass spectrometer, using positive electrospray ionization. LC analysis was undertaken at 254 nm using a UV detector. Samples were eluted on a Phenomenex Luna C18(2) column (5 μm, 4.6 mm × 150 mm) using a linear gradient of 15–99% solvent A in solvent B over 10 min. The solvent A was 100% acetonitrile, solvent B was 0.01% formic acid, which was observed to have no noticeable effect on sample retention time, in water.

IR spectra were recorded on a Perkin-Elmer Spectrum 1000 FT-IR spectrometer as thin films using diffuse reflectance.

^{1}H NMR and ^{13}C NMR spectra were recorded with Varian instruments (400 or 200 MHz for ^{1}H, 100.6 or 50.3 MHz for ^{13}C) at ambient temperature with TMS (for ^{1}H) or the residual solvent peak (for ^{13}C) as internal standards. The line positions or multiplets are given in ppm (δ) and the coupling constants (J) are given as absolute values in Hertz, while the signal multiplicities are abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet), m_c (centered multiplet), br (broadened).

All melting points were determined with a Mel-Temp II apparatus and are uncorrected.

Elemental analyses were carried out at Atlantic Microlab Inc., Norcross, GA.

Commercially available anhydrous solvents and HPLC-grade solvents were used without further purification.

2.3. Pharmacological methods

The human A_{2B} receptor was cloned and expressed in Human Embryonic Kidney cells (HEK-293) in our laboratory using standard techniques [17]. Cells stably expressing the human A_{2B} receptor were grown to confluency in DMEM containing 10% fetal bovine serum (FBS), penicillin/neomycin/streptomycin antibiotic (PNS) and 0.1 mg/ml G418. Cells were washed once with phosphate-buffered saline (PBS) and harvested by scraping and centrifugation for 10 min at 1000 × *g*. Cell pellets were resuspended in lysis buffer (5 mM Tris containing 5 mM EDTA and 5 mM EGTA, pH 7.4 at 4 °C), homogenized and centrifuged for 30 min at 30,000 × *g*. This process was repeated with the final pellet being resuspended in ice-cold 10 mM HEPES, pH 7.4 at 4 °C and frozen at −80 °C until use. Protein concentration was determined using a standardized Coomassie Blue protocol (Pierce Life Sciences).

The methods used for radioligand binding were modified from those described by Stehle et al. [19]. All assays were performed in 10 mM HEPES containing 1 mM EDTA, 2 U/ml adenosine deaminase (ADA), 0.2 mM benzamidine, and 5 mg/ml fatty acid free BSA (pH 7.4 at 22 °C). A total assay volume of 100 μl was employed in which final protein levels varied between 1 and 5 μg per well depending upon protocol. The time to equilibrium and the association rate constant at 22 °C were determined by incubating 25 μl of 1 nM [^{3}H]OSIP339391 with 50 μl of adenosine A_{2B} receptor membrane homogenate for various durations. The dissociation rate constant was determined by adding 10 μM DPCPX to the assay mixture following an initial 60 min incubation period at 22 °C. This excess concentration of unlabeled competitor acts to initiate radioligand dissociation and effectively displaces all radioligand from the receptor over time. Total and non-specific binding was determined in quadruplicate in both association and dissociation experiments at time points ranging from 1 to 180 min. Saturation assays were performed by measuring specific binding at concentrations ranging from 0.02 to 12 nM with total and non-specific binding being determined in duplicate. NSB was defined using an excess of cold OSIP339391 (10 μM). Competition assays were performed with 0.5 nM [^{3}H]OSIP339391 using 10 concentrations of test compounds. All binding assays were initiated by addition of membrane homogenate and were terminated by rapid vacuum filtration using a Packard UNIFILTER-96 cell harvester through GF/C grade filters presoaked in ice-cold 0.5% PEI. Trapped membrane was then given 6 μl × 300 μl washes with ice-cold 10 mM HEPES (pH 7.4 at 4 °C). Filters were dried and receptor bound radioligand was quantified using

liquid scintillation counting on a Packard TopCount NXT. Radioligand concentrations were determined by adding an aliquot of the working solution to 5 ml of Ecoscint, capped, and counted using a tritium dpm program on a Beckman LS6500 Liquid Scintillation Counter.

