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Potent Adenosine Receptor Antagonists that are Selective for the A_1 Receptor Subtype

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SUMMARY

The xanthines currently represent the most potent class of adenosine receptor antagonists. However, known derivatives of xanthine show little difference in antagonist potency between the two putative adenosine receptor subtypes, A_1 and A_2. We conducted a systematic study of xanthine structure-activity relationships that compared antagonist potency at the A_1 receptor of adipocytes with potency at the A_2 receptor of platelets. Since adenosine receptors are coupled to adenylate cyclase in these tissues, inhibition of adenylate cyclase via A_1 receptors and stimulation via A_2 receptors were used as models of receptor activation. Antagonist potency was quantitated by Schild analysis, which yields an estimate of affinity (K_i) for the drug-receptor interaction. K_i values of a series of xanthine analogues enabled us to identify structural modifications than enhanced antagonist selectivity for one receptor subtype over the other. We found that changes in the substituent at position 8 of the xanthine nucleus influenced antagonist potency at the A_1 adenosine receptor more than at the A_2 receptor. In particular, an 8-cyclohexyl or 8-cyclopentyl substituent promoted antagonist selectivity for the A_1 receptor subtype. Thus, 1,3-dipropyl-8-cyclopentylxanthine had comparatively high affinity ($K_i = 0.47 \pm 2$ nM) at the A_1 receptor, and was roughly 150-fold more potent as an antagonist of the A_1- than of the A_2-adenosine receptor subtype. In addition, the cycloalkylxanthines were relatively ineffective as inhibitors of cyclic nucleotide phosphodiesterases when used at concentrations that produce marked adenosine receptor antagonism.

Adenosine has long been known to produce physiological effects, and antagonism of the actions of endogenous adenosine may be an important mechanism for the pharmacological action of methylxanthines (1, 2). Cell surface receptors for adenosine have been classified into A_1 and A_2 subtypes (also referred to as R_i and R_a, respectively) (3, 4). This distinction was based on 1) whether adenosine receptor agonists promote inhibition (via A_1) or activation (via A_2) of adenylate cyclase or 2) agonist potency orders, including the degree of stereoselectivity of certain N^6-substituted agonists (3–5).

Classification of receptors on the basis of type of effector or agonist potencies, however, is not without shortcomings. The role of adenylate cyclase and cAMP in adenosine action is unclear, and several reports have described adenosine receptor-mediated responses that appear to be independent of tissue cAMP levels (6–8). Additionally, agonist potency ratios can depend on tissue-specific factors such as receptor number or receptor-effector coupling efficiency (9). An alternate method of receptor classification is based on comparison of antagonist affinity, a property that is independent of agonist potencies or the biochemical mechanism behind the response.

The use of antagonist affinities as a means of detecting receptor subtypes is not yet practicable in the case of adenosine receptors owing to the lack of subtype-selective antagonists. We have undertaken studies designed to define the SARs for substituted xanthines as antagonists at two subtypes of adenosine receptor in an effort to develop subtype-selective antagonists. As a model response from which antagonist affinities could be derived by Schild analysis, we employed assays of adenosine receptor-coupled adenylate cyclase activity in membrane preparations from rat fat cells and human platelets. These tissues have cell surface adenosine receptors with characteristics of the A_1 and A_2 subtypes, respectively (4, 10–12). Our results show that antagonist affinity at the A_1 subtype is enhanced by addition of a cycloalkyl moiety to position 8 of the alkylxanthine nucleus. This modification yielded compounds having up to 150-fold greater potency at the A_1-adenosine receptor subtype than at the A_2 subtype.

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ABBREVIATIONS: SAR, structure-activity relationship; DPCHX, 1,3-dipropyl-8-cyclohexylxanthine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; DPX, 1,3-dipropylxanthine; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; MIX, 1-methyl-3-isobutylxanthine; NECA, 5′-N-ethylcarboxamidoadenosine; PACPX, 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine; R-PIA, (−)-N^6-(*R*-phenylisopropyl)adenosine.

