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KF26777 (2-(4-bromophenyl)-7,8-dihydro-4-propyl-1*H*-imidazo[2,1-*i*]purin-5(4*H*)-one dihydrochloride), a new potent and selective adenosine A_3 receptor antagonist

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Abstract

We investigated the biochemical and pharmacological properties of a new adenosine A_3 receptor antagonist, KF26777 (2-(4-bromophenyl)-7,8-dihydro-4-propyl-1*H*-imidazo[2,1-*i*]purin-5(4*H*)-one dihydrochloride). This compound was characterized using N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]AB-MECA) or [^{35}S]guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) binding to membranes from human embryonic kidney 293 (HEK293) cells expressing human adenosine A_3 receptors. KF26777 showed a K_i value of 0.20 ± 0.038 nM for human adenosine A_3 receptors labeled with [^{125}I]AB-MECA and possessed 9000-, 2350- and 3100-fold selectivity vs. human adenosine A_1, A_{2A} and A_{2B} receptors, respectively. The inhibitory mode of binding was competitive. KF26777 inhibited the binding of [^{35}S]GTPγS stimulated by 1 μM 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (Cl-IB-MECA). The IC_{50} value was 270 ± 85 nM; the compound had no effect on basal activity. Dexamethasone treatment for HL-60 cells, human promyelocytic leukemia, up-regulated functional adenosine A_3 receptors expression, and resulted in the enhanced elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via the adenosine A_3 receptor. KF26777 antagonized this $[Ca^{2+}]_i$ mobilization induced by Cl-IB-MECA, with a K_B value of 0.42 ± 0.14 nM. These results indicate that KF26777 is a highly potent and selective antagonist of the human adenosine A_3 receptor.

Keywords: Adenosine A_3 receptor; KF26777 (2-(4-bromophenyl)-7,8-dihydro-4-propyl-1*H*-imidazo[2,1-*i*]purin-5(4*H*)-one dihydrochloride); Binding assay; Ca^{2+}; Guanine nucleotide

1. Introduction

Adenosine modulates many cellular functions via G protein-coupled receptors. Adenosine receptors are classified into A_1, A_{2A}, A_{2B} and A_3 receptors, which is based on cDNA cloning, affinities for agonists and antagonists and the mechanism of signal transduction (Linden et al., 1991; Zhou et al., 1992; Palmer and Stiles, 1995). Activation of adenosine A_3 receptors inhibits adenylyl cyclase and stimulates phospholipase C (Abbracchio et al., 1995) and D (Ali et al., 1996). The adenosine A_3 receptor cDNAs have been cloned from multiple species. The homology of amino acid sequence in adenosine A_3 receptors between rat (Zhou et al., 1992) and human (Salvatore et al., 1993) is only 74%. The difference between rat and human receptors seems to contribute to a marked difference in the affinity of ligands to these adenosine A_3 receptors (Linden, 1994). Also, the tissue distribution of the adenosine A_3 receptor varies across species: the rat mRNAs are detected in testis, lung, heart, kidney, brain and circulating inflammatory cells (Zhou et al., 1992); human mRNAs are widespread but the most abundant expression is in the lung, liver (Salvatore et al., 1993) and eosinophils (Walker et al., 1997).

Compared with adenosine A_1 and A_{2A} receptors, activation of adenosine A_3 receptors requires a relatively high concentration of adenosine, which occurs during hypoxic stress and cellular damage such as ischemia and inflammation. The effect of the adenosine A_3 receptor on brain ischemia is complicated: chronic preadministration of an adenosine A_3 receptor agonist had a highly neuroprotective postischemic effect, but acute administration of the same agonist during ischemia exacerbated histological and functional damage (von Lubitz et al., 1994). With respect to inflammation, function of adenosine A_3 receptors is controversial. In lung of patients with airway inflammation, the mRNA level of adenosine A_3 receptors increased (Walker et al., 1997). In addition, stimulation of adenosine A_3 receptors

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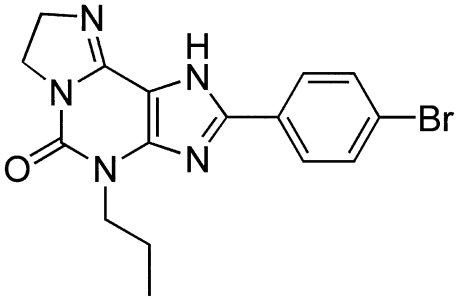

Fig. 1. Structure of KF26777.

induces bronchospasm (Meade et al., 1996), enhances antigen-dependent degranulation (Ramkumar et al., 1993) and protects rat basophilic leukemia RBL-2H3 cells from apoptosis (Gao et al., 2001). Conversely, adenosine A_3 receptor activation inhibits production of tumor necrosis factor-α (TNF-α) and inflammatory cytokines (Hasko et al., 1998; McWhinney et al., 1996; Szabo et al., 1998), chemotaxis (Walker et al., 1997), degranulation and superoxide anion release (Ezeamuzie and Philips, 1999). In adenosine A_3 receptor knockout mice, an adenosine A_3 receptor selective agonist, 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (Cl-IB-MECA) exhibited no potentiation of antigen-dependent degranulation of bone marrow-derived mast cells, and also the ability of Cl-IB-MECA to inhibit lipopolysaccharide-induced TNF-α production in vivo was decreased remarkably in contrast to wild type mice (Salvatore et al., 2000). These data support the finding that adenosine A_3 receptors play a role in both anti- and proinflammatory responses. Thus, it is not clear whether an adenosine A_3 receptor agonist or antagonist is a potential target for the treatment of brain ischemia and inflammatory diseases. In order to elucidate the physiological function of adenosine A_3 receptors and to clarify the therapeutic benefit of drugs that act on the receptor, potent and selective adenosine A_3 receptor antagonists are required.

