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ADENOSINE ANTAGONISM BY PURINES, PTERIDINES AND BENZOPTERIDINES IN HUMAN FIBROBLASTS*

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Abstract—Theophylline (K_i 5 μM) is a competitive inhibitor of the increase in cyclic AMP caused by adenosine in the VA13 fibroblast line. More than 100 purine bases and structurally related heterocycles were tested as adenosine antagonists. Three families of adenosine antagonists were found: xanthines, benzo[*g*]pteridines, and 9-substituted adenines. For the xanthines, the optimal group at the 1-position was butyl (5-fold improvement versus methyl), at the 7-position was 2-chloroethyl (5-fold improvement versus hydrogen), and at the 8-position was *p*-bromophenyl (100-fold improvement versus hydrogen). The receptors appeared to have butyl- and phenyl-sized "pockets" at the 1- and 8-positions, respectively, since compounds with larger groups had greatly reduced activity.

Although the ability of methylxanthines to inhibit responses to adenosine has been known for more that a decade [1], only a few, small-scale structure-activity relationship studies of methylxanthines as adenosine receptor antagonists have been reported [2–5]. In contrast, the central nervous system stimulant properties of caffeine and theophylline have been know for centuries. Other properties of the xanthines, such as diuresis, smooth muscle relaxation, and phosphodiesterase inhibition have made them popular agents for pharmacological screens [6–9]. Since a large-scale SAR‡ study of adenosine receptor antagonism by xanthines might help to answer the question of whether the known pharmacological actions of the xanthines are related to adenosine antagonism, 110 xanthines and other purine, pyrimidine, pteridine, and benzo[*g*]pteridine bases were tested for the ability to antagonize the increase in cyclic AMP caused by adenosine in the VA13 human fibroblast cell line. Two new families of adenosine antagonists (9-substituted adenines and benzo[*g*]pteridines) were found, and members of the xanthine family with greatly increased affinity were discovered. The results of this study have subsequently been applied in the design of the first xanthine-type adenosine receptor ligand, [^{3}H]-1,3-diethyl-8-phenylxanthine [10].

METHODS

Bases. Sources of bases are listed in Table 1. Some representative structures are in Fig. 1. If possible, bases were dissolved at 1 mM in Tyrode–HEPES buffer (Tyrode's balanced salt solution without bicarbonate and with 15 mM HEPES, pH 7.2). Those that were insoluble at 1 mM were usually dissolved at their highest soluble concentration. 8-Phenyltheophylline derivatives had extremely poor solubility in water at pH 7.2. Those that had a hydrogen at position 7 were dissolved in 10 mM or 100 mM NaOH. A few compounds were dissolved at high concentrations in dimethylformamide and then diluted into Tyrode–HEPES.

Several methylxanthines that are formed by condensation of theophylline with formaldehyde and an aliphatic amine were found to decompose into the reactants on contact with water. These were 7-morpholinomethyltheophylline, piperazine-di-(7-methyltheophylline), 7-pyrrolidinomethyltheophylline, and 7-hydroxymethyltheophylline.

Synthesis of bases. 8-Phenyltheophylline derivatives were synthesized by the methods of Hager *et al.* [6]. 1,3-Diethylalloxazine (G105) was prepared from alloxazine and ethyl iodide. Structures were confirmed by Dr. John W. Daly, NIAMDD, by mass spectroscopy.

Incubations. VA13 cells (WI-38 VA13 2RA) are an SV40-transformed version of WI-38 human fetal lung fibroblasts. Cells were grown for 2–3 days in 35 mm or 60 mm dishes. Incubations were initiated by removing the medium, washing the monolayers once with 0.9% NaCl, and adding the test solution. Papaverine (250 μM) was added to the test solutions immediately before the incubations. After 2 min at 33.5°, the test solution was removed and 1 ml of 5% trichloroacetic acid was added.

Measurement of cyclic AMP. The trichloroacetic acid extracts were applied directly to AG1 × 8 200–

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‡ Abbreviations used are: SAR, structure–activity relationship; cyclic AMP, adenosine cyclic 3′,5′-monophosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesufonic acid; and EHNA, 9-(*erythro*-2-hydroxy-3-nonyl)adenine. Abbreviations used solely in the tables are: C, caffeine; T, theophylline; and X, xanthine.

