

# Anilide Derivatives of an 8-Phenylxanthine Carboxylic Congener Are Highly Potent and Selective Antagonists at Human A<sub>2B</sub> Adenosine Receptors

Yong-Chul Kim,<sup>†</sup> Xiao-duo Ji,<sup>†</sup> Neli Melman,<sup>†</sup> Joel Linden,<sup>‡</sup> and Kenneth A. Jacobson<sup>\*,†</sup>

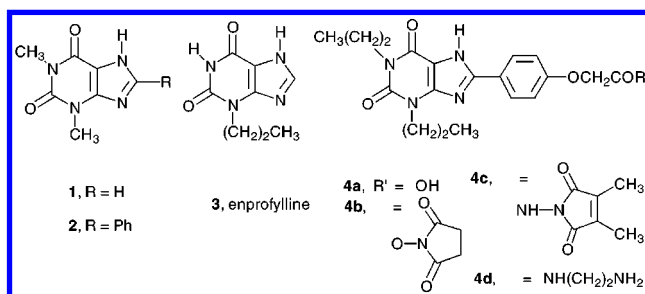
Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0810, and Department of Internal Medicine and Molecular Physiology & Biological Physics, University of Virginia, Box MR4 6012, Health Science Center, Charlottesville, Virginia 22908

Received August 19, 1999

No highly selective antagonists of the A<sub>2B</sub> adenosine receptor (AR) have been reported; however such antagonists have therapeutic potential as antiasthmatic agents. Here we report the synthesis of potent and selective A<sub>2B</sub> receptor antagonists. The structure–activity relationships (SAR) of 8-phenyl-1,3-di-(*n*-propyl)xanthine derivatives in binding to recombinant human A<sub>2B</sub> ARs in HEK-293 cells (HEK-A<sub>2B</sub>) and at other AR subtypes were explored. Various amide derivatives of 8-[4-[[carboxymethyl]oxy]phenyl]-1,3-di-(*n*-propyl)xanthine, **4a**, were synthesized. A comparison of aryl, alkyl, and aralkyl amides demonstrated that simple anilides, particularly those substituted in the *para*-position with electron-withdrawing groups, such as nitro, cyano, and acetyl, bind selectively to human A<sub>2B</sub> receptors in the range of 1–3 nM. The unsubstituted anilide **12** had a *K<sub>i</sub>* value at A<sub>2B</sub> receptors of 1.48 nM but was only moderately selective versus human A<sub>1</sub>/A<sub>2A</sub> receptors and nonselective versus rat A<sub>1</sub> receptors. Highly potent and selective A<sub>2B</sub> antagonists were a *p*-aminoacetophenone derivative **20** (*K<sub>i</sub>* value 1.39 nM) and a *p*-cyanoanilide **27** (*K<sub>i</sub>* value 1.97 nM). Compound **27** was 400-, 245-, and 123-fold selective for human A<sub>2B</sub> receptors versus human A<sub>1</sub>/A<sub>2A</sub>/A<sub>3</sub> receptors, respectively, and 8.5- and 310-fold selective versus rat A<sub>1</sub>/A<sub>2A</sub> receptors, respectively. Substitution of the 1,3-dipropyl groups with 1,3-diethyl offered no disadvantage for selectivity, and high affinities at A<sub>2B</sub> receptors were maintained. Substitution of the *p*-carboxymethyloxy group of **4a** and its amides with acrylic acid decreased affinity at A<sub>2B</sub> receptors while increasing affinity at A<sub>1</sub> receptors. 1,3-Di-(cyclohexylmethyl) groups greatly reduced affinity at ARs, although the *p*-carboxymethyloxy derivative **9** was moderately selective for A<sub>2B</sub> receptors. Several selective A<sub>2B</sub> antagonists inhibited NECA-stimulated calcium mobilization in HEK-A<sub>2B</sub> cells.

## Introduction

The alkylxanthine theophylline, **1** (Figure 1), a weak nonselective adenosine antagonist,<sup>1</sup> is effective when used therapeutically for the treatment of asthma, but its use is associated with unpleasant side effects, such as insomnia and diuresis.<sup>2</sup> In recent years, use of theophylline as a bronchodilator for relief of asthma has been supplanted by drugs of other classes, i.e. selective  $\beta_2$ -adrenergic agonists, corticosteroids, and recently leukotriene antagonists.<sup>3</sup> All of these compounds have limitations, so the development of a theophylline-like drug with reduced side effects is still desirable. The mechanism of action of theophylline in asthma has long been the subject of controversy. It is recognized that at therapeutically relevant doses, theophylline and its closely related analogue caffeine block endogenous adenosine acting as a local modulator in the brain and other organs. Adenosine activates four subtypes of G protein-coupled adenosine receptors (ARs): A<sub>1</sub>/A<sub>2A</sub>/A<sub>2B</sub>/A<sub>3</sub>.<sup>4</sup> In comparison to the other known actions of theophylline, e.g. inhibition of phosphodiesterases, it is more potent in antagonism of ARs. Although lung tissue from asthmatic patients is hyperresponsive in adeno-



**Figure 1.** Structures of various xanthines that act as antagonists at A<sub>2B</sub> receptors.

sine-induced contraction,<sup>5</sup> the adenosine antagonist properties of theophylline had been doubted as an explanation of the therapeutic properties,<sup>6</sup> until relatively recently. One reason was that enprofylline, 3-propylxanthine, **3**, formerly used clinically as an antiasthmatic in Europe, is much weaker than theophylline as an antagonist of two AR subtypes previously well-defined pharmacologically, i.e. A<sub>1</sub>/A<sub>2A</sub> ARs. With the recent focus on A<sub>2B</sub> ARs,<sup>7</sup> it has been noted that therapeutic concentrations of enprofylline block human A<sub>2B</sub> receptors and proposed that antagonists selective for this subtype may have potential use as antiasthmatic agents.<sup>8,9</sup> Enprofylline has a *K<sub>i</sub>* value of 7  $\mu$ M and is somewhat selective in binding to human A<sub>2B</sub> ARs.<sup>9,10</sup> A<sub>2B</sub> ARs are expressed in some mast cells, such as canine BR mastocytoma cells, in which they appear

\* Correspondence to: Dr. K. A. Jacobson, Bldg. 8A, Rm. B1A-19, NIH, NIDDK, LBC, Bethesda, MD 20892-0810. Tel: (301) 496-9024. Fax: (301) 480-8422. E-mail: kajacobs@helix.nih.gov.

<sup>†</sup> National Institute of Diabetes, Digestive and Kidney Diseases.

<sup>‡</sup> University of Virginia.

to be responsible for triggering acute  $\text{Ca}^{2+}$  mobilization and degranulation.<sup>11,12</sup>  $\text{A}_{2\text{B}}$  ARs also trigger  $\text{Ca}^{2+}$  mobilization<sup>10</sup> and participate in a delayed IL8 release from human HMC-1 mast cells.<sup>13</sup> Other functions associated with the  $\text{A}_{2\text{B}}$  AR are the control of cell growth and gene expression,<sup>14</sup> endothelial-dependent vasodilation,<sup>15</sup> and fluid secretion from intestinal epithelia.<sup>16</sup> Adenosine acting through  $\text{A}_{2\text{B}}$  ARs can stimulate chloride permeability in cells expressing the cystic fibrosis transport regulator.<sup>17</sup>

Although AR subtype-selective probes are available for the  $\text{A}_1/\text{A}_{2\text{A}}/\text{A}_3$  ARs,<sup>18</sup> only few weakly selective antagonists<sup>19,20</sup> and no selective agonists<sup>21,22</sup> are known for the  $\text{A}_{2\text{B}}$  receptor. Recently radioligand binding assays<sup>9,10,23</sup> have been reported which will aid in the identification of selective antagonists. Although there are several classes of non-xanthine adenosine antagonists<sup>24–26</sup> that have been found to be potent and slightly selective  $\text{A}_{2\text{B}}$  receptor antagonists, we have selected xanthines as a suitable lead for the development of structure–activity relationships (SAR). Among xanthines, an 8-phenyl group is associated with increased affinity at  $\text{A}_{2\text{B}}$  receptors.<sup>7,27</sup> The 8-phenyl analogue of theophylline, **2**, displayed a 22-fold enhancement of binding affinity at  $\text{A}_{2\text{B}}$  receptors.<sup>19</sup> Leads for achieving moderate selectivity (at least 20-fold versus  $\text{A}_1/\text{A}_{2\text{A}}/\text{A}_3$  ARs) have recently been identified among derivatives of 8-[4-[(carboxymethyl)oxy]phenyl]-1,3-dipropylxanthine,<sup>19,20,27</sup> **4a**. Compound **4b** (MRS 1204, *N*-hydroxysuccinimide ester of **4a**) displayed approximately 20-fold selectivity for human  $\text{A}_{2\text{B}}$  receptors versus  $\text{A}_1/\text{A}_{2\text{A}}/\text{A}_3$  ARs.<sup>19</sup> A 1,2-dimethylmaleimide derivative, **4c** (MRS 1595), bound to human  $\text{A}_{2\text{B}}$  receptors with a  $K_i$  of 19 nM and was selective versus human  $\text{A}_1/\text{A}_{2\text{A}}/\text{A}_3$  receptors by 160-, 100-, and 35-fold, respectively.<sup>20</sup> The  $\text{A}_{2\text{B}}$  receptor selectivity of enprofylline was lost in its 8-aryl-substituted analogues.<sup>20</sup> Aryl amide derivatives were previously reported to be highly potent antagonists at  $\text{A}_{2\text{A}}$  receptors in human platelets.<sup>28</sup> In the present study, these and additional amide derivatives of **4a** were synthesized, to identify novel, selective  $\text{A}_{2\text{B}}$  receptor antagonists.

