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Inhibition of human mast cell activation with the novel selective adenosine A_{2B} receptor antagonist 3-isobutyl-8-pyrrolidinoxanthine (IPDX)

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Abstract

The antiasthmatic drug enprofylline was the first known selective, though not potent, A_{2B} antagonist. On the basis of structure–activity relationships (SARs) of xanthine derivatives, we designed a novel selective adenosine A_{2B} receptor antagonist, 3-isobutyl-8-pyrrolidinoxanthine (IPDX), with potency greater than that of enprofylline. IPDX displaced [^{3}H]ZM241385 ([^{3}H]4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*]-[1,3,5]triazin-5-ylamino]ethyl)phenol) from human A_{2B} adenosine receptors with a K_i value of 470 ± 2 nM and inhibited A_{2B}-dependent cyclic AMP (cAMP) accumulation in human erythroleukemia (HEL) cells with a K_B value of 625 ± 71 nM. We found that IPDX was more selective than enprofylline toward human A_{2B} receptors. It was 38-, 55-, and 82-fold more selective for human A_{2B} than for human A_1 (K_i value of 24 ± 8 μM), human A_{2A} (K_B value of 36 ± 8 μM), and human A_3 (K_i value of 53 ± 10 μM) adenosine receptors, respectively. IPDX inhibited NECA (5′-*N*-ethylcarboxamidoadenosine)-induced interleukin-8 secretion in human mast cells (HMC-1) with a potency close to that determined for A_{2B}-mediated cAMP accumulation in HEL cells, thus confirming the role of A_{2B} adenosine receptors in mediating human mast cell activation. Since adenosine triggers bronchoconstriction in asthmatic patients through human mast cell activation, IPDX may become a basis for the development of new antiasthmatic drugs with improved properties compared with those of enprofylline. Our data demonstrate that IPDX can be used as a tool to differentiate between A_{2B} and other adenosine receptor-mediated responses.

Keywords: Receptors purinergic; P1; Mast cells; Asthma; Adenosine; Xanthines; Interleukin-8

1. Introduction

The endogenous nucleoside adenosine can be released to, or formed in, the extracellular space under hypoxic and inflammatory conditions. Once generated, adenosine acts as an autocoid by interacting with adenosine receptors belonging to the seven transmembrane G-protein-coupled group of cell-surface receptors. Four subtypes of adenosine receptors have been cloned: A_1, A_{2A}, A_{2B}, and A_3. Significant advancement has been made in the understanding of the molecular pharmacology and the physiological relevance of adenosine receptors, but our knowledge of A_{2B} receptors lags behind that of other receptor subtypes. Lack of selective pharmacological probes has hindered research in this area. NECA, a non-selective adenosine analog remains the most potent A_{2B} agonist. Characterization of A_{2B} receptors

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Abbreviations: cAMP, cyclic, AMP; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; DPSPX, 1,3-dipropyl-8-*p*-sulfophenylxanthine; FBS, fetal bovine serum; IB-MECA, N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine; IL-8, interleukin-8; IPDX, 3-isobutyl-8-pyrrolidinoxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; SAR, structure–activity relationship.

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relies mostly on the lack of effectiveness on these receptors of compounds that are selective agonists or antagonists of other adenosine receptor subtypes. Applying this approach, A_{2B} adenosine receptors have been implicated in mast cell activation and asthma, vasodilation and angiogenesis, regulation of cell growth, intestinal function, and modulation of neurosecretion (for review, see Ref. 1).

The ability of extracellular adenosine to modulate mast cell function has long been recognized [2–4], and activation of mast cells by adenosine has been implicated in the pathophysiology of asthma [5,6]. Inhaled adenosine, or its precursor AMP, provokes bronchoconstriction in asthmatic patients via activation of mast cells [7]. Whereas the adenosine A_3 receptor has been shown to modulate rat mast cell function [8], it appears that the A_{2B} adenosine receptor subtype regulates murine [9], canine [10], and human [11] mast cell activation. We have shown previously that a human mast cell line, HMC-1, expresses functional A_{2A} and A_{2B} receptors [6,11]. Both A_2 subtypes of adenosine receptors activate adenylate cyclase in HMC-1 cells. However, only the non-selective A_{2A}/A_{2B} adenosine receptor agonist NECA, and not the selective A_{2A} agonist CGS 21680, induced secretion of IL-8 from HMC-1 cells, suggesting that A_{2B} adenosine receptors mediate mast cell activation in this cell line, through mechanisms other than adenylate cyclase [11].

Pharmacological characterization of receptors based upon apparent agonist potencies is far from ideal, because it depends not only on agonist binding to the receptor but also on multiple processes involved in signal transduction. Potent and selective A_{2B} antagonists would be preferable for receptor subtype identification. Xanthine derivatives are known to inhibit A_{2B} adenosine receptor-dependent signaling. Some xanthines are at least as potent antagonists at A_{2B} receptors as at other adenosine receptors [12–14]. For example, DPCPX has a high affinity at human A_1 adenosine receptors (K_i =3.6 nM), and is also a very potent inhibitor of human A_{2B} adenosine receptor-dependent cAMP accumulation (K_B = 18.4 nM) [14]. Enprofylline (3-propylxanthine), an antiasthmatic drug, is a selective A_{2B} antagonist [1,11], but its potency is much lower (K_B = 7 μM) than that of DPCPX [11,12]. The low potency of enprofylline limits its use in the pharmacological characterization of A_{2B} receptor-mediated processes. In the present study, on the basis of SARs of xanthine derivatives, we designed a novel selective adenosine A_{2B} receptor antagonist, IPDX, with potency greater than that of enprofylline. We demonstrated that IPDX selectively inhibits A_{2B} receptor-dependent secretion of IL-8, but not A_{2A}-mediated cAMP stimulation in human mast cells.

