Published in final edited form as:

Life Sci. 1986 March 3; 38(9): 797–807.

FUNCTIONALIZED CONGENERS OF 1,3-DIPROPYL-8-PHENYLXANTHINE: POTENT ANTAGONISTS FOR ADENOSINE RECEPTORS THAT MODULATE MEMBRANE ADENYLATE CYCLASE IN PHEOCHROMOCYTOMA CELLS, PLATELETS AND FAT CELLS

Dieter Ukena^{1,2}, John W. Daly¹, Kenneth L. Kirk³, and Kenneth A. Jacobson³

¹Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205 USA

³Laboratory of Chemistry, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205 USA

Summary

Six amine, amino acid and peptide derivatives derived from 1,3-dipropyl-8-(p-carboxymethylphenyl)xanthine, a functionalized congener of 1,3-dipropyl-8-phenylxanthine, have been investigated as antagonists at A_{2A} adenosine receptors stimulatory to adenylate cyclase in membranes from rat pheochromocytoma PC 12 cells and human platelets and at A₁ adenosine receptors inhibitory to adenylate cyclase from rat fat cells. The functionalized congeners and conjugates have affinity constants ranging from 80 to 310 nM at A_{2A} receptors of PC 12 cells and from 25 to 135 nM at those of platelets. The affinity of the xanthine derivatives at A₁ receptors of fat cells are in the 15 to 30 nM range. Thus, the amino acid and peptide conjugates have high potencies at both receptor subclasses and show some selectivity toward A₁ adenosine receptors. Derivatives of the congeners should be useful as receptor probes and as radioiodinated ligands.

Alkylxanthines represent the major class of antagonists for adenosine receptors (1). Classical antagonists, such as theophylline and caffeine, are relatively weak, having affinity constants in the range of 10–50 μ M. Furthermore, they are virtually nonselective for the A₁ and A₂ adenosine receptors, where adenosine agonists can cause inhibition and stimulation of adenylate cyclase activity, respectively (1). Adenosine receptors sensitive to xanthines, but not linked to adenylate cyclase, may also exist. Recently developed xanthine derivatives with 1,3-dipropyl and 8-phenyl substituents showed high potencies in the nanomolar range and some selectivity for A₁ adenosine receptors (2,3). However the low water solubility and high lipophilicity of such xanthines have impeded their widespread use *in vivo* (2).

In order to circumvent these problems, we have developed functionalized congeners as antagonists for adenosine receptors. In this approach, 1,3-dipropyl-8-phenylxanthine has been modified with a functionalized chain for covalent attachment to amines, amino acids

²To whom correspondence should be sent.

and oligopeptides (4,5,6). This approach yields a versatile class of adenosine receptor antagonists in which distal structural changes of the attached amino acid or peptide moieties can serve to improve the pharmacodynamic and pharmacokinetic properties (4,5,6). In the present study we have investigated the potencies of these xanthine conjugates as antagonists of adenosine receptor-modulated adenylate cyclase activity in different cell types.

Materials and Methods

Materials

The synthesis and chemical properties of the xanthine derivatives are described elsewhere (4,6). [α - 32 P]ATP (31 μ Cl/mmol) was purchased from Amersham (Arlington Heights, IL). Other compounds used in this study were adenosine deaminase (200 U/mg), GTP, creatine kinase, cyclic AMP, ATP (Boehringer Mannheim Biochemicals, Indianapolis, IN), theophylline, isoproterenol, neutral alumina WN-3, creatine phosphate as Tris-salt, crude bacterial collagenase (Sigma Chemical Company, St Louis, MO), N⁶-cyclohexyladenosine (CHA), 5'-N-ethyl-carboxamidoadenosine (NECA), N⁶-R-phenylisopropyladenosine (R-PIA), N⁶-S-phenylisopropyladenosine (S-PIA), 2-chloroadenosine (Research Biochemicals Inc., Wayland, MA). Rolipram was from A G Schering Laboratories (Berlin, Fed Rep Germany). All other chemicals were of analytical grade or best commercially available.