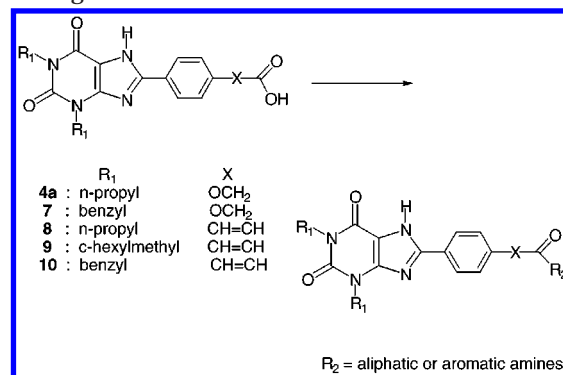
## Results

The structures of the xanthine derivatives **4a–40** tested for affinity in radioligand binding assays at ARs are shown in Tables 1–3. Most of the xanthines are derivatives of the carboxylic congener **4a**<sup>27</sup> in which the carboxylic acid group has been condensed to various amines through amide coupling reactions shown in Scheme 1. This approach was taken based on the high potency in the  $\text{A}_{2\text{B}}$  receptor binding assay of an *N*-hydroxysuccinimide ester **4b** ( $K_i$  value of 9.75 nM)<sup>19</sup> and related acyl hydrazides,<sup>20</sup> such as **4c**.

Besides **4a**, various other analogues of xanthine carboxylic acid congeners, **7–10**, were synthesized according to established synthetic procedures of xanthines,<sup>29</sup> and their corresponding amide or acyl hydrazide conjugates, **34–38**, were also prepared and tested for comparison. The yields and chemical characterization of all new compounds are reported in Table 4.

The potency of the xanthine derivatives at human  $\text{A}_{2\text{B}}$  receptors was evaluated using two binding assays

**Scheme 1.** Synthesis of Amide Derivatives of Xanthine Carboxylic Acid Congeners as Potentially Selective  $\text{A}_{2\text{B}}$  AR Antagonists<sup>a</sup>



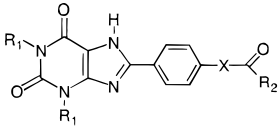
<sup>a</sup> Reagents: (1) EDAC, DMAP, amines in DMF/ $\text{CH}_2\text{Cl}_2$ ; (2) BOP-Cl, triethylamine, amines in  $\text{CH}_2\text{Cl}_2$ ; or (3)  $\text{SOCl}_2$ , amines in pyridine/ $\text{CH}_2\text{Cl}_2$ .

(Tables 1–3) and a functional assay (Figure 2).  $K_i$  values of xanthine derivatives were determined in displacement of binding of two nonselective radioligands: [ $^3\text{H}$ ]-ZM241385 (4-(2-[7-amino-2-furyl][1,2,4]triazolo[2,3-*a*]-[1,3,5]triazin-5-yl]aminoethyl)phenol)<sup>23</sup> and [ $^{125}\text{I}$ ]IABOPX (3-(4-amino-3-iodobenzyl)-8-phenyloxyacetate-1-propylxanthine),<sup>10</sup> at human  $\text{A}_{2\text{B}}$  receptors stably expressed in HEK-293 cell membranes.<sup>10</sup> Results with these two radioligands were identical. To determine selectivity, the xanthines were evaluated using standard binding assays at  $\text{A}_1/\text{A}_{2\text{A}}/\text{A}_3$  receptors. The initial screening utilized rat brain  $\text{A}_1/\text{A}_{2\text{A}}$  receptors (with radioligands [ $^3\text{H}$ ]-*R*-PIA and [ $^3\text{H}$ ]-CGS21680), and selected compounds were examined at the recombinant human subtypes, using [ $^3\text{H}$ ]-CPX (8-cyclopentyl-1,3-dipropylxanthine) ( $\text{A}_1$ )<sup>30</sup> and [ $^{125}\text{I}$ ]-ZM241385 ( $\text{A}_{2\text{A}}$ ).<sup>31</sup> Affinity at cloned human  $\text{A}_3$  receptors expressed in HEK-293 cells was determined using [ $^{125}\text{I}$ ]-IABA (*N*-(4-amino-3-[ $^{125}\text{I}$ ]iodobenzyl)-adenosine)<sup>32</sup> or [ $^{125}\text{I}$ ]-IAB-MECA (*N*-(4-amino-3-iodobenzyl)adenosine-5'-*N*-methyluronamide).<sup>33</sup>

A series of xanthine carboxylic acid derivatives **4a–10** allowed comparison of the effects of substitutions at the 1- and 3-positions and variation of the linkage between the carboxylate group and the 8-phenyl ring. Among 8-(4-carboxymethyloxyphenyl) derivatives differing only in the 1- and 3-substituents, **4a–7**, affinity at  $\text{A}_{2\text{B}}$  receptors decreased in the order: 1,3-dipropyl  $\geq$  1,3-di-*n*-butyl > 1,3-diallyl > 1,3-dibenzyl. The diallyl derivative was more  $\text{A}_{2\text{B}}$  receptor-selective but less potent than the dipropyl derivative. Thus, **4a** was selected for further derivatization as amides.

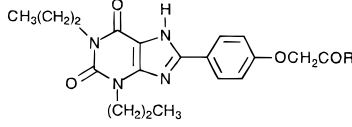
8-(4-Phenylacrylic) acid derivatives **8–10** tended to be more potent at  $\text{A}_1$  receptors and less potent at  $\text{A}_{2\text{B}}$  receptors than the 8-(4-carboxymethyloxyphenyl) derivatives. The 1,3-dicyclohexylmethyl derivative **9** was 25-fold selective for  $\text{A}_{2\text{B}}$  receptors. A primary carboxamide **11** was more potent than the carboxylic acid **4a** at  $\text{A}_1$  (3-fold) and  $\text{A}_{2\text{A}}$  (29-fold) receptors and equipotent at  $\text{A}_{2\text{B}}$  receptors.

The AR affinities of aryl, **12** and **18–33**, alkyl, **17**, and aralkyl, **13–16**, amides of **4a** were compared. A benzyl amide **13** and simple anilides had the highest affinity of binding, in the nanomolar range, to human  $\text{A}_{2\text{B}}$  receptors. Selectivities for the human  $\text{A}_{2\text{B}}$  versus rat  $\text{A}_1$  receptors ranged from 1-fold (**30**) to 27-fold (**20**),

**Table 1.** Affinities or Antagonistic Activities of Xanthine Carboxylic Acid Derivatives in Radioligand Binding Assays at A<sub>1</sub>/A<sub>2A</sub>/A<sub>2B</sub>/A<sub>3</sub> Receptors


compd	R <sub>1</sub>	X	R <sub>2</sub>	K <sub>i</sub> (nM)				hA <sub>1</sub> /hA <sub>2B</sub>	hA <sub>2A</sub> /hA <sub>2B</sub>	hA <sub>3</sub> /hA <sub>2B</sub>
				rA <sub>1</sub> <sup>a</sup>	rA <sub>2A</sub> <sup>b</sup>	hA <sub>2B</sub> <sup>c</sup>	hA <sub>3</sub> <sup>d</sup>			
<b>4a</b>	<i>n</i> -Pr	OCH <sub>2</sub>	OH	58	2200	40 ± 4	3910 ± 2140	4.4	15	98
				175 ± 57 (h)	595 ± 128 (h)		75700 ± 6500 (r)			
<b>4b</b>	<i>n</i> -Pr	OCH <sub>2</sub>	<i>O</i> -succinimide	153	127	9.75 ± 4.80	227			
<b>4c</b>	<i>n</i> -Pr	OCH <sub>2</sub>	NHN-dimethylmaleyl	11.1 ± 2.4	126 ± 41	26.6 ± 4.0	670 ± 154	110	74	25
<b>4d</b>	<i>n</i> -Pr	OCH <sub>2</sub>	NH(CH <sub>2</sub> ) <sub>2</sub> NH <sub>2</sub>	3030 ± 1110 (h)	1970 ± 550 (h)	7.75 ± 0.14	25.6 ± 5.0	0.9	2.4	3.3
				1.2	63					
				6.82 ± 1.57 (h)	18.4 ± 0.03 (h)					
<b>5</b>	allyl	OCH <sub>2</sub>	OH	756 ± 147	4290 ± 570	141 ± 29 (h)	816 ± 91	12	17	5.8
				1660 ± 580 (h)	2370 ± 290 (h)		173000 ± 18000 (r)			
<b>6</b>	<i>n</i> -butyl	OCH <sub>2</sub>	OH	43.1 ± 9.9	874 ± 107	48.0 ± 16.9	90.3 ± 14.2	3.1	53	1.9
				149 ± 83 (h)	2540 ± 1250 (h)		27500 ± 2500 (r)			
<b>7</b>	Bn	OCH <sub>2</sub>	OH	679 ± 190	25 ± 3% (10 <sup>-4</sup> ) <sup>e</sup>	1760 ± 110				
<b>8</b>	<i>n</i> -Pr	CH=CH	OH	15	800	60 ± 2	30 ± 14	2.3	3.2	0.5
				140 ± 3 (h)	190 ± 71 (h)		15000 ± 1700 (r)			
<b>9</b>	<i>c</i> -HexMe	CH=CH	OH	602 ± 24	<10% (10 <sup>-5</sup> ) <sup>e</sup>	199 ± 52	922 ± 399	25	7.6	4.6
				4890 ± 530 (h)	1518 ± 980 (h)					
<b>10</b>	Bn	CH=CH	OH	201 ± 23	4450 ± 1230	469 ± 23				

<sup>a</sup> Displacement of specific binding in rat brain membranes ([<sup>3</sup>H]*R*-PIA) or recombinant human A<sub>1</sub> receptors in HEK-293 cells ([<sup>125</sup>I]IABA), expressed as K<sub>i</sub> ± SEM (*n* = 3–5). <sup>b</sup> Displacement of specific binding in rat striatal membranes ([<sup>3</sup>H]CGS21680) or recombinant human A<sub>2A</sub> receptors in HEK-293 cells ([<sup>125</sup>I]iodo-ZM241385), expressed as K<sub>i</sub> ± SEM (*n* = 3–5). <sup>c</sup> Displacement of specific [<sup>3</sup>H]ZM241385 or [<sup>125</sup>I]IABOPX binding at human A<sub>2B</sub> receptors expressed in HEK-293 cells, in membranes, expressed as K<sub>i</sub> ± SEM (*n* = 3–4). <sup>d</sup> Displacement of specific [<sup>125</sup>I]IAB-MECA or [<sup>125</sup>I]IABA binding at human A<sub>3</sub> receptors expressed in HEK cells, in membranes, expressed as K<sub>i</sub> ± SEM (*n* = 3–4). <sup>e</sup> % Displacement of specific binding at the designated molar concentration.

