Articles

Structure−Activity Relationships at Human and Rat A_{2B} Adenosine Receptors of Xanthine Derivatives Substituted at the 1-, 3-, 7-, and 8-Positions

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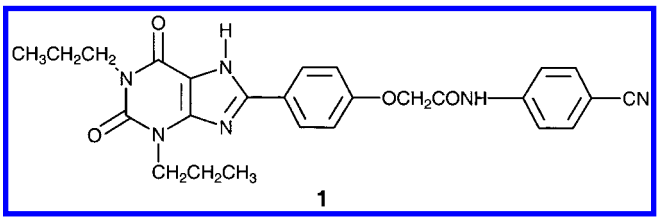
In the search for improved selective antagonist ligands of the A_{2B} adenosine receptor, which have the potential as antiasthmatic or antidiabetic drugs, we have synthesized and screened a variety of alkylxanthine derivatives substituted at the 1-, 3-, 7-, and 8-positions. Competition for ^{125}I-ABOPX (^{125}I-3-(4-amino-3-iodobenzyl)-8-(phenyl-4-oxyacetate)-1-propylxanthine) binding in membranes of stably transfected HEK-293 cells revealed uniformly higher affinity (<10-fold) of these xanthines for human than for rat A_{2B} adenosine receptors. Binding to rat brain membranes expressing A_1 and A_{2A} adenosine receptors revealed greater A_{2B} selectivity over A_{2A} than A_1 receptors. Substitution at the 1-position with 2-phenylethyl (or alkyl/olefinic groups) and at N-3 with hydrogen or methyl favored A_{2B} selectivity. Relative to enprofylline **2b**, pentoxifylline **35** was equipotent and 1-propylxanthine **3** was >13-fold more potent at human A_{2B} receptors. Most N-7 substituents did not enhance affinity over hydrogen, except for 7-(2-chloroethyl), which enhanced the affinity of theophylline by 6.5-fold to 800 nM. The A_{2B} receptor affinity-enhancing effects of 7-(2-chloroethyl) vs 7-methyl were comparable to the known enhancement produced by an 8-aryl substitution. Among 8-phenyl analogues, a larger alkyl group at the 1-position than at the 3-position favored affinity at the human A_{2B} receptor, as indicated by 1-allyl-3-methyl-8-phenylxanthine, with a K_i value of 37 nM. Substitution on the 8-phenyl ring indicated that an electron-rich ring was preferred for A_{2B} receptor binding. In conclusion, new leads for the design of xanthines substituted in the 1-, 3-, 7-, and 8-positions as A_{2B} receptor-selective antagonists have been identified.

Introduction

Four extracellular G protein-coupled receptors for adenosine have been identified as follows: A_1, A_{2A}, A_{2B}, and A_3.[1] A_{2B} receptors, which are coupled to stimulation of adenylyl cyclase[2,3] and also lead to a rise in intracellular calcium,[4] are involved in the control of vascular tone,[5] hepatic glucose balance,[6] cell growth and gene expression,[7] mast cell degranulation,[8] and intestinal water secretion.[9] Activation of A_{2B} receptors in human retinal endothelial cells may lead to neovascularization by a mechanism involving increased vascular endothelial growth factor expression.[7] Selective xanthine antagonists of the A_{2B} receptor have recently been reported.[10,11] Such antagonists are potentially useful in the treatment of asthma,[12,13] intestinal disorders,[9] and diabetes (through improved glucose uptake in skeletal muscle and suppression of glucose production in hepatocytes).[6]

On the basis of adenosine receptor binding assays, we have identified several new xanthines with improved potency and selectivity for human A_{2B} receptors.[10,14] A *p*-cyanoanilide derivative in this series, *N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide) **1**, was shown to be 204-, 255-, and 289-fold selective for human A_{2B} receptors vs human A_1, A_{2A}, and A_3 receptors, although less selective vs rat A_1 and A_{2A} receptors. Compound **1** (100 nM) was shown to completely inhibit calcium mobilization stimulated by 1 μM NECA in HEK-293 (human embryonic kidney) cells expressing human A_{2B} receptors.[10]



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Nonselective radioligands have been used to characterize binding to recombinant human A_{2B} receptors overexpressed in cell lines such as HEK-293. These include ^{125}I-ABOPX (^{125}I-3-(4-amino-3-iodobenzyl)-8-(phenyl-4-oxyacetate)-1-propylxanthine),[15] [^{3}H]8-cyclo-

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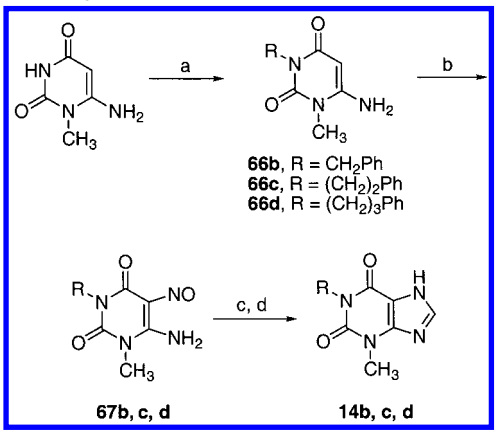
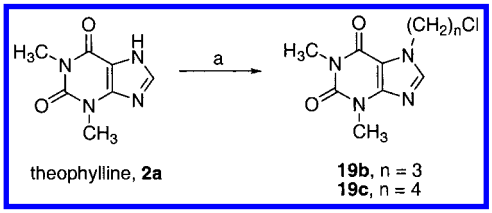
Scheme 1. Synthesis of Xanthine Derivatives **14b**−**d**[a]



[a] Reagents: (a) 10% NaOH, EtOH, R−Br; (b) $NaNO_2$, 6 N HCl, AcOH; (c) $Na_2S_2O_4$; (d) $HC(OMe)_3$.