Table 1 Potencies of bases as adenosine antagonists*

No.	Compound	Source†,‡	Apparent K_i (μM)
	Xanthines		
A1§	xanthine (X)	SIG	130 ± 20
A2	9-methylX	TDF	> 1000
A3	7-methylX	VFX	97 ± 10
A4	3-methylX	ALD	87
A5	3,7-dimethylX (alt: theobromine)	SIG	130
A6	8-chloromethyl-3,7-dimethylX	B	46
A7	8-hydroxymethyl-3,7-dimethylX	B	130
A8	3,7-diethylX	X	44 ± 18
A9	3,7-bis-(2-hydroxyethyl)X	Y	630
A10	3-propyl-7-(dimethylaminoethyl)X	Y	260
A11	1-methylX	TDF	6.6 ± 0.3
A12	1,9-dimethylX	TDF	630
A13	1-methyl-8-methylthioX	Z	2.4
A14	8-phenyl-1-methylX	AA	0.15 ± 0.01
A15	1,7-dimethylX	SIG	4.5
A16	1,7-dimethyl-8-oxoX (alt: 1,7-dimethyluric acid)	TDF	140
A17	1,3-dimethylX [alt: theophylline (T)]	SIG	4.8 ± 0.8 (4)
A18	1,3,9-trimethylX (alt: isocaffeine)	TDF	> 1000
A19	8-fluoroT	N	> 500
A20	8-chloroT	ALD	300
A21	8-bromoT	ALD	130
A22	8-thioT	B	220
A23	8-methylthioT	B	2.4 ± 0.1
A24	8-ethylthioT	B	5.8 ± 0.7
A25	8-nitroT	Y	3.6
A26	8-methylaminoT	B	1.7 ± 0.5
A27	8-dimethylaminoT	B	5.5
A28	8-methylT	B	7.2 ± 0.2
A29	8-ethylT	BB	2.5 ± 0.1
A30	8-propylT	BB	1.3 ± 0.1
A31	8-cyclopropylT	BB	1.6 ± 0.2
A32	theophylline-8-propionate, ethyl ester	B	86
A33	8-benzylT	B,AA	5.2
A34	8-cyclopentylT	BB	0.71 ± 0.14
A35	8-cyclohexylT	B	1.1 ± 0.1
A36	8-(3-indolyl)T	CC	> 1
A37	8-phenylT	CC,AA,DD	0.18 ± 0.02 (3)
A38	9-methyl-8-phenylT	AA	160
A39	8-(*p*-chlorophenyl)T	DD,AA	0.068 ± 0.021
A40	8-(*p*-bromophenyl)T	DD	0.052 ± 0.002
A41	8-(*p*-methoxyphenyl)T	CC	0.12 ± 0.03 (3)
A42	8-(*p*-nitrophenyl)T	CC	0.26 ± 0.01
A43	8-(*p*-dimethylaminophenyl)T	DD	0.26 ± 0.03
A44	8-(*p*-methylphenyl)T	DD,AA	0.14 ± 0.01
A45	8-(3,4-dichlorophenyl)T	AA	> 0.2
A46	8-(*m*-nitrophenyl)T	A	0.56 ± 0.18
A47	8-(*o*-nitrophenyl)T	CC	1.6 ± 0.1
A48	8-(*o*-carboxyphenyl)T	A	> 1
A49	8-(1-naphthyl)T	CC	2.1 ± 0.8
A50	8-(2,6-dimethyl-4-hydroxyphenyl)T	CC	0.86 ± 0.06
A51	7-methoxy-8-phenylT	CC	5.9
A52	1,3,7-trimethylX [alt: caffeine (C)]	SIG	13 ± 2
A53	8-chloroC	KNK	30
A54	8-oxoC (alt: 1,3,7-trimethyluric acid)	TDF	60
A55	8-methoxyC	KNK	38
A56	8-methylaminoC	ALD	11
A57	8-diethylaminoC	ALD	101
A58	8-ethylC	B	12
A59	7-ethylT	X	21
A60	7-(2-chloroethyl)T	ALD	0.98 ± 0.22
A61	7-(2-hydroxyethyl)T	ALD	160

Table 1 (*cont'd*)