Experimental Procedures

Materials. Xanthine derivatives were synthesized from commercially available chemicals by published procedures (13) or were purchased from the indicated commercial sources: 1,3-dipropyl-8-(*p*-sulfophenyl)xanthine, PACPX, Research Biochemicals, Inc. (Wayland, MA); theophylline and xanthine, Sigma Chemical Co. (St. Louis, MO). [α-^{32}P]ATP and [^{3}H]cAMP were purchased from New England Nuclear (Boston, MA). R-PIA was from Boehringer-Mannheim (Indianapolis, IN). The following were obtained from Sigma: NECA, ATP, GTP, papaverine hydrochloride, creatine phosphate, myokinase (adenylate kinase, grade III, 2780 units/mg) adenosine deaminase (type VIII, 250 units/mg), bovine serum albumin (fraction V), and neutral alumina. Creatine kinase (380 units/mg) was from Boehringer-Mannheim, collagenase (138 units/mg) was from Worthington Biochemical Corp. (Freehold, NJ), and forskolin was from Calbiochem (La Jolla, CA). Aqueous counting scintillant (ACS) was purchased from Amersham (Arlington Heights, IL). Dowex AG 50-X8 was from Bio-Rad Laboratories (Richmond, CA). Structures of all new xanthines were consistent with the nuclear magnetic resonance, infrared, ultraviolet, and mass spectra; syntheses and physical characterization will be reported elsewhere.

Membrane preparation. Rat fat cells were isolated by crude collagenase digestion of epididymal fat pads according to the method of Rodbell (14). A partially purified membrane fraction (P-2) was prepared as described by Kono *et al.* (15) and stored at −70° in 250 mM sucrose, 50 mM glycylglycine, pH 7.4.

Membranes from outdated human platelets obtained from the American Red Cross were prepared as follows. Platelets were suspended in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM EDTA) and centrifuged at 1500 × *g* for 20 min. Platelets were scraped from the top of the cell pellet with a spatula and resuspended in buffer A, and the suspension was centrifuged. The pellet was resuspended in a solution containing 16 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride and frozen in liquid nitrogen. After being thawed, crude membranes were washed once in 10 mM triethanolamine, pH 7.5, 2 mM EDTA and stored at −70° in the same medium. The adenylate cyclase activities of platelet and adipocyte membrane preparations were stable for at least 6 months under these storage conditions.

Adenylate cyclase assays. The conditions used for assay of adipocyte adenylate cyclase activity were based on those of Londos *et al.* (16). Briefly, incubations were initiated by the addition of [α-^{32}P]ATP (1 μCi) to each assay tube. Each reaction mixture (100 μl) contained 100 μM [α-^{32}P]ATP, 100 mM NaCl, 50 mM triethanolamine hydrochloride, pH 7.5, 4 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 100 μM papaverine hydrochloride, 1 μM forskolin, 10 μM GTP, 2 mM creatine phosphate, 40 units/ml creatine kinase, 5 units/ml adenosine deaminase, and 2–5 μg of membrane protein. Reactions were carried out for 30 min at 24° and stopped by the sequential addition of zinc acetate (containing [^{3}H]cAMP) and Na_2CO_3 to form a $ZnCO_3$ precipitate, as described by Jakobs *et al.* (17). The cAMP in the supernatant fraction was purified by sequential chromatography over Dowex-50 resin and neutral alumina (18), and the ^{32}P and ^{3}H content were determined by liquid scintillation spectrometry. The [^{32}P]cAMP content was corrected for recovery based on [^{3}H]cAMP content. Adenylate cyclase from platelet membranes was assayed in a similar manner with the following differences or additions: the reaction mixture contained 2.1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EGTA, 40 units/ml myokinase, and 20–40 μg of membrane protein without forskolin or NaCl. Assays of platelet adenylate cyclase activity were conducted for 20 min at 30°. Enzyme activity was linear with time and membrane protein concentration in both membrane preparations. Protein was determined by the Bio-Rad protein assay with bovine gamma globulin standards.

Antagonist K_i determination. Inhibition constants were obtained using the method of Arunlakshana and Schild (19). Thus, a plot of log (CR−1) on log [antagonist], where CR represents agonist EC_{50} ratio (EC_{50} in presence of divided by EC_{50} in absence of antagonist), was derived by linear least squares analysis of data from each experiment. From the abscissae, a K_i value was calculated for a series of experiments ($N \geq 3$). K_i values for a single antagonist were derived from experiments with at least two different membrane preparations. Adenosine receptor agonists employed were NECA (platelet) and R-PIA (adipocyte).