Scheme 2. Synthesis of Xanthine Derivatives **19b**,**c**[a]



[a] Reagents: (a) NaH, $Br(CH_2)_nCl$, DMF.

pentyl-1,3-dipropylxanthine,[13] and [^{3}H]4-(2-[7-amino-2-{furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-ylaminoethyl)phenol.[16] [^{3}H]*N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide) **1** was recently introduced as a new selective radioligand that bound to human A_{2B} receptors with a K_D of 1 nM.[17]

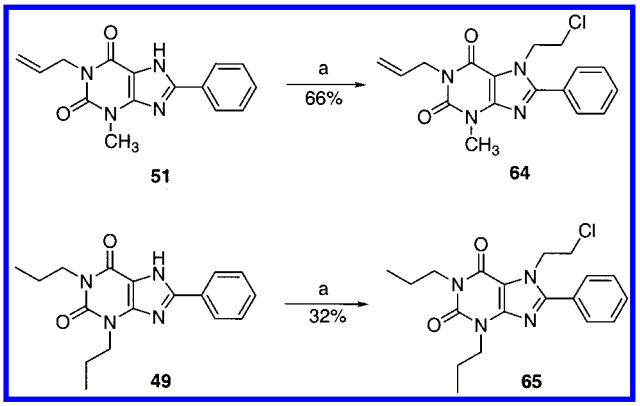
In the present study, in an effort to better understand the structure−activity relationships (SAR) of antagonist ligands of the A_{2B} adenosine receptor, we have screened a variety of alkylxanthine derivatives substituted at the 1-, 3-, 7-, and 8-positions and synthesized some derivatives that were designed by analysis of the biological results obtained from screening. In this study, previously uncharacterized patterns in the SAR of rat and human A_{2B} receptors have been identified.

Results

Chemistry. Sixty-five xanthine derivatives (Table 1) were examined as antagonists of binding at the human A_{2B} adenosine receptor. Many of these ligands had been investigated in earlier studies of A_1 and A_{2A} receptors.[14,18−25,37] Other compounds were synthesized, based on SAR indications that arose during the course of the present study.

Compounds **14b**−**d**, which were substituted with various phenylalkyl groups in the 1-position, were synthesized in four steps (Scheme 1). Alkylation of 6-amino-1-methyluracil was performed with 10% aqueous NaOH and the appropriate alkyl halide. Compounds **66b**−**d** were converted to xanthines **14b**−**d** according to standard procedures as reported.[10,25] The synthesis of several 8-H and 8-aryl derivatives alkylated at the 7-position, **19b**, **19c**, **64**, and **65**, was performed using NaH and the alkyl halide in dimethylformamide (DMF)

Scheme 3. Synthesis of Xanthine Derivatives **64** and **65**[a]



[a] Reagents: (a) NaH, $Cl_2(CH_2)_2$, DMF.

(Schemes 2 and 3). The yields and characterization of the xanthine derivatives prepared are listed in Table 2.

Biology. Binding experiments were carried out using ^{125}I-ABOPX[15] for binding to recombinant human or rat A_{2B} receptors overexpressed in HEK-293 cells (Tables 1 and 3). The xanthines examined included those substituted at the 1-position alone (**3**−**8**), the 3-position alone (**2b**), the 1- and 3-positions (**2a** and **9**−**17**), the 1-, 3-, and 7-positions (**18**−**36**), the 1- and 8-positions (**37**), the 1-, 3-, and 8-positions (**38**−**62**), and the 1-, 3-, 7-, and 8-positions (**63**−**65**). At the 8-position, cycloalkyl or aryl substituents were included. For selected derivatives, binding to membranes expressing rat A_1 and A_{2A} adenosine receptors as an indication of rat A_{2B} selectivity was also carried out (Table 3).

Enprofylline (3-propylxanthine **2b**) was previously identified as being roughly 1 order of magnitude selective for the human A_{2B} vs human A_1 and A_{2A} receptors.[13] Surprisingly, the A_{2B} affinity of the enprofylline isomer (1-propylxanthine **3**) was 13-fold greater than enprofylline itself. Another monosubstitution was examined at N-1: propargyl, allyl, and *n*-butyl analogues **4**−**6** displayed similar affinity with K_i values (equilibrium inhibition constant) of approximately 0.5 μM, and even the more bulky substitutents, 2-phenylethyl **7** and cyclopentyl **8**, were relatively potent with K_i values of 0.4 and 1.0 μM, respectively.

Symmetrical 1,3-substitution of xanthine with methyl **2a**, ethyl **9**, *n*-Pr **10**, or allyl **11** resulted in K_i values of roughly 1 μM at A_{2B} receptors, while the 1,3-di-*n*-hexyl derivative **12** was less potent. Among asymmetrically substituted xanthines, the 1-ethyl-3-methyl derivative **13** was equipotent to the 1,3-diethyl analogue **9** and elongation at the 1-position, as in **16**, did not have a significant effect. The 1-propargyl-3-methyl derivative **14a** was equipotent to the 1-propargyl derivative **4**. Other 1-position substitutions were included (**14b**−**d**) and studied in binding (Table 3). Branched alkyl chains, in **15** and **17**, reduced affinity of A_{2B} binding. A comparison of **6** and **16** indicated that addition of a 3-ethyl group reduced affinity 4-fold.

Most N-7 substituents did not enhance affinity of alkylxanthines over hydrogen at this position. For example, theophylline **2a** and caffeine **18** were equipotent with K_i values of approximately 10 μM for the human A_{2B} receptor. Most other 7-position substitutions