Although several classes of adenosine A_3 receptor antagonists have been reported including dihydropyridines, triazoloquinazolines, flavonoids, pyridines, isoquinolines and triazolonaphthyridines (Baraldi et al., 2000), the majority of these antagonists are not characterized sufficiently. We have identified a novel imidazopurine derivative, KF26777, 2-(4-bromophenyl)-7,8-dihydro-4-propyl-1*H*-imidazo[2,1-*i*]-purin-5(4*H*)-one dihydrochloride (Fig. 1), as a potent and selective adenosine A_3 receptor antagonist, and we report here the biochemical and pharmacological characterization of KF26777.

2. Materials and methods

2.1. Materials

N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]AB-MECA, specific activity, 74 TBq/mmol) was purchased from Amersham (Buckinghamshire, England). [^{3}H]N^6-cyclohexyladenosine ([^{3}H]CHA, 0.925–1.85 TBq/mmol), [^{3}H]-2-[*p*-(2-carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]CGS21680, 1.11–2.22 TBq/mmol), 8-[dipropyl-2,3,4,5-^{3}H(*N*)]cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX, 2.96–4.44 TBq/mmol) and [^{35}S]guanosine 5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS, 46.2 TBq/mmol) were from New England Nuclear (Boston, MA). (*R*)-N^6-phenylisopropyladenosine [(*R*)-PIA], N^6-cyclopentyladenosine (CPA), adenosine deaminase and dexamethasone were from Sigma (St. Louis, MO). CGS21680 was from Research Biochemicals (Natick, MA). 3-(3-iodo-4-aminobenzyl)-8-(4-oxoacetate)phenyl-1-propylxanthine (I-ABOPX), 8-(3-chlorostyryl)caffeine (CSC), 1,3-dipropyl-7-methyl-8-(3,4-dimethoxystyryl)xanthine (KF17837), 8-noradamantan-3-yl-1,3-dipropylxanthine (KW-3902), DPCPX, Cl-IB-MECA, 3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191) and KF26777 were synthesized in the Medicinal Chemistry Division of Kyowa Hakko Laboratories. Fura 2-acetoxymethyl ester (Fura 2-AM) was from Dojindo (Kumamoto, Japan). Medium and supplements used for cell culturing were from GIBCO BRL (Rockville, MD). Fetal calf serum was from Intergen (Purchase, NY).

2.2. Cell and culture

Cell lines transfected with recombinant human adenosine receptors were obtained from Dr. Linden (Virginia University, VA). One cell line, Chinese hamster ovary-K1 (CHO-K1) cells, was transfected with recombinant human adenosine A_1 receptors (hA_1R/CHO). The other three cell lines were human embryonic kidney 293 (HEK293) cells transfected with recombinant human adenosine A_{2A}, A_{2B} or A_3 receptors ($hA_{2A}R$/HEK293, $hA_{2B}R$/HEK293, hA_3R/HEK293, respectively). In the case of transfection with adenosine A_1, A_{2A} and A_{2B} receptors, the vector pSVL (Pharmacia, Piscataway, NJ) containing the sequence encoding for the respective receptor was used. In the case of transfection with adenosine A_3 receptors, pCMV5 (Mumby et al., 1990) containing the sequence for the adenosine A_3 receptor was used. In all cases, cells were cotransfected with pWLneo (Stratagene, La Jolla, CA) containing neomycin gene. HL-60 cells, human promyelocytic leukemia, were from the National Cancer Center (Tokyo, Japan).

The hA_1R/CHO cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.6 mg/ml G418. HEK293 cells transfected with each adenosine receptor subtype were cultured in DMEM/F-12 medium, supplemented with the above mentioned ingredients, except using 1 mg/ml G418 for human adenosine A_3 receptors. HL-60 cells were grown in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. In the case of treatment with dexamethasone, HL-

60 cells were cultured with dexamethasone (1 μM) for 16 h in RPMI medium.

2.3. Receptor binding assays

2.3.1. Membrane preparation

Cultured cells were washed with phosphate-buffered saline (PBS) and harvested using a cell scraper. Cells were homogenized in ice-cold lysis buffer [5 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$] using a Teflon homogenizer. The homogenate was centrifuged at 40,000 × *g* for 20 min at 4 °C and then the pellet was suspended in assay buffer [50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$] with the homogenizer and stored frozen at − 80 °C until use.