2. Materials and methods

2.1. Cell culture and reagents

Human mast cells (HMC-1), a gift from Dr. J.H. Butterfield (Mayo Clinic), were maintained in a suspension culture at a density between 3 and 9 $\times$ 10^5 cells/mL by dilution with Iscove's medium supplemented with 10% (v/v) FBS, 2 mM glutamine, antibiotics (penicillin/streptomycin), and 1.2 mM α-thioglycerol. Human erythroleukemia (HEL) cells were obtained from the American Type Culture Collection (TIB 180) and maintained in suspension culture at a density between 3 and 9 $\times$ 10^5 cells/mL by dilution with RPMI 1640 medium supplemented with 10% (v/v) FBS, 10% (v/v) newborn calf serum, antibiotics, and 2 mM glutamine. Chinese hamster ovary CHO-K1 cells were obtained from the American Type Culture Collection (CRL-9618) and were maintained in Ham's F12 medium supplemented with 10% (v/v) FBS and antibiotics. All cells were kept under a humidified atmosphere of air/CO_2 (19:1) at 37°. CGS 21680 (4-[(*N*-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl]-phenylpropionic acid), NECA, IB-MECA, and enprofylline were purchased from Research Biochemicals, Inc. [^{3}H]NECA (28 Ci/mmol) and [^{3}H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine) (109 Ci/mmol) were obtained from the Amersham Corp. ZM241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*]-[1,3,5]triazin-5-ylamino]ethyl) phenol) and [^{3}H]ZM241385 were purchased from Tocris Cookson, Inc. Other xanthine derivatives were synthesized following published procedures [15–17].

2.2. Synthesis of IPDX

3-Isobutylxanthine was prepared as described by Wooldridge and Slack [18]. 3-Isobutylxanthine was brominated as described [19]. Briefly, bromine (5 mmol) was added to a warm [50°] solution of 5 mmol of the xanthine and 5 mmol of sodium acetate in 40 mL of glacial acetic acid. The solution was maintained at 50° for 30 min and then chilled [4°] for 18 hr. The frozen mixture was thawed, and the crystalline solid was collected by filtration and recrystallized from ethanol and water. Yield: 1.1 g (78%), m.p. > 370°.

A mixture of 3-isobutyl-8-bromoxanthine (0.7 mmol) and pyrrolidine (1.3 g) in a pressure tube was heated at 120° for 8 hr. The volatiles were evaporated under a stream of N_2, and the resulting solid was washed extensively with water. Yield: 168 mg (86%), m.p. 340° d. The product migrated as a single peak when subjected to HPLC on a C_{18} column with 40% methanol as the mobile phase. The λ_{max} values of 3-isobutylxanthine and 3-isobutyl-8-bromoxanthine were 278 and 280 nm, respectively, while the pyrrolidino compound had a λ_{max} of 303 nm, typical of 8-aminoxanthines. NMR and mass spectrometry data were consistent with the structure.

2.3. Transfection of CHO-K1 cells

The cDNAs encoding the human A_1 and A_3 adenosine receptors in the pRc/CMV expression vector (Invitrogen) were a gift from Drs. P.R. Schofield and A. Townsend-Nicholson (Garvan Institute). The CHO-K1 cells were

transfected using Fugene 6 transfection reagent (Boehringer Mannheim). Ten micrograms of plasmid DNA was mixed with 500 μL of serum-free Ham's F12 medium containing 30 μL of Fugene 6. After a 15-min incubation at room temperature, the transfection mixture was added to cells growing on 150-mm culture dishes at 40–60% confluency. Then the cells were incubated for 48 hr under a humidified atmosphere of air/CO_2 (19:1) at 37°.

2.4. Preparation of A_1 or A_3 adenosine receptor-transfected CHO-K1 membranes

CHO-K1 cells were confluent on 150-mm culture dishes approximately 2 days after transfection. Each dish was rinsed once with 10 mL of PBS at room temperature and transferred to ice. Cells were covered with 10 mL of ice-cold 10 mM EDTA, 10 mM HEPES, pH 7.4, buffer containing 3.3 μL/mL of Protease Inhibitor Cocktail (Sigma) and scraped from the plate with a rubber policeman. After another scraping cycle, cells were combined and pelleted by centrifugation at 34,540 *g* for 25 min at 4°. The pellet was resuspended in 3 mL of ice-cold 1 mM EDTA, 10 mM HEPES, pH 7.4 (HE buffer), containing 3.3 μL/mL of Protease Inhibitor Cocktail by repeatedly aspirating/expelling the mixture through a 22-gauge needle with a plastic syringe. The mixture was diluted to 15 mL total volume with HE buffer and recentrifuged. The pellet was resuspended in 500 μL HE buffer containing protease inhibitors by aspirating through the needle and stored at –70°. Protein concentration was determined with a Protein Assay Kit (Pierce) using bovine serum albumin as a standard.

2.5. Preparation of A_{2B} adenosine receptor-transfected HEK-A_{2B} membranes

HEK-293 cells were stably transfected with human A_{2B} adenosine receptor cDNA. Monolayers of HEK-A_{2B} cells were washed once with PBS and harvested in a buffer containing 10 mM HEPES (pH 7.4), 10 mM EDTA, and protease inhibitors. These cells were homogenized in a polytron for 1 min at setting 4 and centrifuged at 29,000 *g* for 15 min at 4°. The cell pellets were washed once with a buffer containing 10 mM HEPES (pH 7.4), 1 mM EDTA, and protease inhibitors, and were resuspended in the same buffer supplemented with 10% sucrose. Frozen aliquots were kept at –80°.

2.6. Radioligand binding

Dissociation constants of radioligands (K_d) were estimated from saturation binding experiments. The K_i values for adenosine receptor antagonism were determined in radioligand competition experiments by the method of Cheng and Prusoff [20]. All binding data were calculated by the non-linear curve fitting program GraphPad Prism (GraphPad Software, Inc.).

A_1 adenosine receptor binding was performed using [^{3}H]DPCPX as a radioligand. Membranes were treated for 20 min at 30° with 1 U of adenosine deaminase/80 μg of protein to reduce the concentration of endogenous adenosine. Assays were carried out in a 250-μL reaction volume in 12 × 75 mm polypropylene tubes. Incubation ingredients included 40–60 μg of CHO membranes, HE buffer, various amounts of competitor in competition binding assays, 10 mM theophylline in nonspecific binding tubes, and an appropriate concentration of [^{3}H]DPCPX. The radioligand concentration in saturation experiments ranged from 0.1 to 30 nM. The concentration of [^{3}H]DPCPX remained constant at 0.6 nM in competition binding assays. Tubes were incubated at 25° for 90 min, and the incubation was terminated by quenching with 4 mL of ice-cold HE buffer. Bound and free radioligand were separated by filtration through GF/C filters on a Brandel cell harvester. The filters were washed three times with 3.5 mL of ice-cold HE buffer, and air dried; their radioactivity was determined by liquid scintillation counting.