No.	Compound	Source†,‡	Apparent K_i (μM)
	Xanthines (cont'd)		
A62	7-(carboxymethyl)T	ALD	250
A63	7-(carboxymethyl)T, ethyl ester	ALD	> 1000
A64	7-(2-hydroxypropyl)T	ALD	130
A65	7-(2,3-dihydroxypropyl)T	ALD	> 1000
A66	7-β-D-ribofuranosylT	EE	1000
A67	7-(*glycero*-pent-2-enopyranosyl)T	B	120
A68	7-phenylT	CC	39
A69	7,8-diphenylT	CC	> 20
A70	1-methyl-3,7-diethylX	X	28
A71	1-methyl-3-isobutylX	ALD	3.5 ± 1.0
A72	1-ethyl-3,7-dimethylX	FF	4.1 ± 0.1
A73	1,3-diethylX	Y	1.2 ± 0.2
A74	1,3,7-triethylX	X	2.5
A75	1-ethyl-3-propyl-7-butyl-8-methylX	Y	2.6 ± 0.7
A76	1,3-dipropylX	Y	0.68 ± 0.03
A77	1,3-diallylX	Y	0.82
A78	1-butyl-3,7-dimethylX	FF	2.8 ± 0.1
A79	1-hexyl-3,7-dimethylX	GG	28
A80	1-(5-oxohexyl)-3,7-dimethylX	GG	400
	Non-xanthine purine bases		
A201	purine	SIG	> 1000
A202	1-methyl-2-oxopurine	Z	> 100
A203	hypoxanthine	SIG	550
A204	1-methylhypoxanthine	SIG	110
A205	1-methyl-8-methylthiohypoxanthine	Z	79
A206	2-thioT	Z	8.6
A207	1-methyl-2-methylthioX	Z	8.6
A208	1-methylguanine	VFX	> 200
A209	6-thioT	Z	14
A210	6-thioC	Z	38
A211	1-methyl-3-isobutyl-6-thioX	HH	8.6
A212	6-methylthioT	Z	44 ± 34
A213	6-selenoT	Z	43
A214	adenine	SIG	200
A215	9-methyladenine	VFX	55 ± 17 (3)
A216	9-(*erythro*-2-hydroxy-3-nonyl)adenine	II	160
A217	1-methyladenine	SIG	1200
	Ring-modified purine bases		
A301	9-oxa-8-phenylT	B	> 100
A302	8-azaT	HTC	> 1000
A303	1-ethyl-3-propyl-7-thiaX	Y	11
	Pyrimidine bases		
B1	1,3-dimethyluracil	KNK	> 1000
B2	1,3-dimethyl-6-aminouracil	SIG	> 1000
B3	1,3-dimethyl-5,6-diaminouracil	ALD	> 1000
	Pteridine bases		
G1§	1-propyl-3-ethyl-6,7-dimethyllumazine	Y	37 ± 1
G2	1,3-dimethyl-7-azalumazine (alt: fervenulin)	ALD	> 1000
	Benzo[g]*pteridine bases*		
G101§	2,4-dioxobenzo[g]pteridine (alt: alloxazine)	SIG	1.1 ± 0.4
G102	7,8-dimethylalloxazine (alt: lumichrome)	ALD	5.2
G103	10-ribityllumichrome (alt: riboflavin)	SIG	> 100
G104	1,3-dimethylalloxazine	CC	> 1
G105	1,3-diethylalloxazine	A	9.1

* Apparent k_i values are means ± S.E. of determinations from separate experiments. A sample number is given in parentheses if greater than 2. When a compound was received from more than one source, all of the sources are given, with the source actually used listed first. 8-Phenyltheophylline (A37) is available from Calbiochem (La Jolla, CA).

xanthines (cont'd)

†Commercial sources:

ALD Aldrich Chemical Co. Milwaukee, WI, U.S.A.
HTC Het-Chem, Harrisonville, MO, U.S.A.
KNK K & K Rare Chemicals, Plainview, NY, U.S.A.
SIG Sigma Chemical Co., St. Louis, MO, U.S.A.
TDF Tridom-Fluka, Hauppauge, NY, U.S.A.
VFX Vega-Fox, Tucson, AZ, U.S.A.

‡Non-commercial sources:

A Synthesized by the author

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§Structure illustrated in Fig. 1.

¶Non-competitive inhibitor, apparent K_i value is calculated from the competitive inhibitor equation and should serve as a rough approximation to the apparent non-competitive K_i value.

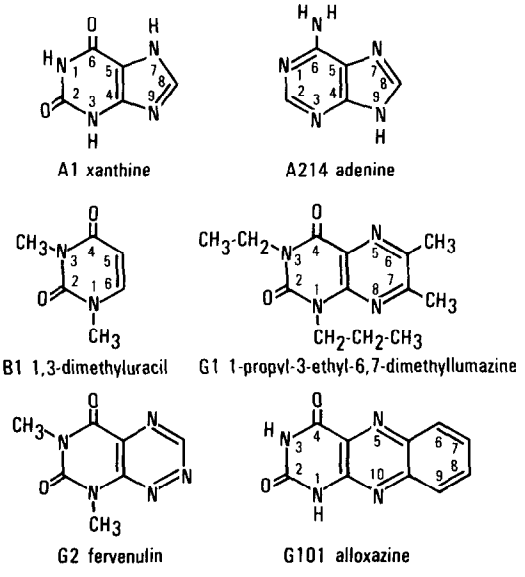

Fig. 1. Structures of representative bases.