2.4. Materials

All standard compounds were purchased from Sigma (St. Louis, MO, USA). All compounds were initially prepared as 10 mM stocks in DMSO with subsequent dilutions performed in assay buffer containing 4% DMSO, hence 1% final DMSO concentration.

2.5. Data analysis

Data are reported as mean ± S.E. mean of at least three experiments. Equilibrium and kinetic constants were obtained by fitting to the appropriate nonlinear regression functions within PRISM (GraphPad Software, San Diego, CA, USA). The equilibrium dissociation constant (K_D) and the binding site density (B_{max}) were derived from radioligand saturation curves using the Langmuir equation $RL = R_t L/(K_D + L)$ where L is the free ligand concentration, RL is the concentration of receptor-bound ligand at equilibrium, and R_t is the total receptor concentration. Since these studies revealed that the cell line employed had a very high level of receptor expression (~21 pmol/mg protein, see Section 3) the methods employed here resulted in a modest extent of ligand depletion. Hence, we used the free concentration of radioligand to analyse saturation data which is one approach to minimize the effects of depletion in such analyses [11]. Saturation curves were routinely analyzed by fitting to one- and two-site isotherms but the data were never adequately described by the latter model. As with the analyses of saturation curve data, competition curve data were routinely fitted to one- and two-site models; invariably the data for all compounds were described better by a one-site model and hence the data reported are from such analyses. IC_{50} (the concentration of compound producing 50% inhibition of specific binding) and Hill Coefficients were derived from fitting to a four-parameter logistic equation. Curve fitting was not constrained when data fully reached maximum and minimum plateaus but was constrained where compounds had low affinity and did not fully inhibit binding. Apparent K_i values were derived using the equation of Cheng and Prusoff [6]: $K_i = IC_{50}/(1 + [L]/K_D)$ where $[L]$ is the free concentration of radioligand in each assay and K_D is the appropriate equilibrium dissociation constant derived from the saturation analysis. In kinetic experiments, the association rate constant (k_{on} in units of min^{-1} nM^{-1}) was derived by the pseudo first-order method [20] which takes into account the concentration of radioligand used, the observed association rate constant (k_{obs} in units of min^{-1}) and the dissociation rate constant (k_{off} in units of min^{-1}) such that

$$k_{on} = \frac{k_{obs} - k_{off}}{[L]}$$

The K_D can then be derived from k_{off}/k_{on}. The k_{off} and k_{obs} are derived directly from the association and dissociation curves generated in PRISM.

3. Results

Using membranes from parental (non-transfected) HEK-293 cells, we failed to detect any specific binding using 10 nM [^{3}H]OSIP339391. Hence, the presence of very low levels of the endogenous A_{2B} receptor [7] would not prove an interfering factor in assay conditions (0.5 nM [^{3}H]OSIP339391) routinely employed to screen compounds in competition assays.

Initial experiments revealed a discrepancy between the concentration of [^{3}H]OSIP339391 achieved and that expected. A common cause of such a difference is the adsorption of the radioligand into plastic labware, such as the 96-well plates or pipette tips, and can often be minimized by the addition of an anti-adsorbant such as bovine serum albumin (BSA). Table 1 shows the extent and the time dependent nature of the adsorption together with the protective effects of increasing BSA concentrations. BSA (0.5%), was therefore, routinely included in the assay buffer for all subsequent experiments.

Specific binding of [^{3}H]OSIP339391 to the human A_{2B} receptor was time dependent with equilibrium established after 30 min at 22 °C with a half-life of approximately 4.5 min and an association rate constant (k_{on}) of 0.095 min^{-1} nM^{-1} (Fig. 1A). The standard time of incubation for subsequent experiments was 60 min. At equilibrium, non-specific binding did not exceed 20% of the total [^{3}H]OSIP339391 bound. Specific binding remained stable for up to 180 min (the longest time point tested) and

Table 1
Influence of BSA on the recovery of [^{3}H]OSIP339391 from the assay medium