A_3 adenosine receptor binding was performed using [^{3}H]NECA as a radioligand, according to previously published work [14]. The conditions for A_3 adenosine receptor binding were essentially the same as for the A_1 receptor except that the buffer was 1 mM EDTA, 10 mM HEPES, 10 mM $MgCl_2$, pH 8.2. The concentration of radioligand ranged from 1 to 55 nM in saturation binding assays and remained constant at 12.5 nM in competition binding assays.

A_{2B} adenosine receptor binding was performed using [^{3}H]ZM214385 as a radioligand. Competition assays were started by mixing 10 nM [^{3}H]ZM214385 with various concentrations of test compounds and 25 μg membrane proteins in 1 mM EDTA, 50 mM Tris, pH 8.0, supplemented with 1 U/mL of adenosine deaminase. The assays were incubated for 90 min, stopped by filtration using a Packard Harvester, and washed four times with ice-cold 1 mM $MgCl_2$, 10 mM Tris, pH 7.4. Nonspecific binding was determined in the presence of 10 μM ZM214385.

2.7. Measurement of cAMP

Before each experiment, HMC-1 or HEL cells were harvested, washed by centrifugation (100 *g* for 10 min at room temperature), and resuspended in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2P0_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/L of d-glucose, 10 mM HEPES-NaOH, pH 7.4, and 1 U/mL of adenosine deaminase, to a concentration of 3×10^6 cells/mL. Cells were preincubated for 15 min at 37° in the same buffer containing the cAMP phosphodiesterase inhibitor papaverine (1 mM). Adenosine agonists and antagonists were added to cells as indicated. Cells were suspended in a total volume of 200 μL, mixed by vortexing, and the incubation was allowed to proceed for 2 min at 37°. The reaction was stopped by the addition of 50 μL of 25% trichloroacetic acid (TCA) to the cell suspen-

sions. TCA-treated extracts were washed five times with 10 vol. of water-saturated ether. Cyclic AMP concentrations were determined using a cAMP assay kit (TRK.432; Amersham Corp.).

2.8. Inhibition of phosphodiesterase (PDE) enzyme activities

Measurements of phosphodiesterase enzyme activities were performed at MDS Pharma Service using either [^{3}H]cAMP (PDE I, II, III and IV) or [^{3}H]cGMP (PDE V and VI) as substrates.

2.9. Measurement of IL-8 secretion

HMC-1 cells were harvested and resuspended to a concentration of 10^5 cells/mL in serum-free Iscove's medium containing 1 U/mL of adenosine deaminase. Cells were incubated for 3 hr under a humidified atmosphere of air/CO_2 (19:1) at 37° with the reagents indicated in Results. At the end of this incubation period the culture medium was collected by centrifugation at 12,000 *g* for 1 min at 4°. IL-8 concentrations were measured using an ELISA method (DPC Biermann GmbH).

3. Results

3.1. SARs of xanthine derivatives as A_{2B} adenosine receptor antagonists

We conducted a systematic study of over 100 mono-, di- and trisubstituted xanthine compounds to understand the structural requirements of a selective A_{2B} antagonist. Table 1 shows the structures of representative xanthine derivatives and compares their potencies as adenosine A_{2B} receptor antagonists estimated by their ability at 1 μM to inhibit cAMP accumulation in HEL cells induced by 2 μM NECA. We have shown previously that these cells express only A_{2B} adenosine receptors, and CGS 21680 has no effect on cAMP accumulation at concentrations up to 1 mM [12]. The naturally occurring xanthine antagonist theophylline (**1**) is representative of the group of 1,3-disubstituted xanthine derivatives (Table 1A). The affinity of this non-selective antagonist is low (>5 μM) at all subtypes of adenosine receptors [21]. Extension of the 1,3-substituents of theophylline to either 1,3-dipropylxanthine (**2**) or 1-propyl-3-methylxanthine (**6**) increases the effectiveness at A_{2B} adenosine receptors. These compounds, at a concentration of 1 μM, inhibited A_{2B} adenosine receptor-dependent cAMP accumulation by approximately 50% in our assay. The further extension of 1,3-alkyl groups in 1,3-dibutylxanthine (**3**) decreased the activity at A_{2B} receptors. Replacement of one methyl group of theophylline with isobutyl in the 3-position (**4**) appeared to increase the inhibitory potency, whereas further extension of the isobutyl group in the 3-position to isoamyl (**5**) decreased this activity. Substitutions at the 7-position with a methyl or propargyl group (**7, 9**) did not affect A_{2B} receptor-mediated antagonism, whereas substitution with a benzyl group (**8**) decreased this activity (Table 1B).

Substitutions at the 8-position in combination with substitutions at the 1- and 3-positions at the xanthine ring (**10–21**) yielded compounds that were considerably more potent than 1,3-disubstituted or 1,3,7-trisubstituted compounds (Table 1C). Among them are derivatives of 1,3-dipropylxanthine with cyclic aliphatic (**10, 11, 13–15, 18–21**) or aromatic (**12, 17**) 8-substituents. We found that compounds with cyclic aliphatic 8-substituents containing a nitrogen atom adjacent to the xanthine imidazole (**18, 20, 21**) were potent A_{2B} antagonists, whereas the compound with an 8-substituent containing only a secondary amine within the cyclohexyl ring (**19**) was not an effective inhibitor of A_{2B} receptor-mediated response at a concentration of 1 μM.

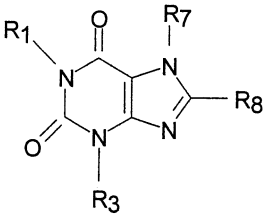
Within the group of 3-substituted xanthines (Table 1D), enprofylline (**22**) is a relatively weak A_{2B} antagonist. Change of the 3-propyl group of enprofylline to a 3-isobutyl group (**25**) increased the effectiveness as an antagonist at A_{2B} receptors, but further extension of isobutyl to isoamyl decreased the potency of A_{2B} antagonism (**23**). Replacing of the 3-isobutyl in compound **25** with 3-benzyl (**24**) did not increase the activity at the A_{2B} receptor further; however, substitution with 3-methylcyclopropyl (**26**) resulted in the loss of A_{2B} antagonism at a concentration of 1 μM.

