Anilide Derivatives of an 8-Phenylxanthine Carboxylic Congener Are Highly Potent and Selective Antagonists at Human A_{2B} Adenosine Receptors

Yong-Chul Kim,[†] Xiao-duo Ji,[†] Neli Melman,[†] Joel Linden,[‡] and Kenneth A. Jacobson*,[†]

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

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No highly selective antagonists of the A_{2B} 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_{2B} receptor antagonists. The structure−activity relationships (SAR) of 8-phenyl-1,3-di-(*n*-propyl)xanthine derivatives in binding to recombinant human A_{2B} ARs in HEK-293 cells (HEK-A_{2B}) 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_{2B} receptors in the range of 1−3 nM. The unsubstituted anilide **12** had a K_i value at A_{2B} receptors of 1.48 nM but was only moderately selective versus human A_1/A_{2A} receptors and nonselective versus rat A_1 receptors. Highly potent and selective A_{2B} antagonists were a *p*-aminoacetophenone derivative **20** (K_i value 1.39 nM) and a *p*-cyanoanilide **27** (K_i value 1.97 nM). Compound **27** was 400-, 245-, and 123-fold selective for human A_{2B} receptors versus human $A_1/A_{2A}/A_3$ receptors, respectively, and 8.5- and 310-fold selective versus rat A_1/A_{2A} receptors, respectively. Substitution of the 1,3-dipropyl groups with 1,3-diethyl offered no disadvantage for selectivity, and high affinities at A_{2B} receptors were maintained. Substitution of the *p*-carboxymethyloxy group of **4a** and its amides with acrylic acid decreased affinity at A_{2B} receptors while increasing affinity at A_1 receptors. 1,3-Di-(cyclohexylmethyl) groups greatly reduced affinity at ARs, although the *p*-carboxymethyloxy derivative **9** was moderately selective for A_{2B} receptors. Several selective A_{2B} antagonists inhibited NECA-stimulated calcium mobilization in HEK-A_{2B} cells.

Introduction

The alkylxanthine theophylline, **1** (Figure 1), a weak nonselective adenosine antagonist,[1] is effective when used therapeutically for the treatment of asthma, but its use is associated with unpleasant side effects, such as insomnia and diuresis.[2] In recent years, use of theophylline as a bronchodilator for relief of asthma has been supplanted by drugs of other classes, i.e. selective β_2-adrenergic agonists, corticosteroids, and recently leukotriene antagonists.[3] 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): $A_1/A_{2A}/A_{2B}/A_3$.[4] 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 adenosine-induced contraction,[5] the adenosine antagonist properties of theophylline had been doubted as an explanation of the therapeutic properties,[6] 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_1/A_{2A} ARs. With the recent focus on A_{2B} ARs,[7] it has been noted that therapeutic concentrations of enprofylline block human A_{2B} receptors and proposed that antagonists selective for this subtype may have potential use as antiasthmatic agents.[8,9] Enprofylline has a K_i value of 7 μM and is somewhat selective in binding to human A_{2B} ARs.[9,10] A_{2B} ARs are expressed in some mast cells, such as canine BR mastocytoma cells, in which they appear

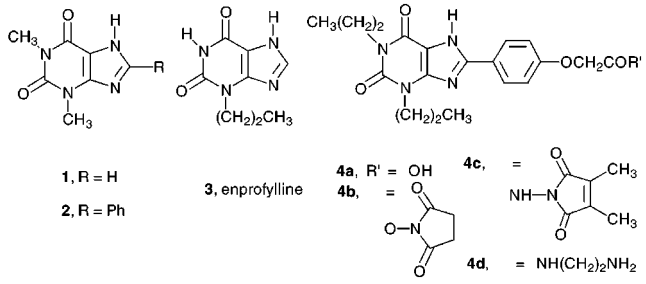

Figure 1. Structures of various xanthines that act as antagonists at A_{2B} receptors.

* 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.
[†] National Institute of Diabetes, Digestive and Kidney Diseases.
[‡] University of Virginia.