Agonist and antagonist stock solutions (10 mM in DMSO), were diluted into Millipore water such that the vehicle was present in the assay at 1–2%. Notable exceptions were compounds **1** and **2** which were dissolved in water alone, the acidic compounds (**8, 10, 15, 26**) which were added as the sodium salts, and secondary amines (**18, 19**) which were used as hydrochlorides (see Tables 1–3). Stock solutions of several xanthines (**20–22, 25**) were 1 mM in 10% DMSO containing 10 mM NaOH. Solvents had either slight (<5%) inhibitory (1% DMSO) or stimulatory (1% DMSO, 1 mM NaOH) effects of adenylate cyclase, but in every experiment, appropriate solvents were included in control tubes containing agonist but no antagonist. In addition, we ascertained that DMSO and NaOH had no effect on the K_i value derived for theophylline.

Phosphodiesterase activities. A crude supernatant fraction from porcine coronary artery was prepared according to the method of Wells *et al.* (20). cAMP and cGMP hydrolysis was determined by the method of Keravis *et al.* (21).

Results

Adenosine receptors. Adenosine and its derivatives are known to stimulate platelet and to inhibit adipocyte adenylate cyclases, presumably through A_2 and A_1 receptors, respectively (4, 11, 16, 22). NECA caused a 3- to 4-fold elevation in adenylate cyclase activity of the platelet membrane preparation, with an EC_{50} of 0.23 μM (Fig. 1A). NECA was more potent than R-PIA in this system, which is consistent with the presence of an A_2-adenosine receptor (R-PIA EC_{50} = 3 μM, data not shown). Conversely, R-PIA produced a 50–60% reduction in forskolin-stimulated adenylate cyclase activity of adipocyte membranes with an EC_{50} of 16 nM (Fig. 1B).

The structure of xanthine, on which our study of SARs was based, is shown in Fig. 2. Addition of xanthine derivatives such as DPX to NECA-stimulated adenylate cyclase in platelet membranes produced parallel rightward shifts in NECA concentration-response curves (Fig. 1A). DPX and other xanthine analogues antagonized R-PIA-mediated inhibition of adenylate cyclase in adipocyte membranes in a similar fashion (Fig. 1B). There was no significant effect of the xanthines we tested on basal adenylate cyclase activity from either membrane preparation.

The data for DPX in Fig. 1 were transformed by Schild analysis to give the plot shown in Fig. 3. K_i values for antagonism by DPX of platelet and adipocyte membrane adenosine receptors were 1.8 μM and 0.78 μM, respectively, for the experiments shown. Also shown are data obtained for DPCHX, which had a greater difference in K_i value between the two assay systems than did DPX. For each xanthine we tested, the slope of the Schild regression was not significantly different from unity.

Table 1 shows a group of derivatives of xanthine modified at positions 1 and 3 with K_i values calculated from data like those shown in Figs. 1 and 3. Alkyl substitution at positions 1 and 3 enhanced antagonist potency at both A_1 and A_2 subtypes of the adenosine receptor. Replacing the 3-methyl group of theophylline (compound **2**) with the larger isobutyl group of MIX (compound **4**) enhanced antagonism, whereas making a similar substitution at position 1 did not (compare compounds **2** and **5**). The effect of an ester in either position 1 (compound **9**) or