To explore the SARs of 3,8-disubstituted xanthines, we chose compound **25** as a parent compound for comparison. Table 1E compares the activities of representative 8-alkyl (**27**), 8-aromatic (**28**), and 8-cycloalkyl (**29**) derivatives of 3-isobutylxanthine at the A_{2B} receptor. 3-Isobutyl-8-*n*-butylxanthine (**27**) inhibited A_{2B} adenosine receptor-dependent cAMP accumulation by 38 ± 10% at a concentration of 1 μM. Substitution of 8-butyl in compound **27** with 8-phenyl (**28**) decreased its activity at the A_{2B} receptor, and substitution with 8-cyclohexyl (**29**) resulted in the loss of A_{2B} antagonism at a concentration of 1 μM.

Compounds were selected based on their inhibitory potency of A_{2B} receptor-mediated cAMP accumulation and tested for their selectivity against A_1 and A_{2A} adenosine receptors. As seen in Table 2, the group of 1,3,8-trisubstituted xanthines, representing the most potent A_{2B} antagonists (**10–15, 18, 20, 21**), also had a high affinity at A_1 receptors. These compounds, generally, had lower affinity at A_{2A} receptors in comparison with A_1 adenosine receptors, but some of them were particularly ineffective at A_{2A} adenosine receptors. For example, the potent A_1 antagonist 1,3-dipropyl-8-pyrrolidinoxanthine (**21**) inhibited A_{2B} adenosine receptor-dependent cAMP accumulation in HEL cells by 77 ± 3% at a concentration of 1 μM, but had no effect on A_{2A}-dependent stimulation of adenylate cyclase in human platelets at concentrations up to 3 μM.

We have reported previously that enprofylline, a 3-substituted xanthine, is a selective A_{2B} antagonist [11]. Among

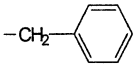
Table 1
SARs of adenosine A_{2B} receptor antagonists



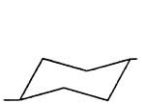
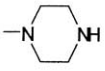
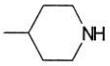
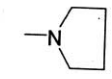
(A) 1,3-Disubstituted xanthines

Compound No.	R_1	R_3	R_7	R_8	A_{2B} antagonism (% inhibition)
1	$-CH_3$	$-CH_3$	–H	–H	0[a]
2	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H	–H	49 ± 6
3	$-(CH_2)_3CH_3$	$-(CH_2)_3CH_3$	–H	–H	10 ± 15
4	$-CH_3$	$-CH_2CH(CH_3)_2$	–H	–H	35 ± 8
5	$-CH_3$	$-(CH_2)_2CH(CH_3)_2$	–H	–H	7 ± 17
6	$-(CH_2)_3CH_3$	$-CH_3$	–H	–H	50 ± 18

(B) 1,3,7-Trisubstituted xanthines

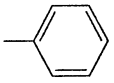
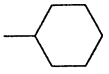
7	$-CH_3$	$-CH_2CH(CH_3)_2$	$-CH_2C{\equiv}CH$	–H	36 ± 13
8	$-CH_3$	$-CH_2CH(CH_3)_2$		–H	7 ± 5
9	$-CH_3$	$-CH_2CH(CH_3)_2$	$-CH_3$	–H	39 ± 14

(C) 1,3,8-Trisubstituted xanthines

10	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H		100 ± 10
11	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H		100 ± 5
12	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H		100 ± 15
13	$-(CH_2)_2CH_3$	$(CH_2)_2CH_3$	–H		70 ± 4
14	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H		91 ± 1
15	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H		87 ± 3
16	$-CH_3$	$-CH_2CH(CH_3)_2$	–H	$-NHCH_3$	65 ± 9
17	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H		72 ± 3
18	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H		75 ± 18
19	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H		0
20	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H		92 ± 5
21	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	–H		77 ± 3

(continued on next page)

Table 1 *(continued)*

Compound No.	R_1	R_3	R_7	R_8	A_{2B} antagonism (% inhibition)
(D) 3-Monosubstituted xanthines					
22	–H	$-(CH_2)_2CH_3$	–H	–H	13 ± 12[b]
23	–H	$-(CH_2)_2CH(CH_3)_2$	–H	–H	26 ± 16
24	–H		–H	–H	46 ± 12
25	–H	$-CH_2CH(CH_3)_2$	–H	–H	59 ± 7
26	–H		–H	–H	0
(E) 3,8-Disubstituted xanthines					
27	–H	$-CH_2CH(CH_3)_2$	–H	$-(CH_2)_3CH_3$	38 ±10
28	–H	$-CH_2CH(CH_3)_2$	–H		18 ± 4
29	–H	$-CH_2CH(CH_3)_2$	–H		0

Adenosine A_{2B} receptor antagonism was estimated by the ability of 1 μM xanthines to inhibit cAMP accumulation in HEL cells induced by 2 μM NECA, and is expressed as percent inhibition. Data are presented as means ± SEM of three separate experiments performed in triplicate.

[a,b] K_B values for [a]theophylline (13 μM) and [b]enprofylline (7 μM) were determined in the same cells by Schild analysis and published previously [11].

the 3-substituted xanthines, 3-isobutylxanthine (**25**) was the most potent A_{2B} antagonist (Table 1). That is, the inhibition potency of this compound at A_{2B} receptors was higher than that of 1-methyl-3-isobutylxanthine (**4**). However, similar to enprofylline, compound **25** was a very poor A_1 and A_{2A} antagonist (Table 2). These data indicate that the lack of 1-alkyl substituents in the xanthine moiety renders the group of 3-substituted xanthines poor inhibitors of A_1 and A_{2A} adenosine receptor subtypes while retaining their inhibitory potency at the A_{2B} receptor. Based upon these data we chose, therefore, to prepare IPDX (**30,** Table 3) as a new selective A_{2B} antagonist. The choice of the pyrrolidino moiety at the 8-position was based upon the relative lower affinity of compound **21** at human A_{2A} adenosine receptors (Table 2). As indicated above, the absence of the 1-substituent and the incorporation of the isobutyl group at the 3-position (e.g. **25**) yielded compounds with a low affinity at the A_1 as well as the A_{2A} adenosine receptors (Table 2). In either the mono-, di-, or trisubstituted series of compounds investigated, incorporation of an isobutyl group at the 3-position was tolerated and appeared to be preferred in yielding potent A_{2B} antagonists. Furthermore, the inclusion of a substituent at the 8-position further increased the potency of the xanthine class of A_{2B} receptor antagonists. By incorporating the features of these trends observed in the SARs of the xanthine class of inhibitors, compound **30** was prepared.