2.3.2. Human adenosine A_3 receptor binding assay

[^{125}I]AB-MECA binding to the membrane fraction of hA_3R/HEK293, HL-60 cells or dexamethasone-treated HL-60 cells was measured by a modification of the method described by Salvatore et al. (1993). The reaction mixture contained assay buffer, the membrane suspension (20 μg hA_3R/HEK293 or 100 μg HL-60), 0.1 nM [^{125}I]AB-MECA and 2 U/ml adenosine deaminase. The binding assays were performed in the presence of 1% of final concentration of dimethyl sulfoxide (DMSO). Incubation was for 120 min at 25 °C. Nonspecific binding was determined in the presence of 100 μM (*R*)-PIA. Binding reactions were terminated by filtration through 0.3% polyethyleneimine pretreated Whatman GF/B filters (Maidstone, UK) under reduced pressure using a MT-24 cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with ice-cold assay buffer. Radioactivity was determined in the COBRA γ-counter (Packard, Downers Grove, IL). The concentration–response curves were obtained from assays performed in duplicate at seven concentrations of each drug. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.3.3. Human adenosine A_1, A_{2A} or A2B receptor binding assay

Radioligand binding assays for human adenosine A_1, A_{2A} and A_{2B} receptors were performed as described by Salvatore et al. (1993). Binding assays for adenosine A_1 receptors were performed in assay buffer containing hA_1R/CHO cell membranes (10 μg), 3 nM [^{3}H]CHA and 2 U/ml adenosine deaminase. Nonspecific binding was determined in the presence of 10 μM (*R*)-PIA. Binding assays for adenosine A_{2A} receptors were performed in assay buffer containing $hA_{2A}R$/HEK293 cell membranes (10 μg), 5 nM [^{3}H]CGS21680 and 2 U/ml adenosine deaminase. Nonspecific binding was measured in the presence of 100 μM CPA. Binding assays for adenosine A_{2B} receptors were performed in assay buffer containing $hA_{2B}R$/HEK293 cell membranes (70 μg), 10 nM [^{3}H]DPCPX and 2 U/ml adenosine deaminase. Nonspecific binding was assessed in the presence of 10 μM DPCPX. All these binding reactions were performed in the presence of 1% DMSO (final concentration) and at 25 °C for 90 min. The incubations were terminated by filtration through Whatman GF/C filters under reduced pressure using a MT-24 cell harvester. Filters were washed three times with ice-cold assay buffer and placed in scintillation vials. Bound radioactivity was determined using a liquid scintillation counter, Packard TRI-CARB 4530.

2.3.4. Binding of other radioligands

Binding of [^{3}H]prazosin, [^{3}H]rauwolscine, [^{3}H]dihydroalprenolol, [^{3}H](*R*)-(+)8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepin-7-ol hemimaleate (SCH23390), [^{3}H]spiperone, [^{3}H]pyrilamine, [^{125}I]iodoaminopotentidine, [^{3}H]*N*-methylscopolamine, [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin and [^{3}H]ketanserin to the α_1-adrenoceptors (Greengrass and Bremner, 1979), α_2-adreno-ceptors (Boyajian and Leslie, 1987), and β-adrenoceptors (U'Prichard et al., 1978), dopamine D_1 receptors (Dearry et al. 1990) and dopamine D_{2L} receptors (Grandy et al., 1989), histamine H_1 receptors (Hill et al., 1978) and histamine H_2 receptors (Traiffort et al., 1991), muscarinic M_1 receptors (Buckley et al., 1989), 5-HT_{1A} receptors (Hall et al., 1985) and 5-HT_2 receptors (Leysen et al., 1982), respectively, were measured according to the methods described previously.

2.4. Binding assay of [^{35}S]GTPγS

The binding of [^{35}S]GTPγS was carried out using the hA_3R/HEK293 cell membranes. The membrane fraction was prepared as described previously (Cohen et al., 1996; Jacobson et al., 1997). Cultured cells were washed with PBS and harvested using a cell scraper. Cells were homogenized in ice-cold lysis buffer [5 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$] using a Polytron homogenizer. The homogenate was centrifuged at 40,000 × *g* for 20 min at 4 °C. Then, the pellet was suspended in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA in the presence of 300 μg/ml saponin and centrifuged at 40,000 × *g* for 20 min at 4 °C. The pellet was homogenized in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA and stored frozen at − 80 °C until use. For assay, membranes (50 μg) were suspended in a buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 3 U/ml adenosine deaminase, 1 mM dithiothreitol, 100 mM NaCl, 25 mM $MgCl_2$ and 3 μM GDP. The membrane fraction and 0.1 nM [^{35}S]GTPγS in the presence or absence of drug were incubated for 20 min at 30 °C. Nonspecific binding was determined in the presence of 10 μM GTPγS. These binding reactions were performed in the presence of 1% DMSO (final concentration). Incubation of the reaction mixture was terminated by filtration through a GF/B filter using a MT-24 cell harvester. Filters were washed three times with ice-cold assay buffer and placed in scintillation vials, and bound radioactivity was determined using a Packard TRI-CARB 4530 liquid scintillation counter.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cell lines by Isogen (Wako, Osaka, Japan), a phenol-based reagent capable of extracting RNA, according to the manufacture's instructions. Briefly, cells were homogenized in Isogen (10^7 cells/ml) and treated by chloroform and isopropanol stepwise. Precipitation of the obtained fraction by 70% ethanol yielded total RNA. The first-strand cDNAs were synthesized by reverse transcription of total RNA using SUPERSCRIPT Preamplification System (Life Technologies, Rockville, MD), according to the manufacture's instructions. The PCR primers were synthesized as described previously for human adenosine A_1 receptors (sense primer [5′-CAACATTGGGCCACAGACCT-3′] and antisense primer [5′ TAGGTAAGGATGCTGGGCTT-3′], which amplify a 606-bp fragment), human adenosine A_{2A} receptors (sense primer [5′-AGATGGAGAGCCAGCCTCTGC-3′] and antisense primer [5′-GCTAAGGAGCTCCACGTCTGG-3′], which amplify a 535-bp fragment, Suzuki et al., 1998), human adenosine A_{2B} receptors (sense primer [5′-CAGACGCCCACCAACTACTT-3′] and antisense primer [5′-GCCACCAGGAAGATCTTAATG-3′], which amplify a 513-bp fragment, Fredholm et al., 1996), human adenosine A_3 receptors (sense primer [5′-ACCCCCATGTTTGGCTG-3′] and antisense primer [5′-GCACAAGCTGTGGTACCTCA-3′], which amplify a 361-bp fragment, Kohno et al., 1996) or human β-actin (sense primer [5′-GACTACCTCATGAAGATCCT-3′] and antisense primer [5′-ATCTGCTGGAAGGTGGACAG-3′], which amplify a 506-bp fragment). Primers were synthesized by Lifetech Oriental (Tokyo, Japan). PCR was carried out using Ampli Taq Gold Kit (Perkin-Elmer, Foster City, CA) in a Takara PCR Thermal Cycler 480 (Takara, Kyoto, Japan). PCR conditions for adenosine receptor subtypes were as follows: the initial denaturing step at 95 °C for 10 min, followed by 28 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s. PCR conditions for β-actin were as follows: the initial denaturing step at 95 °C for 10 min, followed by 22 cycles of 94 °C for 1 min, 55 °C for 1 min, the last elongation at 72 °C for 10 min. The PCR products were run on 0.8% agarose gels and visualized with ethidium bromide. These samples were quantified as control of β-actin using lumino imageanalyzer LAS-1000plus (Fuji Film, Tokyo, Japan).