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

Although AR subtype-selective probes are available for the $A_1/A_{2A}/A_3$ ARs,[18] only few weakly selective antagonists[19,20] and no selective agonists[21,22] are known for the A_{2B} receptor. Recently radioligand binding assays[9,10,23] have been reported which will aid in the identification of selective antagonists. Although there are several classes of non-xanthine adenosine antagonists[24−26] that have been found to be potent and slightly selective A_{2B} 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 A_{2B} receptors.[7,27] The 8-phenyl analogue of theophylline, **2**, displayed a 22-fold enhancement of binding affinity at A_{2B} receptors.[19] Leads for achieving moderate selectivity (at least 20-fold versus $A_1/A_{2A}/A_3$ ARs) have recently been identified among derivatives of 8-[4-[(carboxymethyl)oxy]phenyl]-1,3-dipropylxanthine,[19,20,27] **4a**. Compound **4b** (MRS 1204, *N*-hydroxysuccinimide ester of **4a**) displayed approximately 20-fold selectivity for human A_{2B} receptors versus $A_1/A_{2A}/A_3$ ARs.[19] A 1,2-dimethylmaleimide derivative, **4c** (MRS 1595), bound to human A_{2B} receptors with a K_i of 19 nM and was selective versus human $A_1/A_{2A}/A_3$ receptors by 160-, 100-, and 35-fold, respectively.[20] The A_{2B} receptor selectivity of enprofylline was lost in its 8-aryl-substituted analogues.[20] Aryl amide derivatives were previously reported to be highly potent antagonists at A_{2A} receptors in human platelets.[28] In the present study, these and additional amide derivatives of **4a** were synthesized, to identify novel, selective A_{2B} receptor antagonists.

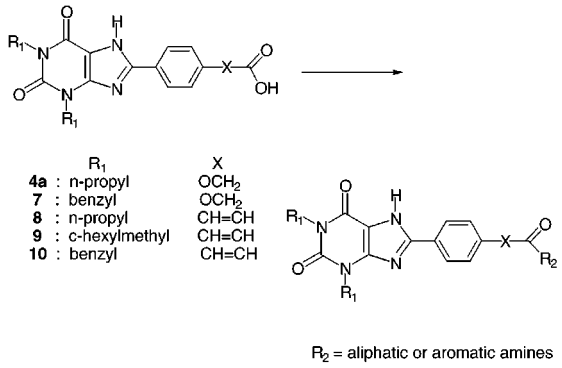
Results

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**[27] 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 A_{2B} receptor binding assay of an *N*-hydroxysuccinimide ester **4b** (K_i value of 9.75 nM)[19] and related acyl hydrazides,[20] such as **4c**.

Besides **4a**, various other analogues of xanthine carboxylic acid congeners, **7**−**10,** were synthesized according to established synthetic procedures of xanthines,[29] 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 A_{2B} receptors was evaluated using two binding assays

Scheme 1. Synthesis of Amide Derivatives of Xanthine Carboxylic Acid Congeners as Potentially Selective A_{2B} AR Antagonists[a]



[a] Reagents: (1) EDAC, DMAP, amines in DMF/CH_2Cl_2; (2) BOP-Cl, triethylamine, amines in CH_2Cl_2; or (3) $SOCl_2$, amines in pyridine/CH_2Cl_2.

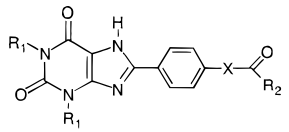
(Tables 1−3) and a functional assay (Figure 2). K_i values of xanthine derivatives were determined in displacement of binding of two nonselective radioligands: [^{3}H]-ZM241385 (4-(2-[7-amino-2-furyl[1,2,4]triazolo[2,3-*a*]-[1,3,5]triazin-5-yl]aminoethyl)phenol)[23] and [^{125}I]IABOPX (3-(4-amino-3-iodobenzyl)-8-phenyloxyacetate-1-propylxanthine),[10] at human A_{2B} receptors stably expressed in HEK-293 cell membranes.[10] Results with these two radioligands were identical. To determine selectivity, the xanthines were evaluated using standard binding assays at $A_1/A_{2A}/A_3$ receptors. The initial screening utilized rat brain A_1/A_{2A} receptors (with radioligands [^{3}H]*R*-PIA and [^{3}H]CGS21680), and selected compounds were examined at the recombinant human subtypes, using [^{3}H]CPX (8-cyclopentyl-1,3-dipropylxanthine) (A_1)[30] and [^{125}I]ZM241385 (A_{2A}).[31] Affinity at cloned human A_3 receptors expressed in HEK-293 cells was determined using [^{125}I]IABA (N^6-(4-amino-3-[^{125}I]iodobenzyl)-adenosine)[32] or [^{125}I]IAB-MECA (N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide).[33]