Table 2
Potency of adenosine A_{2B} receptor antagonists at A_1 and A_{2A} receptors

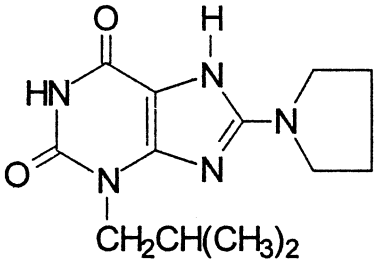
Compound No.	K_i for A_1 (nM)	K_i for A_{2A} (nM)	Reference	A_{2B} (% inhibition)
2	940 ± 250	1900 ± 500	[15]	49 ± 6
4	1600 ± 800	1800 ± 500	[15]	35 ± 8
6	7500 ± 1800	9800 ± 1300	[15]	50 ± 18
10	0.47 ± 0.02	69 ± 13	[15]	100 ± 10
11	2.5 ± 0.5	120 ± 30	[15]	100 ± 5
12	37 ± 9	560 ± 11	[15]	100 ± 15
13	42 ± 9	240 ± 40	[15]	70 ± 4
14	8 ± 2	20 ± 10	[17]	91 ± 1
15	85 ± 5	> 3000	[17]	87 ± 3
18	690 ± 130	2100 ± 200	[15]	75 ± 18
20	22 ± 4	490 ± 70	[15]	92 ± 5
21	76 ± 13	> 3000	[17]	77 ± 3
25	14,000 ± 1000	22,000 ± 8000	[15]	59 ± 7

Adenosine A_{2B} receptor antagonism was estimated by ability of 1 μM xanthines to inhibit cAMP accumulation in HEL cells induced by 2 μM NECA and is expressed as percent inhibition. Data are presented as means ± SEM of three separate experiments performed in triplicate. K_i values for rat adipocyte A_1 and human platelet A_{2A} adenosine receptors have been published previously [15, 17].

3.2. Characterization of IPDX as an antagonist of human adenosine receptors

To determine the affinity of IPDX at human A_1 and A_3 receptors, we used a model system described previously by Klotz *et al.* [14]. CHO cells were transfected with human A_1

Table 3
Adenosine receptor subtype selectivity of IPDX



Compound No.	K_i for A_1 (nM)	K_B for A_{2A} (nM)	K_B for A_{2B} (nM)	K_i for A_3 (nM)
30	24,000 ± 8,000	36,000 ± 8,000	625 ± 71	53,000 ± 10,000

K_i values for A_1 and A_3 receptors were determined by radioligand binding in membranes of CHO-K1 cells expressing human recombinant receptors. K_B values for A_{2A} and A_{2B} receptors were determined by Schild analysis of inhibition of CGS21680- or NECA-induced cAMP stimulation in HMC-1 or HEL cells, respectively. Data are means ± SEM of three separate experiments performed in triplicate.

or A_3 receptors, and membranes from cells transiently expressing adenosine receptors were used in radioligand binding assays. Figure 1A shows a representative saturation curve of human A_1 adenosine receptors with [^{3}H]DPCPX. Saturation analysis of the human A_1 adenosine receptors expressed in CHO cells, conducted in three independent experiments done in triplicate, revealed an expression level of 1.2 ± 0.2 pmol/mg membrane protein and a K_d value of 1.03 ± 0.26 nM for [^{3}H]DPCPX. These data are in close agreement with previously published characteristics of human A_1 adenosine receptors [14].

Antagonist affinity of IPDX, determined in three independent competition binding experiments done in triplicate, was low at human A_1 adenosine receptors with a K_i value of 24 ± 8 μM (Table 3). Figure 1B shows a representative IPDX competition curve for [^{3}H]DPCPX radioligand binding. The affinity of IPDX at A_1 adenosine receptors was lower than that of theophylline (K_i of 11.9 μM) or DPSPX (K_i of 1.3 μM).

Saturation analysis of [^{3}H]NECA binding to the human A_3 receptors, transiently expressed in CHO cells, gave a K_d value of 12.2 ± 2.3 nM and an expression level of 257 ± 34 fmol/mg membrane protein, as determined in five independent experiments done in triplicate (Fig. 2A). Antagonist affinity of IPDX at human A_3 adenosine receptors, determined in three independent competition binding experiments done in triplicate, was low, with a K_i value of 53 ± 10 μM (Table 3). For comparison, the selective A_3 adenosine receptor agonist IB-MECA displaced [^{3}H]NECA in a competition binding assay with a K_i of 1.89 nM (Fig. 2B), in close agreement with previous reports [14].

IPDX antagonist potency on human A_{2B} receptors was determined by Schild analysis of inhibition of NECA-induced cAMP accumulation in HEL cells. IPDX appeared to be a potent A_{2B} antagonist. Increasing concentrations of IPDX, from 2 to 10 μM, produced rightward shifts of the concentration–response curve of NECA (Fig. 3A). Schild analysis of three independent experiments, done in triplicate, yielded a dissociation constant of the antagonist–receptor complex (K_B) of 625 ± 71 nM (Table 3). Figure 3B shows a representative Schild plot for IPDX inhibition of A_{2B}-dependent cAMP accumulation in HEL cells, com-

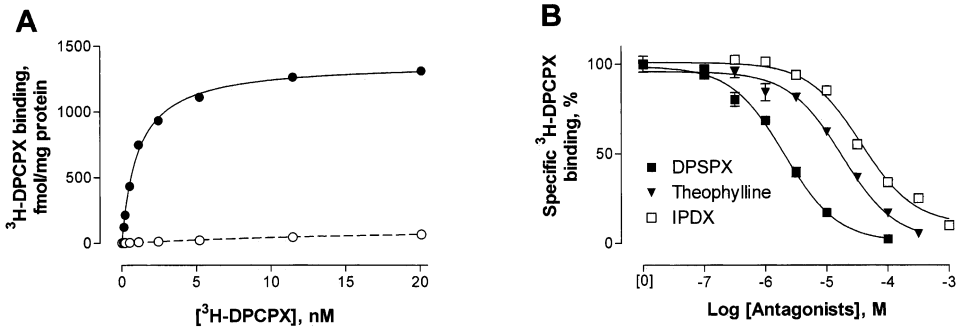


Fig. 1. [^{3}H]DPCPX radioligand binding to human A_1 adenosine receptors. (A) Saturation binding of [^{3}H]DPCPX to A_1 adenosine receptors. Specific (●) and nonspecific (○) binding of [^{3}H]DPCPX from a representative experiment is shown. (B) Competition of DPSPX, 1,3-dimethylxanthine (theophylline), and IPDX for specific binding of [^{3}H]DPCPX to A_1 adenosine receptors. Results (means ± SEM) are representative of three independent experiments.