2.6. Measurement of cytosolic Ca^{2+} concentration in HL-60 cells

The nontreated or dexamethasone-treated HL-60 cells (2×10^6 cells/ml) were preincubated with 3 μM Fura 2-AM and 0.003% pluronic F-127 (Asahidenka Kogyo, Tokyo, Japan) in Hanks' balanced salt solution (HBSS) buffer for 1 h at 37 °C in the dark. After washing three times with HBSS buffer to remove extracellular dye, the cells were suspended in HBSS buffer at 2×10^6 cells/ml and kept in the dark at room temperature. The cell suspension was transferred to each cuvette and stirred with a magnetic stirrer, and the fluorescence intensity of Fura 2 was quantified using a Ca^{2+} analyzer CAF-100 (Japan Spectroscopic, Tokyo, Japan). For measurement of antagonist activity, antagonists were added to each cuvette 2 min before addition of agonist, Cl-IB-MECA. The maximum and minimum fluorescence were determined in the presence of 0.1% Triton-X and 3 mM EGTA, respectively. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to the method by Grynkiewicz et al. (1985). These experiments were performed in the presence of 0.1% DMSO (final concentration).

2.7. Data analysis

Computer analysis [EBDA and LIGAND (Munson and Rodbard, 1980)] was used to evaluate the dissociation constant (K_d value) and receptor density (B_{max} value). IC_{50} values were determined from computerization of logit–log curve. The Cheng–Prusoff equation (Cheng and Prusoff, 1973) was used to calculate K_i values from IC_{50} values. Statistical significance of the K_d and B_{max} changes was determined using a one-way ANOVA test. In measurement of $[Ca^{2+}]_i$, $\Delta[Ca^{2+}]_i$ was the change of the $[Ca^{2+}]_i$. K_B values were estimated using the Schild equation (Arunlakushana and Schild, 1959). Data represent means ± S.E.M.

3. Results

3.1. High affinity and selectivity to human adenosine A_3 receptors of KF26777

As reported previously (Salvatore et al., 1993), [^{125}I]AB-MECA bound with high affinity to membranes prepared from HEK293 cells expressing cloned human adenosine A_3 receptors (clone HS-21a). KF26777 potently inhibited the [^{125}I]AB-MECA binding to human adenosine A_3 receptors in a dose dependent manner. The K_i value was 0.20 ± 0.038 nM. Affinities of binding to other human adenosine receptor

Table 1
Affinities of adenosine A_3 receptor antagonists for human adenosine receptor subtypes

	K_i (nM)			
	A_3	A_1	A_{2A}	A_{2B}
KF26777	0.20 ± 0.038	1800 ± 170	470 ± 72	620 ± 170
I-ABOPX	6.8 ± 1.7	38 ± 8.3	36 ± 2.6	39 ± 0.67
MRS1191	32 ± 3.0	>10000	>10000	>10000

Binding assays were performed using membranes prepared from cells stably expressing each human adenosine receptor subtypes (A_1: CHO cells, A_{2A}, A_{2B}, A_3: HEK293 cells). Values were means ± S.E.M. of three separate experiments.

Table 2
Radioligand binding profile of KF26777

Receptor	Percent of inhibition (10 μM)
Adrenaline α_1	11
Adrenaline α_2	18
Adrenaline β	15
Dopamine D_1	42
Dopamine D_{2L}	7
Histamine H_1	8
Histamine H_2	44
Muscarine M_1	11
5-HT_{1A}	15
5-HT_2	11

Assays were carried out according to the standard protocol documented in the references. All assays were validated using appropriate reference standards. Values were means from one experiment performed in duplicate.

subtypes by KF26777 was much lower than that to the adenosine A_3 receptors. The K_i values of KF26777 for the human adenosine A_1, A_{2A} and A_{2B} receptors were 1800 ± 170, 470 ± 72 and 620 ± 170 nM, respectively (Table 1). Thus, the selectivity ratios for the adenosine A_1, A_{2A} and A_{2B} receptors vs. adenosine A_3 receptors were 9000-, 2350- and 3100-fold, respectively. Compared with the well-known adenosine A_3 receptor antagonists, I-ABOPX and MRS1191, KF26777 had higher affinity and selectivity for the adenosine A_3 receptors.