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-substitutents, **4a**−**7**, affinity at A_{2B} receptors decreased in the order: 1,3-dipropyl ≥ 1,3-di-*n*-butyl > 1,3-diallyl > 1,3-dibenzyl. The diallyl derivative was more A_{2B} 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 A_1 receptors and less potent at A_{2B} receptors than the 8-(4-carboxymethyloxyphenyl) derivatives. The 1,3-dicyclohexylmethyl derivative **9** was 25-fold selective for A_{2B} receptors. A primary carboxamide **11** was more potent than the carboxylic acid **4a** at A_1 (3-fold) and A_{2A} (29-fold) receptors and equipotent at A_{2B} 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 A_{2B} receptors. Selectivities for the human A_{2B} versus rat A_1 receptors ranged from 1-fold (**30**) to 27-fold (**20**),

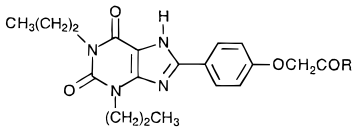
Table 1. Affinities or Antagonistic Activities of Xanthine Carboxylic Acid Derivatives in Radioligand Binding Assays at $A_1/A_{2A}/A_{2B}/A_3$ Receptors



compd	R_1	X	R_2	K_i (nM) rA_1[a]	rA_{2A}[b]	hA_{2B}[c]	hA_3[d]	hA_1/hA_{2B}	hA_{2A}/hA_{2B}	hA_3/hA_{2B}
4a	*n*-Pr	OCH_2	OH	58	2200	40 ± 4	3910 ± 2140	4.4	15	98
				175 ± 57 (h)	595 ± 128 (h)		75700 ± 6500 (r)			
4b	*n*-Pr	OCH_2	*O*-succinimide	153	127	9.75 ± 4.80	227			
4c	*n*-Pr	OCH_2	NHN-dimethylmaleyl	11.1 ± 2.4	126 ± 41	26.6 ± 4.0	670 ± 154	110	74	25
				3030 ± 1110 (h)	1970 ± 550 (h)					
4d	*n*-Pr	OCH_2	$NH(CH_2)_2NH_2$	1.2	63	7.75 ± 0.14	25.6 ± 5.0	0.9	2.4	3.3
				6.82 ± 1.57 (h)	18.4 ± 0.03 (h)					
5	allyl	OCH_2	OH	756 ± 147	4290 ± 570	141 ± 29 (h)	816 ± 91	12	17	5.8
				1660 ± 580 (h)	2370 ± 290 (h)		173000 ± 18000 (r)			
6	*n*-butyl	OCH_2	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)			
7	Bn	OCH_2	OH	679 ± 190	25 ± 3% (10^{-4})[e]	1760 ± 110				
8	*n*-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)			
9	*c*-HexMe	CH=CH	OH	602 ± 24	<10% (10^{-5})[e]	199 ± 52	922 ± 399	25	7.6	4.6
				4890 ± 530 (h)	1518 ± 980 (h)					
10	Bn	CH=CH	OH	201 ± 23	4450 ± 1230	469 ± 23				

[a] Displacement of specific binding in rat brain membranes ([^{3}H]*R*-PIA) or recombinant human A_1 receptors in HEK-293 cells ([^{125}I]IABA), expressed as K_i ± SEM (n = 3−5). [b] Displacement of specific binding in rat striatal membranes ([^{3}H]CGS21680) or recombinant human A_{2A} receptors in HEK-293 cells ([^{125}I]iodo-ZM241385), expressed as K_i ± SEM (n = 3−5). [c] Displacement of specific [^{3}H]ZM241385 or [^{125}I]IABOPX binding at human A_{2B} receptors expressed in HEK-293 cells, in membranes, expressed as K_i ± SEM (n = 3−4). [d] Displacement of specific [^{125}I]IAB-MECA or [^{125}I]IABA binding at human A_3 receptors expressed in HEK cells, in membranes, expressed as K_i ± SEM (n = 3−4). [e] % Displacement of specific binding at the designated molar concentration.

