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Adenosine receptor binding: Structure–activity analysis generates extremely potent xanthine antagonists

(N^6-cyclohexyladenosine/theophylline)

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ABSTRACT **Structure–activity analysis of alkylxanthine derivatives at adenosine receptor binding sites has been employed to design more potent adenosine receptor antagonists. Receptor affinities of xanthines were determined by measuring inhibition of the binding of N^6-[^{3}H]cyclohexyladenosine to bovine brain membranes. 1,3-Dipropyl substitutions enhance potency compared to the 1,3-dimethyl substitution in theophylline. An 8-phenyl substituent produces a considerable increase in potency, which is augmented by certain *para* substitutions on the 8-phenyl ring. Combining an *ortho* amino with a *para*-chloro substituent on the 8-phenyl ring affords further increases in potency. Combining all of these substituents results in 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine, a compound of extraordinary receptor affinity, with a K_i for adenosine A_1 receptors of 22 pM. It is 4,000,000 times more potent than xanthine itself and 70,000 times more potent than theophylline.**

Structure–activity analysis to synthesize progressively more potent drugs has been a major thrust of pharmaceutical researchers. In some cases, as with enzyme inhibitors where *in vitro* assays are employed, very potent and selective agents can be designed. For most pharmacologic effects, especially in the psychotropic area, less effective *in vivo* screening tests have been employed in which drug potency may vary because of differences in absorption, metabolism, and penetration to the target organ as well as differences at receptors.

The advent of simple, sensitive, and specific binding assays for drug and neurotransmitter receptors has permitted a more efficient approach to the design of pharmacologic agents. It is now possible to measure in binding assays more than 20 types of neurotransmitter receptors in the brain and peripheral tissues (1). The influences of ions and guanine nucleotides upon receptor binding permit one to discriminate between experimental compounds as pure agonists, pure antagonists, and mixed agonist-antagonists.

Adenosine may be a neuromodulator in the brain and regulate various biological activities in the periphery (2–5). Adenosine and its derivatives inhibit platelet aggregation (6), dilate coronary blood vessels (7, 8), and have locomotor (9, 10) and cardiac (8) depressant effects. Xanthines, which block adenosine receptors, have behavioral stimulant, cardiac stimulant, and bronchodilatory actions. Central stimulant effects and other pharmacological actions of the xanthines may involve blockade of adenosine receptors (2, 10, 11). Several different radioactive ligands label adenosine receptors (12–15). We have labeled adenosine receptors with the agonist N^6-[^{3}H]cyclohexyladenosine ([^{3}H]CHA) and the xanthine antagonist 1,3-diethyl-8-[^{3}H]phenylxanthine ([^{3}H]DPX) (12). Both of these ligands bind to the A_1 subtype of adenosine receptor (14), which is localized to synaptic zones in the brain associated in part with excitatory axonal projections (16, 17). A_1 receptors inhibit adenylate cyclase, as reflected by their GTP regulation (18, 19), have a nanomolar affinity for adenosine, and have a higher affinity for N^6(L-phenylisopropyl)adenosine (L-PIA) than for 5′-*N*-ethylcarboxamide adenosine, whereas A_2 receptors stimulate adenylate cyclase, have micromolar affinity for adenosine, and have a higher affinity for 5′-*N*-ethylcarboxamide adenosine than for L-PIA (20, 21). Alternatively, A_1 receptors are designated R_i and A_2 receptors, R_a (21). Binding studies suggest heterogeneity among A_1 receptors (22).

Until recently, the most potent adenosine antagonist has been theophylline, which has K_i values of about 10 μM at most A_1 and A_2 receptors (2, 12, 23, 24). At slightly higher concentrations, theophylline inhibits phosphodiesterase (24) and affects calcium transport (25). Adenosine antagonists with greater potency and specificity might have enhanced therapeutic selectivity.

In the present study we describe systematic variations in the structure of alkyl-substituted xanthines resulting in the development of xanthines up to 70,000 times more potent than theophylline in competing for adenosine A_1 receptor-binding sites in bovine brain membranes.

MATERIALS AND METHODS

Materials. [^{3}H]CHA (11 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) was from New England Nuclear. L-PIA was from Boehringer Mannheim. 1,3-Diethylxanthine and 1,3-dipropylxanthine were from Searle (Chicago), 8-(*p*-chlorophenyl)theophylline and 8-(*p*-bromophenyl)theophylline were from Keitaro Senga (Pharmaceutical Institute, Keio University, Tokyo), and the *p*-nitro, *p*-methoxy, *p*-methyl, and *o*-nitro derivatives of 8-phenyltheophylline were from Edward C. Taylor (Department of Chemistry, Princeton University, Princeton, NJ). Other 8-phenylxanthines were synthesized in our laboratories.