Even at a concentration of 10 μM, KF26777 had very little effect on the bindings for α_1- and α_2-adrenoceptors, and β-adrenoceptors, dopamine D_1 receptors and dopamine D_{2L} receptors, histamine H_1 receptors and histamine H_2 receptors, muscarinic M_1 receptors, 5-HT_{1A} receptors and 5-HT_2 receptors (Table 2).

Scatchard analysis of [^{125}I]AB-MECA binding to human adenosine A_3 receptors showed that KF26777 caused significant change in the K_d value ($P < 0.05$) without change of the B_{max} value, indicating that the inhibition mode is competitive (Fig. 2A). On the other hand, inhibition mode of I-ABOPX is noncompetitive, since significant change in

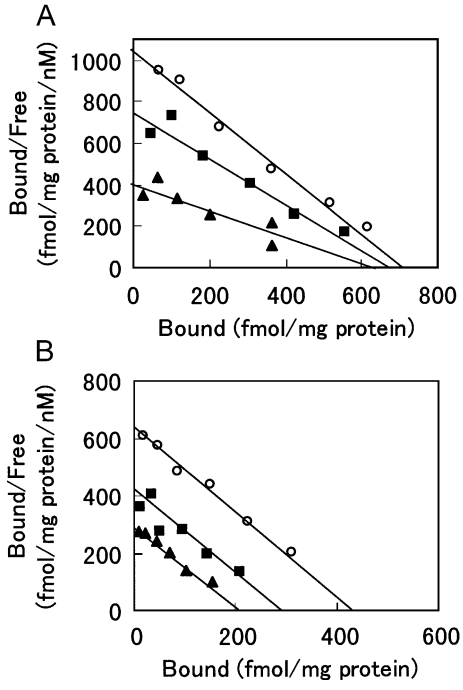

Fig. 2. Scatchard plots for the binding of [^{125}I]AB-MECA in the absence or presence of KF26777 (A) and I-ABOPX (B) in membranes prepared from HEK293 cells expressing human adenosine A_3 receptors. (A) K_d values were 0.67 ± 0.026 nM (KF26777: 0 nM, open circle), 0.93 ± 0.096 nM (KF26777: 0.3 nM, closed square) and 1.14 ± 0.10 nM (KF26777: 1 nM, closed triangle). (B) K_d values were 0.55 ± 0.071 nM (I-ABOPX: 0 nM, open circle), 0.54 ± 0.15 nM (I-ABOPX: 10 nM, closed square) and 0.67 ± 0.072 nM (I-ABOPX: 30 nM, closed triangle). Values were means ± S.E.M. of three separate experiments.

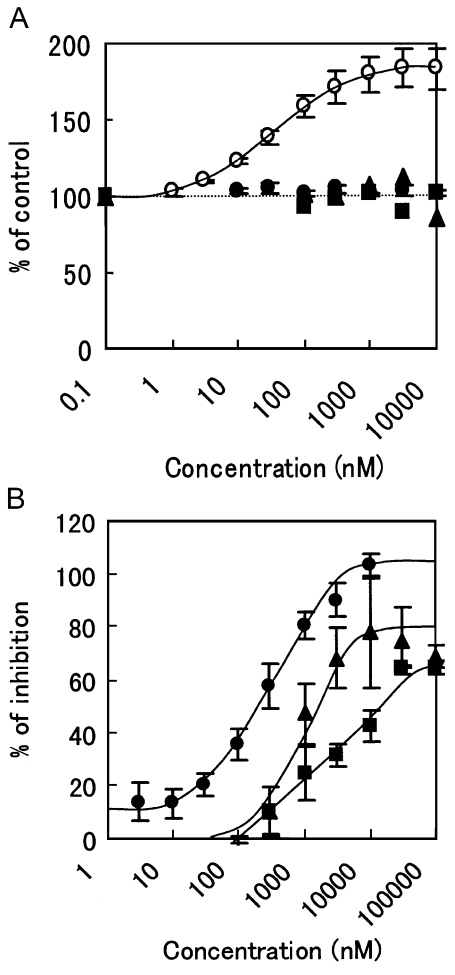

Fig. 3. Effects of adenosine A_3 receptor ligands on [^{35}S]GTPγS binding in membranes prepared from HEK293 cells expressing human adenosine A_3 receptors. Binding of [^{35}S]GTPγS is performed in the absence (A) or presence (B) of 1 μM Cl-IB-MECA. Open circle: Cl-IB-MECA, closed circle: KF26777, closed square: I-ABOPX, closed triangle: MRS1191. Data (A) were expressed as percentage of the basal [^{35}S]GTPγS binding (8600 ± 860 dpm). EC_{50} value of Cl-IB-MECA was 33 ± 6.0 nM. IC_{50} values were 270 ± 85 nM (KF26777), 2200 ± 1100 nM (MRS1191) and 13,000 ± 2300 nM (I-ABOPX). Values were means ± S.E.M. of three separate experiments.