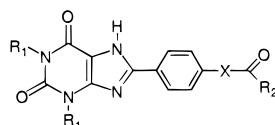
**Table 2.** Affinities or Antagonistic Activities of Xanthine Amide Derivatives in Radioligand Binding Assays<sup>a</sup> at A<sub>1</sub>/A<sub>2A</sub>/A<sub>2B</sub>/A<sub>3</sub> Receptors


compd	R	K <sub>i</sub> (nM)						hA <sub>1</sub> /hA <sub>2B</sub>	hA <sub>2A</sub> /hA <sub>2B</sub>	hA <sub>3</sub> /hA <sub>2B</sub>
		rA <sub>1</sub>	rA <sub>2A</sub>	hA <sub>1</sub>	hA <sub>2A</sub>	hA <sub>2B</sub>	hA <sub>3</sub>			
<b>11</b>	NH <sub>2</sub>	20.0 ± 3.8	76.3 ± 14.0			16.3 ± 4.2				
<b>12</b>	NH-Ph	4.22 ± 0.88	45.6 ± 1.4	40.1 ± 5.1	25.8 ± 4.5	1.48 ± 0.63	137 ± 54	27	17	93
<b>13</b>	NH-CH <sub>2</sub> Ph	5.02 ± 0.55	25.9 ± 7.6	54.7 ± 21.2	23.8 ± 5.71	2.04 ± 0.17	79.2 ± 17.8	27	12	39
<b>14</b>	NH-CH(Ph) <sub>2</sub>	120 ± 21	20 ± 8% (10 <sup>-6</sup> )			33.7 ± 17.0				
<b>15</b>	N(CH <sub>2</sub> Ph) <sub>2</sub>	167 ± 49	2750 ± 800	690 ± 98	642 ± 198	9.88 ± 1.05	284 ± 14	70	65	29
<b>16</b>	N(CH <sub>3</sub> )Ph	218 ± 80	497 ± 250			5.42 ± 1.71				
<b>17</b>	N(CH <sub>2</sub> COOEt) <sub>2</sub>	26.8 ± 2.4	999 ± 144			43.4 ± 8.4				
<b>18<sup>b</sup></b>	NH-Ph(2-COCH <sub>3</sub> )	27.9 ± 1.6	434 ± 129	335 ± 64	431 ± 176	2.74 ± 1.01	61.9 ± 3.4	120	160	23
<b>19</b>	NH-Ph(3-COCH <sub>3</sub> )	439 ± 111	949 ± 394	234 ± 28	58.9 ± 7.1	4.92 ± 0.55	352 ± 69	48	12	72
<b>20<sup>b</sup></b>	NH-Ph(4-COCH <sub>3</sub> )	37.6 ± 4.0	548 ± 183	157 ± 8	112 ± 37	1.39 ± 0.30	230 ± 23	110	81	170
<b>21</b>	NH-Ph(4-COOCH <sub>3</sub> )	38.4 ± 3.9	541 ± 128	225 ± 9	3100 ± 1540	3.93 ± 1.35	363 ± 148	57	790	92
<b>22</b>	NH-Ph(4-CONH <sub>2</sub> )	10.2 ± 2.5	683 ± 167			7.75 ± 1.11				
<b>23</b>	NH-Ph(4-CONHCH <sub>3</sub> )	24.8 ± 1.8	98.1 ± 49.6			3.34 ± 0.51				
<b>24</b>	NH-Ph(4-COOH)	145 ± 28	220 ± 79	161 ± 53	39.0 ± 21.5	16.1 ± 4.7	>5000	10	2.4	>300
<b>25</b>	NH-Ph(4-CH <sub>3</sub> )	17.5 ± 5.0	126 ± 38			1.88 ± 0.76				
<b>26</b>	NH-Ph(4-OH)	5.88 ± 1.06	63.3 ± 20.4			3.71 ± 0.76				
<b>27<sup>b</sup></b>	NH-Ph(4-CN)	16.8 ± 3.6	612 ± 287	403 ± 194	503 ± 10.8	1.97 ± 0.31	570 ± 184	210	260	290
<b>28</b>	NH-Ph(4-NO <sub>2</sub> )	13.1 ± 3.9	1180 ± 360	57.0 ± 3.1	70.0 ± 10.7	1.52 ± 0.24	138 ± 17.1	38	46	91
<b>29</b>	NH-Ph(4-CF <sub>3</sub> )	44.6 ± 6.5	917 ± 258	61.2 ± 8.2	238 ± 28	2.14 ± 0.47	213 ± 94	29	110	100
<b>30</b>	NH-Ph(4-F)	2.72 ± 0.51	988 ± 518	17.9 ± 4.5	16.6 ± 3.6	2.22 ± 0.19	391 ± 147	8.1	7.5	176
<b>31</b>	NH-Ph(4-Cl)	6.35 ± 1.47	995 ± 550	49.7 ± 14.2	187 ± 38	2.47 ± 0.71	1870 ± 370	20	400	760
<b>32</b>	NH-Ph(4-Br)	7.46 ± 2.66	221 ± 36	73.5 ± 23.3	1640 ± 660	2.35 ± 0.01	2300 ± 420	31	700	980
<b>33</b>	NH-Ph(4-I)	15.7 ± 4.2	152 ± 47	293 ± 67	5140 ± 540	2.13 ± 0.12	1270 ± 130	140	2400	600

<sup>a</sup> The methods of each binding assay are shown in Table 1. <sup>b</sup> **18**, MRS 1668; **20**, MRS 1706; **27**, MRS 1754.

while comparisons within the same species (human) generally led to greater selectivities. Anilides substituted in the *para*-position with groups such as nitro, cyano, and acetyl displayed the highest selectivity. An

*N*-methylanilide of **4a**, **16**, was 40- and 92-fold selective for human A<sub>2B</sub> receptors versus rat A<sub>1</sub>/A<sub>2A</sub> receptors; thus the *N*-methylation reduced affinity by 3.7-fold but increased selectivity. An *ortho*-substituted acetophenone

**Table 3.** Affinities or Antagonistic Activities of Miscellaneous Xanthine Derivatives in Radioligand Binding Assays<sup>a</sup> at A<sub>1</sub>/A<sub>2A</sub>/A<sub>2B</sub>/A<sub>3</sub> Receptors

compd	R <sub>1</sub>	X	R <sub>2</sub>	K <sub>i</sub> (nM)			
				rA <sub>1</sub>	rA <sub>2A</sub>	hA <sub>2B</sub>	hA <sub>3</sub>
<b>34</b>	<i>n</i> -Pr	CH=CH	NHN-dimethylmaleyl	3.94 ± 1.20 105 ± 5 (h)	406 ± 105 223 ± 55 (h)	16.7 ± 3.0	31.0 ± 3.1
<b>35</b>	<i>n</i> -Pr	CH=CH	NH-Ph(2-COCH <sub>3</sub> )	7.67 ± 2.20	143 ± 50	3.65 ± 0.98	121 ± 138
<b>36</b>	<i>c</i> -HexMe	CH=CH	NH-Ph(2-COCH <sub>3</sub> )	<i>b</i> (10 <sup>-5</sup> )	<i>b</i> (10 <sup>-5</sup> )	<i>b</i> (10 <sup>-5</sup> )	
<b>37</b>	Bn	CH=CH	NH-Ph(2-COCH <sub>3</sub> )	34300	<i>b</i> (10 <sup>-5</sup> )	<i>b</i> (10 <sup>-5</sup> )	
<b>38</b>	Bn	OCH <sub>2</sub>	NH-Ph(2-COCH <sub>3</sub> )	<i>b</i> (10 <sup>-5</sup> )	<i>b</i> (10 <sup>-5</sup> )	<i>b</i> (10 <sup>-5</sup> )	
<b>39</b>	Et	OCH <sub>2</sub>	NH-Ph(4-CH <sub>3</sub> )	34.9 ± 0.3	71.1 ± 7.7	1.78 ± 0.43	<i>b</i> (10 <sup>-6</sup> )
<b>40</b>	Et	OCH <sub>2</sub>	NH-Ph(4-CH <sub>2</sub> CONH(CH <sub>2</sub> ) <sub>2</sub> NH <sub>2</sub> )	65.0 ± 15.4	1370 ± 490	15.2 ± 6.8	

<sup>a</sup> The methods of each binding assay are shown in Table 1. <sup>b</sup> <10% Displacement of specific binding at the designated molar concentration.