	Assay buffer − BSA	Assay buffer + 0.2% BSA	Assay buffer + 0.5% BSA
Expected dpm	38,500	38,500	38,500
Obtained dpm at time			
0 min	19,641 ± 300	25,578 ± 369	33,447 ± 1831
8 min	12,825 ± 285	25,695 ± 504	38,432 ± 146
16 min	8,996 ± 70	25,837 ± 594	38,175 ± 714
24 min	7,545 ± 91	26,346 ± 286	38,291 ± 597

Aliquots (25 μl) of 8 nM [^{3}H]OSIP339391 were removed and counted at various time points after preparation (see Section 2 for details). The data are expressed as mean ± S.E. mean of four individual determinations. The expected dpm was calculated using the specific activity (87 Ci $mmol^{-1}$) of the radioligand.

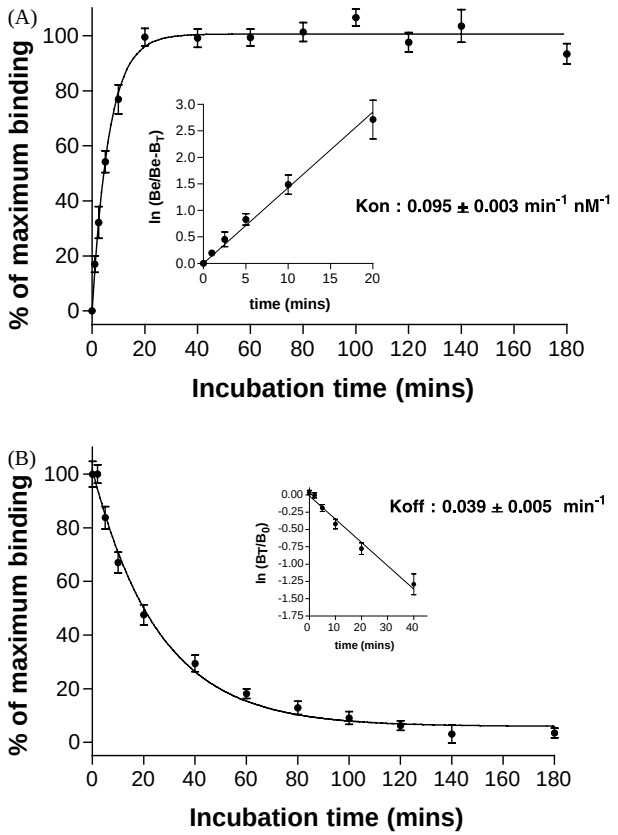


Fig. 1. Binding kinetics of [^{3}H]OSIP339391 to HEK-293 cell membranes expressing the human recombinant A_{2B} receptor. (A) Association of 1 nM [^{3}H]OSIP339391 with 1 μg receptor protein per well. (B) Dissociation of [^{3}H]OSIP339391 initiated by 10 μM DPCPX. Insets show the logarithmic transformations of the kinetic data where ln is natural logarithm, Be is specific binding at equilibrium, BT is specific binding at each time point and B0 is specific binding at time 0. Linear relationships are generally indicative of a non-complex binding interaction. The data are expressed as mean ± S.E. mean of three individual experiments.