400 columns, and eluted as described [11]. Cyclic AMP was measured by the method of Gilman [12].

Estimation of K_i *values.* K_i values were estimated by a weighted non-linear least squares method [11]. First, the adenosine dose-response curve was found by fitting to a Langmuir isotherm. Then the dose–inhibition data for antagonist versus 10 μM adenosine was fit to a standard non-cooperative competitive inhibition equation, with the best fit parameters for the adenosine dose–response curve substituted into the equation as constants.

Three experiments were generally needed to determine the apparent K_i value for an inhibitor. The compound was first tested at a single concentration to obtain a rough estimate of the K_i. In a second experiment, the compound was tested at three or more concentrations bracketing the estimated K_i. Finally, the compound was tested at one or two concentrations at or slightly above the K_i to confirm the estimate.

RESULTS

Increases in cyclic AMP elicited by adenosine. Adenosine causes a 20-fold increase in intracellular

cyclic AMP during a 2-min incubation with VA13 cells. The dose–response curve is non-cooperative and has an ED_{50} for adenosine of 15 μM [11]. Unless otherwise indicated, all experiments reported in the present paper used short 2-min incubations to minimize potential cumulative non-specific effects, such as metabolic inhibition by test agents. The phosphodiesterase inhibitor papaverine was included at 250 μM to prevent effects due to phosphodiesterase inhibition by methylxanthines.

Lack of agonist activity of bases. Representative bases from each structural class (about twenty-five compounds) were tested for agonist activity and found to have none.

Competitive inhibition by theophylline. Theophylline was a classic competitive inhibitor of the response to adenosine (Fig. 2). The adenosine dose–response curve was shifted to the right by theophylline, but the maximum response was unaffected.

Specificity of inhibitors. Like theophylline, most of the bases inhibited the response to 10 μM adenosine. Previous results [11] indicated that there exist two kinds of blockers of the response to adenosine: specific, competitive inhibitors ("adenosine receptor blockers") and non-specific, non-competitive inhibitors ("adenylate cyclase inhibitors"). The former class of compounds blocks the response to adenosine but not the increases in cyclic AMP caused by isoproterenol or prostaglandin E_1, and can be surmounted by increasing the concentration of adenosine. The latter class blocks the responses to isoproterenol and PGE_1 and cannot be surmounted. The two groups differ considerably in their structural attributes, although there is some overlap. As a measure of specificity, representative compounds from different structural classes were tested at high concentrations as inhibitors of the responses to 1 mM adenosine and 10 μM (*l*)-isoproterenol. As shown in Table 2, all but two of the bases caused little or no inhibition of isoproterenol or 1 mM adenosine and thus were specific, competitive adenosine antagonists. The two exceptions were 9-methyladenine (A215) and 9-(*erythro*-2-hydroxy-3-nonyl)adenine (EHNA, A216). Both compounds are 9-substituted adenines and thus bear some resemblance to 2′, 5′-dideoxyadenosine, which was shown [11] to be a non-competitive inhibitor. Further analysis of 9-methyladenine showed that it blocked the response to adenosine with a K_i of 55 μM, but it reduced the response to isoproterenol by less than 50 per cent at 1 mM. 9-Methyladenine thus appeared to be a specific adenosine receptor blocker at lower concentrations. EHNA had mainly non-specific blocking activity.

All of the more potent competitive inhibitors were able to block completely the response to 10 μM adenosine when tested at high enough concentrations. This was true for alloxazine (G101) and 9-methyladenine as well as the xanthines. Dose–inhibition curves were always monophasic.

Determination of apparent K_i *values for competitive inhibitors.* Due to the large number of compounds tested, it was impossible to determine for each individual compound whether its mode of action was competitive or non-competitive. However, two methods could be used to classify a compound as competitive with reasonable certainty. First, compounds could be classified *a priori* by their structures, since the features necessary for non-competitive inhibition (9-substituted adenine) are highly specific. Second, since representative compounds from each structural subgroup of competitive inhibitors were found to lack non-competitive inhibitory activity even at concentrations 10- or 100-fold higher than those which inhibited the response to 10 μM adenosine, it is reasonable to presume that other very