Table 1. Affinities of Xanthine Derivatives in a Radioligand Binding Assay at Human A_{2B} Receptors

compd	R_1	R_3	R_7	R_8	K_i[a] (nM)	compd	R_1	R_3	R_7	R_8	K_i[a] (nM)
2a (theophylline)	Me	Me	H	H	9070 ± 1490	**31**	CH_2OCH_3	Me	Me	H	14 000 ± 2400
2b (enprofylline)	H	Pr	H	H	4730 ± 270	**32**	CH_2CN	Me	Me	H	21 600 ± 5200
3	Pr	H	H	H	360 ± 70	**33**	$(CH_2)_2OC\text{-}OCH_3$	Me	Me	H	8710 ± 1320
4	propargyl	H	H	H	552 ± 76	**34**	CH_2COCH_3	Me	Me	H	20 800 ± 4800
5	allyl	H	H	H	461 ± 18 (4)	**35** (pentoxifylline)	$(CH_2)_4CO\text{-}CH_3$	Me	Me	H	5180 ± 2010
6	*n*-Bu	H	H	H	421 ± 38 (4)	**36**	Me	propargyl	Me	H	9480 ± 1340
7	2-phenylethyl	H	H	H	408 ± 67 (4)	**37**	Pr	H	H	cyclopentyl	34 ± 10
8	cyclopentyl	H	H	H	965 ± 64 (4)	**38** (CPX)	Pr	Pr	H	cyclopentyl	63.8 ± 8.3
9	Et	Et	H	H	1770 ± 260	**39** (8-PT)	Me	Me	H	C_6H_5	415 ± 219
10	*n*-Pr	*n*-Pr	H	H	1110 ± 330 (4)	**40** (SPT)	Me	Me	H	$C_6H_4\text{-}4\text{-}S\text{-}O_3H$	1330 ± 220
11	allyl	allyl	H	H	1330 ± 240	**41**	Me	Me	H	$C_6H_4\text{-}4\text{-}OH$	60.7 ± 3.1
12	*n*-hexyl	*n*-hexyl	H	H	7580 ± 1900	**42**	Me	Me	H	$C_6H_4\text{-}4\text{-}OCH_3$	394 ± 38
13	Et	Me	H	H	1620 ± 440	**43**	Me	Me	H	$C_6H_4\text{-}4\text{-}NO_2$	6940 ± 1960
14a	propargyl	Me	H	H	511 ± 52 (4)	**44**	Me	Me	H	$C_6H_4\text{-}4\text{-}N\text{-}(CH_3)_2$	289 ± 11
14b	benzyl	Me	H	H	10 200 ± 2000	**45**	Me	Me	H	$C_6H_4\text{-}2\text{-}CO_2H$	11 100 ± 4700
14c	2-phenylethyl	Me	H	H	646 ± 15	**46**	Me	Me	H	$C_6H_4\text{-}3\text{-}CO_2H$	5530 ± 640 (4)
14d	3-phenylpropyl	Me	H	H	2330 ± 250	**47**	Me	Me	H	$C_6H_3\text{-}2,4\text{-}(NO_2)_2$	15 400 ± 6600
15	Me	*i*-Pr	H	H	4890 ± 870	**48** (DPX)	Et	Et	H	C_6H_5	62.0 ± 11.4
16	*n*-Bu	Et	H	H	1720 ± 160	**49**	Pr	Pr	H	C_6H_5	18.9 ± 3.2 (4)
17	*i*-amyl	*i*-Bu	H	H	6240 ± 1340	**50**	Pr	Pr	H	pyrazine	79.6 ± 11.3
18 (caffeine)	Me	Me	Me	H	10 400 ± 1800	**51**	allyl	Me	H	C_6H_5	37 ± 6
19a	Me	Me	2-Cl-ethyl	H	1390 ± 200	**52**	*i*-amyl	*i*-Bu	H	C_6H_5	9890 ± 1440
19b	Me	Me	3-Cl-propyl	H	5620 ± 980	**53**	Pr	Pr	H	$C_6H_3\text{-}2,6\text{-}F_2$	310 ± 42
19c	Me	Me	4-Cl-butyl	H	15 000 ± 1100	**54**	Pr	Pr	H	$C_6H_3\text{-}2,3\text{-}(OH)_2$	125 ± 10
20	Me	Me	propargyl	H	9460 ± 1880	**55**	Pr	Pr	H	$C_6H_3\text{-}2,4\text{-}(OH)_2$	20.7 ± 2.0
21	Me	Me	allyl	H	9490 ± 1220	**56**	Pr	Pr	H	$C_6H_3\text{-}2,5\text{-}(OH)_2$	138 ± 6.9
22	Me	Me	benzyl	H	19 400 ± 4140	**57**	Pr	Pr	H	$C_6H_3\text{-}2\text{-}OH\text{-}4\text{-}OCH_3$	46.5 ± 0.7
23	Me	Me	CH_2COOH	H	27 600 ± 11 400	**58**	Pr	Pr	H	$C_6H_3\text{-}3,5\text{-}(OH)_2\text{-}4\text{-}OCH_3$	59.5 ± 7.0
24	Me	Me	CH_2CN	H	14 500 ± 300	**59**	Pr	Pr	H	$C_6H_4\text{-}4\text{-}NHC\text{-}OCH_3$	36.7 ± 9.5 (4)
25	Me	Me	$(CH_2)_2\text{-}NH_2$	H	23 500 ± 6700	**60**	Pr	Pr	H	$C_6H_3\text{-}2\text{-}OMe\text{-}4\text{-}Cl$	6530 ± 570 (4)
26	Me	Me	$(CH_2)_2OH$	H	17 300 ± 2900	**61** (XCC)	Pr	Pr	H	$C_6H_4\text{-}4\text{-}OCH_2\text{-}CO_2H$	40 ± 4
27	Me	Me	$(CH_2)_2OCO\text{-}CH_3$	H	23 200 ± 8300	**62**	Pr	Pr	H	$C_6H_3\text{-}2\text{-}OH\text{-}4\text{-}OCH_2\text{-}CO_2H$	188 ± 22
28	allyl	allyl	Me	H	3390 ± 760	**63**	allyl	Me	Me	C_6H_5	11 400 ± 1000
29	propargyl	Me	Me	H	4130 ± 1040	**64**	allyl	Me	2-Cl-ethyl	C_6H_5	311 ± 22
30	CH_2COOH	Me	Me	H	23 500 ± 7600	**65**	Pr	Pr	2-Cl-ethyl	C_6H_4	691 ± 72

[a] Displacement of specific [^{125}I]ABOPX binding at human A_{2B} receptors expressed in HEK-293 cells, in membranes, expressed as K_i ± SEM. $n = 3$, unless noted. Cases in which $n = 4$ are noted in parentheses.