Table 2. Affinities or Antagonistic Activities of Xanthine Amide Derivatives in Radioligand Binding Assays[a] at $A_1/A_{2A}/A_{2B}/A_3$ Receptors

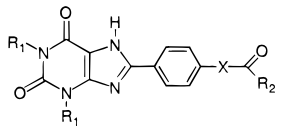


compd	R	K_i (nM) rA_1	rA_{2A}	hA_1	hA_{2A}	hA_{2B}	hA_3	hA_1/hA_{2B}	hA_{2A}/hA_{2B}	hA_3/hA_{2B}
11	NH_2	20.0 ± 3.8	76.3 ± 14.0			16.3 ± 4.2				
12	NH-Ph	4.22 ± 0.88	45.6 ± 1.4	40.1 ± 5.1	25.8 ± 4.5	1.48 ± 0.63	137 ± 54	27	17	93
13	$NH\text{-}CH_2Ph$	5.02 ± 0.55	25.9 ± 7.6	54.7 ± 21.2	23.8 ± 5.71	2.04 ± 0.17	79.2 ± 17.8	27	12	39
14	$NH\text{-}CH(Ph)_2$	120 ± 21	20 ± 8% (10^{-6})			33.7 ± 17.0				
15	$N(CH_2Ph)_2$	167 ± 49	2750 ± 800	690 ± 98	642 ± 198	9.88 ± 1.05	284 ± 14	70	65	29
16	$N(CH_3)Ph$	218 ± 80	497 ± 250			5.42 ± 1.71				
17	$N(CH_2COOEt)_2$	26.8 ± 2.4	999 ±144			43.4 ± 8.4				
18[b]	NH-Ph(2-$COCH_3$)	27.9 ± 1.6	434 ± 129	335 ± 64	431 ± 176	2.74 ± 1.01	61.9 ± 3.4	120	160	23
19	NH-Ph(3-$COCH_3$)	439 ± 111	949 ± 394	234 ± 28	58.9 ± 7.1	4.92 ± 0.55	352 ± 69	48	12	72
20[b]	NH-Ph(4-$COCH_3$)	37.6 ± 4.0	548 ± 183	157 ± 8	112 ± 37	1.39 ± 0.30	230 ± 23	110	81	170
21	NH-Ph(4-$COOCH_3$)	38.4 ± 3.9	541 ± 128	225 ± 9	3100 ± 1540	3.93 ± 1.35	363 ± 148	57	790	92
22	NH-Ph(4-$CONH_2$)	10.2 ± 2.5	683 ± 167			7.75 ± 1.11				
23	NH-Ph(4-$CONHCH_3$)	24.8 ± 1.8	98.1 ± 49.6			3.34 ± 0.51				
24	NH-Ph(4-COOH)	145 ± 28	220 ± 79	161 ± 53	39.0 ± 21.5	16.1 ± 4.7	>5000	10	2.4	>300
25	NH-Ph(4-CH_3)	17.5 ± 5.0	126 ± 38			1.88 ± 0.76				
26	NH-Ph(4-OH)	5.88 ± 1.06	63.3 ± 20.4			3.71 ± 0.76				
27[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
28	NH-Ph(4-NO_2)	13.1 ± 3.9	1180 ± 360	57.0 ± 3.1	70.0 ± 10.7	1.52 ± 0.24	138 ± 17.1	38	46	91
29	NH-Ph(4-CF_3)	44.6 ± 6.5	917 ± 258	61.2 ± 8.2	238 ± 28	2.14 ± 0.47	213 ± 94	29	110	100
30	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
31	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
32	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
33	NH-Ph(4-I)	15.7 ± 4.2	152 ± 47	293 ± 67	5140 ± 540	2.13 ± 0.12	1270 ± 130	140	2400	600