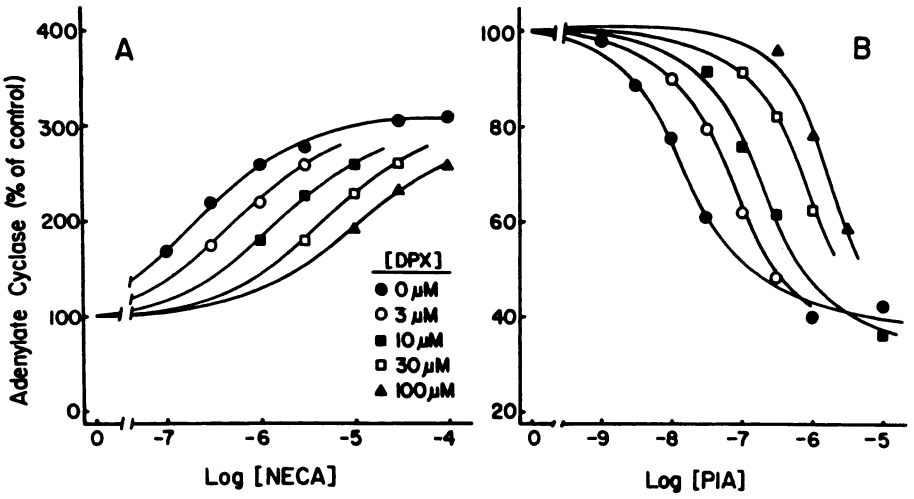

Fig. 1. Effect of adenosine analogues and DPX on platelet and adipocyte adenylate cyclase activities. A. Stimulation of platelet adenylate cyclase by the indicated molar concentrations of NECA (EC_{50} = 0.23 μM). DPX was present at the indicated concentrations. Incubations were for 20 min at 30°. B. Inhibition of forskolin-stimulated adipocyte adenylate cyclase by R-PIA (EC_{50} = 16 nM). DPX was present at concentrations given in A. Incubations were for 30 min at 24° in the presence of the indicated concentrations of R-PIA. Details of assay conditions are given in Experimental Procedures. Adenylate cyclase activity is expressed as percentage of control where control represents activity in the absence of agonist. Control activities were 7.8 and 200 pmol of cAMP/min/mg of membrane protein for platelet and adipocyte adenylate cyclase, respectively. Data are means of duplicates from a single experiment and are representative of at least three experiments.

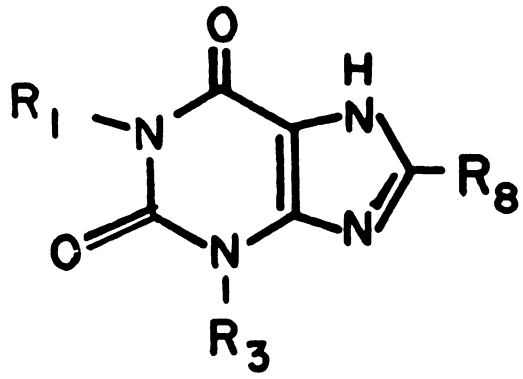

Fig. 2. Structure of xanthine parent compound, showing the substitution pattern employed for SAR determination.

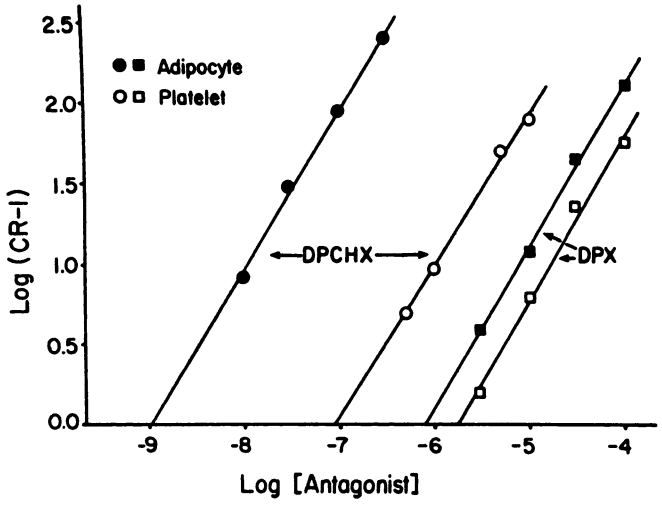

Fig. 3. Schild plot of antagonism of fat and platelet adenosine receptors by DPCHX and DPX. Data are derived from four separate experiments like those in Fig. 1 where antagonists were present during the adenylate cyclase assays at the molar concentrations indicated on the *abcissa*. *CR* represents the ratio of agonist EC_{50} in the presence of antagonist to its EC_{50} in the absence of antagonist.

3 (compound **7**) was not as drastic as that of a stark negative charge; antagonistic activities of compounds **8** and **10** were very low. None of the substitutions at positions 1 and 3 of the xanthine nucleus altered the ratio of K_i values for the two receptor subtypes.

