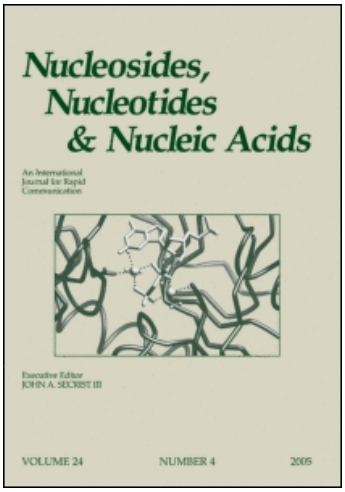

Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Survey of Nonxanthine Derivatives as Adenosine Receptor Ligands

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To cite this Article Siddiqi, Suhaib M. , Ji, Xiao-duo , Melman, Neli , Olah, Mark E. , Jain, Rahul , Evans, Patricia , Glashofer, Marc , Padgett, William L. , Cohen, Louis A. , Daly, John W. , Stiles, Gary L. and Jacobson, Kenneth A.(1996) 'Survey of Nonxanthine Derivatives as Adenosine Receptor Ligands', Nucleosides, Nucleotides and Nucleic Acids, 15: 1, 693 — 717
To link to this Article: DOI: 10.1080/07328319608002416
URL: http://dx.doi.org/10.1080/07328319608002416

NUCLEOSIDES & NUCLEOTIDES, 15(1-3), 693-717 (1996)

A SURVEY OF NONXANTHINE DERIVATIVES AS ADENOSINE RECEPTOR LIGANDS[1]

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Abstract: The binding affinities at rat A_1, A_{2a}, and A_3 adenosine receptors of a wide range of heterocyclic derivatives have been determined. Mono-, bi-, tricyclic and macrocyclic compounds were screened in binding assays, using either [^{3}H]PIA or [^{3}H]CGS 21680 in rat brain membranes or [^{125}I]AB-MECA in CHO cells stably transfected with rat A_3 receptors. Several new classes of adenosine antagonists (*e.g.* 5-oxoimidazopyrimidines and a pyrazoloquinazoline) were identified. Various sulfonylpiperazines, 11-hydroxytetrahydrocarbazolenine, 4H-pyrido[1,2-a]pyrimidin-

[1]Dedicated to Prof. Yoshihisa Mizuno on the occasion of his 75th birthday.

one, folic acid, and cytochalasin H and J bound to A_3 receptors selectively. Moreover, cytochalasin A, which bound to A_1 adenosine receptors with K_i value of 1.9 μM, inhibited adenylyl cyclase in rat adipocytes, but not via reversible A_1 receptor binding.

Introduction

The A_1, the A_{2a}, the A_{2b} and the A_3 adenosine receptors are members of the G-protein-coupled superfamily and have now been defined both on the basis of pharmacological differences[1] and on the basis of distinct amino acid sequences.[2] Adenosine receptors mediate a wide variety of physiological functions[3-5]. These include: Inhibition of neurotransmitter release from nerve endings, vasoconstriction in the kidney, cardiac depression and inhibition of lipolysis via A_1 receptors; vasodilatation, inhibition of platelet aggregation, and inhibition of lymphocyte function via A_2 receptors; potentiation of histamine release from mast cells[6] resulting in hypotension[7] via A_3 receptors. The A_1 and A_3 receptors cause inhibition of adenylyl cyclase and activation of phospholipase C. A_1 receptors also couple to activation of potassium channels and inhibition of calcium channels. The A_{2a} and A_{2b} receptors activate adenylyl cyclase.

Numerous structure-activity relationship studies at A_1 and A_2 adenosine receptors, aimed at increasing potency and selectivity of agonists and antagonists, have been published (see ref. 1). Although xanthines are the classical adenosine antagonists, numerous classes of nonxanthine antagonists,[1,8] mainly fused nitrogen-containing heterocyclic structures, have been reported. Xanthines have, however, proven to be either inactive at cloned rat A_3 receptors, or relatively inactive at the sheep and human cloned A_3 receptors. The lack of any selective antagonist for the rat A_3 adenosine receptor[9, 27] prompted us to undertake a detailed examination of a variety of nonxanthine derivatives as ligands for A_3 and other adenosine receptors. Ligands selective for A_3 receptors[10] have

promise as agents for treating ischemia of the brain[11] and heart,[3] inflammation,[5] and asthma.[5] The present study is a survey of some known and some novel classes of nonxanthine adenosine ligands of widely varying structure.