Table 2. Synthetic Yields and Characterization of Xanthine Derivatives

compd	formula	yield (%)	analysis
14b	$C_{13}H_{12}N_4O_2$	44	C, H, N
14c	$C_{14}H_{14}N_4O_2 \cdot 0.2H_2O$	32	C, H, N
14d	$C_{15}H_{16}N_4O_2$	60	C, H, N
19b	$C_{10}H_{13}ClN_4O_2$	88	C, H, N
19c	$C_{11}H_{15}ClN_4O_2$	95	C, H, N
64	$C_{17}H_{18}ClN_4O_2$	66	HRMS[a]
65	$C_{19}H_{24}ClN_4O_2$	32	HRMS[a]

[a] High-resolution mass in positive ion FAB mode determined to be within acceptable limits. Compound **64**: calcd, 345.1118; found, 345.1110. Compound **65**: calcd, 375.1588; found, 375.1573. HPLC demonstrated >95% purity, retention times (mobile phase, min): **64**, (A) 22.69; (B) 23.63; **65**, (A) 23.58; (B) 30.18.

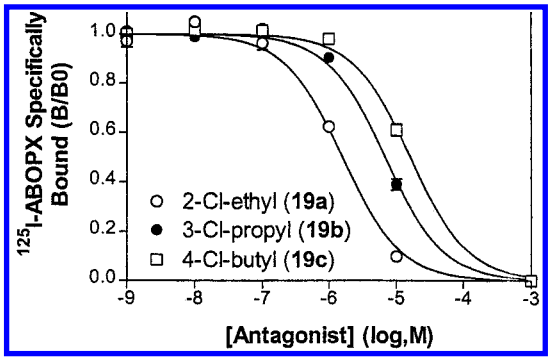


Figure 1. Competition by 7-substituted xanthines for ^{125}I-ABOPX binding to recombinant human A_{2B} receptors. The indicated antagonists (see Table 1) were incubated to equilibrium (2 h) in 100 μL with radioligand (0.7 nM ^{125}I-ABOPX) and human HEK-A_{2B} cell membranes (25 μg of membrane protein). Each point is the mean ± SEM of triplicate determinations. Where not shown, the SEM is smaller than the symbol. K_i values derived from triplicate experiments are summarized in Table 1.

of theophylline examined, i.e., **20**−**27**, including charged and heteroatom substitution, produced K_i values of 10−20 μM. The notable exception to this pattern was 2-chloroethyltheophylline **19a** (K_i value of 1.4 μM), which displayed a 7.5-fold greater affinity than caffeine. Homologues of **19a** were also prepared and studied in binding (Table 3). As illustrated in Figure 1, the potency order of 7-substituted compounds was 2-Cl-ethyl **19a** > 3-Cl-propyl **19b** > 4-Cl-butyl **19c**. A 7-methyl group slightly reduced the affinity of 1,3-diallylxanthine (**28** vs **11**). Among caffeine analogues in which the 1-substituent was modified, including charged and heteroatom substitution in **29**−**35**, some variation in affinity was observed (K_i values of 4−21 μM), with the 1-propargyl analogue **29** as the most potent. The relatively high affinity of pentoxifylline **35** (K_i = 5.2 μM) is notable since this compound is used therapeutically to treat intermittent claudication. An isomer **36** was less potent than **29**.

For the 8-cyclopentyl analogues **37** and **38**, the absence of a 3-alkyl group slightly increased affinity. An 8-phenyl substituent, well-explored as a means of enhancing affinity at A_1 and A_{2A} receptors,[19−21] generally enhanced affinity at the human A_{2B} receptor by

Table 3. Affinities of Xanthine Derivatives in Radioligand Binding Assays at Rat A_1 and A_{2A} Receptors and Selected Compounds at Other Subtypes[a−d]

	K_i (nM)			K_i ratio	
compd	rA_1[a]	rA_{2A}[b]	rA_{2B}[c]	rA_1/rA_{2B}	rA_{2A}/rA_{2B}
1	16.8 ± 3.6[f]	612 ± 287[f]	12.8 ± 1.1	1.3	48
2a	6920 ± 160[f]	6700 ± 320[f]	15 100 ± 2700	0.46	0.44
3	3310 ± 580	<10% (10^{-5})	1880 ± 190	1.8	>10
4	12 700 ± 5600	<10% (10^{-5})			
5[e]	24 300 ± 4300	<10% (10^{-5})			
6	8890 ± 1090	<10% (10^{-5})			
7[e]	37 600 ± 8900	<10% (10^{-5})			
8	5570 ± 760	<10% (10^{-4})			
13	24 700 ± 8100	9780 ± 220			
14a[e]	5830 ± 250	33 600 ± 7700	2150 ± 260	2.7	16
14b	1660 ± 620	3190 ± 1970	10 200 ± 1500	0.16	0.31
14c[e]	27 600 ± 3900	<10% (10^{-4})	5530 ± 610	5.0	>20
14d	24 400 ± 4800	<10% (10^{-4})	15 300 ± 2000	1.6	>10
16	2170 ± 940	<10% (10^{-4})			
19a	15 200 ± 1100	4230 ± 1580	5390 ± 780	2.8	0.78
19b	23 600 ± 3900	<10% (10^{-4})	14 200 ± 100	1.7	>10
19c	17 700 ± 3700	33 300 ± 4300	18 700 ± 500	0.95	1.8
37	7.68 ± 1.46				
38	1.0	500	186 ± 5	0.005	3
51[e]	302 ± 49	1920 ± 400	174 ± 35	1.7	11
54	39.0 ± 5.2	<10% (10^{-5})			
55	220 ± 11	107 ± 10			
56	7.46 ± 0.58	17 300 ± 6500			
57	97.7 ± 2.7	5500 ± 760			
58	30.3 ± 3.5	1340 ± 680			
59	39.2 ± 10.8	<10% (10^{-5})			
62	757 ± 109	2720 ± 470			
63	<10% (10^{-5})	23 500 ± 9800			
64[e]	35 ± 4% (10^{-5})	3570 ± 1360	1700 ± 90	>6	2.1
65[e]	1660 ± 130	16 700 ± 4300	3360 ± 530	0.49	5.0