[a] The methods of each binding assay are shown in Table 1. [b] **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_{2B} receptors versus rat A_1/A_{2A} 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[a] at $A_1/A_{2A}/A_{2B}/A_3$ Receptors



compd	R_1	X	R_2	K_i (nM) rA_1	rA_{2A}	hA_{2B}	hA_3
34	*n*-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
35	*n*-Pr	CH=CH	NH-Ph(2-$COCH_3$)	7.67 ± 2.20	143 ± 50	3.65 ± 0.98	121 ± 138
36	*c*-HexMe	CH=CH	NH-Ph(2-$COCH_3$)	*b* (10^{-5})	*b* (10^{-5})	*b* (10^{-5})	
37	Bn	CH=CH	NH-Ph(2-$COCH_3$)	34300	*b* (10^{-5})	*b* (10^{-5})	
38	Bn	OCH_2	NH-Ph(2-$COCH_3$)	*b* (10^{-5})	*b* (10^{-5})	*b* (10^{-5})	
39	Et	OCH_2	NH-Ph(4-CH_3)	34.9 ± 0.3	71.1 ± 7.7	1.78 ± 0.43	*b* (10^{-6})
40	Et	OCH_2	NH-Ph(4-$CH_2CONH(CH_2)_2NH_2$)	65.0 ± 15.4	1370 ± 490	15.2 ± 6.8	

[a] The methods of each binding assay are shown in Table 1. [b] <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.
7	13	>310	EI: 482	$C_{27}H_{22}N_4O_5$	HRMS[a]
10	40	>310	FAB: 479	$C_{28}H_{22}N_4O_4$	C,H,N
12	71	301−302	CI: 462	$C_{25}H_{27}N_5O_4$	C,H,N
13	41	268	CI: 476	$C_{26}H_{29}N_5O_4$	C,H,N
14	46	269−270	EI: 551	$C_{32}H_{33}N_5O_4$	C,H,N
15	55	230	EI: 565	$C_{33}H_{35}N_5O_4$	C,H,N
16	49	215	FAB: 476	$C_{26}H_{29}N_5O_4$	C,H,N
17	13	225	CI: 558	$C_{27}H_{35}N_5O_8$	C,H,N
18	68	294	EI: 503	$C_{27}H_{29}N_5O_5$	C,H,N
19	29	269−270	EI: 503	$C_{27}H_{29}N_5O_5$	HRMS[a]
20	29	309−310	EI: 503	$C_{27}H_{29}N_5O_5 \cdot 0.23H_2O$	C,H,N
21	56	>310	CI: 520	$C_{27}H_{29}N_5O_6$	C,H,N
22	19	>310	CI: 505	$C_{26}H_{28}N_6O_5$	C,H,N
23	45	>310	FAB: 519	$C_{27}H_{30}N_6O_5 \cdot 1.8CH_2Cl_2$	C,H,N
24	51	>310	FAB: 506	$C_{26}H_{27}N_5O_6 \cdot 0.60CH_2Cl_2$	C,H,N
27	44	>310	CI: 487	$C_{26}H_{26}N_6O_4$	C,H,N
28	31	307	CI: 507	$C_{25}H_{26}N_6O_6 \cdot 0.43CH_3OH$	C,H,N
29	44	>310	CI: 530	$C_{26}H_{26}F_3N_5O_4 \cdot 0.26CH_3OH$	C,H,N
30	48	298	CI: 480	$C_{25}H_{26}FN_5O_4$	C,H,N
31	31	309	CI: 496	$C_{25}H_{26}ClN_5O_4 \cdot 0.26(CH_3)_2CO$	C,H,N
32	36	>310	CI: 540	$C_{25}H_{26}BrN_5O_4$	C,H,N
33	13	>310	CI: 588	$C_{25}H_{26}IN_5O_4 \cdot 0.60CH_3OH$	C,H,N
4c	79	>310	FAB: 509	$C_{25}H_{28}N_6O_6$	C,H,N
34	49	302−303	EI: 504	$C_{26}H_{28}N_6O_5$	C,H,N
35	42	281−283	CI: 500	$C_{28}H_{29}N_5O_4 \cdot 0.27MeOH$	C,H,N
36	33	305	FAB: 608	$C_{36}H_{41}N_5O_4$	HRMS[a]
37	76	308−309	CI: 596	$C_{36}H_{29}N_5O_4 \cdot 0.60H_2O$	C,H,N
38	18	284	EI: 599	$C_{35}H_{29}N_5O_5$	HRMS[a]