Preparation of pheochromocytoma (PC 12) cell membranes

PC 12 cells, derived from a pheochromocytoma tumor of the rat adrenal medulla, were obtained from Or G Guroff (National Institutes of Health, Bethesda, Maryland, USA). The cells were grown in plastic tissue culture flasks in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) with 6% fetal calf serum, 6% horse serum and a penicillin-streptomycin mixture. The cells were kept at 37°C in an atmosphere enriched in CO₂. After washing the cells twice with buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4), membranes were prepared by homogenizing cells in 5 mM Tris-HCl, 1 mM EDTA, pH 7.4, using a Polytron homogenizer at a setting of 6 for 10 sec. The homogenate was centrifuged at 1,000 $\times$ g for 10 min and the supernatant again centrifuged at 39,000 $\times$ g for 20 min. The pellet was resuspended in 5 mM Tris-HCl, 1 mM EDTA, pH 7.4, and centrifuged at 39,000 $\times$ g for 20 min. Finally, the membranes were resuspended in 50 mM Tris-HCl, pH 7.4, frozen in liquid nitrogen and stored at -70°C Protein was measured according to the method of Lowry (7).

Preparation of human platelet membranes

Platelet membranes were prepared as described by Tsai and Lefkowitz (8).

Preparation of rat fat cell membranes

Isolated rat fat cells were prepared according to the method of Rodbell (9) Plasma membranes were prepared as described by McKeel and Jarett (10).

Adenylate cyclase assay

Adenylate cyclase activity was assayed in a medium containing 0.1 mM [α - 32 P]ATP (0.3–0.4 μ Cl/tube), 0.1 mM cyclic AMP, 1 μ g/ml adenosine deaminase, 0.1 mM rolipram (4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone; ZK 62,711), 0.2 mM EGTA, 5 mM creatine phosphate as Tris-salt, 0.4 mg/ml creatine kinase, 2 mg/ml bovine serum albumin and 50 mM Tris-HCl, pH 7.4, in a total volume of 100 μ l. The concentrations of GTP and $MgCl_2$ were 10 μ M and 0.5 mM for PC 12 cell membranes, 1 μ M and 1 mM for platelet membranes and 10 μ M and 1 mM for fat cell membranes, respectively. In the case of fat cell membranes, 150 mM NaCl was included in the assay. Incubations were initiated by the addition of PC 12 cell membranes (approximately 5–10 μ g protein/tube), human platelet membranes (approximately 10–15 μ g protein/tube) or rat fat cell membranes (approximately 5 μ g protein/tube) to reaction mixtures that had been preincubated for 5 min at 37°C and were conducted for 10 min at 37°C. Reactions were stopped by addition of 0.4 ml 125 mM zinc acetate and 0.5 ml 144 mM Na_2CO_3 . Under these conditions, cyclic AMP formation was linear as a function of time for at least 10 min. Cyclic AMP was purified as described previously (11).

Data analysis

EC_{50} and IC_{50} values were obtained from the concentration-response curves by linear regression analysis after logit-log transformation. K_B values (affinity constants) of the antagonists were calculated using the Schild equation $K_B = C/(CR-1)$, where C denotes the concentration of the competitor and CR the ratio of the EC_{50} or IC_{50} values in the presence and absence, respectively, of the competitor.

Results

A₂ Adenosine receptors

Membranes from pheochromocytoma PC 12 cells contain adenylate cyclases that can be activated by adenosine analogs (12). The agonist profile for this adenosine receptor was determined from the effects of several adenosine agonists on activity of adenylate cyclase in PC 12 membranes: 5'-N-Ethylcarboxamido-adenosine (NECA) increased basal adenylate activity about 6 to 7-fold over basal values with an EC_{50} of 100 nM (Fig 1). 2-Chloroadenosine (EC_{50} 460 nM) was about 5-fold less potent than NECA and had almost the same efficacy. The R-isomer of N⁶-phenylisopropyladenosine (R-PIA; EC_{50} 970 nM) was about 4-fold more potent than the S-isomer (S-PIA; EC_{50} 3,900 nM). This order of potency (NECA > 2-chloroadenosine > R-PIA) as well as the degree of stereoselectivity for R- and S-PIA are characteristic for stimulatory A₂ adenosine receptors (1).