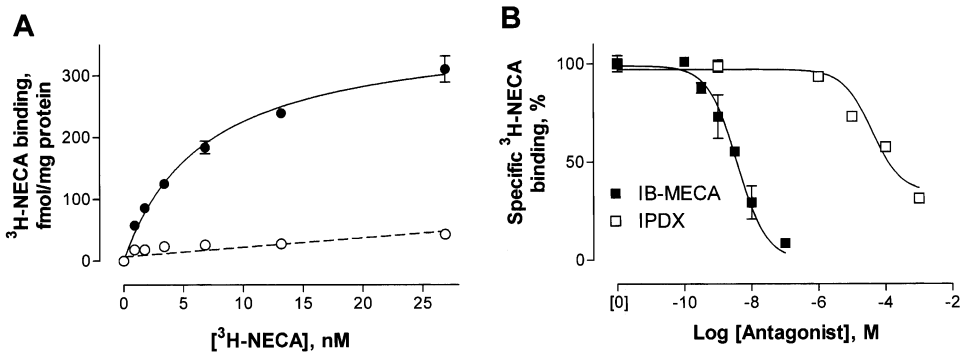


Fig. 2. [^{3}H]NECA radioligand binding to human A_3 adenosine receptors. (A) Saturation binding of [^{3}H]NECA to A_3 adenosine receptors. Specific (●) and nonspecific (○) binding of [^{3}H]NECA from a representative experiment is shown. (B) Competition of IB-MECA and IPDX for specific binding of [^{3}H]NECA to A_3 adenosine receptors. Results (means ± SEM) are representative of five independent experiments.

pared with enprofylline. The antagonist potency of IPDX at A_{2B} adenosine receptors was 11 times higher than that of the selective A_{2B} antagonist enprofylline (K_B of 7 μM).

To confirm the K_B values obtained by Schild plot analysis for IPDX at A_{2B} receptors, we performed radioligand binding in membranes from HEK-A_{2B} cells permanently overexpressing human A_{2B} adenosine receptors. Saturation analysis of [^{3}H]ZM241385 binding to the human A_{2B} adenosine receptors gave a K_d value of 13.3 ± 0.6 nM and a B_{max} value of 11.1 ± 2.1 pmol/mg of membrane protein (N = 2, Fig. 4A). We then compared the affinity of IPDX with that of other adenosine antagonists. K_i values were 470 ± 27 nM for IPDX (N = 5), 5.2 ± 0.9 μM for theophylline (N = 3) and 12.5 ± 0.5 μM for enprofylline (N = 3, Fig. 4B). These data show that IPDX has a higher affinity to the human A_{2B} adenosine receptor than either enprofylline or theophylline.

Human mast cells (HMC-1) express both A_{2A} and A_{2B} adenosine receptors. The selective A_{2A} agonist CGS 21680 stimulates cAMP accumulation with an EC_{50} value of 61 ± 18 nM, and the selective A_{2A} antagonist ZM241385 inhibits this effect with a K_B value of 0.3 ± 0.07 nM [22]. These are typical characteristics of A_{2A} receptors [21]. As seen in Fig. 5A, IPDX appeared to be a poor A_{2A} antagonist. Only high concentrations of IPDX, from 20 to 100 μM, produced rightward shifts in the concentration–response curve of CGS 21680. Figure 5B shows a representative Schild plot of antagonism of A_{2A} adenosine receptors by IPDX. Schild analysis of three independent experiments, done in triplicate, yielded a K_B value of 36 ± 8 μM (Table 3).

Some xanthines are known inhibitors of PDEs (EC 3.1.4.17). We, therefore, tested whether IPDX has any inhibitory activity on PDE I-VI isozymes. At a concentration of 10 μM (approximately 20-fold higher than the K_i value at A_{2B} receptors), IPDX inhibited PDE II by 53%, and it did not have significant inhibition against any of the other PDE isozymes tested (data not shown).

Our data show that the affinity of IPDX at human A_{2B} receptors is 38–82 times higher than at other human adenosine receptor subtypes (Table 3). Considering this high selectivity at human A_{2B} receptors and its greater potency compared with enprofylline, we propose that IPDX can be a valuable tool in the characterization of A_{2B}-dependent processes.

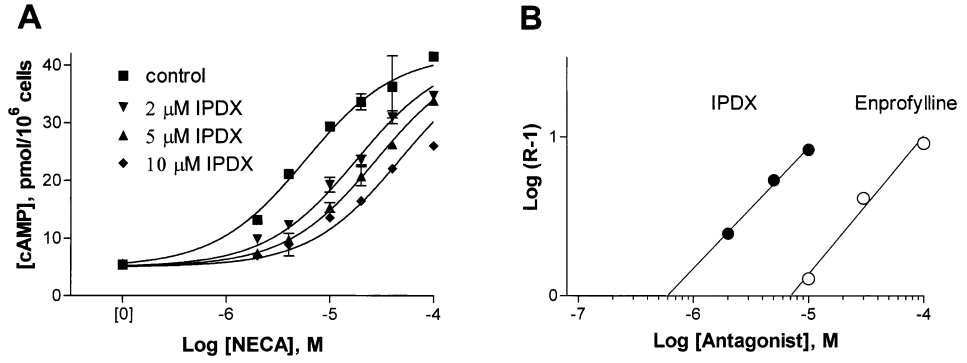


Fig. 3. Antagonistic effects of IPDX on A_{2B}-mediated cAMP accumulation in HEL cells induced by NECA. (A) Concentration–response curves were repeated in the absence and in the presence of increasing concentrations of IPDX, which produced a progressive shift to the right. Results (means ± SEM) are representative of three independent experiments. (B) Schild plot of antagonism of human A_{2B} receptors by IPDX (●) and enprofylline (○). R represents the ratio of the agonist EC_{50} in the presence of antagonist to its EC_{50} in the absence of antagonist. Results are representative of three independent experiments.