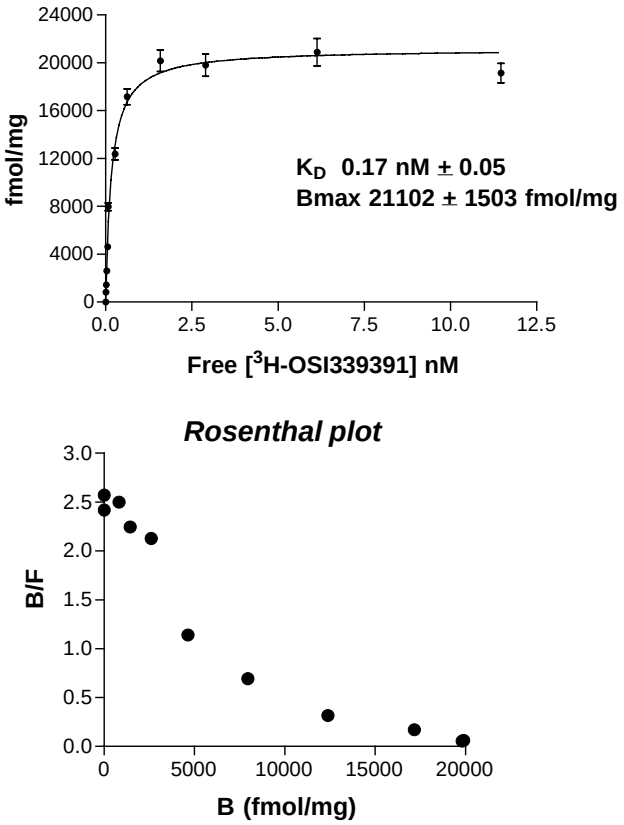


Fig. 2. Mean saturation isotherm of [^{3}H]OSIP339391 binding to HEK-293 cell membranes expressing the human A_{2B} receptor. The data are expressed as mean ± S.E. mean of three individual experiments performed in triplicate.

was fully reversible as measured by addition of 10 μM DPCPX to the assay mixture once equilibrium had been achieved. The dissociation half life was approximately 19 min and the slow dissociation rate constant of 0.039 min^{-1} confirmed the suitability of termination of the binding assay by vacuum filtration without significant loss of specific binding (Fig. 1B). From k_{on} and k_{off} values a K_D of 0.41 ± 0.06 nM was derived.

Saturation assays confirmed the high affinity nature of the interaction between [^{3}H]OSIP339391 and the human A_{2B} receptors yielding a K_D value of 0.17 ± 0.05 nM, a value less than three fold different from that derived from the kinetic constants (Fig. 2). High receptor expression was reflected in a B_{max} value of over 20 pmol/mg of protein.

Table 2 and Fig. 3 shows a range of adenosine receptor ligands tested for their ability to inhibit 0.5 nM specific [^{3}H]OSIP339391 binding to the human A_{2B} receptor. The most potent inhibitor of [^{3}H]OSIP339391 was OSIP339391 itself with a K_i value of 0.5 nM consistent with the K_D values for the radiolabeled version derived from the saturation and kinetic studies. This is indicative of good internal consistency within the assay. In general, the affinity of the ligands employed in this study is consistent with previously reported data (see Section 4).

OSIP339391 is selective for the human adenosine A_{2B} over A_1, A_{2A} or A_3 receptors; in radioligand binding assays for these receptors employing [^{3}H]-DPCPX, [^{3}H]ZM241385 and [^{125}I]AB-MECA, respectively, the

Table 2
Apparent equilibrium inhibition constants (K_i) and slope factors for compounds at the human A_{2B} receptor using [^{3}H]OSIP339391

Compound	K_i (nM)	nH
OSIP339391	0.5 ± 0.1	1.17
XAC	1.6 ± 0.4	0.82
ZM 241385	5.8 ± 1.3	0.85
CGS15943	7.6 ± 1.5	0.83
DPCPX	51.1 ± 11.5	1.09
MRS-1220	332 ± 29	0.68
NECA	489 ± 109	0.81
Theophylline	17 ± 1% inhibition @ 10 μM	–
Enprofylline	18 ± 2% inhibition @ 10 μM	–
CPA	No inhibition at 5 μM	–
CHA	No inhibition at 5 μM	–
CGS-21680	No inhibition at 10 μM	–

The data are expressed as mean ± S.E. mean of at least three individual experiments.