[a] Displacement of specific [^{3}H]*R*-PIA binding in rat brain membranes in HEK-293 cells, expressed as K_i ± SEM or percent displacement at the indicated concentration (n = 3−5). [b] Displacement of specific [^{3}H]CGS 21680 binding in rat striatal membranes, expressed as K_i ± SEM or percent displacement at the indicated concentration (n = 3−5). [c] Rat A_{2B} receptor expressed in HEK cells, using [^{125}I]I-ABOPX as radioligand. [d] Human A_3 receptor, K_i ± SEM (μM), using [^{125}I]I-AB-MECA as radioligand: **3**, 2.37 ± 0.08; **4**, 3.08 ± 0.43; **5**, 16.1 ± 2.9; **6**, 4.61 ± 1.26; **7**, 7.51 ± 1.64; **8**, 0.639 ± 0.158; **14a**, 10.9; **14b**, 4.44 ± 0.89; **14c**, 6.88 ± 0.47; **14d**, 8.65 ± 2.73. [e] **5**, MRS 1973; **7**, MRS 1975; **14a**, MRS 1980; **14c**, MRS 3005; **51**, MRS 1916; **64**, MRS 3000; **65**, MRS 1995. [f] Values from refs 10 and 14.

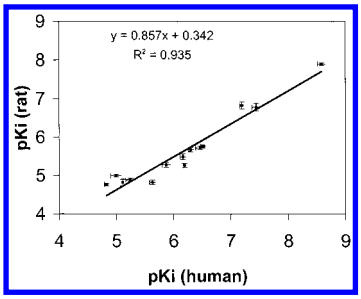


Figure 2. Correlation in the affinities of various xanthines for binding to human and rat A_{2B} adenosine receptors. The pK_i values of compounds that were evaluated for binding to rat and human receptors (Table 2) are plotted and fit by linear regression.

roughly 20-fold. 8-Phenyltheophylline **39** (K_i value of 415 nM) displayed a 22-fold greater human A_{2B} receptor affinity than theophylline **2a**. Substitution of the 8-phenyl ring indicated that an electron-rich ring was preferred in receptor binding. The rank order of potency of substitution at the 8-aryl ring in theophylline analogues **39**−**47** was 4-hydroxy > 4-dimethylamino > 4-methoxy > 4-sulfo > 3-carboxy and 4-nitro > 2-carboxy and 2,4-dinitro.

Homologation of 1,3-dialkyl groups in 8-phenylxanthines enhanced affinity, by 22-fold in the case of 1,3-dipropyl analogue **49** in comparison to **39**. As with 8-H xanthines, a 1-allyl substitutent greatly increased affinity at the A_{2B} receptor in the 8-aryl series. Thus, 1-allyl-3-methyl-8-phenylxanthine **51** displayed a K_i value of 37 nM. Branched substitution, in **52** as before, greatly decreased affinity. Phenyl ring-substituted analogues of **49** were also tested: The electron-withdrawing 2,6-difluoro and 4-chloro groups on the 8-aromatic ring, in **53** and **60**, reduced affinity. Various di- and trisubstituted phenyl rings, containing electron-donating groups, displayed considerable affinity but not exceeding that of **49**. The functionalized congener XCC (xanthine carboxylic congener, 8-[4-[(carboxymethyl)oxy]phenyl]-1,3-dipropylxanthine, **61**), which served as the basis of A_{2B} receptor-selective amide derivatives such as **1**,[10] was nearly as potent as **49**.

The 7-(2-chloroethyl) group of **65** decreased the affinity of compound **49** by 36-fold to a K_i value of 690 nM. However, in comparison to the 7-methyl modification of 8-phenylxanthines, this group increased affinity. Compound **64**, the 7-(2-chloroethyl) derivative of the 8-phenylxanthine derivative **63**, displayed a 37-fold greater affinity at human A_{2B} receptors.

Selected compounds were tested in binding experiments at rat A_{2B} receptors (Table 3). A close correlation between relative potencies at human and rat A_{2B} receptors was found (Figure 2). Affinities were usually higher by 2−9-fold at the human vs the rat A_{2B} receptor, with more pronounced differences observed for the more potent compounds. Compound **14c**, a 1-phenylethyl derivative, showed slight A_{2B} receptor selectivity in the rat. The reference compound **1** was still highly potent at the rat A_{2B} receptor, with a K_i value of 12.8 nM, although not selective for A_{2B} vs A_1 receptors in the rat. Several other compounds were selective for A_{2B} vs A_{2A} but not A_1 receptors in the rat: **3**, **14a**, **14d**, **19b**, and **51**. Selected compounds were also tested at human A_3 receptors (Table 3, footnote e), and most were found to be approximately an order of magnitude more selective for human A_{2B} vs A_3 receptors, except compound **8**, which was equipotent at the two subtypes.

Discussion

Theophylline **2a** is widely used as an antiasthmatic drug, although its mechanism of action is uncertain. The related xanthine, enprofylline[13,14] **2b**, which is also therapeutically efficacious in the treatment of asthma, was earlier thought to act through a nonadenosine receptor-mediated mechanism due to its low affinity at A_1 and A_{2A} receptors. However, the discovery that enprofylline has greater than anticipated affinity and slight selectivity at the A_{2B} subtype[13] supports the hypothesis that A_{2B} receptor antagonism may contribute to antiasthmatic activity of xanthines.[12,26,27] This hypothesis was strengthened by functional effects of A_{2B} receptor activation observed in mast cells of dog and human.[8,12,28] Thus, potent and/or selective A_{2B} receptor antagonists may provide new therapeutic agents.

The SAR of xanthines in binding to A_{2B} adenosine receptors was investigated in the present study. Substitution at the 1-position by a group larger than at the 3-position (H or Me) favored affinity at the A_{2B} receptor. The affinity of xanthines in binding to the human A_{2B} receptor was enhanced by substitution with propyl, butyl, allyl, propargyl, or 2-phenylethyl groups at N-1, while hydrogen or methyl at N-3 favored affinity at this subtype.