We also determined affinities for a series of derivatives of MIX (compound **4**) modified at position 8 (Table 2). A methyl group at this position (compound **11**) did not have a great effect on potency, but the presence of a bulky *tert*-butyl moiety (**12**) increased potency significantly. The affinity of MIX at the A_1 receptor in fat cell membranes was increased roughly 18-fold by the 8-*tert*-butyl substitution, but the affinity at the A_2 subtype was increased only by a factor of 3. Thus, the K_i ratio (K_i at A_2/K_i at A_1) was increased to approximately 7 by this modification. The presence of a highly electronegative trifluoromethyl moiety at position 8 (compare compounds **11** and **13**) drastically reduced potency at the adenosine receptors (Table 2). Again, as with positions 1 and 3, an ester group was well tolerated (**14**) but the corresponding carboxylic acid (compound **15**) caused a loss of affinity.

To further explore the SARs at the highly sensitive position 8 on the xanthine nucleus, we chose DPX (compound **16**) as a parent compound since the 1,3-dipropyl configuration appeared to be one of the most favorable for antagonistic potency at either receptor. In addition, the 1,3-dipropyl arrangement allowed us to compare our findings with the results of a number of recent studies of adenosine receptor antagonism that have used 1,3-dipropylxanthines (12, 23–26) (see Discussion). Table 3 gives the K_i values for a series of derivatives of DPX.

By comparing the K_i values for the series **17–20**, it can be seen that the presence of a nitrogen atom adjacent to the xanthine imidazole decreased antagonist potency (compound **17** versus **20**). A secondary amine within the 6-atom ring also reduced potency (compare **18** with **17** and **19** with **20**). In fact, within this series of cyclic aliphatic substituents, we found that when nitrogen was omitted from the ring, antagonist potency was dramatically enhanced (compound **20**). K_i values at the A_1 receptor varied greatly within this series, and DPCHX (compound **20**) had a 48-fold lower K_i at the A_1 than at the A_2 receptor. In addition, we determined affinity constants for other 8-cycloalkylxanthines, and found that DPCPX (compound **21**), was approximately 150-fold selective for the A_1 receptor. Exchanging a cyclopentyl for a cyclopropyl group at position 8 greatly reduced potency at A_1, resulting in a compound with relatively little selectivity (compound **22**).

Among the several 8-aromatic substituents we investigated, the 2-amino-4-chlorophenyl moiety of PACPX increased antagonist potency markedly at each adenosine receptor subtype

TABLE 1

K_i values for antagonism at A_1- and A_2-adenosine receptors by xanthine derivatives modified at positions 1 and 3

Antagonist affinities at the A_1- and A_2-adenosine receptors were determined by Schild analysis of adenylate cyclase activity as modulated by R-PIA (adipocyte membranes) and NECA (platelet membranes). See Experimental Procedures for details.

No.	Substituent		K_i[a]	
	R_1	R_3	A_1	A_2
			μM	
1	—H	—H	>100[b]	>100
2	—CH_3	—CH_3	4.8 ± 0.6	5.7 ± 0.3
3	—H	—$CH_2CH(CH_3)_2$	14 ± 1	22 ± 8
4	—CH_3	—$CH_2CH(CH_3)_2$	1.6 ± 0.8	1.8 ± 0.5
5	—$CH_2CH_2CH(CH_3)_2$	—CH_3	7.5 ± 1.8	9.8 ± 1.3
6	—$CH_2CH_2CH(CH_3)_2$	—$CH_2CH(CH_3)_2$	2.5 ± 0.4	2.3 ± 0.3
7	—CH_3	—$CH_2CH_2OCOCH_3$	4.9 ± 1.2	9.0 ± 0.8
8	—CH_3	—$CH_2CH(CH_3)CH_2SO_3^-$	>100	>100
9	—CH_2COOCH_3	—$CH_2CH(CH_3)_2$	3.7 ± 1.5	7.4 ± 0.7
10	—CH_2COO^-	—$CH_2CH(CH_3)_2$	>100	>100

[a] K_i values represent the mean ± standard error of at least three separate determinations, utilizing Schild analysis of agonist EC_{50} values in the presence of four different concentrations of antagonist.

[b] K_i values of analogues with very low affinity or solubility were estimated using one concentration of antagonist in an abbreviated Schild analysis, where a slope of unity was assumed for log (*CR*-1) on log [antagonist].