**Table 4.** Yields and Chemical Characterization of Xanthine Derivatives

compd	% yield	mp (°C)	MS	formula	anal.
<b>7</b>	13	>310	EI: 482	C <sub>27</sub> H <sub>22</sub> N <sub>4</sub> O <sub>5</sub>	HRMS <sup>a</sup>
<b>10</b>	40	>310	FAB: 479	C <sub>28</sub> H <sub>22</sub> N <sub>4</sub> O <sub>4</sub>	C, H, N
<b>12</b>	71	301–302	CI: 462	C <sub>25</sub> H <sub>27</sub> N <sub>5</sub> O <sub>4</sub>	C, H, N
<b>13</b>	41	268	CI: 476	C <sub>26</sub> H <sub>29</sub> N <sub>5</sub> O <sub>4</sub>	C, H, N
<b>14</b>	46	269–270	EI: 551	C <sub>32</sub> H <sub>33</sub> N <sub>5</sub> O <sub>4</sub>	C, H, N
<b>15</b>	55	230	EI: 565	C <sub>33</sub> H <sub>35</sub> N <sub>5</sub> O <sub>4</sub>	C, H, N
<b>16</b>	49	215	FAB: 476	C <sub>26</sub> H <sub>29</sub> N <sub>5</sub> O <sub>4</sub>	C, H, N
<b>17</b>	13	225	CI: 558	C <sub>27</sub> H <sub>35</sub> N <sub>5</sub> O <sub>8</sub>	C, H, N
<b>18</b>	68	294	EI: 503	C <sub>27</sub> H <sub>29</sub> N <sub>5</sub> O <sub>5</sub>	C, H, N
<b>19</b>	29	269–270	EI: 503	C <sub>27</sub> H <sub>29</sub> N <sub>5</sub> O <sub>5</sub>	HRMS <sup>a</sup>
<b>20</b>	29	309–310	EI: 503	C <sub>27</sub> H <sub>29</sub> N <sub>5</sub> O <sub>5</sub> ·0.23H <sub>2</sub> O	C, H, N
<b>21</b>	56	>310	CI: 520	C <sub>27</sub> H <sub>29</sub> N <sub>5</sub> O <sub>6</sub>	C, H, N
<b>22</b>	19	>310	CI: 505	C <sub>26</sub> H <sub>28</sub> N <sub>6</sub> O <sub>5</sub>	C, H, N
<b>23</b>	45	>310	FAB: 519	C <sub>27</sub> H <sub>30</sub> N <sub>6</sub> O <sub>5</sub> ·1.8CH <sub>2</sub> Cl <sub>2</sub>	C, H, N
<b>24</b>	51	>310	FAB: 506	C <sub>26</sub> H <sub>27</sub> N <sub>5</sub> O <sub>6</sub> ·0.60CH <sub>2</sub> Cl <sub>2</sub>	C, H, N
<b>27</b>	44	>310	CI: 487	C <sub>26</sub> H <sub>26</sub> N <sub>6</sub> O <sub>4</sub>	C, H, N
<b>28</b>	31	307	CI: 507	C <sub>25</sub> H <sub>26</sub> N <sub>6</sub> O <sub>6</sub> ·0.43CH <sub>3</sub> OH	C, H, N
<b>29</b>	44	>310	CI: 530	C <sub>26</sub> H <sub>26</sub> F <sub>3</sub> N <sub>5</sub> O <sub>4</sub> ·0.26CH <sub>3</sub> OH	C, H, N
<b>30</b>	48	298	CI: 480	C <sub>25</sub> H <sub>26</sub> FN <sub>5</sub> O <sub>4</sub>	C, H, N
<b>31</b>	31	309	CI: 496	C <sub>25</sub> H <sub>26</sub> ClN <sub>5</sub> O <sub>4</sub> ·0.26(CH <sub>3</sub> ) <sub>2</sub> CO	C, H, N
<b>32</b>	36	>310	CI: 540	C <sub>25</sub> H <sub>26</sub> BrN <sub>5</sub> O <sub>4</sub>	C, H, N
<b>33</b>	13	>310	CI: 588	C <sub>25</sub> H <sub>26</sub> IN <sub>5</sub> O <sub>4</sub> ·0.60CH <sub>3</sub> OH	C, H, N
<b>4c</b>	79	>310	FAB: 509	C <sub>25</sub> H <sub>28</sub> N <sub>6</sub> O <sub>6</sub>	C, H, N
<b>34</b>	49	302–303	EI: 504	C <sub>26</sub> H <sub>28</sub> N <sub>6</sub> O <sub>5</sub>	C, H, N
<b>35</b>	42	281–283	CI: 500	C <sub>28</sub> H <sub>29</sub> N <sub>5</sub> O <sub>4</sub> ·0.27MeOH	C, H, N
<b>36</b>	33	305	FAB: 608	C <sub>36</sub> H <sub>41</sub> N <sub>5</sub> O <sub>4</sub>	HRMS <sup>a</sup>
<b>37</b>	76	308–309	CI: 596	C <sub>36</sub> H <sub>29</sub> N <sub>5</sub> O <sub>4</sub> ·0.60H <sub>2</sub> O	C, H, N
<b>38</b>	18	284	EI: 599	C <sub>35</sub> H <sub>29</sub> N <sub>5</sub> O <sub>5</sub>	HRMS <sup>a</sup>

<sup>a</sup> High-resolution mass in EI or FAB<sup>+</sup> mode (*m/z*) determined to be within acceptable limits: **7**: calcd, 482.1590; found, 482.1597; **19**: calcd, 503.2169; found, 503.2169; **36**: calcd, 608.3237; found, 608.3251; **38**: calcd, 599.2169; found, 599.2171. HPLC demonstrated >95% purity, retention times (mobile phase, min): **7**: A, 9.94; B, 10.14; **19**: A, 14.76; B, 17.52; **36**: A, 23.97; B, 24.79; **38**: A, 17.58; B, 24.20. Mobile phases consisted of: (A) 0.1 M TEAA (pH = 5.0)/CH<sub>3</sub>CN, 80:20 to 20:80, in 30 min; and (B) H<sub>2</sub>O/MeOH, 80:20 to 20:80, in 30 min, both with a flow rate of 1 mL/min.

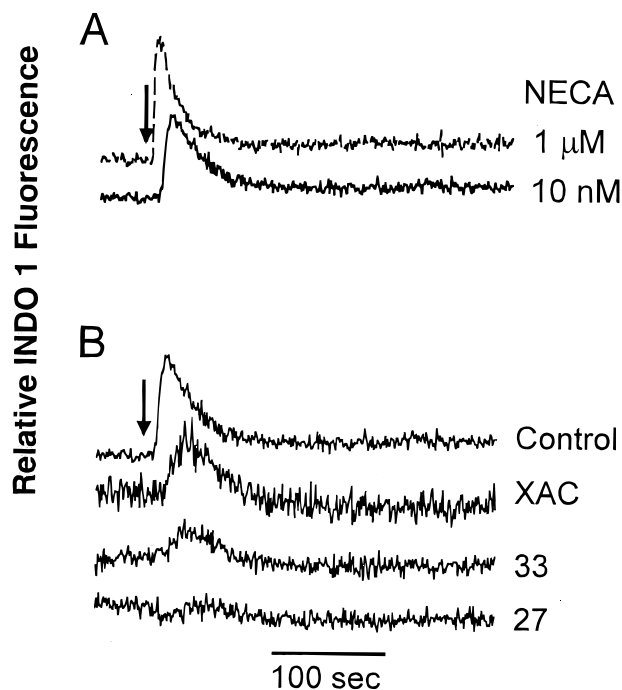
**18** was 120-, 160-, and 23-fold selective for human A<sub>2B</sub> receptors versus human A<sub>1</sub>/A<sub>2A</sub>/A<sub>3</sub> receptors and 10- and 160-fold selective versus rat A<sub>1</sub>/A<sub>2A</sub> receptors. The *para*-substituted acetophenone **20** was more potent at A<sub>2B</sub> receptors than the corresponding *ortho*- and *meta*-isomers. Other highly potent and moderately selective A<sub>2B</sub> antagonists were a *p*-trifluoromethyl derivative, **29** (*K<sub>i</sub>* value 2.14 nM), and a *p*-cyanoanilide, **27** (*K<sub>i</sub>* value 1.97 nM), which was highly selective versus the other human subtypes but only 8.5-fold selective versus rat A<sub>1</sub> receptors. A *p*-nitro derivative, **28**, bound to human A<sub>2B</sub> receptors with a *K<sub>i</sub>* of 1.52 nM but was only 35-fold selective versus human A<sub>1</sub> receptors. A *p*-iodo derivative, **33** (*K<sub>i</sub>* value 2.13 nM), was 140-, 2400-, and 600-fold selective for human A<sub>2B</sub> receptors versus human

A<sub>1</sub>/A<sub>2A</sub>/A<sub>3</sub> receptors. A *p*-toluide of **4a**, **25**, was reported previously<sup>27</sup> and displayed a *K<sub>i</sub>* value at human A<sub>2B</sub> receptors of 1.88 nM.

The introduction of the dimethylmaleimido group<sup>20</sup> in **34** and in the *p*-acryloyl derivative **35** resulted in moderate selectivity for A<sub>2B</sub> receptors versus other human but not rat ARs.