A 7-(2-chloroethyl) substituent strikingly favored A_{2B} receptor selectivity. Most N-7 substituents did not enhance affinity over hydrogen, except for 2-chloroethyl, which enhanced the human A_{2B} receptor affinity of theophylline by 6.5-fold. Thus, 7-(2-chloroethyl)theophylline displayed a K_i value of 800 nM. Previously, 7-(2-chloroethyl)theophylline was noted to be slightly selective for adenosine A_{2A} vs A_1 receptors.[29] At the A_{2B} receptor, the effects of the 7-(2-chloroethyl) group were compatible with the enhancement produced by 8-aryl substitution. For example, the affinity of an 8-aryl-7-chloroethyl analogue **64** was intermediate between those of the corresponding 7-H and 7-methyl analogues. Compound **64** was roughly 35-fold more potent at the human A_{2B} receptor than the 7-methyl analogue **63** and 8-fold less potent than the 7-H analogue, **51**.

An 8-phenyl substituent, well-explored as a means of enhancing affinity at A_1 and A_{2A} receptors,[19−24] enhanced affinity at A_{2B} receptors by roughly 20-fold. The detail in the present study concerning 8-aryl ring substitution and compatibility with other xanthine substituents greatly extended previous findings.[10,14] As for simpler xanthines, even in the 8-phenyl series, a larger alkyl group at the 1-position than at the 3-position favored affinity at the A_{2B} receptor. This was illustrated by 1-allyl-3-methyl-8-phenylxanthine, **51**, which displayed a K_i value of 37 nM at A_{2B} receptors. Substitution of the 8-phenyl ring indicated that an electron-rich ring (e.g., 4-hydroxy substitution, **41**) was preferred in receptor binding. Consistent with this finding, nitro and other electron-withdrawing substituents greatly reduced affinity.

The initial screen of A_{2B} receptor affinity in the present study utilized the human subtype, and selected compounds were also evaluated at rat A_{2B} receptors. We

found uniformly higher affinity of substituted xanthines for human than for rat A_{2B} adenosine receptors, but these differences were consistently less than 10-fold. Compounds having various degrees of selectivity for rat A_{2B} receptors were **3**−**7** (1-substitituted xanthines), **14a**−**d** (1-substitituted theophyllines), **51** (1-allyl-3-methyl-8-phenylxanthine), and **64** (1-allyl-7-chloroethyl-3-methyl-8-phenylxanthine). The 1-phenylethyl group appeared to enhance A_{2B} receptor selectivity in the rat.

Selectivity for the human A_{2B} receptor has been easier to achieve than selectivity for the rat homologue, mainly due to the large species differences at A_1 and A_{2A} receptors.[10] To further define the selectivity of A_{2B} receptor antagonists, additional characterization at receptor homologues of various species will be needed.

In conclusion, we have identified new leads for the design of xanthine derivatives as selective antagonists of the A_{2B} receptor, including asymmetric substitution at the 1- and 3-positions, 7-haloalkyl groups, and 8-aryl ring substitution patterns. Furthermore, the affinities at rat and human A_{2B} receptors have been compared. Optimal combinations of substituents on the 1-, 3-, 7-, and 8-positions of xanthines may lead to more potent and selective antagonists. Such selective compounds will aid in the elucidation of the physiological role of this receptor and possibly lead to therapeutically useful agents for treating asthma, diabetes, and other diseases.[6,10,30,31]

Experimental Section

Synthetic Methods. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained with a Varian Gemini-300 spectrometer using $CDCl_3$ or DMSO-d_6 as solvent. The chemical shifts are expressed as parts per million (ppm) downfield from tetramethylsilane or as relative ppm from $CDCl_3$ (7.27 ppm) and dimethyl sulfoxide (DMSO) (2.5 ppm). FAB (fast atom bombardment) mass spectrometry was performed with a JEOL SX102 spectrometer using 6-kV Xe atoms. CI-NH_3 (chemical ionization) mass spectra were carried out with a Finnigan 4600 mass spectrometer. 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 thin-layer chromatography (TLC) (MK6F silica, 0.25 mm, glass backed; Whatman, Clifton, NJ). Where needed, determinations of purity were performed with a Hewlett-Packard 1090 high-performance liquid chromatography (HPLC) system using an SMT OD-5−60 C18 analytical column (250 mm × 4.6 mm, Separation Methods Technologies, Inc., Newark, DE) in two different linear gradient solvent systems. One solvent system (A) was 0.1 M triethylammonium acetate buffer:CH_3CN in ratios of 80:20 to 20:80 in 30 min with a flow rate of 1 mL/min. The other (B) was H_2O:CH_3OH in ratios of 60:40 to 10:90 in 30 min, 10:90 after 30 min, with a flow rate of 1 mL/min. Peaks were detected by UV absorption (250 nm) using a diode array detector.

6-Amino-1-methyl-3-benzyl-1*H*-pyrimidine-2,4-dione (66b). To a suspension of 6-amino-1-methyluracil (706 mg, 5 mmol) in methanol (15 mL) was added 10% aqueous NaOH solution (2 mL, 5 mmol) and benzyl bromide (0.65 mL, 5.5 mmol). The reaction mixture was refluxed for 3 h and quenched by the addition of saturated NH_4Cl solution. The solvent was evaporated under reduced pressure, and the residue was diluted with chloroform (50 mL). The organic phase was washed with water and brine and dried over sodium sulfate, filtered, and evaporated under reduced pressure. The residue was purified by flash column chromatography (silica, chloroform:methanol = 10:1) to give 97 mg of **66b** (33%, based on recovered starting material) as a white foaming solid. ^{1}H NMR ($CDCl_3$): δ 3.33 (s, 3H), 5.00 (s, 1H), 5.08 (s, 2H), 7.23−7.42 (m, 5H). MS (positive ion FAB): 232 $[M + H]^+$.

6-Amino-1-methyl-5-nitroso-3-benzyl-1*H*-pyrimidine-2,4-dione (67b). Compound **66b** (53 mg, 0.23 mmol) was dissolved in a mixed solvent of water (1 mL), acetic acid (0.2 mL), and 6 N HCl (0.05 mL) and treated with $NaNO_2$ (24 mg, 0.35 mmol) in small portions over a period of 10 min. The mixture was filtered, and the filter cake was washed with water to afford **67b** (30 mg, 50%) as a violet solid. ^{1}H NMR (DMSO-d_6): δ 3.25 (s, 3H), 5.10 (s, 2H), 7.23−7.40 (m, 5H). MS (positive ion FAB): 261 $[M + H]^+$.