The general structure of the functionalized congeners of 1,3-dipropyl-8-phenylxanthine is shown in Fig. 2 (see Table I for structures). The potencies of these congeners and other xanthines as antagonists at A₂ adenosine receptors of PC 12 membranes was determined versus the NECA-stimulated adenylate cyclase activity in membranes. The effects of the xanthine amine congener (XAC) 8 and the D-lysine conjugate 9 on the concentration-response curve of NECA for stimulation of adenylate cyclase activity are shown in Fig. 3A. NECA stimulated enzyme activity with an EC_{50} of 170 nM. The amine congener XAC (8)

did not affect basal activity in the absence of NECA, but produced a parallel shift of the concentration-response curve to the right without a change of slope or maximal effect. This is consistent with a competitive antagonism. The EC_{50} of NECA in the presence of $0.5 \mu\text{M}$ of the XAC (8) was $1.25 \mu\text{M}$. The K_B of the antagonist calculated from the Schild equation for this experiment was 179 nM (see Table I). The D-lysine conjugate 9 was somewhat less potent and had a K_B of 152 nM for this experiment.

This method was used to evaluate the potency of the xanthine derivatives as A_2 adenosine receptor antagonists. The K_B values of the antagonists and the structure of the R substituent (see Fig. 2) are listed in Table I. The amine congener XAC 8 was about 25-fold more potent than the carboxylic acid congener 7 which congener had the same potency as the parent 1,3-dipropyl-8-phenylxanthine 4. The latter was as potent as 8-phenyltheophylline 2, which in turn was about 10-fold more potent than theophylline 1. Incorporation of p-sulfo substituents on the 8-phenyl moiety affords water soluble derivatives (3,5), but reduces the potency at the A_2 receptors of PC 12 cells about 3 to 5-fold. Incorporation of a p-methoxy substituent (6) also reduces potency at the A_2 receptors of PC 12 cells in this case by 6-fold.

Coupling of the carboxylic acid congener 8 to various amino acid residues (conjugates 9–15) either did not alter the activity from that of the amine congener 8 or reduced activity at the A_2 receptor of PC 12 cells. These conjugates include the equipotent D- and L-lysine conjugates (9,10) and a phenol derivative (11), suitable for radiolabeling with iodine-125. The latter compound was as potent as the parent D-lysine conjugate (8). The tyrosyl-lysine congener (12) is also suitable for iodine-125 radiolabeling, and was about 4-fold less potent than the parent amine congener (8). The methionine conjugate (13) was about 4-fold less potent than the parent amine congener.

In a similar manner, the potencies of the xanthine derivatives were determined at A_2 adenosine receptors of human platelets. In these cells NECA is a potent agonist at the A_2 receptors that mediate inhibition of aggregation via an activation of adenylate cyclase (11,13). NECA stimulated the activity of adenylate cyclase of platelet membranes with an EC_{50} of 310 nM (Fig. 3B). The concentration-response curve of NECA was shifted to the right in the presence of the carboxylic acid congener (7) and the amine congener XAC (8). The EC_{50} of NECA increased to $0.48 \mu\text{M}$ and $11.5 \mu\text{M}$ in the presence of the carboxylic acid congener and of XAC, respectively. This shift was used to calculate the K_B values of the antagonists (Table I). 8-Phenyltheophylline (2) and 1,3-dipropyl-8-phenylxanthine (4) were nearly equally potent at A_2 receptors of human platelets and of PC 12 cells. However, the presence of p-sulfo or p-methoxy substituents reduced the potencies at A_2 receptors of PC 12 cells, but not of human platelets. Furthermore, the amine congener XAC as well as the amino acid conjugates were 2 to 5-fold more potent at A_2 adenosine receptors of platelets than at those of PC 12 cells, having affinities as low as 25 to 40 nM for the platelet A_2 receptors (Table I).