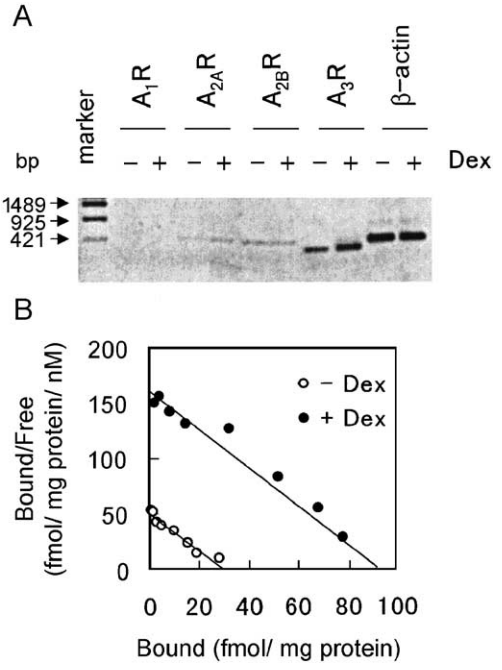

Fig. 4. Increase in expression of adenosine A_3 receptors in HL-60 cells treated with dexamethasone. HL-60 cells were treated with 1 μM dexamethasone for 16 h. (A) RT-PCR analysis of adenosine receptor subtypes in HL-60 cells which were treated with dexamethasone (+) or nontreated (−). (B) Scatchard plot for the binding of [^{125}I]AB-MECA. K_d values were 0.66 ± 0.13 nM (nontreated, open circle) and 0.69 ± 0.054 nM (dexamethasone-treated, closed circle). B_{max} values were 33 ± 4.7 fmol/mg protein (nontreated) and 110 ± 20 fmol/mg protein (dexamethasone-treated). Values were means ± S.E.M. of three separate experiments.

the B_{max} value ($P < 0.05$) and no change in the K_d value were observed (Fig. 2B).

3.2. Effect of KF26777 on [^{35}S]GTPγS binding induced via adenosine A_3 receptor stimulation

The selective adenosine A_3 receptor agonist, Cl-IB-MECA increased dose-dependently the binding of the guanine nucleotide [^{35}S]GTPγS with an EC_{50} value of 33 ± 6.0 nM. No change of [^{35}S]GTPγS binding was detected in the presence of the adenosine A_3 receptor antagonists, KF26777, MRS1191 and I-ABOPX (Fig. 3A). Each of the antagonists inhibited dose-dependently the binding of [^{35}S]GTPγS induced with 1 μM Cl-IB-MECA. The IC_{50} values of KF26777, MRS1191 and I-ABOPX were 270 ± 85, 2200 ± 1100 and 13000 ± 2300 nM, respectively (Fig. 3B).

3.3. Effect of dexamethasone on adenosine A_3 receptor expression and function in HL-60 cells

The RT-PCR analysis for HL-60 cells revealed that dexamethasone treatment increased mRNA levels by a factor of 1.5 in the adenosine A_3 receptors (Fig. 4A). The mRNA of adenosine A_1 receptors was not detected, and the mRNAs of adenosine A_{2A} and A_{2B} receptors were detected but the levels were very low in both dexamethasone-treated and nontreated HL-60 cells (Fig. 4A). Also the binding of [^{125}I]AB-MECA to HL-60 cell membranes was affected by dexamethasone treatment; the corticoid elicited about a threefold increase of B_{max} values from 33 ± 4.7 to 110 ± 20 fmol/mg protein. The K_d values were not different between nontreatment (0.66 ± 0.13 nM) and dexamethasone treatment (0.69 ± 0.054 nM) (Fig. 4B). The maximum $[Ca^{2+}]_i$ level produced by Cl-IB-MECA was approximately fivefold higher in dexamethasone-treated HL-60 cells compared with nontreated cells (Fig. 5). This $[Ca^{2+}]_i$ elevation by Cl-IB-MECA was concentration dependent with an EC_{50} value of 7.1 ± 2.7 nM (Fig. 5B). Adenosine A_1 receptor antagonists (DPCPX or KW-3902) and A_{2A} receptor antagonists (KF17837 or CSC) at concentrations of 1 μM showed less than 50% inhibition of the $[Ca^{2+}]_i$ elevation produced by 10 nM Cl-IB-MECA in dexamethasone-treated HL-60 cells (data not shown). Collectively, these data indicate that the $[Ca^{2+}]_i$ elevation observed in dexamethasone-treated HL-60 cells is mediated via adenosine A_3 receptors.