TABLE 2

K_i values for antagonism of A_1- and A_2-adenosine receptors by derivatives of MIX (compound 4) modified at position 8

No.	Substituent	K_i[a]	
	R_8	A_1	A_2
		μM	
4	—H	1.6 ± 0.5	1.8 ± 0.5
11	—CH_3	2.8 ± 0.7	4.0 ± 0.7
12	—$C(CH_3)_3$	0.089 ± 0.008	0.60 ± 0.08
13	—CF_3	>100[b]	>100
14	—$CH_2CH_2COOCH_3$	2.7 ± 0.3	3.8 ± 0.3
15	—$CH_2CH_2COO^-$	38 ± 13	66 ± 3

[a,b] See footnotes *a* and *b* of Table 1.

TABLE 3

K_i values for antagonism at A_1- and A_2-adenosine receptors by derivatives of DPX (compound 16) modified at position 8

No.	Substituent	K_i[a]	
	R_8	A_1	A_2
		μM	
16	—H	0.94 ± 0.25	1.9 ± 0.5
17		0.022 ± 0.004	0.49 ± 0.07
18		0.69 ± 0.13	2.1 ± 0.2
19		1.1 ± 0.2	3.9 ± 1.2
20		0.0025 ± 0.0005	0.12 ± 0.03
21		0.00047 ± 0.00002	0.069 ± 0.013
22		0.042 ± 0.009	0.24 ± 0.04
23		0.89 ± 0.26	1.4 ± 0.04
24		0.037 ± 0.009	0.56 ± 0.11
25		0.003 ± 0.001	0.022 ± 0.003
26		0.090 ± 0.020	0.18 ± 0.03

[a] See Footnote *a*, Table 1.

(compound **25**, Table 3). In our hands, PACPX had only 7-fold greater affinity at the A_1 than at the A_2 site, whereas other workers (24–26) have reported higher selectivity for this antagonist (see Discussion). Insertion of a methylene unit between the phenyl moiety and the imidazole ring of the DPX parent lead to a reduction in affinity, especially at the A_1 receptor (compound **23** versus **24**). The presence of an acidic sulfonate group in the *para* position of the phenyl ring had little effect on potency at the A_1 receptor and increased potency at the A_2 site (compound **26** versus **24**). Ukena *et al.* (27) have recently reported that the sulfonate moiety had no effect on the potency of 1,3-dipropyl-8-phenylxanthine to antagonize A_2 receptors of platelets, but caused a small reduction in potency at the A_1 receptor of fat cells.

Phosphodiesterase inhibition. Besides measuring antagonist potency at adenosine receptors, we assayed the effects of several of the xanthines on cyclic nucleotide phosphodiesterase activities. Many xanthines are potent inhibitors of phosphodiesterase (13), an interaction that could complicate any study of adenosine receptor antagonism where phosphodiesterase activity is present. Table 4 shows the inhibition of hydrolysis of cAMP and cGMP by a supernatant fraction from porcine coronary artery. The concentrations of cyclic nucleotides used were assumed to approximate cellular levels (28, 29). In order to determine inhibitory potency, the xanthines were used at concentrations that represent the K_i and a 10-fold excess of the K_i at the A_2-adenosine receptor, which appeared to have a lower affinity for the xanthines than the A_1 receptor (Tables 2 and 3). From Table 4, it can be seen that DPCHX (**20**) and DPCPX (**21**) caused relatively less inhibition of cAMP or cGMP hydrolysis than did the well known phosphodiesterase inhibitor, MIX (**4**), when used at similar multiples of its K_i. The 8-cycloalkyl compounds were also weaker inhibitors than 1,3-dipropyl-8-phenylxanthine (**24**), a xanthine that has served as a parent compound for many adenosine receptor antagonists (12, 23, 24, 27). In addition, MIX promoted relaxation of smooth muscle preparations when used at 18 μM, whereas DPCHX and DPCPX (10 μM) had no effect.

TABLE 4

Inhibition of cyclic nucleotide phosphodiesterase activities by xanthine analogues

Hydrolysis of 1 μM cAMP and 0.1 μM cGMP by porcine coronary artery supernatant fraction was assayed as described in Experimental Procedures. Concentrations of xanthines represent the K_i (or a 10-fold excess of the K_i) for antagonism of platelet adenosine receptors.