1-Benzyl-3-methylxanthine (14b). Sodium hydrosulfite (120 mg, 0.69 mmol) was added in small portions to a suspension of **67b** (30 mg, 0.12 mmol) in ethyl acetate (4 mL) and water (2 mL), until the violet color disappeared. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (2 × 10 mL). The combined organic layer was washed with brine and dried over sodium sulfate, filtered, and evaporated under reduced pressure to dryness. The crude product was suspended in trimethylorthoformate (2 mL) and refluxed for 2 h. Trimethylorthoformate was removed by a nitrogen stream, and the residue was purified by preparative TLC (chloroform:methanol = 10:1). The solid was recrystallized from ethyl acetate/hexane to furnish xanthine **14b** (13 mg, 44%) as a white solid having a mp 224−226 °C. ^{1}H NMR ($CDCl_3$): δ 3.65 (s, 3H), 5.28 (s, 2H), 7.27−7.35 (m, 3H) 7.49 (d, 2H, J = 6.6 Hz), 7.73 (s, 1H), 11.93 (bs, 1H). MS (positive ion FAB): 257 $[M + H]^+$.

6-Amino-1-methyl-3-phenethyl-1*H*-pyrimidine-2,4-dione (66c). Compound **66c** was prepared by the procedure described for **66b**, except using ethanol as solvent, instead of methanol, and obtained in 21% yield. ^{1}H NMR ($CDCl_3$): δ 2.91 (m, 2H), 3.42 (s, 3H), 4.12 (m, 2H), 4.56 (bs, 2H), 5.00 (s, 1H), 7.20−7.31 (m, 5H). MS (positive ion FAB): 246 $[M + H]^+$.

6-Amino-1-methyl-5-nitroso-3-phenethyl-1*H*-pyrimidine-2,4-dione (67c). Compound **67c** was prepared by the procedure described for **67b** in 51% yield. ^{1}H NMR (DMSO-d_6): δ 2.88 (t, 2H, J = 7.8 Hz), 3.26 (s, 3H), 4.10 (t, 2H, J = 8.0 Hz), 7.20−7.38 (m, 5H). MS (positive ion FAB): 275 $[M + H]^+$.

3-Methyl-1-(2-phenylethyl)xanthine (14c). Compound **14c** was prepared by the procedure described for **14b** in 32% yield; mp 240−243 °C. ^{1}H NMR ($CDCl_3$): δ 2.97 (m, 2H), 3.66 (s, 3H), 4.29 (m, 2H), 7.22−7.29 (m, 2H) 7.32 (d, 3H, J = 4.4 Hz), 7.78 (s, 1H), 11.45 (bs, 1H). MS (positive ion FAB): 271 $[M + H]^+$.

6-Amino-1-methyl-3-(3-phenyl-propyl)-1*H*-pyrimidine-2,4-dione (66d). Compound **66d** was prepared by the procedure described for **66c** in 45% yield. ^{1}H NMR ($CDCl_3$): δ 1.97 (m, 2H), 2.68 (t, 2H, J = 8.0 Hz), 3.39 (s, 3H), 3.97 (t, 2H, J = 7.4 Hz), 4.45 (bs, 2H), 4.97 (s, 1H), 7.10−7.28 (m, 5H). MS (positive ion FAB): 260 $[M + H]^+$.

6-Amino-1-methyl-5-nitroso-3-(3-phenyl-propyl)-1*H*-pyrimidine-2,4-dione (67d). Compound **67d** was prepared by the procedure described for **67b** in 78% yield. ^{1}H NMR (DMSO-d_6): δ 1.91 (m, 2H), 2.66 (t, 2H, J = 7.7 Hz), 3.22 (s, 3H), 3.95 (t, 2H, J = 7.3 Hz), 7.11−7.30 (m, 5H). MS (CI/NH_3): 289 $[M + H]^+$.

3-Methyl-1-(3-phenylpropyl)xanthine (14d). Compound **14d** was prepared by the procedure described for **14b** in 60% yield; mp 176−180 °C. ^{1}H NMR ($CDCl_3$): δ 2.06 (quintet, 2H, J = 7.4 Hz), 2.75 (t, 2H, J = 7.4 Hz), 3.63 (s, 3H), 4.14 (t, 2H, J = 7.4 Hz), 7.12−7.30 (m, 5H), 7.74 (s, 1H), 12.28 (bs, 1H). MS (positive ion FAB): 285 $[M + H]^+$.

7-(3-Chloropropyl)theophylline (19b). Sodium hydride (24 mg, 60% in mineral oil, 0.61 mmol) was added to a solution of theophylline (100 mg, 0.56 mmol) in DMF (2 mL), and the reaction mixture was stirred at room temperature for 10 min. 1-Bromo-3-chloropropane (0.07 mL, 0.70 mmol) was then added dropwise. The reaction mixture was stirred at room temperature for 24 h, and the solvent was removed by a nitrogen stream. The residue was purified using preparative TLC (ethyl acetate:hexane:methanol = 10:10:1) to give **19b** (126 mg, 88%) as a white solid. The solid was recrystallized from ethyl acetate/*n*-hexane; mp 122−124 °C. ^{1}H NMR

($CDCl_3$): δ 2.39 (m, 2H), 3.42 (s, 3H), 3.50 (t, 2H, J = 5.9 Hz), 3.61 (s, 3H), 4.49 (t, 2H, J = 6.5 Hz), 7.62 (s, 1H). MS (positive ion FAB): 257 $[M + H]^+$.

7-(4-Chlorobutyl)theophylline (19c). Compound **19c** was prepared by the procedure described for **19b** in 95% yield; mp 115−117 °C. ^{1}H NMR ($CDCl_3$): δ 1.81 (m, 2H), 2.07 (m, 2H), 3.42 (s, 3H), 3.57 (t, 2H, J = 6.3 Hz), 3.60 (s, 3H), 4.35 (t, 2H, J = 7.1 Hz), 7.56 (s, 1H). MS (positive ion FAB): 271 $[M + H]^+$.