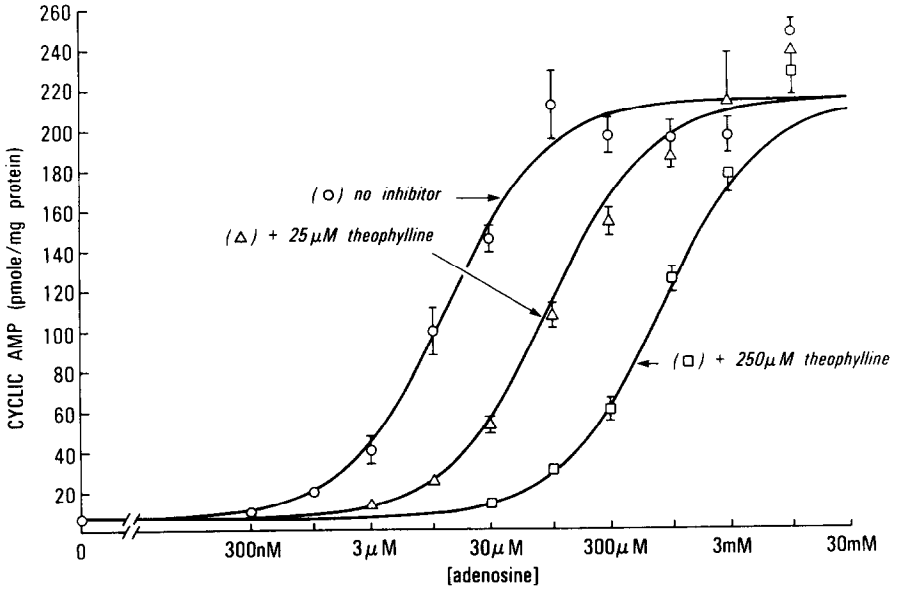

Fig. 2. Competitive adenosine antagonism by theophylline. Data were fit to a competitive inhibition equation by weighted non-linear least squares. The model assumed that the agonist and antagonist were both non-cooperative. χ_v^2 was 0.95, indicating that the model provided a good fit to the data.

Table 2. Specificity of bases as antagonists

No.	Compound	Concn (μM)	Increase in cyclic AMP (% of control increase) Adenosine (10 μM)	Adenosine (1 mM)	Isoproterenol (10 μM)
A1	Xanthine (X)	100	69 ± 9 (4)		98 ± 3 (4)
A5	3,7-DimethylX	500	34 ± 2 (4)	93 ± 1 (4)	100 ± 5 (4)
A17	Theophylline (T)	100	7		101 ± 4 (6)
A17	Theophylline (T)	1000	1		97 ± 1 (4)
A21	8-BromoT	1000	18	117 ± 8 (2)	96 ± 1 (2)
A37	8-PhenylT	1	23	82 ± 2† (4)	
A37	8-PhenylT	10	3		95 ± 4 (6)
A37	8-PhenylT	10	3		121 ± 5 (3)
A60	7-(2-Chloroethyl)T	10	14	103 ± 26 (2)	
A60	7-(2-Chloroethyl)T	100	2		90 ± 5 (6)
A61	7-(2-Hydroxyethyl)T	1000	25 ± 2 (2)		109 ± 1 (2)
A71	1-Methyl-3-isobutylX	100	5		92 ± 3 (6)
A73	1,3-DiethylX	10	16	93 ± 4 (2)	
A73	1,3-DiethylX	100	2		88 ± 3† (6)
A203	Hypoxanthine	1000	54 ± 4 (4)	105 ± 6 (2)	94 ± 4 (2)
A203	Hypoxanthine	5000	14 ± 3 (2)		99 ± 5 (2)
A204	1-Methylhypoxanthine	1000	20 ± 2 (2)		102 ± 1 (2)
A214	Adenine	1000	26 ± 2 (4)	76 ± 3† (4)	96 ± 3 (4)
A215	9-Methyladenine	50	64		95 ± 2 (4)
A215	9-Methyladenine	100	40 ± 3 (2)		94 ± 5 (6)
A215	9-Methyladenine	200	35 ± 1 (2)		88 ± 2† (4)
A215	9-Methyladenine	1000	7 ± 2 (2)	52 ± 3† (4)	87 ± 2† (4)
A216	EHNA	100			88 ± 4† (6)
A216	EHNA	1000	23 ± 4 (2)	55 ± 1† (4)	52 ± 3† (4)
G1	1-propyl-3-ethyl-6,7-dimethyllumazine	100	38		100 ± 12 (3)
G101	Alloxazine	100	2		110 ± 3 (4)

* Purine and pteridine bases were tested as inhibitors of the responses to 10 μM adenosine, 1 mM adenosine, and 10 μM isoproterenol. Increases in cyclic AMP in the presence of inhibitor are expressed as percentages of control increases. Results are means ± S.E (N). In some cases, responses to 10 μM adenosine plus inhibitor were calculated from the apparent K_i of the inhibitor. Calculated responses can be identified by lack of S.E.