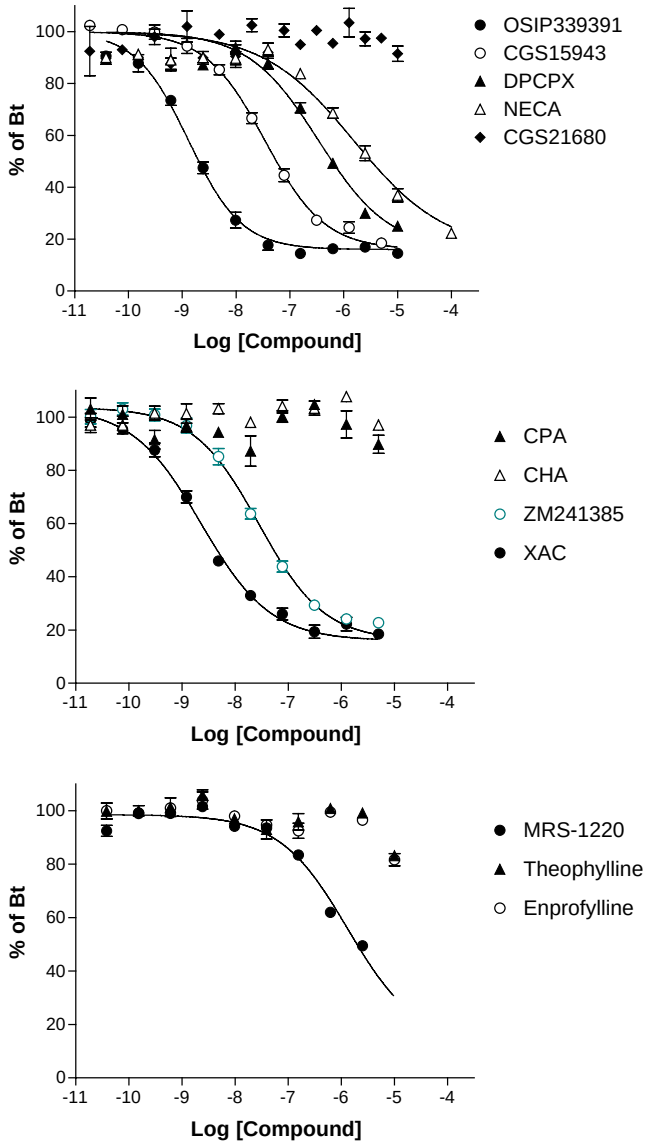


Fig. 3. Inhibition of [^{3}H]OSIP339391 binding to HEK-293 cell membranes expressing the human A_{2B} receptor by various adenosine receptor ligands. The data are expressed as mean ± S.E. mean of at least three individual experiments performed in triplicate. Bt is total bound radioactivity.

affinity for these human subtypes is 37 ± 7, 328 ± 75, and 450 ± 55 nM (authors' unpublished data). This suggests that OSIP339391 is at least 70-fold selective for the A_{2B} receptor over other adenosine receptor subtypes.

4. Discussion

We have shown that [^{3}H]OSIP339391 binds with high affinity (K_D = 410 pM in kinetic studies) to a homogenous population of human recombinant A_{2B} receptors stably expressed in membranes prepared from HEK-293 cells. This high affinity makes it a more attractive radioligand than [^{3}H]-NECA or [^{3}H]-DPCPX, when characterizing this receptor subtype or evaluating potential novel ligands. Specifically, its high affinity (slow dissociation rate) makes it suitable for a filtration based binding assay and is likely to produce more robust, reliable, and reproducible K_i values than the lower affinity [^{3}H]-DPCPX or [^{3}H]-NECA, both of which suffered marked dissociation during the assay termination process. This characteristic could also make it the radioligand of choice in systems where low expression of A_{2B} receptors is suspected. Furthermore, the selectivity of OSIP339391 among adenosine receptor subtypes (greater than 70-fold) further points to a utility in tissues or cell lines with multiple receptor subtypes. This receptor selectivity is not shared by DPCPX, NECA or ZM241385, whose radiolabeled derivatives have been used before now for adenosine A_{2B} receptor studies. The recently described radiolabeled derivative of MRS1754 [12] appears to have similar attributes with respect to high affinity and selectivity. Although saturation data fitted well to the one-site model, and did not fit adequately to the two-site model, a slight curvilinearity was observed in the derived Rosenthal plot. This analysis might suggest binding to the endogenous A_{2B} receptors in HEK-293 cells at very high radioligand concentrations. However, initial experiments using non-transfected HEK-293 cells does not support this idea. Further, the presence of a second binding component is difficult to reconcile given that the association and dissociation kinetics were apparently monophasic. However, it is possible that significant receptor occupancy of a lower affinity component, which would have allowed detection, may not have been achieved, given that the kinetic assays were performed using a single, low concentration of [^{3}H]OSIP339391. Hence, the curvilinearity may simply reflect that lower radioligand concentrations had not achieved equilibrium during the incubation period employed [15].