1-Allyl-7-chloroethyl-3-methyl-8-phenylxanthine (64). Sodium hydride (10 mg, 60% in mineral oil, 0.25 mmol) was added dropwise to a solution of 1-allyl-3-methyl-8-phenylxanthine **51**[36] (6 mg, 0.02 mmol) in DMF (1 mL), and the reaction mixture was stirred at room temperature for 10 min. 1,2-Dichloroethane (0.05 mL, 0.6 mmol) was added, and the reaction mixture was stirred at 70 °C for 36 h. The solvent was removed by a nitrogen stream. The residue was purified using preparative TLC (dichloromethane:2-propanol = 100:1) to give **64** (4.8 mg, 70%) as a white solid; mp 130−133 °C. ^{1}H NMR ($CDCl_3$): δ 3.66 (s, 3H), 3.94 (t, 2H, J = 6.0 Hz) 4.66 (t, 2H, J = 6.3 Hz) 4.68 (d, 2H, J = 5.5 Hz) 5.22 (dd, 1H, J = 10.2, 1.1 Hz) 5.31 (dd, 1H, J = 17.3, 1.1 Hz) 5.96 (m, 1H) 7.54−7.56 (m, 3H) 7.64−7.68 (m, 2H). High-resolution MS (positive ion FAB) calcd for $C_{17}H_{18}ClN_4O_2$ $[M + H]^+$, 345.1118; found, 345.1110. HPLC indicated 99% purity (retention times (min): A, 22.7; B, 23.6).

7-Chloroethyl-1,3-dipropyl-8-phenylxanthine (65). To a solution of 1,3-dipropyl-8-phenylxanthine **49**[25] (13 mg, 0.042 mmol) in DMF (2 mL) was added NaH (18 mg, 60% in mineral oil, 0.45 mmol), and the reaction mixture was stirred for 10 min at room temperature. 1,2-Dichloroethane (0.10 mL, 1.27 mmol) was added dropwise, and the reaction mixture was stirred at 70 °C for 48 h. The solvent was removed by a nitrogen stream. The residue was purified using preparative TLC (dichloromethane:2-propanol = 100:1) to give **65** (5.0 mg, 32%) as a white solid; mp 128−132 °C. ^{1}H NMR ($CDCl_3$): δ 1.09 (t, 3H, J = 7.4 Hz), 1.10 (t, 3H, J = 7.7 Hz), 1.82 (m, 2H), 1.95 (m, 2H), 4.06 (t, 2H, J = 6.3 Hz), 4.11 (dd, 2H, J = 7.4, 7.7 Hz), 4.23 (dd, 2H, J = 7.7, 7.4 Hz), 4.74 (t, 2H, J = 6.2 Hz), 7.64−7.66 (m, 3H), 7.74−7.77 (m, 2H). High-resolution MS (positive ion FAB) calcd for $C_{19}H_{24}ClN_4O_2$ $[M + H]^+$, 375.1588; found, 375.1573. HPLC indicated 96% purity (retention times (min): A, 23.6; B, 30.2).

Pharmacological Methods. Cloning of A_{2B} Receptor. The rat A_{2B} receptor was cloned by Dr. Eric Yuan-Ji Day (University of Virginia) by the following method: Sprague−Dawley rats were euthanized with an overdose of pentobarbital. The urinary bladders were removed and stored in RNALater (Qiagen). mRNA was extracted from rat bladder tissue using a Qiagen mRNA extraction kit. The rat A_{2B} receptor was amplified by RT-polymerase chain reaction (PCR) with the forward primer GGCCATGCAGCTAGAGACGCAGGAC and reverse primer TAGGTCACAAGCTCAGACTGA and then cloned into TOPO2.1 and confirmed by sequencing. The confirmed PCR product was then subcloned in a pDoubleTrouble vector using the restriction enzyme sites for HindIII and ECoRV.

Stable Transfection. HEK-293 cells were grown in DMEM/F12 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin to 50% confluence in six well dishes. Cells were transfected with 2 μg of plasmid DNA and 5 μL of lipofectamine in accordance with the manufacturer's instructions (GibcoBRL). Transfected cells were grown for 48 h prior to selection by the addition of 1 mg/mL G418 in the medium. Resistant colonies were isolated and screened using radioligand binding with ^{125}I-ABOPX. Clonal lines with high specific binding were expanded and maintained in 0.5 mg/mL G418. Several clones with expression levels of approximately 20 000 fmol/mg were preserved. The average K_D for ^{125}I-ABOPX for the rat A_{2B} receptor was determined to be 40.9 nM (SEM = 4.4, n = 7).

Binding Assays. Membranes from HEK-293 cells stably expressing the human or rat A_{2B} receptor were used for competition binding assays with ^{125}I-ABOPX (2200 Ci/mmol).[10,32] 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 (10 mM HEPES and 1 mM EDTA, pH 7.4) supplemented with 1 U/mL adenosine deaminase and 5 mM $MgCl_2$. Nonspecific binding was measured in the presence of 100 μM NECA. Xanthine derivatives for competition assays were diluted in HE buffer with 10% DMSO. The incubation time was 3 h at 21 °C. Competition experiments were carried out using between 0.5 and 1.0 nM ^{125}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). K_i values for different compounds were derived from IC_{50} values as described, assuming a K_D value for ^{125}I-ABOPX of 36 nM at the human A_{2B} receptor. Data from replicate experiments were tabulated as means ± SEM. Nonspecific binding, measured in the presence of 100 μM NECA (RBI-Sigma, St. Louis, MO), was 25% of total binding. All nonradioactive compounds were initially dissolved in DMSO and diluted with buffer to the final concentration, with the amount of DMSO in the final assay tubes consistently ≤0.5%.

For competition experiments, 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 the Prism program (GraphPAD, San Diego, CA), were converted to apparent K_i values.[33] Equilibrium binding competition experiments at rat A_1, rat A_{2A}, and human A_3 adenosine receptors were carried out as previously reported.[34−36]

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