† $P < 0.05$ vs control, one-sided t-test.

similar compounds in the same subgroups are also specific. Both of these arguments apply best to high-affinity blockers and are less applicable to low-affinity blockers with K_i values around 1 mM, since nonspecific effects could appear at the higher concentrations. For this reason, calculated K_i values are called "apparent" K_i values.

The method used for estimating apparent K_i values has been described (see Methods and Ref. 11). If the inhibitor is competitive, this method should give the same K_i value as standard null methods [13]. Apparent affinity constants are given in Table 1. When apparent K_i values were determined for the same compound in more than one experiment, results usually agreed well even if different inhibitor concentrations were used. The K_i value of 4.8 μM for theophylline is in reasonable agreement with values in other systems of 2.5 μM [14], 12 μM [2], 20 μM [15], 25 μM [16], 28 μM [17], and 35 μM [18].

Families of inhibitors. In addition to the well-known xanthine family, two new families of adenosine antagonists were found: benzo[*g*]pteridines and 9-substituted adenines. As shown below, the three families had very different SARs.

Xanthine SAR. The simplest xanthine derivative with blocking activity was xanthine itself, which was a weak but specific adenosine antagonist. The imidazole ring was necessary for activity since theophylline derivatives that lacked all or part of the ring (pyrimidines B1, B2, and B3) were inert.

1-Position. All of the xanthines with high inhibitory potency were alkylated at the 1-position. As shown in Table 3, changing the 1-position substituent from hydrogen to methyl increased activity about 20-fold. Lengthening the alkyl chain to butyl resulted in an additional 5-fold increase in activity, but further lengthening to hexyl caused a large decrease in activity. These results suggest that there is a butyl-sized hydrophobic pocket or groove on the receptor near the 1-position.

3-Position. Alkylation at the 3-position was not necessary for high activity. Methylation at the 3-position slightly increased or decreased activity, depending on the 7- and 8-position substituents. Enlarging the methyl group to isobutyl produced a slight increase in activity. The lack of influence of the 3-position alkyl group on affinity may mean that it is only loosely bound to the receptor.

7-Position. With one exception, substitution at the 7-position of theophylline reduced activity. 7-(2-Chloroethyl)theophylline (A60) had an anomalously high activity that was not shared by the ethyl (A59)

Table 3. 1-Position SAR of xanthines*

Modification	Parent base			
	Xanthine	3-MethylX	7-MethylX	3,7-DimethylX
1-H	130	87	97	130
1-Methyl	6.6	4.8	4.5	13
1-Ethyl				4.1
1-Butyl				2.8
1-Hexyl				28

* Values are apparent K_i values in μM.

or butyl (A75) substitutions. Although 2-chloroethyl compounds sometimes act as alkylating agents, this did not appear to be the case with 7-(2-chloroethyl)theophylline since its action was reversible. Hydrophilic substituents at the 7-position drastically reduced activity.

1-Ethyl-3-propyl-7-thiaxanthine (A303) was quite active. This compound has a sulfur in place of the 7-nitrogen and lacks a hydrogen at the 7-position.

8-Position. Changes at the 8-position had dramatic effects on affinity. The 8-carbon was essential for activity since 8-azatheophylline (A302) was inert.

Halogenation at the 8-position drastically reduced activity (see A19–A21, Table 1). The low activity was not due to electron-withdrawing effects, since 8-nitrotheophylline (A25) was as active as theophylline.

The receptor had a distinct preference for hydrophobic substituents at the 8-position. This can be seen in the series fluoro-chloro-bromo (A19–A21) and in the series of alkyl and aryl substituents in Table 1. The phenyl group caused the greatest increase in affinity. 8-(*p*-Bromophenyl)theophylline (A40) was 100 times as active as theophylline and was the most potent antagonist in this study. The *p*-chloro and *p*-methoxy derivatives (A39 and A41) were also highly active.

9-Position. Small changes at the 9-position reduce affinity about 1000-fold. This is true for 9-methyl substitution (A2, A12, A18, A38) and for replacement of N^9 with an oxygen (A301). It should be noted that 9-oxa-8-phenyltheophylline (A301) lacks a hydrogen or alkyl group at N^7. However, compounds A212 and A303 also lack 7-position hydrogens but retain activity.

The low activity of 8-oxa- and 8-thioxanthines (A16, A22, A54) may be due to 9-position effects, since these compounds exist in the lactam form and have hydrogens on N^9.

Additivity and interactions between positions. Changes at the 1-position appear to be additive with changes at other positions. For instance, methylating the 1-position has about the same effect regardless of whether the 3- and/or 7-positions are alkylated (Table 3).