[^{3}H]CHA Binding Assay. [^{3}H]CHA binding to bovine brain membranes was performed essentially as described (12). Briefly, 10 mg of original tissue wet weight of bovine brain membranes was incubated for 2 hr at 20°C with drug and 1 nM [^{3}H]CHA in 2 ml of 50 mM Tris·HCl, pH 7.7. Samples were collected on GF/B filters under reduced pressure, washed

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Abbreviations: PACPX, 1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine; DPX, 1,3-diethyl-8-phenylxanthine; CHA, N^6-cyclohexyladenosine; L-PIA, N^6-(L-phenylisopropyl)adenosine (formal name, N^6-[(*R*)-1-methyl-2-phenethyl]adenosine).

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three times, and assayed for radioactivity in a liquid scintillation counter. Dose–inhibition curves were generated with four to eight concentrations of drug in triplicate incubations. IC_{50} values were computed from total binding (no drug), nonspecific binding (10 μM L-PIA), and the dose–inhibition data by using a nonlinear least-squares fit to a competitive inhibition model. K_i values were calculated from the Cheng–Prusoff equation (26). Compounds with K_i values below 0.5 nM were tested in binding assays with only 2.5 mg of wet weight of tissue to avoid conditions in which the receptor concentration exceeded the K_i.

Xanthine Solutions. 8-Phenylxanthines were dissolved at 1 mM in dimethylformamide or 0.1 M KOH (we now recommend 10 mM in dimethyl sulfoxide). These solutions were stored for up to 2 weeks at 4°C. Stock solutions were diluted in one step to 1 μM or 10 μM in water and then, if necessary, were rapidly diluted further.

General Xanthine Synthesis. Syntheses of 8-phenylxanthines will be reported in detail elsewhere. For most xanthines, a 1,3-dialkyl-5,6-diaminouracil was acylated with a substituted benzoic acid to form a 1,3-dialkyl-5-acylamino-6-aminouracil, which was ring-closed by boiling for 5 min in 2.5 M NaOH. 8-(*o*-Hydroxyphenyl)theophylline was ring-closed by heating 1,3-dimethyl-5-(acetylsalicyloyl)-amino-6-aminouracil for 20 min in $POCl_3$.

Synthesis of 1,3-Dipropyl-8-(2-Amino-4-Chlorophenyl)Xanthine (PACPX). PACPX was synthesized by a modification of the method of Pfleiderer and Kempter (27). 2-Nitro-4-chlorobenzoic acid (0.02 mol) was dissolved in 30 ml of MeOH. 1,3-Dipropyl-5-nitroso-6-aminouracil (0.01 mol) was added with stirring, followed by 0.02 mol of diisopropylcarbodiimide. After 10 min, the white precipitate, 1,3-dipropyl-5-[(2-nitro-4-chlorobenzoyl)oxy]imino-6-(2-nitro-4-chlorobenzoyl)-iminouracil, was collected by filtration. To the dried intermediate was added 15 ml of 22% ammonium sulfide with stirring. After 10 min, concentrated HCl was added to pH 8 in a hood and the precipitate was collected by filtration. The product was roughly a 50:50 mixture of PACPX and 1,3-dipropyl-5-[(2-amino-4-chlorobenzoyl)-amino]-6-aminouracil. To complete the cyclization, the crude product was boiled in 2.5 M KOH for 20 min, neutralized, and filtered. The product was purified once by dissolving in KOH and precipitating with HCl and again by recrystallizing from dimethylformamide. The product was identified by chemical ionization mass spectrometry and elemental analyses. Yield was 2.1%.

Table 1. Adenosine receptor affinities of xanthines with varying alkyl substituents

Substituent	K_i for inhibition of [^{3}H]CHA binding, nM
None (xanthine)	99,000
1-Methyl	2,600
1,7-Dimethyl	7,400
1,3-Dimethyl (theophylline)	1,600
3,7-Dimethyl (theobromine)	68,000
1,3,7-Trimethyl (caffeine)	11,000
1,3-Diethyl	1,400
1,3-Dipropyl	100
1,3-Dimethyl-8-phenyl	1.2
1,3-Diethyl-8-phenyl (DPX)	2.0
1,3-Dipropyl-8-phenyl	0.12

Affinity constants for antagonism of [^{3}H]CHA binding to A_1 adenosine receptors in bovine brain membranes were determined as described in *Materials and Methods*.