Introduction of a *p*-acrylic acid group in an anilide derivative, e.g. **36** versus **18**, decreased selectivity. Bulky 1,3-di(cyclohexylmethyl) groups or 1,3-dibenzyl groups in anilide derivatives **37** and **38**, respectively, greatly diminished binding to ARs. Substitution of the 1,3-dipropyl groups with ethyl, as in **40** and **41**, offered no disadvantage for selectivity, and high affinities were maintained.





**Figure 2.** Inhibition by several selective  $A_{2B}$  AR antagonists of NECA-stimulated calcium mobilization in HEK- $A_{2B}$  cells. Cells were loaded with Indo-1 for 1 h: (A) calcium mobilization in response to 10 nM and 1  $\mu$ M NECA added at the arrow; (B) calcium mobilization in response to 10 nM NECA added at the arrow in cells pretreated for 2 min with 1% DMSO (control) or with 100 nM of the indicated antagonists. The results are typical of replicate experiments.

The functional effects of several selective  $A_{2B}$  antagonists in inhibiting the effects of NECA in HEK- $A_{2B}$  cells were examined (Figure 2). Several selective  $A_{2B}$  AR antagonists at 100 nM nearly completely inhibited NECA-stimulated calcium mobilization. In comparison, XAC (8-[4-[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine), which has a  $K_i$  value of 12.3 nM in binding to human  $A_{2B}$  ARs,<sup>21</sup> inhibited the effect by roughly one-half. Thus, the potency of the xanthines in the functional assay was parallel to results from the binding assay.

## Discussion

We have identified amide derivatives of **4a**, such as **20**, as adenosine antagonists which are potent and selective for human  $A_{2B}$  receptors. High affinity for the receptor has been achieved through the formation of anilides and a benzyl amide of **4a**. To increase selectivity, substitution of the aryl ring in the anilide derivatives was carried out. It appears that electron-withdrawing substituents at the 2- or 4-position are best suited for  $A_{2B}$  receptor selectivity. Further SAR studies are in progress to enhance the pharmacological profile of these xanthine derivatives as  $A_{2B}$  receptor antagonists. Although this study reveals xanthines having high selectivity for the human subtypes, there is still a need for improving  $A_{2B}$  receptor selectivity in the rat.

There is also a need to enhance water solubility in potent and selective antagonists such as **18**, **27**, and **33**, which are highly hydrophobic. An attempt to introduce the charged *p*-carboxylate group, in **24**, resulted in lower affinity, although selectivity was maintained.

High-affinity compounds, such as **18** and **27**, may be the first selective pharmacological probes needed to investigate the physiological role of this AR subtype and in tritiated form may be suitable as selective radioligands for the  $A_{2B}$  receptor. There is evidence that both  $A_{2B}/A_3$  ARs may play a role in asthma.<sup>12</sup> The  $A_3$  AR mediates the degranulation of rat RBL mast-like cells<sup>34</sup> and is present in high density in human blood eosinophils.<sup>35</sup> The availability of antagonists selective for the  $A_{2B}$  receptor should provide an opportunity to explore the importance of these two receptor subtypes in asthma.

## Materials and Methods

**Materials.** Compounds **4a**, **11**, **25**, and **26** were synthesized as reported.<sup>27</sup> Compounds **5–7** and **10** were synthesized as reported.<sup>29</sup> Compounds **39** and **40** were synthesized as reported.<sup>28</sup> Compounds **8** and **9** were obtained from Dr. Susan Daluge, Glaxo, Research Triangle, NC. *R*-PIA, and 2-chloroadenosine were purchased from Research Biochemicals International (Natick, MA). All other agents were purchased from Aldrich (St. Louis, MO).

**Synthesis.** Proton nuclear magnetic resonance spectroscopy was performed on a Varian GEMINI-300 spectrometer and spectra were taken in DMSO- $d_6$  or CDCl<sub>3</sub>. Unless noted, chemical shifts are expressed as ppm downfield from tetramethylsilane or relative ppm from DMSO (2.5 ppm). Chemical ionization (CI) mass spectrometry was performed with a Finnigan 4600 mass spectrometer and electron impact (EI) mass spectrometry with a VG7070F mass spectrometer at 6 kV for high-resolution mass. FAB (fast atom bombardment) mass spectrometry was performed with a JEOL SX102 spectrometer using 6-kV Xe atoms. Elemental analysis ( $\pm 0.4\%$  acceptable) was performed by Atlantic Microlab Inc. (Norcross, GA). All melting points were determined with a Unimelt capillary melting point apparatus (Arthur H. Thomas Co., PA) and were uncorrected. All xanthine derivatives were homogeneous as judged using TLC (MK6F silica, 0.25 mm, glass backed; Whatman Inc., Clifton, NJ). Where needed, evaluation of purity was done on a Hewlett-Packard 1090 HPLC system using an OD-5-60 C18 analytical column (150 mm  $\times$  4.6 mm; Separation Methods Technologies, Inc., Newark, DE) in two different linear gradient solvent systems, at a flow rate of 1 mL/min. One solvent system (A) was 0.1 M TEAA (pH = 5.0)/CH<sub>3</sub>CN, 80:20 to 20:80, in 30 min, and the other (B) was H<sub>2</sub>O/MeOH, 80:20 to 20:80, in 30 min. Peaks were detected by UV absorption using a diode array detector.

### General Procedure for the Preparation of Amide Derivatives of **4a** and **7–10**. Method A (Carbodiimide).

A solution of a **4a** analogue (0.0517 mmol), the desired amine compound (0.103 mmol), EDAC (20 mg, 0.103 mmol), and DMAP (4 mg, 0.032 mmol) in 2 mL of anhydrous DMF/CH<sub>2</sub>Cl<sub>2</sub> (1:1 v/v) was stirred at room temperature for 24 h. The mixture was evaporated to dryness under reduced pressure, and the residue was purified by preparative silica gel TLC (CHCl<sub>3</sub>:MeOH = 20:1) and crystallization in MeOH/ether or MeOH/CH<sub>2</sub>Cl<sub>2</sub> to afford the desired compounds (**12–14**, **18**, **36**).

**Method B (BOP-Cl).** A solution of a **4a** analogue (0.0517 mmol), the desired amine compound (0.103 mmol), BOP-Cl (14 mg, 0.0517 mmol), and triethylamine (20  $\mu$ L, 0.206 mmol) in 2 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> was stirred at room temperature for 24 h. The mixture was treated with the same procedure as method A for purification of the desired compounds (**15**, **17**, **19**, **20**, **38**).

**Method C (Acid Chloride).** A solution of a **4a** analogue (0.0517 mmol) in 1 mL of thionyl chloride was stirred at 70  $^{\circ}$ C for 4 h and the excess thionyl chloride was removed by nitrogen stream. To the residue was added a solution of the desired amine compound (0.103 mmol) in 1 mL of anhydrous pyridine and 1 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub>. The mixture was stirred at room temperature for 24 h, then subjected to the same procedure as method A for purification of the desired compounds (**16**, **21**, **22**, **27–35**, **37**).

**8-[4-[(Carboxymethyl)oxy]phenyl]-1,3-dibenzylxanthine (7):**  $^1\text{H}$  NMR (DMSO- $d_6$ ) 4.23 (s, 2H,  $-\text{OCH}_2-$ ), 5.10 (s, 2H,  $-\text{NCH}_2-$ ), 5.23 (s, 2H,  $-\text{NCH}_2-$ ), 6.88 (d, 2H,  $J = 8.8$  Hz, Ar), 7.22–7.41 (m, 10H,  $2 \times$ -Ph), 8.01 (d, 2H,  $J = 8.8$  Hz, Ar).

**8-(4-(2-Carboxy-*trans*-vinyl)phenyl)-1,3-dibenzylxanthine (10):**  $^1\text{H}$  NMR (DMSO- $d_6$ ) 5.12 (s, 2H,  $-\text{NCH}_2-$ ), 5.26 (s, 2H,  $-\text{NCH}_2-$ ), 6.63 (d, 1H,  $J = 15.6$  Hz,  $-\text{CH}=\text{CH}_2$ ), 7.22–7.43 (m, 10H,  $2 \times$ -Ph), 7.63 (d, 1H,  $J = 15.6$  Hz,  $-\text{CH}=\text{CH}_2$ ), 7.84 (d, 2H,  $J = 8.8$  Hz, Ar), 8.17 (d, 2H,  $J = 8.8$  Hz, Ar).

**8-[4-[(Phenylcarbamoylmethyl)oxy]phenyl]-1,3-di(*n*-propyl)xanthine (12):**  $^1\text{H}$  NMR (DMSO- $d_6$ ) 0.89 (2t, 6H,  $J = 7.8$  Hz,  $2 \times$ -CH $_3$ ), 1.58 and 1.74 (2m, 4H,  $2 \times$ -CH $_2$ -), 3.87 and 4.02 (2t, 4H,  $J = 6.8$  Hz,  $2 \times$ -NCH $_2$ -), 4.80 (s, 2H,  $-\text{OCH}_2-$ ), 7.06–7.12 (m, 1H,  $-\text{Ph}$ ), 7.14 (d, 2H,  $J = 8.8$  Hz, Ar), 7.33 (t, 2H,  $J = 7.8$  Hz,  $-\text{Ph}$ ), 7.64 (d, 2H,  $J = 7.8$  Hz,  $-\text{Ph}$ ), 8.09 (d, 2H,  $J = 8.8$  Hz, Ar), 10.13 (s, 1H,  $-\text{NH}$ ).