Substitution at the 7-position changes the SAR at the 8-position. For instance, theophylline is very sensitive to changes at the 8-position, but caffeine is indifferent to such changes (Table 4). Receptor-bound caffeine may be tilted in such a way that the 8-position is no longer in close contact with the receptor.

9-Substituted adenines. There were two members of the 9-substituted adenine family: 9-methyladenine (A215) and adenine itself (A214), which exists primarily as the 9-H tautomer. Both compounds had some non-specific inhibitory activity (Table 2), but were specific competitive adenosine antagonists at lower concentrations.

The SAR for adenine derivatives was very different from the SAR for xanthines. Methylation of adenine at N^1 reduced affinity (A217), while methylation at N^9 increased affinity (A215). This was the reverse of the pattern observed for xanthines.

Pteridines and benzo[g]*pteridines.* The benzo[*g*]pteridine alloxazine (G101) was a specific adenosine antagonist. The compound might almost be considered a condensed version of 8-phenylxanthine, but its SAR is very different from the xanthine SAR. Ethylation at N^1 and N^3 enhances the affinity of xanthine 100-fold, but causes a 10-fold reduction in the affinity of alloxazine (G105).

The pteridine 1-propyl-3-ethyl-6,7-dimethyllumazine (G1) was a specific adenosine antagonist. Since it was the only pteridine tested, it was impossible to tell by SAR whether it belonged to the xanthine family, the alloxazine family, or some other family.

Miscellaneous purine bases. Either the 2- or 6-oxygen of xanthine could be replaced by another atom from the oxygen column of the periodic table (sulfur or selenium) without any drastic loss in activity. The 6-thioxanthines belonged to the same family as the xanthines, since their SAR at the 3-position (A209 versus A211) and the 7-position (A209 versus A210) was the same as for xanthines.

Table 4. Effect of methylation at the 7-position on the 8-position SAR of theophylline*

Modification	Parent base	
	1,3-DimethylX	1,3,7-TrimethylX
8-H	4.8	13
8-Chloro	300	30
8-Methylamino	1.7	11
8-Ethyl	2.5	12

* Values are apparent K_i values in μM.

Ability to form the thiolactam structure was unnecessary, since 2- and 6-methylthio derivatives retained activity. The activity of 2-thioxanthines and 6-thioxanthines indicates that the 2- and 6-positions may not function as hydrogen acceptors since thio groups are poor hydrogen acceptors.

Replacement of the 2- or 6-oxygens with hydrogen or nitrogen caused substantial decreases in activity. As mentioned above, adenine belonged to a different SAR family than xanthine. Hypoxanthine belonged to the xanthine family, since methylation at the 1-position enhanced activity. The enhancement was not as great as with xanthine, so hypoxanthine and its derivatives may form a subfamily within the xanthine family.

DISCUSSION

The present study is the first large-scale SAR study of purine bases at the adenosine receptor. Four smaller studies have been reported: Hayashi *et al.* [2] tested ten xanthines that were methylated at the 1-, 3-, and/or 7-positions in guinea pig ileum. The SAR was similar to the results of the present study, except that the effects of methylation at the 1-position were not as pronounced. Scholtholt *et al.* [3] reported that the 1-methyl group but not the 3-methyl group of theophylline was necessary for antagonism versus adenosine in a coronary artery preparation. Green and Stanberry [4] reported similar findings for the neuro-2a cell line. Huang *et al.* [5] reported that xanthine, 1-methylxanthine, and 8-chlorotheophylline did not block adenosine-elicited cyclic AMP increases in slices of guinea pig cerebral cortex. Some differences exist between studies. 1-Methylxanthine was active in the present study and in three of the above-mentioned studies, but not in the Huang *et al.* paper. 1-Butyl-3,7-dimethylxanthine (A78) was more active than caffeine in the present paper but less active in the Scholtholt *et al.* paper. In the present study, xanthine, 3-methylxanthine, and 3,7-dimethylxanthine were not entirely inactive, but they had weak antagonist activity. The discrepancies may be due to differences in species, tissue, or technique.

One *de facto* large-scale methylxanthine SAR exists. Beavo *et al.* [9] studied the lipolytic effects of fifty-seven xanthine derivatives in rat fat cells. Since the antilipolytic activity of adenosine was not known at the time, these effects were ascribed to phosphodiesterase inhibition. Schwabe *et al.* [19] later showed that theophylline-elicited lipolysis was due to blockade against endogenous adenosine in the cell suspension and that phosphodiesterase inhibition was not involved. It is clear that the Beavo *et al.* study can be reinterpreted as an adenosine antagonism study, since the SAR from the Beavo *et al.* study is essentially identical with that found in the present study. Interestingly, fat cells have the A_1 subtype of adenosine receptor, whereas VA13 cells have the A_2 subtype [11, 20, 21]. Thus, the xanthines tested to date do not appear to differentiate between adenosine receptor subtypes. The potencies of xanthines in displacing [^{3}H]-N^6cyclohexyladenosine from A_1 adenosine receptors in guinea pig brain membranes [10] also agree closely with their potencies versus A_2 receptors in the present study.