[a] High-resolution mass in EI or FAB^+ 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_3CN, 80:20 to 20:80, in 30 min; and (B) H_2O/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_{2B} receptors versus human $A_1/A_{2A}/A_3$ receptors and 10- and 160-fold selective versus rat A_1/A_{2A} receptors. The *para*-substituted acetophenone **20** was more potent at A_{2B} receptors than the corresponding *ortho*- and *meta*-isomers. Other highly potent and moderately selective A_{2B} antagonists were a *p*-trifluoromethyl derivative, **29** (K_i value 2.14 nM), and a *p*-cyanoanilide, **27** (K_i value 1.97 nM), which was highly selective versus the other human subtypes but only 8.5-fold selective versus rat A_1 receptors**.** A *p*-nitro derivative, **28**, bound to human A_{2B} receptors with a K_i of 1.52 nM but was only 35-fold selective versus human A_1 receptors. A *p*-iodo derivative, **33** (K_i value 2.13 nM), was 140-, 2400-, and 600-fold selective for human A_{2B} receptors versus human $A_1/A_{2A}/A_3$ receptors. A *p*-toluide of **4a**, **25**, was reported previously[27] and displayed a K_i value at human A_{2B} receptors of 1.88 nM.

The introduction of the dimethylmaleimido group[20] in **34** and in the *p*-acryloyl derivative **35** resulted in moderate selectivity for A_{2B} 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.

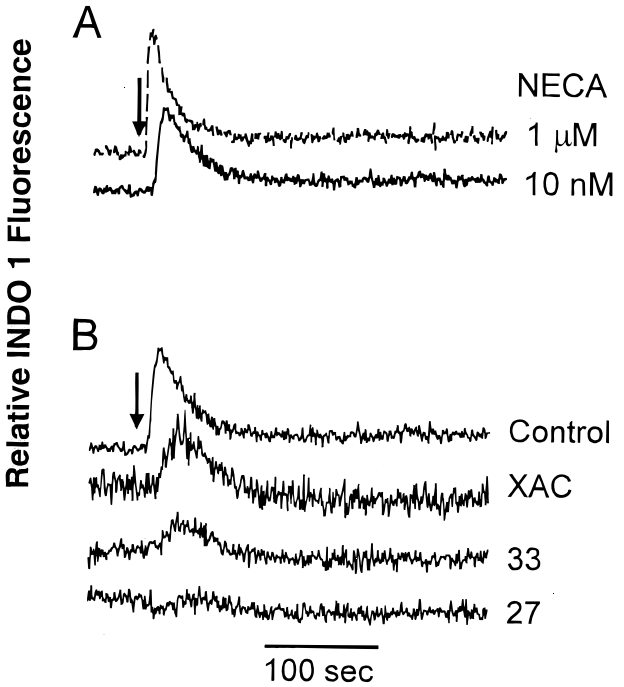


Figure 2. Inhibition by several selective A_{2B} AR antagonists of NECA-stimulated calcium mobilization in HEK-A_{2B} cells. Cells were loaded with Indo-1 for 1 h: (A) calcium mobilization in response to 10 nM and 1 μM NECA added at the arrow; (B) calcium mobilization in response to 10 nM NECA added 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,[21] 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.[12] The A_3 AR mediates the degranulation of rat RBL mast-like cells[34] and is present in high density in human blood eosinophils.[35] 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.[27] Compounds **5**−**7** and **10** were synthesized as reported.[29] Compounds **39** and **40** were synthesized as reported.[28] 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 $CDCl_3$. 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 (±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 × 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_3CN, 80:20 to 20:80, in 30 min, and the other (B) was H_2O/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_2Cl_2 (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 ($CHCl_3$:MeOH = 20:1) and crystallization in MeOH/ether or MeOH/CH_2Cl_2 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 μL, 0.206 mmol) in 2 mL of anhydrous CH_2Cl_2 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 °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_2Cl_2. 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}H NMR (DMSO-d_6) 4.23 (s, 2H, −OCH_2−), 5.10 (s, 2H, −NCH_2−), 5.23 (s, 2H, −NCH_2−), 6.88 (d, 2H, J = 8.8 Hz, Ar), 7.22−7.41 (m, 10H, 2×-Ph), 8.01 (d, 2H, J = 8.8 Hz, Ar).