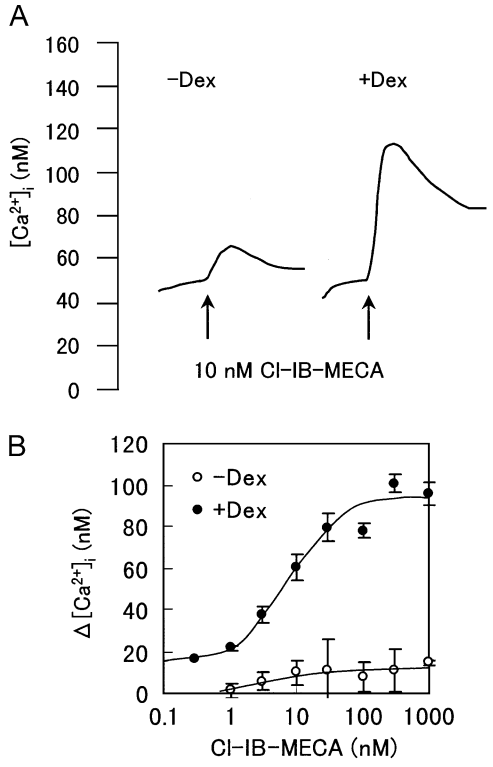

Fig. 5. Enhancement of adenosine A_3 receptor-mediated $[Ca^{2+}]_i$ elevation in HL-60 cells by dexamethasone treatment. HL-60 cells were treated with 1 μM dexamethasone for 16 h. (A) $[Ca^{2+}]_i$ response by 10 nM Cl-IB-MECA in dexamethasone-treated or nontreated HL-60 cells. (B) Concentration–response curve of Cl-IB-MECA for the $[Ca^{2+}]_i$ elevation in dexamethasone-treated (closed circle) or nontreated (open circle) HL-60 cells. EC_{50} value was 7.1 ± 2.7 nM in dexamethasone-treated HL-60 cells. Values were means ± S.E.M. of three separate experiments.

3.4. Effect of KF26777 on intracellular Ca^{2+} elevation induced by adenosine A_3 receptor activation

KF26777 and I-ABOPX inhibited in a concentration dependent manner the $[Ca^{2+}]_i$ elevation produced by 10 nM Cl-IB-MECA in dexamethasone-treated HL-60 cells with IC_{50} values of 1.5 ± 0.37 and 65 ± 7.2 nM, respectively (Fig. 6A). In addition, subnanomolar and nanomolar concentrations of KF26777 shifted the concentration–response curve for Cl-IB-MECA to the right (Fig. 6B). However, at higher concentrations (over 300 nM) of Cl-IB-MECA, the curve in the presence of KF26777 reached plateau at around 80% of the maximal response observed in the absence of the antagonist. Schild plot indicated an apparent K_B value of 0.42 ± 0.14 nM with a slope factor of 1.1 ± 0.086. These values were consistent closely with the respective K_i values obtained in binding assay. These data confirm that KF26777 has potent antagonist activity to human adenosine A_3 receptors.

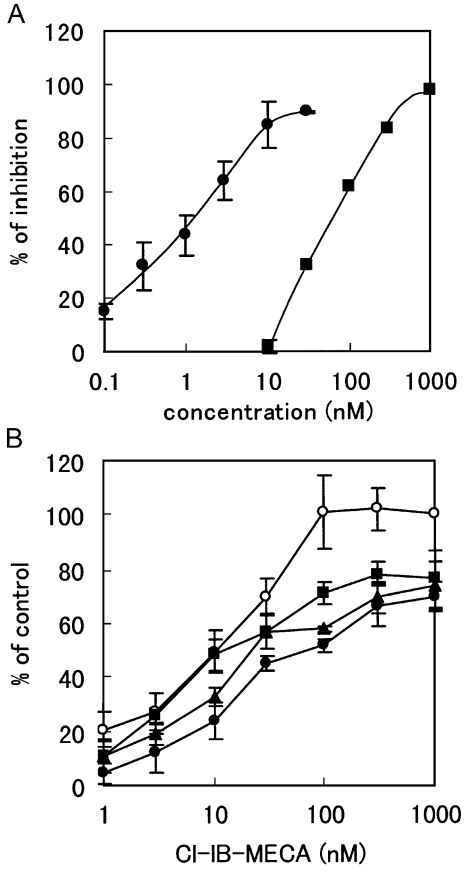

Fig. 6. Effects of adenosine A_3 receptor antagonists on Cl-IB-MECA-induced $[Ca^{2+}]_i$ elevation in dexamethasone-treated HL-60 cells. (A) Concentration–inhibition curves of KF26777 (circle) and I-ABOPX (square) for the $[Ca^{2+}]_i$ elevation induced by 10 nM Cl-IB-MECA in dexamethasone-treated HL-60 cells. IC_{50} values were 1.5 ± 0.37 nM (KF26777) and 65 ± 7.2 nM (I-ABOPX). (B) Concentration–response curves of Cl-IB-MECA for the $[Ca^{2+}]_i$ elevation in dexamethasone-treated HL-60 cells in the absence (open circle) and presence of KF26777 (closed square: 0.3 nM; closed triangle: 1 nM; closed circle: 3 nM). Values were means ± S.E.M. of three separate experiments.

4. Discussion

Compound KF26777 is a potent and selective antagonist for human adenosine A_3 receptors as evidenced by following. First, KF26777 showed a high affinity binding to human adenosine A_3 receptors with a K_i value of 0.20 nM (Table 1). Scatchard analysis indicated that the binding mode of KF26777 was competitive, while that of I-ABOPX is noncompetitive (Fig. 2). Second, it was shown that KF26777 possessed about 9000-, 2350- and 3100-fold selectivity for the adenosine A_3 receptors over the adenosine A_1, A_{2A} and A_{2B} receptors, respectively (Table 1). Third, KF26777 had no or a very low affinity for the ten other G protein-coupled receptors examined (Table 2). Fourth, KF26777 antagonized Cl-IB-MECA-induced [^{35}S]GTPγS binding to hA_3R/HEK293 cell membranes, and had no effect per se on basal binding, which indicates that KF26777 is not an inverse agonist but a neutral antagonist (Fig. 3). Fifth, KF26777 antagonized Cl-IB-MECA-induced $[Ca^{2+}]_i$ elevation in dexamethasone-treated HL-60 cells (Fig. 6A) and it showed no effect on basal $[Ca^{2+}]_i$ (data not shown). However, in the concentration–response curve of Cl-IB-MECA for the $[Ca^{2+}]_i$ elevation, the elevation could not get complete recovery at higher concentrations of Cl-IB-MECA irrespective of rightward shift via KF26777 (Fig. 6B). This data indicates that KF26777 is not a complete competitive antagonist in functional assay. Though several antagonists of G protein-coupled receptors showed the differences of inhibition mode between binding and function (Ring et al., 1992; Wienen et al., 1993), the exact reason of such difference on KF26777 remains to be clarified.