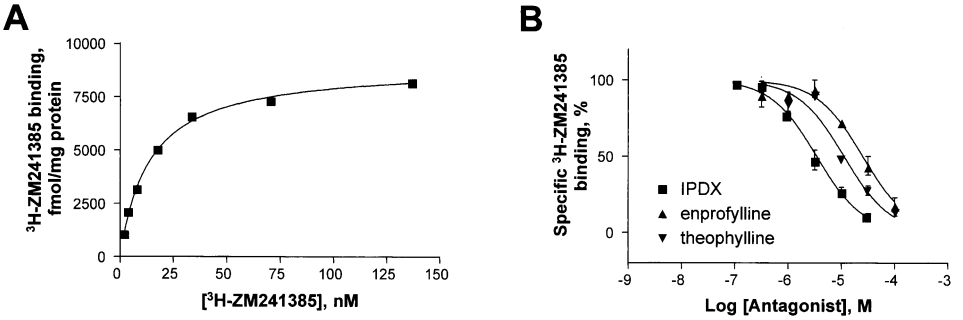


Fig. 4. [^{3}H]ZM241385 radioligand binding to human A_{2B} adenosine receptors. (A) Saturation binding of [^{3}H]ZM241385 to A_{2B} adenosine receptors. Specific binding for a representative experiment is shown. (B) Competition of IPDX, enprofylline, and theophylline for specific binding of [^{3}H]ZM241385 to A_{2B} adenosine receptors. Results (means ± SEM) are representative of 3–5 independent experiments.

3.3. Inhibition of NECA-induced IL-8 secretion by IPDX in human mast cells

We have shown previously that incubation of HMC-1 cells with the non-selective adenosine receptor agonist NECA, but not with the selective A_{2A} agonist CGS 21680, induces secretion of IL-8 [11]. Incubation of HMC-1 cells for 3 hr with increasing concentrations of NECA revealed a sigmoid curve for IL-8 production with an EC_{50} value of 2.3 ± 0.4 μM (Fig. 5C). Increasing concentrations of IPDX, from 1 to 10 μM, produced rightward shifts of the concentration–response curve of NECA. Schild analysis yielded a slope close to unity (1.07 ± 0.7), indicating that IPDX is a simple competitive antagonist, with a K_B value of 1 ± 0.06 μM, close to that found for inhibition of NECA-stimulated cAMP accumulation in HEL cells (Table 3). These data confirm that secretion of IL-8 is an A_{2B}-dependent process in human mast cells. Panel B in Fig. 5 compares Schild plots of antagonism of A_{2B} receptor-dependent secretion of IL-8 and A_{2A}-mediated cAMP stimulation by IPDX in HMC-1 cells.

4. Discussion

Enprofylline was originally developed and found to be clinically useful for the treatment of asthma, but its mechanism of action (and particularly its ability to block adenosine receptors) was initially unclear. In early studies, enprofylline was found to be about 20 times more potent in blocking hippocampal A_2 receptors compared with A_1 receptors [23]. However, enprofylline was then found to be a poor antagonist of A_2 receptors in thymocytes and platelets [24,25]. Enprofylline has also been found to have a low affinity at A_3 receptors [26]. These findings led to the conclusion that enprofylline was not an adenosine antagonist [27]. With the cloning of two subtypes of A_2 adenosine receptors and the recent focus on A_{2B} receptors, a reassessment of these previous results became necessary. It is known now that the presence of A_{2B} adenosine receptors in the hippocampus [28] could explain the effects of enprofylline in that preparation. We found that enprofylline is indeed a selective A_{2B} antagonist, with a K_B value of 7 μM, well within its therapeutic concentration range [11]. Our findings were subsequently confirmed, independently, by others [29]. Enprofylline has been shown to be 22-, 5-, and 6-fold more selective for human A_{2B} than for human A_1, human A_{2A}, and human A_3 adenosine receptors, respectively. Enprofylline, therefore, became the first known selective, though not potent, A_{2B} antagonist.

In the present work, we conducted a systematic SAR study in a search for more potent selective A_{2B} antagonists based on the xanthine nucleus. We found that the most potent, though non-selective, A_{2B} antagonists belong to the group of 1,3,8-trisubstituted xanthines. Some of them, such as 1,3-dipropyl-8-pyrrolidinoxanthine, were of particular interest, because of their selectivity toward A_1 and A_{2B} receptors, and a low affinity at human A_{2A} adenosine receptors. Other compounds of interest were 3-substituted xanthines, a group that includes enprofylline. Analysis of the SARs of 3-substituted xanthines revealed that an isobutyl group in the 3-position was most favorable for antagonistic potency at A_{2B} receptors. The low affinity of 3-isobutylxanthine at A_1 and A_{2A} receptors supported the concept that the lack of 1-alkyl substituents in the xanthine moiety renders the group of 3-substituted xanthines ineffective at other adenosine receptor subtypes.

Our approach to designing a xanthine derivative with an 8-pyrrolidino substituent lacking substituents in the 1-position resulted in the synthesis of a novel selective A_{2B} antagonist, IPDX. This compound displaced [^{3}H]ZM241385 from human A_{2B} adenosine receptors with a K_i value of 470 ± 2 nM, and inhibited A_{2B}-mediated cAMP accumulation in HEL cells with a potency (K_B of 625 nM) 11 times higher than that of the selective A_{2B} antagonist enprofylline (K_B of 7 μM). IPDX also showed improved A_{2B} selectivity compared with enprofylline. Under our experimental conditions, we found IPDX to be 38-, 55-, and 82-fold more selective for human A_{2B} than for human A_1, human A_{2A}, and human A_3 adenosine receptors, respectively. Furthermore, it lacked significant PDE inhibitory activity. Thus, IPDX may be used as a selective A_{2B} antagonist.