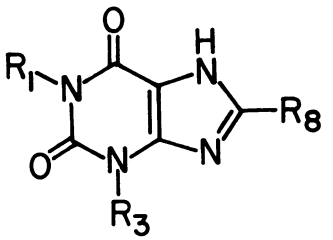

FIG. 1. Structure of substituted xanthines. Xanthine itself has $R_1 = R_3 = R_8 = H$.

RESULTS

Xanthine itself was a relatively weak inhibitor of specific [^{3}H]-CHA binding at adenosine A_1 receptors (Table 1). A 1-methyl substituent increased the affinity of the xanthine molecule for adenosine A_1 receptors about 40-fold (Fig. 1). An additional methyl group at the 3 position results in theophylline, which was about twice as potent as 1-methylxanthine. Interestingly, 7-position substituents counteract the enhanced potency of 1,3-methyl substituents. Thus, caffeine (1,3,7-trimethylxanthine) was 1/5 as potent as theophylline and 1,7-dimethylxanthine was 1/3 as potent as 1-methylxanthine. The low potency of theobromine (3,7-dimethylxanthine) fits with its lack of behavioral stimulant effects (9).

1,3-Diethyl substituents had similar effects on potency as 1,3-dimethyl substituents. However, 1,3-dipropyl substituents enhanced potency almost 20 times compared to theophylline.

The most dramatic increase in potency came with 8-phenyl substituents. Thus, 8-phenyltheophylline was 1,000 times more potent than theophylline. The 8-phenyl and 1,3-dipropyl substituents had an additive effect on potency so that 1,3-dipropyl-8-phenylxanthine was 10,000 times more potent than theophylline.

The great potency of 8-phenyltheophylline prompted us to evaluate the role of varying substituents on the 8-phenyl ring (Table 2). In all cases the most potent compounds had substituents at the *para* position. Uncharged groups such as chloro, bromo, methyl, and methoxy at the *para* position increased affinity by up to 4-fold compared to 8-phenyltheophylline. An amino substituent at the *para* position enhanced affinity slightly, whereas nitro caused a moderate loss of affinity. In contrast, all substituents at the *ortho* or *meta* positions resulted in a loss of affinity. The carboxyl group, which has a negative charge at physiological pH, markedly decreased affinity even when present in the *para* position. This effect was greatest at the *ortho* and *meta* positions.

Table 2. Adenosine receptor affinities of 8-phenyltheophyllines with substituents on the phenyl ring

Substituent on 8-phenyl ring	K_i for inhibition of [^{3}H]CHA binding, nM		
	ortho	*meta*	*para*
H	1.2	1.2	1.2
Bromo	—	4.0	0.34
Methyl	3.6	5.4	0.51
Methoxy	190	8.7	0.63
Chloro	—	—	0.64
Amino	2.3	5.8	0.69
Fluoro	6.8	2.4	1.8
Hydroxy	4.8	3.1	2.0
Nitro	49	22	4.0
Carboxyl	21,000	540	18

See legend to Table 1.

Table 3. Adenosine receptor affinities of compounds that combine two or more variations on the 8-phenyltheophylline structure

8-Phenyl substituent	Xanthine substituent	K_i for inhibition of [^{3}H]CHA binding, nM
H	1,3-Dimethyl	1.2
2-Amino-4-nitro	1,3-Dimethyl	1.2
2,4-Diamino	1,3-Dimethyl	5.9
2-Amino-4-chloro	1,3-Dimethyl	0.20
H	1,3-Diethyl	2.0
2-Amino-4-chloro	1,3-Diethyl	0.32
H	1,3-Dipropyl	0.12
2,4-Diamino	1,3-Dipropyl	0.14
2-Amino-4-chloro	1,3-Dipropyl	0.022

See legend to Table 1.

We next explored the effects of two substituents on the 8-phenyl ring (Table 3). Attention was directed towards *ortho* amino substituents in the hope that these would confer greater water solubility to the very insoluble 8-phenylxanthines. Although individual substitution of amino at the 2 and 4 positions of the phenyl rings had produced agents with high potency (Table 2), 2,4-diamino substitution decreased potency. This reduction in potency could be offset with 1,3-dipropyl substituents in place of 1,3-dimethyl substituents on the xanthine ring. However, the resultant compound 1,3-dipropyl-8-(2,4-diaminophenyl)xanthine was not more potent than 1,3-dipropyl-8-phenylxanthine itself (Table 3). The greatest success came with combinations of 2-amino and 4-chloro substituents. Thus, 8-(2-amino-4-chlorophenyl)theophylline was about 3 times more potent than 8-(4-chlorophenyl)theophylline. We then examined the effects of various 1,3-dialkyl substitutions. The 1,3-diethyl derivative of 8-(2-amino-4-chlorophenyl)xanthine was less potent than the 1,3-dimethyl derivative. However, the 1,3-dipropyl derivative, PACPX, had extremely high potency with a K_i for adenosine receptors of about 20 pM.