8-(4-(2-Carboxy-*trans*-vinyl)phenyl)-1,3-dibenzylxanthine (10): ^{1}H NMR (DMSO-d_6) 5.12 (s, 2H, −NCH_2−), 5.26 (s, 2H, −NCH_2−), 6.63 (d, 1H, J = 15.6 Hz, −CH═), 7.22−7.43 (m, 10H, 2×-Ph), 7.63 (d, 1H, J = 15.6 Hz, −CH═), 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}H NMR (DMSO-d_6) 0.89 (2t, 6H, J = 7.8 Hz, 2×-CH_3), 1.58 and 1.74 (2m, 4H, 2×-CH_2−), 3.87 and 4.02 (2t, 4H, J = 6.8 Hz, 2×-NCH_2−), 4.80 (s, 2H, −OCH_2−), 7.06−7.12 (m, 1H, −Ph), 7.14 (d, 2H, J = 8.8 Hz, Ar), 7.33 (t, 2H, J = 7.8 Hz, −Ph), 7.64 (d, 2H, J = 7.8 Hz, −Ph), 8.09 (d, 2H, J = 8.8 Hz, Ar), 10.13 (s, 1H, −NH).

8-[4-[((4-Acetylphenyl)carbamoylmethyl)oxy]phenyl]-1,3-di(*n*-propyl)xanthine (20): ^{1}H NMR (DMSO-d_6) 0.89 (2t, 6H, J = 7.8 Hz, 2×-CH_3), 1.58 and 1.74 (2m, 4H, 2×-CH_2−), 2.54 (s, 3H, −$COCH_3$), 3.87 and 4.02 (2t, 4H, J = 6.8 Hz, 2×-NCH_2−), 4.85 (s, 2H, −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, −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_2Cl_2 to give 9 mg of **23**: ^{1}H NMR (DMSO-d_6) 0.89 (2t, 6H, J = 7.8 Hz, 2×-CH_3), 1.58 and 1.73 (2m, 4H, 2×-CH_2−), 2.76 (s, 3H, −NHCH_3), 3.86 and 4.01 (2t, 4H, J = 6.8 Hz, 2×-NCH_2−), 4.82 (s, 2H, −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, −NHCH_3), 10.34 (s, 1H, −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_3CN (90/10 to 40/60) to give 10 mg of **24**: ^{1}H NMR (DMSO-d_6) 0.89 (2t, 6H, J = 7.8 Hz, 2×-CH_3), 1.58 and 1.74 (2m, 4H, 2×-CH_2−), 3.87 and 4.01 (2t, 4H, J = 6.8 Hz, 2×-NCH_2−), 4.84 (s, 2H, −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, −NH−).

8-[4-[((4-Cyanophenyl)carbamoylmethyl)oxy]phenyl]-1,3-di(*n*-propyl)xanthine (27): ^{1}H NMR (DMSO-d_6) 0.89 (2t, 6H, J = 7.8 Hz, 2×-CH_3), 1.58 and 1.74 (2m, 4H, 2×-CH_2−), 3.86 and 4.01 (2t, 4H, J = 6.8 Hz, 2×-NCH_2−), 4.85 (s, 2H, −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, −NH−).