**8-[4-[(4-Acetylphenyl)carbamoylmethyl]oxy]phenyl]-1,3-di(*n*-propyl)xanthine (20):**  $^1\text{H}$  NMR (DMSO- $d_6$ ) 0.89 (2t, 6H,  $J = 7.8$  Hz,  $2 \times$ -CH $_3$ ), 1.58 and 1.74 (2m, 4H,  $2 \times$ -CH $_2$ -), 2.54 (s, 3H,  $-\text{COCH}_3$ ), 3.87 and 4.02 (2t, 4H,  $J = 6.8$  Hz,  $2 \times$ -NCH $_2$ -), 4.85 (s, 2H,  $-\text{OCH}_2-$ ), 7.15 (d, 2H,  $J = 8.8$  Hz, Ar), 7.79 (d, 2H,  $J = 7.8$  Hz, Ar), 7.96 (d, 2H,  $J = 7.8$  Hz, Ar), 8.09 (d, 2H,  $J = 8.8$  Hz, Ar), 10.48 (s, 1H,  $-\text{NH}$ ).

**8-[4-[(4-Methylcarbamoyl)phenylcarbamoylmethyl]oxy]phenyl]-1,3-di(*n*-propyl)xanthine (23):** A solution of 20 mg of **21** (0.0358 mmol) in 1 mL of 40% aqueous methylamine was stirred at room temperature for 1 h. The mixture was evaporated to dryness under reduced pressure, and the residue was purified by preparative silica gel TLC (CHCl $_3$ : MeOH = 20:1) and crystallization in MeOH/CH $_2$ Cl $_2$  to give 9 mg of **23**:  $^1\text{H}$  NMR (DMSO- $d_6$ ) 0.89 (2t, 6H,  $J = 7.8$  Hz,  $2 \times$ -CH $_3$ ), 1.58 and 1.73 (2m, 4H,  $2 \times$ -CH $_2$ -), 2.76 (s, 3H,  $-\text{NHCH}_3$ ), 3.86 and 4.01 (2t, 4H,  $J = 6.8$  Hz,  $2 \times$ -NCH $_2$ -), 4.82 (s, 2H,  $-\text{OCH}_2-$ ), 7.14 (d, 2H,  $J = 8.8$  Hz, Ar), 7.71 (d, 2H,  $J = 7.8$  Hz, Ar), 7.81 (d, 2H,  $J = 7.8$  Hz, Ar), 8.09 (d, 2H,  $J = 8.8$  Hz, Ar), 8.33 (m, 1H,  $-\text{NHCH}_3$ ), 10.34 (s, 1H,  $-\text{NH}$ ).

**8-[4-[(4-Carboxyphenyl)carbamoylmethyl]oxy]phenyl]-1,3-di(*n*-propyl)xanthine (24):** A suspension of 20 mg of **21** (0.0385 mmol) in 1 mL of 1 N NaOH solution was stirred for 2 h to turn to a clear solution. The mixture was neutralized by adding 1 mL of 1 N HCl. The precipitate was collected by filtration and purified by low-pressure C18 column chromatography using linear gradient elution of 1 M triethylammoniumacetate buffer (pH = 7.0) and CH $_3$ CN (90/10 to 40/60) to give 10 mg of **24**:  $^1\text{H}$  NMR (DMSO- $d_6$ ) 0.89 (2t, 6H,  $J = 7.8$  Hz,  $2 \times$ -CH $_3$ ), 1.58 and 1.74 (2m, 4H,  $2 \times$ -CH $_2$ -), 3.87 and 4.01 (2t, 4H,  $J = 6.8$  Hz,  $2 \times$ -NCH $_2$ -), 4.84 (s, 2H,  $-\text{OCH}_2-$ ), 7.14 (d, 2H,  $J = 8.8$  Hz, Ar), 7.77 (d, 2H,  $J = 8.8$  Hz, Ar), 7.92 (d, 2H,  $J = 8.8$  Hz, Ar), 8.09 (d, 2H,  $J = 8.8$  Hz, Ar), 10.45 (s, 1H,  $-\text{NH}$ ).

**8-[4-[(4-Cyanophenyl)carbamoylmethyl]oxy]phenyl]-1,3-di(*n*-propyl)xanthine (27):**  $^1\text{H}$  NMR (DMSO- $d_6$ ) 0.89 (2t, 6H,  $J = 7.8$  Hz,  $2 \times$ -CH $_3$ ), 1.58 and 1.74 (2m, 4H,  $2 \times$ -CH $_2$ -), 3.86 and 4.01 (2t, 4H,  $J = 6.8$  Hz,  $2 \times$ -NCH $_2$ -), 4.85 (s, 2H,  $-\text{OCH}_2-$ ), 7.13 (d, 2H,  $J = 8.8$  Hz, Ar), 7.80 (d, 2H,  $J = 7.8$  Hz, Ar), 7.84 (d, 2H,  $J = 7.8$  Hz, Ar), 8.09 (d, 2H,  $J = 8.8$  Hz, Ar), 10.58 (s, 1H,  $-\text{NH}$ ).

**8-(4-(2-Carboxy-*trans*-vinyl)phenyl)-1,3-di(*n*-propyl)xanthine *N,N*-(1,2-dimethylmaleyl)hydrazide (34):**  $^1\text{H}$  NMR (CDCl $_3$ ) 1.01 (2t, 6H,  $J = 7.8$  Hz,  $2 \times$ -CH $_3$ ), 1.72 and 1.89 (2m, 4H,  $2 \times$ -CH $_2$ -), 2.05 (s, 6H,  $2 \times$ -CH $_3$ ), 4.02 and 4.17 (2t, 4H,  $J = 6.8$  Hz,  $2 \times$ -NCH $_2$ -), 6.67 (d, 1H,  $J = 15.6$  Hz,  $-\text{CH}=\text{CH}_2$ ), 7.63 (d, 2H,  $J = 8.8$  Hz, Ar), 7.74 (d, 1H,  $J = 15.6$  Hz,  $-\text{CH}=\text{CH}_2$ ), 8.09 (d, 2H,  $J = 8.8$  Hz, Ar), 9.43 (s, 1H,  $-\text{NH}$ ).

**8-[4-[2-(2-Acetylphenyl)carbamoyl-*trans*-vinyl]phenyl]-1,3-di(*n*-propyl)xanthine (35):**  $^1\text{H}$  NMR (DMSO- $d_6$ ) 0.89 (2t, 6H,  $J = 7.8$  Hz,  $2 \times$ -CH $_3$ ), 1.59 and 1.76 (2m, 4H,  $2 \times$ -CH $_2$ -), 3.88 and 4.04 (2t, 4H,  $J = 6.8$  Hz,  $2 \times$ -NCH $_2$ -), 4.79 (d, 3H,  $J = 4.9$  Hz,  $-\text{COCH}_3$ ), 6.93 (d, 1H,  $J = 15.6$  Hz,  $-\text{CH}=\text{CH}_2$ ), 7.51 (d, 1H,  $J = 15.6$  Hz,  $-\text{CH}=\text{CH}_2$ ), 7.57 (t, 1H,  $J = 7.8$  Hz, Ar), 7.69 (t, 1H,  $J = 7.8$  Hz, Ar), 7.75 (d, 2H,  $J = 7.8$  Hz, Ar), 8.03 (d, 2H,  $J = 7.8$  Hz, Ar), 8.18 (d, 2H,  $J = 7.8$  Hz, Ar), 8.53 (t, 1H,  $J = 5.8$  Hz,  $-\text{NH}$ ).

**8-[4-[2-(2-Acetylphenyl)carbamoyl-*trans*-vinyl]phenyl]-1,3-dibenzylxanthine (37):**  $^1\text{H}$  NMR (DMSO- $d_6$ ) 4.79 (d, 3H,  $J = 5.8$  Hz,  $-\text{COCH}_3$ ), 5.13 (s, 2H,  $-\text{NCH}_2-$ ), 5.27 (s, 2H,  $-\text{NCH}_2-$ ), 6.93 (d, 1H,  $J = 15.6$  Hz,  $-\text{CH}=\text{CH}_2$ ), 7.24–7.41 (m, 10H,  $2 \times$ -Ph), 7.46 (d, 1H,  $J = 15.6$  Hz,  $-\text{CH}=\text{CH}_2$ ), 7.57 (t, 1H,  $J = 8.8$  Hz, Ar), 7.69 (t, 1H,  $J = 7.8$  Hz, Ar), 7.75 (d, 2H,  $J = 8.8$  Hz, Ar), 8.03 (d, 2H,  $J = 7.8$  Hz, Ar), 8.20 (d, 2H,  $J = 7.8$  Hz, Ar), 8.54 (t, 1H,  $J = 5.8$  Hz,  $-\text{NH}$ ).

**8-[4-[(2-Acetylphenyl)carbamoylmethyl]oxy]phenyl]-1,3-dibenzylxanthine (38):**  $^1\text{H}$  NMR (DMSO- $d_6$ ) 4.68 (s, 3H,  $-\text{COCH}_3$ ), 4.71 (s, 2H,  $-\text{OCH}_2-$ ), 5.12 (s, 2H,  $-\text{NCH}_2-$ ), 5.26 (s, 2H,  $-\text{NCH}_2-$ ), 7.15 (d, 2H,  $J = 8.8$  Hz, Ar), 7.23–7.42 (m, 10H,  $2 \times$ -Ph), 7.55 (t, 1H,  $J = 7.8$  Hz, Ar), 7.68 (t, 1H,  $J = 7.8$  Hz, Ar), 8.02 (d, 2H,  $J = 7.8$  Hz, Ar), 8.11 (d, 2H,  $J = 8.8$  Hz, Ar), 8.48 (t, 1H,  $J = 4.8$  Hz,  $-\text{NH}$ ).