A₁ Adenosine receptors

The potency of xanthine derivatives as antagonist at the A_1 receptors of rat fat cells was determined versus the N⁶-substituted adenosine analog R-PIA. R-PIA is a potent A_1 receptor agonist for inhibition of adenylate cyclase and lipolysis in fat cells (14,15). R-PIA

inhibited isoproterenol-stimulated adenylate cyclase activity of rat fat cell membranes with an IC_{50} of 26 nM (Fig. 4). The amine congener XAC (8) shifted the concentration-response curve of R-PIA to the right without changing the slope or the maximal degree of inhibition (Fig 4). From this shift the K_B of the antagonist was calculated with the Schild equation to be 10.8 nM (Table I). 8-Phenyltheophylline (2) was about 6-fold and 1,3-dipropyl-8-phenylxanthine (4) 30-fold more potent at A_1 receptors compared to A_2 receptors. The incorporation of p-sulfo and p-methoxy groups reduced the potencies at A_1 receptors. The carboxylic acid congener of 1,3-dipropyl-8-phenylxanthine (7) was as potent at the parent compound itself, whereas XAC (8) was more potent with a K_B of 15 nM. All the amino acid conjugates (9–13) had K_B values lower at the A_1 receptors of fat cells than those at A_2 receptors of PC 12 cells and platelets (Table I).

Discussion

Theophylline and caffeine are prototypes for adenosine receptor antagonists of the xanthine class. Although both xanthines are relatively weak antagonists, they undoubtedly owe many of their pharmacological effect to blockade of adenosine receptors (1,16). The presence of an 8-phenyl group enhances the potency of theophylline as an adenosine antagonist (17). Even more potent antagonists result from the replacement of the 1,3-dimethyl groups of 8-phenyltheophylline with n-propyl groups and by situating uncharged electron-donating para-substituents on the 8-phenyl ring. Affinity constants of less than 1 nM at A_1 receptors have been attained (2). However, it has been observed that the 8-phenylxanthine analogs are hydrophobic and because of this are probably not effectively absorbed into circulation after intraperitoneal injection (see ref 41). Unfavorable solubility and pharmacokinetics may prevent the widespread *in vivo* use of many 8-phenylxanthines.

The attachment of drugs to carrier molecules is a useful approach in the design of new potent analogs with desirable solubilities and pharmacokinetics (18–20). In an approach to adenosine antagonists, 1,3-dipropyl-8-phenylxanthine has been attached covalently through a functionalized chain to amines, amino acids and oligopeptides (4,5,6). This approach resulted in potent adenosine receptor antagonists with relatively higher water solubilities.

The amine congener XAC (8) is about 30 to 80-fold more potent at A_2 adenosine receptors of PC 12 cells and of platelets than the parent compound 1,3-dipropyl-8-phenylxanthine 4 (Table I). This amine congener is at present the most potent known xanthine antagonist at A_2 adenosine receptors. The attachment of free amino acids to the chain results in conjugates having affinity constants for A_2 -adenosine receptors of 35 to 85 nM in platelets and 130 to 300 nM in PC 12 cells. These compounds will have improved solubility characteristics due to the presence of an amino group, which is predominantly charged at physiological pH. It has been shown that the water solubility of amino acid conjugates can be enhanced by two orders of magnitude over that of uncharged 8-phenylxanthine derivatives (6). By virtue of the attachment of amino acids the undesirable binding to plasma proteins and partition into lipids typical of the hydrophobic 8-phenylxanthines should be greatly reduced. This could lead to improved pharmacokinetic properties of the xanthine derivatives for *in vivo* applications.