We found that the cell line used in these studies had a high level of receptor expression (B_{max} ~ 21 pmol/mg protein) which resulted in a small degree of ligand depletion under the experimental conditions employed here (receptor concentration, R_t = 210 pmol). We do not believe this extent of depletion resulted in a meaningful (greater than two-fold) underestimation of K_D as it has been shown that R_t must at least exceed K_D for this to occur [14]. K_D from kinetic studies was 410 pmol, and hence $R_t = 0.5K_D$. Indeed, K_D from saturation analyses (170 pM) was actually lower then that derived from kinetic studies. With regard to the influence of ligand depletion on Rosenthal plots, it has been shown that this results in a convex relationship [14], whereas we found a concave relationship. Ligand depletion in competition binding studies also leads to underestimation of IC_{50}, but it has been calculated this difference is only two-fold when depletion is 50% [9]. Under the experimental conditions employed here the depletion is ~20%, hence the error in IC_{50} estimation is very minimal, indeed smaller than the standard deviation between experiments.

OSIP339391 is one compound of a series of pyrrolpyrimidines that were synthesized in a program to identify selective A_{2B} receptor antagonists. Many of the compounds in this series, but not OSIP339391, were tested in functional assays to help further characterize their properties. One such assay was A_{2B} receptor-mediated accumulation of cAMP in recombinant cell lines. These compounds neither augmented nor reduced basal levels of cAMP, suggesting this series of compounds behave as neutral antagonists, and blocked NECA-induced cAMP responses with affinities consistent with radioligand binding-derived estimates. Clearly, the characterization of OSIP339391 will be more complete, and its possible utility enhanced, by further characterization in functional assays utilizing endogenous and recombinant receptor systems.

In competition binding experiments, the affinity of test compounds was in good agreement with the previously reported values from studies that have employed different radioligands including [^{3}H]-DPCPX, [^{3}H]MRS1754 and [^{125}I]-ABOPX [2,12,16]. There are some data which do not agree so well and worthy of comment. The high affinity displayed by the antagonist XAC for the human A_{2B} receptor (1.6 nM) is somewhat more potent than that previously reported (16 nM) [12]. The triazolotriazine ZM 241385 had a K_i of 5.8 nM in line with findings employing [^{3}H]ZM241385 as the radioligand [13] but contrasting with the lower affinity obtained against the recently tritiated xanthine derivative [^{3}H]MRS1754 (145 nM) [12]. Theophylline and enprofylline, both A_{2B} antagonists used as antiasthmatic therapies, inhibited [^{3}H]OSIP339391 binding only at 10 μM and not to an extent to permit accurate determination of affinity due to the low solubility of these compounds. Nevertheless, the affinities of these compounds are less than 10 μM, consistent with published data [12] The adenosine A_1 receptor agonists CPA and CHA showed no significant inhibition of specific binding even at 5 μM. Similarly, the selective A_{2A} agonist, CGS 21680 did not inhibit specific [^{3}H]OSIP339391 binding to the human A_{2B} receptor even at a concentration of 10 μM. This observation is consistent with functional studies indicating this agonist to be inactive at A_{2B} receptors and selective for the A_{2A} subtype [10].The choice of HEK-293 cells for transfection with the human A_{2B} gene may be considered somewhat controversial, given that this cell line expresses an endogenous A_{2B} receptor which is coupled to cAMP generation [7]. We attempted to transfect other cell lines such as CHO, which do not appear to express adenosine A_{2B} receptors [18] but without success. Nevertheless, using parental HEK-293 cells we were not able to detect a significant level of specific binding at high concentrations of either [^{3}H]OSIP339391 or [^{3}H]-DPCPX (results not shown). Thus, although there appears to be only a small number of endogenous A_{2B} receptors in HEK-293 cells, these must be efficiently coupled to adenylyl cyclase in order to measure a functional response. Measurement of functional responses in cells due to the activation of endogenous receptors which cannot be detected using radioligands is not without precedent, a similar observation having been made of 5-HT_{1B} receptors in CHO cells [8].

In conclusion, [^{3}H]OSIP339391 represents a novel, selective and high affinity radioligand which will be useful in the further exploration and characterization of recombinant and endogenous adenosine A_{2B} receptors.

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