Inhibition of [^{3}H]CHA binding by theophylline and caffeine was competitive with displacement curves that were parallel to those of CHA itself. To determine whether the major changes in potency with the substituted xanthines involved alterations in the mode of inhibition of receptor binding, we examined displacement curves in detail (Fig. 2). For all xanthines, including the most potent PACPX, displacement curves for [^{3}H]CHA binding were parallel. Pseudo-Hill coefficients computed from these displacement curves were approximately 0.8–0.9, indicating the absence of positive or negative cooperative interactions. Thus, the xanthines evaluated appear to compete directly with [^{3}H]CHA at its recognition site.

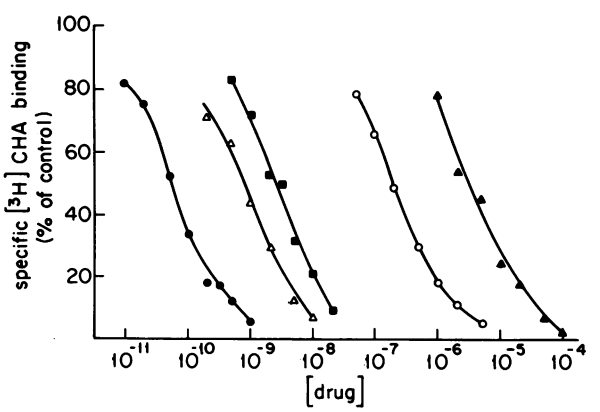

FIG. 2. Displacement curves for xanthines versus [^{3}H]CHA in bovine brain membranes. All incubations were in triplicate. ●, PACPX; △, 8-(*p*-bromophenyl)theophylline; ■, 8-phenyltheophylline; ○, 1,3-dipropylxanthine; ▲, theophylline.

DISCUSSION

Systematic modifications of the xanthine molecule coupled to rapid parallel assays of effects on adenosine receptor binding have made feasible the design of xanthine derivatives with greatly enhanced affinity at adenosine receptors. The most potent agent obtained, PACPX, is about 70,000 times more potent than theophylline and 4,000,000 times more potent than xanthine itself.

The present study utilized bovine brain membranes in which 8-phenylxanthines are more potent than in other species. [^{3}H]-CHA binding sites in rat brain display K_i values of 5 nM for PACPX, 150 nM for 8-phenyltheophylline, and 10 μM for theophylline. Thus, although both 8-phenylxanthines are less potent in rat, PACPX is still about 30-fold more potent than 8-phenyltheophylline and 2,000-fold more potent than theophylline.

The development of xanthines with enhanced adenosine receptor affinity highlights the utility of receptor-binding assays in drug development. This approach has several advantages over *in vivo* screening techniques. An analysis of factors determining variations in potency is simplified, because differences in potency are determined only by receptor affinity and not by differential absorption, metabolism, or accessibility to the target organ. Only milligram amounts of the drug need be synthesized for receptor studies, compared to 1–10 g for *in vivo* screens. Finally, receptor assays can be more efficient than *in vivo* screens. In our laboratory 500 assays are readily conducted in a day, whereas the number of drugs evaluated with *in vivo* screens is much less.

Of course, structures that are potent *in vitro* must be evaluated *in vivo* and, if necessary, modified to ensure bioavailability. For example, because PACPX is poorly soluble in water at neutral pH, it might not be well absorbed. The high nonspecific binding of [^{3}H]PACPX to tissue membranes and filters we have observed in initial experiments appears to be related to these physical properties.

Besides assisting drug development, structure–activity analysis may shed light on chemical features favoring xanthine interactions with adenosine receptors. Decreased potencies of xanthines with bulky *ortho* substituents on the 8-phenyl moiety suggest that the receptor prefers the 8-phenyl ring in the same plane as the xanthine ring. Retention of potency by derivatives with *ortho* amino groups suggest that they hydrogen bond to the N-7 of xanthine, stabilizing a conformation with the 8-phenyl and xanthine rings in the same plane. The very great reduction in potency with *meta* substitution suggests that both *meta* positions on the 8-phenyl ring are important for receptor interactions. On the other hand, most *para* substituents do not markedly alter receptor affinity.

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