Although in the case of theophylline (1), 8-phenyltheophylline (2) or 1,3-dipropyl-8-phenylxanthine (4), the xanthine had equivalent potencies at the A₂ receptors of PC 12 cells and human platelets, this was not true for certain of the other xanthines or for the functionalized congeners (Table I). Introduction of a p-sulfo or p-methoxy substituent into 4 markedly decreased potency at the A₂ receptor of PC 12 cells, while having little effect on potency at the A₂ receptor of human platelets. The resultant xanthines, 5 and 6 respectively, were about 5-fold more potent at platelet receptors than at PC 12 cell receptors. Similarly, all of the functionalized congeners (8–13) were more potent at platelet receptors than at PC 12 cell receptors. The results indicate that the A₂ receptors of PC 12 cells and platelets are not identical. The potencies of adenosine analogs as agonists at these A₂ adenosine receptors also are not identical for these two cell types with, for example, NECA having an EC₅₀ of 310 nM for activation of adenylate cyclase in platelet membranes and an EC₅₀ of 100 nM in PC 12 cell membranes.

Comparison of the potencies of the lysine conjugates (9,10) reveals that the enantiomers are nearly equipotent to each other at both A₁ and A₂ adenosine receptors. This indicates that the chiral center is sufficiently removed from the pharmacophore at the receptor to have no effect on receptor affinity.

In addition to increasing the potency and changing the physicochemical properties of drugs, the functionalized congener approach enables the design of receptor probes and labels. The p-hydroxyphenylpropionyl derivative 10 of the D-lysine conjugate and the tyrosine conjugate 11 can undergo radioiodination by virtue of electron rich aromatic rings. Both have potencies at A₁ receptors in the nanomolar range. Therefore, such compounds have potential for elaboration of radioligands for adenosine receptors. Indeed, [³H]XAC has proven to be a useful radioligand for A₁-adenosine receptors in brain membranes (submitted for publication). It is conceivable that prosthetic groups designed to accept specifically other radioisotopes, such as metal chelators, may also be linked to functionalized congeners as receptor probes (see ref. 6).

Selectivity of xanthine derivatives as antagonists at the A₁ and A₂ receptor subclasses is highly desirable in the development of these drugs as research tools and as therapeutic agents. The present study compares activity at two different A₂ receptors stimulatory to adenylate cyclase and at an A₁ receptor inhibitory to adenylate cyclase. Comparison of the inhibition constants from the PC 12 cell cyclase and the human platelet cyclase with that of fat cell cyclase reveals a selectivity ratio of about 30 for 1,3-dipropyl-8-phenylxanthine (Table I). Thus, the parent compound shows a higher affinity for A₁ receptors. The carboxylic acid congener (7) shows nearly the same degree of selectivity for A₁ receptors as the parent compound. The amine congener XAC (8) and the amino acid conjugates are much less selective. Different results were obtained if the K_i values of the xanthine derivatives for inhibition of [³H]CHA binding to rat brain membranes are used for comparison (Table I). Binding of [³H]CHA to brain membranes has been shown to occur only at A₁ receptors (21). The K_i values of the antagonists for inhibition of [³H]CHA binding to rat brain membranes are much lower – up to 20-fold – than the affinity constants for the A₁ receptor controlled adenylate cyclase in rat fat cells. The K_i value of 8-phenyltheophylline for inhibition of radioligand binding to A₁ adenosine receptors in fat cell membranes (22) is 80–100 nM (22),

about 4-fold lower than the K_B value (350 nM) obtained in adenylate cyclase studies in the same membranes (Table I). It is likely that ratios obtained through comparisons of antagonist activity in biochemical (adenylate cyclase) or physiological assays will be more predictive of the selectivity of xanthines for both *in vivo* and *in vitro* applications than comparison of values obtained in radioligand binding assays. Certain of the present congeners have proven selective in blocking A_1 receptor-mediated cardiac depression *in vivo* compared to A_2 receptor-mediated vasodilation (23).

Acknowledgments

One of the authors (D Ukena) is on leave from the Pharmakologisches Institut der Universität Heidelberg, Fed Rep. Germany, with support of the Deutsche Forschungsgemeinschaft (Uk 4/1-1).

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Fig. 1.