The reported adenosine A_3 receptor antagonists can be classified into five families on the basis of chemical structure: (a) flavonoid derivatives (MRS1067, Karton et al., 1996); (b) dihydropyridine and pyridine derivatives (MRS1191, Jiang et al., 1996; MRS1523, Li et al., 1999); (c) triazoloquinazoline derivatives (MRS1220, Kim et al., 1996); (d) isoquinoline and quinazoline derivatives (VUF8504, van Muijlwijk-Koezen et al., 1998) and (e) others (L-249313, Jacobson et al., 1996; MRE3008-F20, Baraldi et al., 1999). KF26777 has a unique structure (an imidazopurine derivative) as an adenosine A_3 receptor antagonist. Also, it is one of the most potent and selective antagonists of human adenosine A_3 receptors and well characterized pharmacologically.

It was reported that adenosine A_3 receptor stimulation activated phospholipase C and caused $[Ca^{2+}]_i$ elevation. In hA_3R/HEK293 cells, no significant $[Ca^{2+}]_i$ elevation was observed by the selective adenosine A_3 receptor agonist, Cl-IB-MECA (data not shown). A human promyelocytic leukemia cell line, HL-60 is known to express adenosine A_3

receptors and show $[Ca^{2+}]_i$ elevation via adenosine A_3 receptors (Kohno et al., 1996). In our experiments, the $[Ca^{2+}]_i$ elevation induced by adenosine A_3 receptor activation in HL-60 cells was insufficient to enable characterization of the adenosine A_3 receptor ligands. Recently, it was reported that dexamethasone increased adenosine A_3 receptor expression in rat basophilic leukemia RBL-2H3 cells, thereby enhancing $[Ca^{2+}]_i$ mobilization and antigen-induced degranulation via adenosine A_3 receptors (Ramkumar et al., 1995). We found that dexamethasone treatment increased adenosine A_3 receptor mRNA levels in HL-60 cells (Fig. 4A) and binding of [^{125}I]AB-MECA to cell membranes (Fig. 4B) and enhanced $[Ca^{2+}]_i$ elevation through adenosine A_3 receptors activation (Fig. 5). Dexamethasone treatment did not significantly change mRNA levels of adenosine A_3 receptors in the cell lines KU812, THP-1 and U937, which express constitutively adenosine A_3 receptors (data not shown). Thus, this suggests that enhanced expression and function of adenosine A_3 receptors by dexamethasone does not occur in all cells expressing adenosine A_3 receptors.

In dexamethasone-treated HL-60 cells, the $[Ca^{2+}]_i$ elevation via Cl-IB-MECA was inhibited neither by adenosine A_1 receptor antagonists (KW-3902 and DPCPX) nor A_{2A} receptor antagonists (KF17837 and CSC). The effect of adenosine A_{2B} receptor antagonists could not be assessed because no such specific antagonists have been reported. However, information from binding experiments shows that Cl-IB-MECA displays adenosine A_3 receptor selectivity of 2500-fold vs. A_1 and 1600-fold vs. A_{2A} receptors (Kim et al., 1994) and Cl-IB-MECA causes less than 50% of inhibition of adenosine A_{2B} receptor binding at a concentration of 10 μM (data not shown). These results are consistent with the proposal that the Cl-IB-MECA induced $[Ca^{2+}]_i$ elevation in dexamethasone-treated HL-60 cells by an effect on adenosine A_3 receptors. Thus, in HL-60 cells treated with dexamethasone, both mRNA and protein levels of functional adenosine A_3 receptors were increased. An additional mechanism for the observed glucocorticoid-induced increase of adenosine A_3 receptor activity may also be involved because in RBL-2H3 cells, dexamethasone up-regulated the expression level of signal transduction molecules coupled to adenosine A_3 receptors such as G protein α subunits (G_{i2}, G_{i3}) and β subunits which couple to phospholipase C (Ramkumar et al., 1995).

It is important to recognize the species differences in binding affinity; KF26777 showed no affinity for rat adenosine A_3 receptors, which is a property shared by other adenosine A_3 receptor antagonists except for MRS1191 and MRS1523. However, affinities and selectivities of these MRS compounds were much less at the rat adenosine A_3 receptor (K_i: 0.1–1 μM, 20–30-fold selective vs. rat adenosine A_1 or A_{2A} receptors; Jiang et al., 1997; Li et al., 1999). At the present time, no adenosine A_3 receptor antagonists that have high affinity and selectivity for both rat and human are known.

In conclusion, we have demonstrated that KF26777 is one of the most selective, high affinity ligands for the human adenosine A_3 receptor. Therefore, KF26777, which is a well-characterized antagonist, should be a useful tool to aid the pharmacological characterization of the adenosine A_3 receptor and may be a useful prototype drug for the treatment of brain ischemia and inflammatory disease including asthma.

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