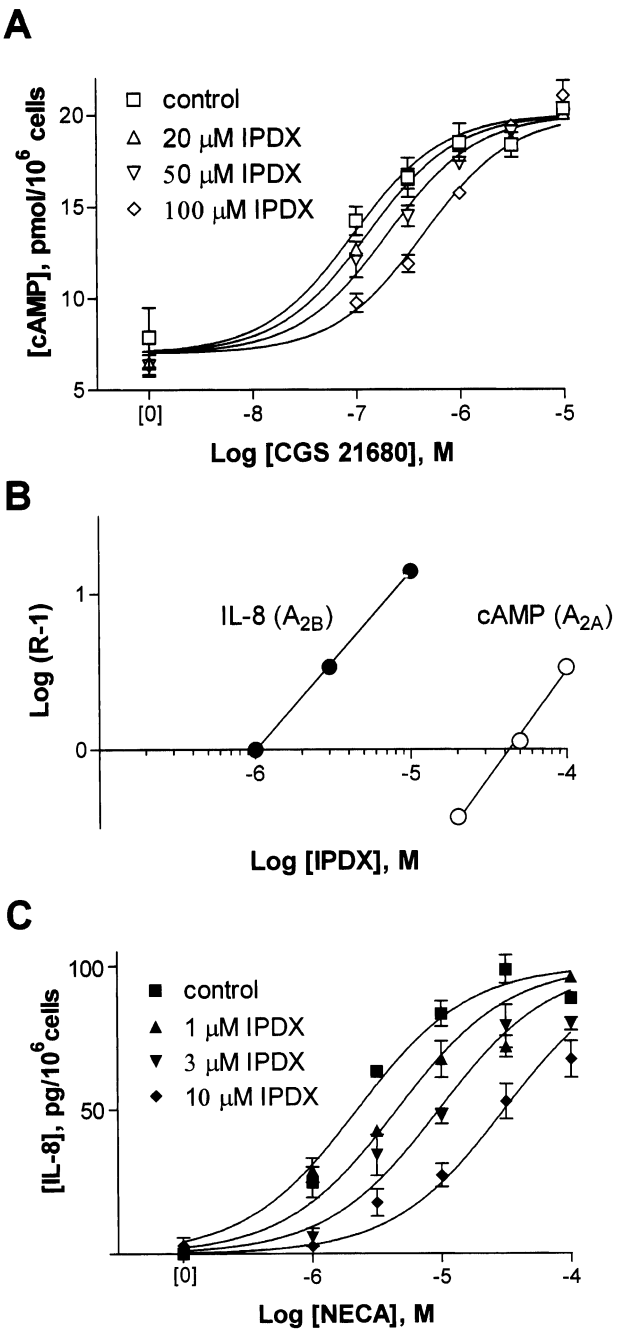


Fig. 5. Antagonistic effects of IPDX on A_{2A}-mediated cAMP accumulation and on A_{2B}-mediated IL-8 secretion in HMC-1 cells. (A) Antagonistic effects of IPDX on A_{2A}-mediated cAMP accumulation in HMC-1 cells induced by CGS 21680. Concentration–response curves were repeated in the absence and in the presence of increasing concentrations of IPDX, which produced a progressive shift to the right. Results (means ± SEM) are representative of three independent experiments. (B) Schild plot of antagonism of A_{2A}-mediated cAMP accumulation (○) and A_{2B}-mediated IL-8 secretion (●) in HMC-1 cells by IPDX. R represents the ratio of agonist EC_{50} in the presence of antagonist to its EC_{50} in the absence of antagonist. Results are representative of three independent experiments. (C) Antagonistic effects of IPDX on A_{2B}-mediated IL-8 secretion in HMC-1 cells induced by NECA. Concentration–response curves were repeated in the absence and in the presence of increasing concentrations of IPDX, which produced a progressive shift to the right. Results are presented as means ± SEM of three experiments.

Various amide derivatives of 1,3-dipropyl-8-[4-[[carboxymethyl]oxy]phenyl] xanthine were reported recently to have a high binding affinity at recombinant human A_{2B} receptors overexpressed in HEK-293 cells, with K_i values in a lower nanomolar range [30]. We are not aware of studies using these compounds to differentiate the effects of A_{2B} adenosine receptors from those of other adenosine receptor subtypes. In the present study we demonstrated that IPDX can be used as a tool to differentiate between A_{2B} and other adenosine receptors, particularly in tissues and cell types expressing more than one adenosine receptor subtype. Our data show that IPDX inhibited A_{2B}-mediated IL-8 secretion with a K_B value of 1 μM, but was virtually ineffective at this concentration on A_{2A}-mediated cAMP accumulation in human mast cells (HMC-1; K_B of 36 μM). We previously reported that A_{2B} adenosine receptors mediate IL-8 secretion in mast cells. This conclusion was based on the observation that the non-selective adenosine receptor agonist NECA, but not the selective A_{2A} agonist CGS 21680, induced secretion of IL-8 in HMC-1 cells, and that this process can be blocked with 300 μM enprofylline [11]. Given the relatively high enprofylline concentrations used, we thought it was important to confirm our previous results with a more potent and selective antagonist. Our new data provide more reliable information. IPDX inhibited NECA-induced IL-8 secretion with a potency in close agreement with that determined for A_{2B}-mediated cAMP accumulation in HEL cells, thus confirming the role of A_{2B} adenosine receptors in mediating human mast cell activation. Considering the proposed role of adenosine in triggering bronchoconstriction in asthmatic patients through human mast cell activation [5], IPDX may become a basis for the development of new antiasthmatic drugs with improved properties compared with those of enprofylline and theophylline.

A_{2B} adenosine receptors have been implicated in endothelial-dependent vasodilation, regulation of cell growth, and gene expression. A potential role of A_{2B} receptors in the intestine received particular attention because of its relevance to diarrheal processes. As part of the pathophysiology of these disorders, neutrophils release AMP, which is then converted to adenosine and activates intestinal epithelium via A_{2B} receptors to stimulate chloride secretion, an important mechanism of diarrheal diseases [31,32]. Also, the A_{2B} adenosine receptor may be one player involved in the pathophysiology of the retinopathy of prematurity or diabetes mellitus [33]. Inhibiting the A_{2B} adenosine receptor may slow abnormal blood vessel growth in the retina, thereby benefitting patients who suffer from retinopathy, a leading cause of blindness. The appreciation of the potential role of A_{2B} receptors in the pathogenesis of different disease processes raises a possibility that IPDX or its derivatives may become a useful tool for the pharmacological characterization of the pathophysiology of these diseases and possibly a basis for drug development for these and other applications.

Acknowledgments

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