Stimulation of adenylate cyclase activity of PC 12 cell membranes by adenosine analogs. Adenylate cyclase activity was determined for 10 min at 37°C. Mean values of duplicate determinations are shown. NECA (○); 2-chloroadenosine (□); R-PIA (△); S-PIA (◇). The EC₅₀ values from three separate experiments are as follows (means with 95% confidence limits in parentheses). NECA 100 nM (45–220); 2-chloroadenosine 460 nM (330–640), R-PIA 970 (600–1,400); S-PIA 3,900 nM (1,000–8,700).

Fig. 2.

Structure of the xanthine amine congener (XAC, R = H, 8). Structures of R in amino acid and peptide conjugates are in Table I.

FIG. 3.

Inhibition of NECA-stimulated adenylyl cyclase activity by xanthines in membranes from (A) PC 12 cells and (B) human platelets. (A) EC₅₀ values for NECA are 0.17 μM without antagonist (0); 0.73 μM with 0.5 μM D-lysine conjugate (9) (Δ); and 1.25 μM with 0.5 μM XAC (8) (□). (B) EC₅₀ values for NECA are 0.31 μM without antagonist (○); 0.48 μM with 1 μM carboxylate congener (8) (□); and 11.5 μM with 1 μM XAC (8) (Δ). Mean values of duplicate determinations are shown.

FIG 4.

Antagonism by a xanthine of the inhibition by R-PIA of isoproterenol-stimulated adenylyl cyclase activity of rat fat cell membranes. Basal enzyme activity was 120 pmol cyclic AMP per min per mg protein. The IC_{50} values for R-PIA for inhibition of isoproterenol (10 μ M)-stimulated adenylyl cyclase activity was 26 nM in the absence (○) and 146 nM in the presence of 50 nM XAC (□). Mean values of duplicate determinations are shown.

Table IPotencies of xanthine derivatives as antagonists at A_{2A} and A₁ adenosine receptors.

Xanthine	K _B (nM) versus NECA-Stimulation		K _B (nM) versus PIA-Inhibition Rat Fat Cells	K _I , (nM) versus [³ H]CHA Binding* Rat Brain
	Rat PC 12 Cells	Human Platelets		
Theophylline (1)	17,000 (15,800–19,000)	13,800 (4,000–17,900)	8,700 (5,100–14,800)	14,000
8-Phenyltheophylline (2)	1,600 (400–6,100)	1,900 (500–6,800)	350 (200–600)	400
8-(p-Sulfophenyl)theophylline (3)	5,000 (1,400–10,000)	5,500 (1,600–9,000)	2,500 (1,600–3,900)	4,500
1,3-Dipropyl-8-phenylxanthines para-substituent				
None (4)	2,300 (600–8,700)	2,100 (1,300–3,600)	80 (24–290)	13
HO ₃ S– (5)	11,100 (3,100–29,900)	1,900 (1,300–3,900)	430 (280–660)	210
CH ₃ O– (6)	13,900 (6,600–28,900)	2,900 (2,200–3,900)	160 (130–210)	14
HO ₂ CCH ₂ –O– (7)	2,000 (1,300–3,150)	2,400 (1,500–3,900)	83 (38–180)	58
RNH(CH ₂) ₂ NHCOCH ₂ O–				
R = H– (8) (XAC)	83 (72–96)	25 (21–30)	15 (8.1–29)	1.2
R = D-Lys- (9)	130 (100–160)	37 (35–40)	19.6 (13.7–28)	0.87
R = L-Lys- (16)	110 (180–150)	35 (29–42)	27.5 (21.0–36)	1.0
R = ε-(p-HOC ₆ H ₅ (CH ₂) ₂ CO)-D-Lys (11)	140 (70–290)	85 (60–130)	28.4 (15.7–51)	1.9
R = D-Tyr-D-Lys- (12)	310 (250–390)	135 (130–141)	28.6 (22–37)	2.4
R = L-Met- (13)	300 (220–410)	60 (30–120)	18 (12.7–25.5)	1.5

K_B values are means (n = 3) with 95% confidence limits in parentheses.* K_I values were from ref 4,5,6.