No.	Compound	Concentration	% Inhibition of Hydrolysis[a]	
			cAMP	cGMP
		μM		
4	MIX	1.8	50 ± 1	44 ± 2
		18	70 ± 1	85 ± 1
20	DPCHX	0.12	1.3 ± 0.7	2.1 ± 2.1
		1.2	8.7 ± 2.4	6.6 ± 0.2
21	DPCPX	0.069	2.5 ± 1.2	2.0 ± 1.4
		0.69	2.3 ± 0.6	2.0 ± 1.5
24	DPPX[b]	0.56	11 ± 1	2.5 ± 1.6
		5.6	30 ± 3	20 ± 2

[a] Per cent inhibition was determined by comparing enzyme activity in the presence of drug to that in the presence of drug vehicle. Values represent mean ± standard error of at least three separate determinations done in duplicate. Data were obtained from experiments using two different preparations of porcine coronary artery.

[b] DPPX, 1,3-dipropyl-8-phenylxanthine.

Discussion

Since the first demonstration by Sattin and Rall (30) in 1970, that methylxanthines reduced the effects of adenosine on cAMP metabolism, interest in the development of more potent adenosine receptor antagonists has increased considerably. Efforts are especially being aimed toward development of subtype-selective antagonists. To this end, our approach has been to evaluate the SARs of a series of derivatives of xanthines as antagonists at A_1- and A_2-adenosine receptors. Among the analogues we tested are some of the most potent and subtype-selective antagonists known. We explored characteristics such as polarity or charge distribution, electronegativity, and the size or bulk of substituents at various positions on the xanthine nucleus. Utilizing this approach, we arrived at the following set of observations concerning the structural requirements for antagonism at adenosine receptors.

Substitution at positions 1 and 3 of the xanthine nucleus with an alkyl moiety of one to five carbons in chain length appeared to be necessary for adenosine receptor antagonism. DPX was one of the most potent analogues in the 1- and 3-modified series. A similar observation was made by Bruns *et al.* (23), who evaluated competition by xanthine analogues for [^{3}H]cyclohexyladenosine ([^{3}H]CHA) binding at apparent A_1 sites in bovine brain. In addition, limited polarity in positions 1 and 3 did not destroy antagonist activity; xanthines having ester functions at these positions had inhibition constants in the low μM range. Variation of alkyl group size or substituent polarity, however, had no effect on antagonist selectivity. Therefore, we directed further efforts toward studying SARs at position 8.

Substitution of a phenyl ring at position 8 is known to increase adenosine receptor antagonism by alkylxanthines (23, 31), and results we obtained for both A_1 and A_2 receptor subtypes supported this observation. The separation of the phenyl ring from the xanthine by a methylene group eliminates resonance interaction between the ring systems, but the lower potency of 1,3-dipropyl-8-benzylxanthine (compound **23**) probably results from the fact that the phenyl ring of the 8-benzylxanthine is located in a position far removed from that occupied by the 8-phenyl or 8-cycloalkyl moieties. The 8-phenyl moiety also conferred 15-fold antagonist selectivity to the xanthines by virtue of enhancing potency more at subtype A_1 than at A_2. Indeed, we noted that antagonist affinity at the A_1 receptor was more susceptible to modifications at position 8 than was affinity at the A_2 receptor.

The hypothesis that the A_1 receptor is most sensitive to changes in the 8-position of the xanthine was further supported by data obtained for the 8-*tert*-butyl analogue of MIX, and the series of cyclic aliphatic substituents (compounds **12** and **17–22**). From the observation that the presence of a nitrogen atom in the ring of 8-piperidine or 8-piperazine derivatives was detrimental to antagonist potency at the A_1 receptor, we concluded that 8-cycloalkyl xanthines would have the lowest K_i values at the A_1 receptor and therefore have the highest degree of selectivity. DPCHX was roughly 48-fold selective for the A_1 receptor of adipocytes over the A_2 receptor of platelets, and had a K_i of 2.5 ± 0.5 nM for antagonism at A_1. The corresponding 8-cyclopentyl compound (**21**) displayed even more selectivity (150-fold) by virtue of increased potency to antagonize the A_1 subtype. Interestingly, these findings for 8-cycloalkyl compounds demonstrate that aromaticity at position 8 of the xanthine parent compound is not required for potent antagonism at adenosine receptors.