**Pharmacology.** The human A $_{2B}$  receptor cDNA was subcloned into the expression plasmid pDoubleTrouble.<sup>36</sup> The plasmid was amplified in competent JM109 cells and plasmid DNA isolated using Wizard Megaprep columns (Promega Corp., Madison, WI). A $_{2B}$  ARs were introduced into HEK-293 cells by means of Lipofectin.<sup>37</sup>

**Cell Culture.** Transfected HEK cells were grown under 5% CO $_2$ /95% O $_2$  humidified atmosphere at a temperature of 37 °C. Colonies were selected by growth of cells in 0.6 mg/mL G418. Transfected cells were maintained in DMEM supplemented with Hams F12 nutrient mixture (1/1), 10% newborn calf serum, 2 mM glutamine, and containing 50 IU/mL penicillin, 50  $\mu$ g/mL streptomycin, and 0.2 mg/mL Geneticin (G418, Boehringer Mannheim). Cells were cultured in 10-cm diameter round plates and subcultured when grown confluent (approximately after 72 h).

**Radioligand Binding Studies. At A $_{2B}$  receptors:** Confluent monolayers of HEK-A $_{2B}$  cells were washed with PBS followed by ice-cold buffer A (10 mM HEPES, 10 mM EDTA, pH 7.4) with protease inhibitors (10 mg/mL benzamidine, 100 mM phenylmethanesulfonyl fluoride, and 2 mg/mL of each aprotinin, pepstatin, and leupeptin). The cells were homogenized in a polytron (Brinkmann) for 20 s and centrifuged at 30000g and the pellets washed twice with buffer HE (10 mM HEPES, 1 mM EDTA, pH 7.4 with protease inhibitors). The final pellet was resuspended in buffer HE, supplemented with 10% sucrose, and frozen in aliquots at  $-80$  °C. For binding assays membranes were thawed and diluted 5–10-fold with HE to a final protein concentration of approximately 1 mg/mL. To determine protein concentrations, membranes and bovine serum albumin standards were dissolved in 0.2% NaOH/0.01% SDS and protein was determined using fluorescence fluorescence.<sup>38</sup>

To prepare [ $^{125}\text{I}$ ]ABOPX, 10 mL of 1 mM ABOPX in methanol/1 M NaOH (20:1) was added to 50 mL of 100 mM phosphate buffer, pH 7.3. One or 2 mCi of Na $^{125}\text{I}$  was added, followed by 10 mL of 1 mg/mL chloramine-T in water. After a 20-min incubation at room temperature, 50 mL of 10 mg/mL Na-metabisulfite in water was added to quench the reaction. The reaction mixture was applied to a C18 HPLC column, eluting with a mixture of methanol and 4 mM phosphate, pH 6.0. After 5 min at 35% methanol, the methanol concentration was ramped to 100% over 15 min. Unreacted ABOPX eluted in 11–12 min; [ $^{125}\text{I}$ ]ABOPX eluted at 18–19 min in a yield of 50–60% with respect to the initial  $^{125}\text{I}$ .

Saturation binding assays for human A $_{2B}$  ARs were performed with [ $^3\text{H}$ ]ZM214385 (17 Ci/mmol; Tocris Cookson, Bristol, U.K.)<sup>23</sup> or [ $^{125}\text{I}$ ]ABOPX (2200 Ci/mmol).

In equilibrium binding assays the ratio of [ $^{127}\text{I}$ ]/[ $^{125}\text{I}$ ]ABOPX was 10–20/1. Radioligand binding experiments were performed in triplicate with 20–25  $\mu$ g of membrane protein in a total volume of 0.1 mL of HE buffer supplemented with 1 U/mL adenosine deaminase and 5 mM MgCl $_2$ . The incubation time was 3 h at 21 °C. Nonspecific binding was measured in the presence of 100  $\mu$ M NECA. Competition experiments were carried out using 0.6 nM [ $^{125}\text{I}$ ]ABOPX. Membranes were filtered on Whatman GF/C filters using a Brandel cell harvester (Gaithersburg, MD) and washed three times during 15–20 s with ice-cold buffer (10 mM Tris, 1 mM MgCl $_2$ , pH 7.4).



$B_{\max}$  and  $K_D$  values were calculated by Marquardt's nonlinear least-squares interpolation for single-site binding models.<sup>39</sup>  $K_i$  values for different compounds were derived from  $IC_{50}$  values as described, assuming a  $K_D$  value for [<sup>125</sup>I]IABOPX of 36 nM.<sup>40</sup> Data from replicate experiments are tabulated as means  $\pm$  SEM.

**At other ARs:** [<sup>3</sup>H]CPX,<sup>30</sup> [<sup>125</sup>I]iodo-ZM241385, and [<sup>125</sup>I]-IABA were utilized in radioligand binding assays to membranes derived from HEK-293 cells expressing recombinant human  $A_1/A_{2A}/A_3$  ARs, respectively. Binding of [<sup>3</sup>H]*R*-N<sup>6</sup>-phenylisopropyladenosine<sup>41</sup> ([<sup>3</sup>H]*R*-PIA; Amersham, Chicago, IL) to  $A_1$  receptors from rat cerebral cortical membranes and of [<sup>3</sup>H]CGS21680<sup>42</sup> (NEN, Boston, MA) to  $A_{2A}$  receptors from rat striatal membranes was performed as described. Adenosine deaminase (3 units/mL) was present during the preparation of the brain membranes, in a preincubation of 30 min at 30 °C, and during the incubation with the radioligands. All nonradioactive compounds were initially dissolved in DMSO and diluted with buffer to the final concentration, where the amount of DMSO never exceeded 2%. Incubations were terminated by rapid filtration over Whatman GF/B filters, using a Brandell cell harvester (Brandell, Gaithersburg, MD). The tubes were rinsed three times with 3 mL of buffer each.

At least six different concentrations of competitor, spanning 3 orders of magnitude adjusted appropriately for the  $IC_{50}$  of each compound, were used.  $IC_{50}$  values, calculated with the nonlinear regression method implemented in Graph-Pad (Prism, San Diego, CA), were converted to apparent  $K_i$  values as described.<sup>40</sup> Hill coefficients of the tested compounds were in the range of 0.8–1.1.

**Functional assay:** HEK- $A_{2B}$  cells from one confluent T75 flask were rinsed with  $Ca^{2+}$ - and  $Mg^{2+}$ -free Dulbecco's phosphate-buffered saline (PBS) and then incubated in  $Ca^{2+}$ - and  $Mg^{2+}$ -free HBSS with 0.05% trypsin and 0.53 mM EDTA until the cells detached. The cells were rinsed twice by centrifugation at 250g in PBS and resuspended in 10 mL of HBSS composed of 137 mM NaCl, 5 mM KCl, 0.9 mM  $MgSO_4$ , 1.4 mM  $CaCl_2$ , 3 mM  $NaHCO_3$ , 0.6 mM  $Na_2HPO_4$ , 0.4 mM  $KH_2PO_4$ , 5.6 mM glucose, and 10 mM HEPES, pH 7.4, and the  $Ca^{2+}$ -sensitive fluorescent dye Indo-1-AM (5  $\mu$ M) 37 °C for 60 min. The cells were rinsed once and resuspended in 25 mL dye-free HBSS supplemented with 1 U/mL adenosine deaminase and held at room temperature. Adenosine receptor antagonists prepared as 100 $\times$  stocks in DMSO or vehicle was added and the cells were transferred to a 37 °C bath for 2 min. Then the cells (1 million in 2 mL) were transferred to a stirred cuvette maintained at 37 °C within an Aminco SLM 8000 spectrofluorometer (SLM Instruments, Urbana IL). The ratios of Indo-1 fluorescence obtained at 400 and 485 nm (excitation, 332 nm) was recorded using a slit width of 4 nm. NECA was added after a 100-s equilibration period.

**Acknowledgment.** We thank Melissa Marshall for technical assistance with the binding assays and acknowledge the grant support from NIH HL37942 and HL56111.

**Supporting Information Available:** Characterization of xanthine derivatives by proton NMR and elemental analyses. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References