Use of xanthines as phosphodiesterase inhibitors. The dangers of using xanthines as phosphodiesterase inhibitors are apparent from the Beavo *et al.* [9] paper. Xanthines can cause powerful pharmacodynamic effects via adenosine blockade, and these effects may or may not be of the same magnitude and sign as phosphodiesterase inhibition effects. Xanthines are in general much better adenosine blockers than phosphodiesterase inhibitors. Theophylline, for instance, has a K_i value of 4.8 μM versus adenosine in the VA13 cell line, but it has K_i values between 50 μM and 10 mM versus various phosphodiesterases [20, 23]. Even 1-methyl-3-isobutylxanthine (A71) cannot be considered a specific phosphodiesterase inhibitor, since its adenosine K_i (3.5 μM) is toward the low end of its range of phosphodiesterase K_i values [22–24].

Binding sites. Since dose-inhibition curves were monophasic and obeyed the competitive inhibitor equation, it is likely that each antagonist interacted with a single type of receptor site. Whether the xanthine, alloxazine, and 9-substituted adenine families each bind to the same site, and if so, whether that site is the same as the adenosine binding site, is impossible to determine from the evidence available in the present paper and the literature.

Green and Stanberry [4] reported that the SAR for the xanthines was completely different from the SAR for adenosine and cited this as evidence that the xanthine site was not the adenosine site. The same SAR differences were found in the present study. The xanthine SAR at the 2- and 6-positions was completely incompatible with the SAR for nucleoside agonists and antagonists [11]. The SARs at the 8-position were similar when variations that forced adenosine into the inactive *syn* conformation were omitted from the comparison; not enough comparisons were available, however, to determine whether the similarities were meaningful. The competitivity of theophylline does not contradict the separate-site model, since it is possible to have two allosteric sites whose occupation is mutually exclusive. It is also possible that the xanthines do bind to the same site as adenosine, but in a different binding mode. In this case, xanthine would line up with moieties on the receptor in a different way than adenosine, and thus would have a different SAR.

It is an interesting coincidence that theophylline and adenosine are both purines. In addition, all of the adenosine agonists and antagonists are flat, relatively rigid compounds. In the most active antagonists (8-phenyltheophylline, alloxazine, and 8,5′-*S*-cycloadenosine), the area of flatness is extended beyond the 8-position. These similarities provide some very tentative evidence that the bases may bind to the same site as the nucleosides.

If the same-site hypothesis is correct, certain conclusions can be drawn about base–receptor interactions. Since the three families of bases have different SARs, they must have different binding modes. The xanthine family is further divided into three submodes: theophyllines, caffeines, and hypoxanthines. The existence of so many binding modes implies a rather loose fit of base to receptor. This

is consistent with the same-site hypothesis: if the receptor site is large enough to accept nucleosides, it should fit the smaller bases rather loosely. In addition, the freedom of position of the bases is not hampered by attachment to a ribose ring.

Central nervous system stimulation. One of the objectives of this study was to examine the possibility that some of the well-known pharmacological actions of the xanthines are mediated by blockade of the action of endogenous adenosine. Adenosine causes sedation on intraventricular or intrahypothalamic injection [25], and Sattin and Rall [26] suggested that the mild central nervous system stimulant activity of caffeine and theophylline may be due to adenosine antagonism. Lack of behavioral data makes it difficult to evaluate this hypothesis. Caffeine begins to show stimulant activity in humans at 2 mg/kg [27], corresponding to a concentration of 10 μM in the body water, which is in reasonable agreement with caffeine's K_i value of 13 μM in VA13 cells. Theophylline is also active as a stimulant [28].

Other pharmacodynamic actions of xanthines. In addition to CNS stimulation, xanthines cause diuresis, smooth muscle relaxation, and contracture of skeletal muscle. These actions appear to be unrelated to adenosine antagonism. 1,3-Dialkyl-6-aminouracils (e.g. B2) are diuretics but not adenosine antagonists; 7-(2,3-dihydroxypropyl)theophylline is a bronchodilator but does not block adenosine, and a number of contracture-inducing purines [29] have little adenosine antagonist activity.

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