Recently, there have been several comparisons of antagonist activities at A_1 and A_2 receptor subtypes (24–27). Daly *et al.* (24) utilized [^{3}H]CHA binding in rat cerebral cortex to evaluate antagonist affinity at A_1, and they determined potency at A_2 by measuring antagonism of [^{3}H]cAMP accumulation elicited by 2-chloroadenosine in guinea pig cerebral cortical slices. Under these conditions, they reported a high degree of selectivity (A_2/A_1 K_i potency ratio = 400) for PACPX, which differs greatly from the results we obtained in the present study. We observed only a 7-fold higher potency at the A_1 than at the A_2 receptor. A possible reason for the difference in selectivity obtained in the two studies is that their measure of antagonist potency at the A_2 site was based on an IC_{50}, or the concentration of antagonist that inhibits an agonist-mediated response by 50%. An IC_{50} value depends heavily on the amount of agonist used in the experiment, whereas a true K_i (which we derived by Schild analysis) is independent of agonist concentration. Later, Schild analysis of antagonist potency was utilized by Schwabe *et al.* (25), who observed 55-fold selectivity of PACPX for the A_1 receptor subtype over the A_2 subtype. Thus, the K_i for antagonism by PACPX of NECA-stimulated adenylate cyclase (at the A_2 receptor in human platelets) was higher than the value we obtained from data generated under very similar assay conditions. We are currently unable to explain this discrepancy.

Finally, Bruns *et al.* (26) have used competition binding to compare K_i values for xanthine analogues as antagonists at A_1 and A_2 receptors in brain. The degree of selectivity (or K_i ratio A_2/A_1) they observed for several compounds, including PACPX, was higher than the selectivity that we determined by analysis of antagonism of receptor function. However, this group reported that 8-cyclopentyltheophylline displayed greater than 100-fold higher affinity at the A_1 receptor than at the A_2 receptor, which is in keeping with our observations for DPCHX and DPCPX.

Comparison of the data presented here and elsewhere (14–27) indicates that the extent of antagonist selectivity (A_2/A_1)

may differ depending upon the preparation of receptors and/or methods used to define antagonist potency. This is most dramatically demonstrated when the highly potent and lipophilic 8-phenylxanthines are used as antagonists. For example, Schwabe *et al.* (25) reported that the A_2/A_1 K_i ratio was about 700 for 8-phenyltheophylline when competition binding in a bovine brain preparation (A_1) was compared with adenylate cyclase modulation in human platelets (A_2). The extent of the apparent selectivity dropped to 26-fold when rat adipocyte adenylate cyclase modulation (A_1) was compared to the human platelet system. Daly *et al.* (24) have reported only a 1.4-fold difference between apparent K_i values of 8-phenyltheophylline as an inhibitor of radioligand binding at the A_1 receptors in a membrane preparation from rat cerebral cortex and as an inhibitor of 2-chloroadenosine-stimulated [^{3}H]cAMP accumulation in guinea pig cerebral cortical slices. Ukena *et al.* (27), using apparently identical preparations and techniques to those used by Schwabe *et al.* (25), reported that 8-phenyltheophylline is 5-fold more potent as an antagonist of A_1 than of A_2 receptors. These confusing discrepancies may arise from species variation and/or from invalid assumptions concerning the calculation of K_i values.

An important additional consideration in evaluating the usefulness of adenosine receptors antagonists is the effect of structural modifications on the ability of xanthine analogues to inhibit cyclic nucleotide phosphodiesterases. The possibility that the pharmacological effects of a xanthine-based adenosine receptor antagonist are due, at least in part, to inhibition of phosphodiesterases would complicate interpretation of adenosine receptor studies. When used at concentrations that caused marked adenosine receptor antagonism, the two 8-cycloalkylxanthines DPCHX and DPCPX had little effect on phosphodiesterase activities relative to the inhibition caused by MIX. Thus, besides being receptor subtype-selective antagonists, DPCPX and DPCHX have the additional advantage of having much greater activity as adenosine receptor antagonists than as inhibitors of the ubiquitous cyclic nucleotide phosphodiesterases.

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