- Linden, J.; Jacobson, K. A. Molecular biology of recombinant adenosine receptors. In *Cardiovascular Biology of Purines*; Burnstock, G., Dobson, J. G., Liang, G. T., Linden, J., Eds.; Kluwer: The Netherlands, 1998; pp 1–20.
- Vassallo, R.; Lipsky, J. J. Theophylline: recent advances in the understanding of its mode of action and uses in clinical practice. *Mayo Clin. Proc.* **1998**, *73*, 346–354.
- Drazen, J. M.; Israel, E.; O'Byrne, P. M. Treatment of asthma with drugs modifying the leukotriene pathway. *N. Engl. J. Med.* **1999**, *340*, 197–206.
- Fredholm, B. B.; Battig, K.; Holmen, J.; Nehlig, A.; Zwartau, E. E. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* **1999**, *51*, 83–133.
- Björck, T.; Gustafsson, L. E.; Dahlen, S. E. Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. *Am. Rev. Respir. Dis.* **1992**, *145*, 1087–1091.
- Chapman, K. R.; Ljungholm, K.; Kallen, A. Long-term xanthine therapy of asthma. Enprofylline and theophylline compared. *Int. Enprofylline Study Group Chest* **1994**, *106*, 1407–1413.
- Daly, J. W.; Butts-Lamb, P.; Padgett, W. Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. *Cell Mol. Neurobiol.* **1983**, *3*, 69–80.
- Feoktistov, I.; Biaggioni, I. Adenosine  $A_{2B}$  Receptors. *Pharmacol. Rev.* **1997**, *49*, 381–402.
- Robeva, A. S.; Woodard, R.; Jin, X.; Gao, Z.; Bhattacharya, S.; Taylor, H. E.; Rosin, D. L.; Linden, J. Molecular characterization of recombinant human adenosine receptors. *Drug Dev. Res.* **1996**, *39*, 243–252.
- Linden, J.; Thai, T.; Figler, H.; Jin, X. W.; Robeva, A. S. Characterization of human  $A_{2B}$  adenosine receptors: Radioligand binding, Western blotting, and coupling to G(q) in human embryonic kidney 293 cells and HMC-1 mast cells. *Mol. Pharmacol.* **1999**, *56*, 705–713.
- Auchampach, J. A.; Jin, J.; Wan, T. C.; Caughey, G. H.; Linden, J. Canine mast cell adenosine receptors: cloning and expression of the  $A_3$  receptors and evidence that degranulation is mediated by the  $A_{2B}$  receptor. *Mol. Pharmacol.* **1997**, *52*, 846–860.
- Forsyth, P.; Ennis, M. Adenosine, mast cells, and asthma. *Inflamm. Res.* **1999**, *48*, 301–307.
- Feoktistov, I.; Goldstein, A. E.; Biaggioni, I. Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase in adenosine  $A_{2B}$  receptor-mediated interleukin-8 production in human mast cells. *Mol. Pharmacol.* **1999**, *55*, 726–734.
- Neary, J.; Rathbone, M. P.; Cattabeni, F.; Abbracchio, M. P.; Burnstock, G. Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. *Trends Neurosci.* **1996**, *19*, 13–18.
- Martin, P. L.; Ueeda, M.; Olsson, R. A. 2-Phenylethoxy-9-methyladenine: an adenosine receptor antagonist that discriminates between  $A_2$  adenosine receptors in the aorta and the coronary vessels from the guinea pig. *J. Pharmacol. Exp. Ther.* **1993**, *265*, 248–253.
- Strohmeier, G. R.; Reppert, S. M.; Lencer, W. I.; Madara, J. L. The  $A_{2B}$  adenosine receptor mediates cAMP responses to adenosine receptor agonists in human intestinal epithelia. *J. Biol. Chem.* **1995**, *270*, 2387–2394.
- Clancy, J. P.; Ruiz, F. E.; Sorscher, E. J. Adenosine and its nucleotides activate wild-type and R117H CFTR through an  $A_{2B}$  receptor-coupled pathway. *Am. J. Physiol.* **1999**, *276*, C361–C369.
- Jacobson, K. A.; Knutsen, L. P1 and P2 purine and pyrimidine receptor ligands. *Handbk. Exp. Pharmacol.* **1999**, in press.
- Jacobson, K. A.; IJzerman, A. P.; Linden, J. 1,3-Dialkylxanthine derivatives having high potency as antagonists at human  $A_{2B}$  adenosine receptors. *Drug Dev. Res.* **1999**, *47*, 45–53.
- Kim, Y.-C.; Kardon, Y.; Ji, X.-d.; Linden, J.; Jacobson, K. A. Acylhydrazide derivatives of a xanthine carboxylic congener (XCC) as selective antagonists at human  $A_{2B}$  adenosine receptors. *Drug Dev. Res.* **1999**, *47*, 178–188.
- de Zwart, M.; Link, R.; von Frijtag Drabbe Künzel, J. K.; Cristalli, G.; Jacobson, K. A.; Townsend-Nicholson, A.; IJzerman, A. P. A screening of adenosine analogues on the human adenosine  $A_{2B}$  receptor as part of a search for potent and selective agonists. *Nucleosides Nucleotides* **1998**, *17*, 969–986.
- Cristalli, G.; Camaioni, E.; Costanzi, S.; Vittori, S.; Volpini, R.; Klotz, K. N. Characterization of potent ligands at human recombinant adenosine receptors. *Drug Dev. Res.* **1998**, *45*, 176–181.
- Ji, X.-D.; Jacobson, K. A. Use of the triazolotriazine [<sup>3</sup>H]-ZM241385 as a radioligand at recombinant human  $A_{2B}$  adenosine receptors. *Drug Des. Discov.* **1999**, *16*, 89–98.
- Brackett, L. E.; Daly, J. W. Functional characterization of the  $A_{2B}$  adenosine receptor in NIH 3T3 fibroblasts. *Biochem. Pharmacol.* **1994**, *47*, 801–814.
- Kim, Y.-C.; de Zwart, M.; Chang, L.; Moro, S.; von Frijtag Drabbe Künzel, J. K.; Melman, N.; IJzerman, A. P.; Jacobson, K. A. Derivatives of the triazoloquinazoline adenosine antagonist (CGS15943) having high potency at the human  $A_{2B}$  and  $A_3$  receptor subtypes. *J. Med. Chem.* **1998**, *41*, 2835–2841.
- de Zwart, M.; Vollinga, R. C.; von Frijtag Drabbe Künzel, J. K.; Beukers, M.; Slegers, D.; IJzerman, A. P. Potent antagonists for the  $A_{2B}$  receptor. II. Triazolotriazines with high affinity. *Drug Dev. Res.* **1998**, *43*, 28.
- Jacobson, K. A.; Kirk, K. L.; Padgett, W. L.; Daly, J. W. Functionalized congeners of 1,3-dialkylxanthines: Preparation of analogues with high affinity for adenosine receptors. *J. Med. Chem.* **1985**, *28*, 1334–1340.

- (28) Jacobson, K. A.; Ukena, D.; Padgett, W. L.; Daly, J. W.; Kirk, K. L. Xanthine functionalized congeners as potent ligands at A<sub>2</sub>-adenosine receptors. *J. Med. Chem.* **1987**, *30*, 211–214.
- (29) Kim, H. O.; Ji, X.-D.; Melman, N.; Olah, M. E.; Stile, G. L.; Jacobson, K. A. Structure–activity relationships of 1,3-dialkyl-xanthine derivatives at rat A<sub>3</sub> adenosine receptors. *J. Med. Chem.* **1994**, *37*, 3373–3382.
- (30) Bruns, R. F.; Fergus, J. H.; Badger, E. W.; Bristol, J. A.; Santay, L. A.; Hartman, J. D.; Hays, S. J.; Huang, C. C. Binding of the A<sub>1</sub>-selective adenosine antagonist 8-cyclopentyl-1,3-dipropyl-xanthine to rat brain membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1987**, *335*, 59–63.
- (31) Palmer, T. M.; Poucher, S. M.; Jacobson, K. A.; Stiles, G. L. <sup>125</sup>I-4-(2-[7-Amino-2-(furyl){1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-ylaminoethyl)phenol (<sup>125</sup>I-ZM241385), a high affinity antagonist radioligand selective for the A<sub>2a</sub> adenosine receptor. *Mol. Pharmacol.* **1996**, *48*, 970–974.
- (32) Salvatore, C. A.; Jacobson, M. A.; Taylor, H. E.; Linden, J.; Johnson, R. G. Molecular cloning and characterization of the human A<sub>3</sub> adenosine receptor. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10365–10369.
- (33) Olah, M. E.; Gallo-Rodriguez, C.; Jacobson, K. A.; Stiles, G. L. <sup>125</sup>I-4-Aminobenzyl-5'-N-methylcarboxamidoadenosine, a high affinity radioligand for the rat A<sub>3</sub> adenosine receptor. *Mol. Pharmacol.* **1994**, *45*, 978–982.
- (34) Ramkumar, V.; Stiles, G. L.; Beaven, M. A.; Ali, H. The A<sub>3</sub> adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. *J. Biol. Chem.* **1993**, *268*, 16887–16890.
- (35) Kohno, Y.; Ji, X.-d.; Mawhorter, S. D.; Koshiba, M.; Jacobson, K. A. Activation of adenosine A<sub>3</sub> receptors on human eosinophils elevates intracellular calcium. *Blood* **1996**, *88*, 3569–3574.
- (36) Robeva, A.; Woodard, R.; Luthin, D. R.; Taylor, H. E.; Linden, J. Double tagging recombinant A<sub>1</sub>- and A<sub>2A</sub>-adenosine receptors with hexahistidine and the FLAG epitope—Development of an efficient generic protein purification procedure. *Biochem. Pharmacol.* **1996**, *51*, 545–555.
- (37) Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielsen, M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7413–7417.
- (38) Stowell, C. P.; Kuhlenschmidt, T. G.; Hoppe, C. A. A fluorescamine assay for submicrogram quantities of protein in the presence of Triton X-100. *Anal. Biochem.* **1978**, *85*, 572–580.
- (39) Marquardt, D. M. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Indust. Appl. Math.* **1963**, *11*, 431–441.
- (40) Linden, J. Calculating the dissociation constant of an unlabeled compound from the concentration required to displace radiolabel binding by 50%. *J. Cyclic Nucleotide Res.* **1982**, *8*, 163–172.
- (41) Schwabe, U.; Trost, T. Characterization of adenosine receptors in rat brain by (–)[<sup>3</sup>H]N<sup>6</sup>-phenylisopropyladenosine. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1980**, *313*, 179–187.
- (42) Jarvis, M. F.; Schutz, R.; Hutchison, A. J.; Do, E.; Sills, M. A.; Williams, M. [<sup>3</sup>H]CGS 21680, an A<sub>2</sub> selective adenosine receptor agonist directly labels A<sub>2</sub> receptors in rat brain tissue. *J. Pharmacol. Exp. Ther.* **1989**, *251*, 888–893.

JM990421V