8-(4-(2-Carboxy-*trans*-vinyl)phenyl)-1,3-di(*n*-propyl)-xanthine *N*′,*N*′-(1,2-dimethylmaleyl)hydrizide (34): ^{1}H NMR ($CDCl_3$) 1.01 (2t, 6H, J = 7.8 Hz, 2×-CH_3), 1.72 and 1.89 (2m, 4H, 2×-CH_2−), 2.05 (s, 6H, 2×-CH_3), 4.02 and 4.17 (2t, 4H, J = 6.8 Hz, 2×-NCH_2−), 6.67 (d, 1H, J = 15.6 Hz, −CH═), 7.63 (d, 2H, J = 8.8 Hz, Ar), 7.74 (d, 1H, J = 15.6 Hz, −CH═), 8.09 (d, 2H, J = 8.8 Hz, Ar), 9.43 (s, 1H, −NH−).

8-[4-[2-(2-Acetylphenyl)carbamoyl-*trans*-vinyl]phenyl]-1,3-di(*n*-propyl)xanthine (35): ^{1}H NMR (DMSO-d_6) 0.89 (2t, 6H, J = 7.8 Hz, 2×-CH_3), 1.59 and 1.76 (2m, 4H, 2×-CH_2−), 3.88 and 4.04 (2t, 4H, J = 6.8 Hz, 2×-NCH_2−), 4.79 (d, 3H, J = 4.9 Hz, −$COCH_3$), 6.93 (d, 1H, J = 15.6 Hz, −CH═), 7.51 (d, 1H, J = 15.6 Hz, −CH═), 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, −NH−).

8-[4-[2-(2-Acetylphenyl)carbamoyl-*trans*-vinyl]phenyl]-1,3-dibenzylxanthine (37): ^{1}H NMR (DMSO-d_6) 4.79 (d, 3H, J = 5.8 Hz, −$COCH_3$), 5.13 (s, 2H, −NCH_2−), 5.27 (s, 2H, −NCH_2−), 6.93 (d, 1H, J = 15.6 Hz, −CH═), 7.24−7.41 (m, 10H, 2×-Ph), 7.46 (d, 1H, J = 15.6 Hz, −CH═), 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, −NH−).

8-[4-[[(2-Acetylphenyl)carbamoylmethyl]oxy]phenyl]-1,3-dibenzylxanthine (38): ^{1}H NMR (DMSO-d_6) 4.68 (s, 3H, −$COCH_3$), 4.71 (s, 2H, −OCH_2−), 5.12 (s, 2H, −NCH_2−), 5.26 (s, 2H, −NCH_2−), 7.15 (d, 2H, J = 8.8 Hz, Ar), 7.23−7.42 (m, 10H, 2×-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, −NH−).

Pharmacology. The human A_{2B} receptor cDNA was subcloned into the expression plasmid pDoubleTrouble.[36] 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.[37]

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 μg/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 30000*g* 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 fluorescamine fluorescence.[38]

To prepare [^{125}I]IABOPX, 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}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}I]IABOPX eluted at 18−19 min in a yield of 50−60% with respect to the initial ^{125}I.

Saturation binding assays for human A_{2B} ARs were performed with [^{3}H]ZM214385 (17 Ci/mmol; Tocris Cookson, Bristol, U.K.)[23] or [^{125}I]IABOPX (2200 Ci/mmol).

In equilibrium binding assays the ratio of [^{127}I/^{125}I]ABOPX was 10−20/1. Radioligand binding experiments were performed in triplicate with 20−25 μg 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 μM NECA. Competition experiments were carried out using 0.6 nM [^{125}I]IABOPX. 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.[39] K_i values for different compounds were derived from IC_{50} values as described, assuming a K_D value for [^{125}I]IABOPX of 36 nM.[40] Data from replicate experiments are tabulated as means ± SEM.

At other ARs: [^{3}H]CPX,[30] [^{125}I]iodo-ZM241385, and [^{125}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 [^{3}H]*R*-*N*^{6}-phenylisopropyladenosine[41] ([^{3}H]*R*-PIA; Amersham, Chicago, IL) to A_1 receptors from rat cerebral cortical membranes and of [^{3}H]CGS21680[42] (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.[40] 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 250*g* 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_3PO_4, 5.6 mM glucose, and 10 mM HEPES, pH 7.4, and the Ca^{2+}-sensitive fluorescent dye Indo-1-AM (5 μM) 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× 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 (SML 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.

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

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