Results and Discussion

Xanthines, the best known class of adenosine antagonists, contain fused 5:6 heterocyclic rings. Caffeine and theophylline, two well known xanthine antagonists have K_i values of approximately 40 and 15 μM, respectively, at both the A_1 and A_{2a} receptor.[1] No significant antagonist activity has been observed for xanthines or nonxanthine derivatives at the rat A_3 adenosine receptor. Thus, an A_3 antagonist is actively being sought. In addition to screening this diverse group of compounds at the cloned A_3 receptor, they were also tested at rat A_1 and rat A_{2a} receptors.

Table 1 shows the results of radioligand binding competition experiments at rat brain adenosine receptors for 110 cyclic compounds. Structures of selected compounds of interest, having K_i values at A_1 receptors in the 10^{-6} to 10^{-4} M range, are shown in Figure 1. Potential antagonism of adenylyl cyclase inhibition mediated by A_1 receptors in rat adipocytes (Table 2) and by cloned rat A_3 receptors expressed in CHO cells[9] was also examined. Several compounds that inhibited A_1 receptor binding were shown to antagonize the inhibitory effects of N^6-phenylisopropyladenosine (*R*-PIA) on adenylyl cyclase in adipocyte membranes (Table 2), with K_B values of ≥ 1 μM, as calculated using Schild analysis (see below).[9] Functional assays at A_{2a} receptors were not carried out. At rat A_3 receptors, although numerous compounds showed K_i values in the range of 10^{-5} to 10^{-4} M, two of these compounds (**6** and **47**), examined in the functional assay at cloned rat A_3 receptors coupled to adenylyl cyclase in CHO cells, appeared to have no antagonist activity. The dose-response curve for IB-MECA[39] was not shifted in the presence of 40

Table 1. Affinities of heterocyclic derivatives in radioligand binding assays at rat brain A_1, A_{2a}, and A_3 receptors[a-d].

Compound	Source[f]	Name	K_i (μM) or % inhibition[d] (A_1)[a]	(A_{2a})[b]	(A_3)[c]
		Non-Fused Rings			
1	F	N,N'-(2-Chloro-5-cyano-1,3-phenylene)dioxamic acid (Lodoxamide)	n.d.	n.d.	17%
2	T	N,N-Diethylaminoethyl-2,2-diphenylvalerate (SKF-525A, Proadifen)	11±6%[d]	e	49.3 ±10.3
3	A	2,6-*bis*[(4S)-Isopropyl-2-oxazolin-2-yl]pyridine	90.9±5.8	19±5%[d]	46.2 ± 0.3
4	A	6-(4-Chlorophenyl)-4,5-dihydro-2-(2-hydroxybutyl)-3(2*H*)-pyridazinone	75.1±20.8	e	22.4 ± 10.0
		Fused Bicyclics (6:5)			
5	A	(-)-5-Bromo-4-chloro-3-indolyl-β-D-glucoside	25±7%[d]	e	37.0 ± 9.4
6	A	5-Bromo-4-chloro-3-indolyl-β-D-galactoside	18±14%[d]	e	18.1±3.8
7	R	5-Chloro-2(3H)-benzoxazolone (Chlorzoxazone)	21±3%	15±4%	34±5%
8	R	Calcimycin	29± 4%	e	24±8%
9	A	1-Methyl-2-phenylbenzimidazole	[23][j]	[99][k]	19±8% (10^{-5})
10	R	4-[3-[(1,1-Dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one hydrochloride ((±)-CGP-12177A)	23±3%	40±3%	109±5
11	Bo	2-[2-Methoxy-4-(methylsulfinyl)phenyl]-1H-imidazo[4,5-*b*]pyridine (Sulmazole, ARL115)	[52]j	22.7±8.6	15±0% (10^{-5})
12	D	1,4,6-Trimethyl-1*H*-pyrazolo-pyridine	[>100]j	e	22±0% (10^{-5})
13	D	Ethyl 4-phenylamino-1-methyl-pyrazolopyridine-5-carboxylate	[1.1][i]	[2.4][i]	43±2%

Table 1. Continued

14	D	Ethyl 4-benzylamino-1-methyl-pyrazolopyridine-5-carboxylate	[1.0][i]	[2.5][i]	29±3% (10^{-5})
15	D	Ethyl 4-phenylethylamino-1-methyl-pyrazolopyridine-5-carboxylate	[4.3][i]	[12][i]	26±6%
16	D	Ethyl 4-ethoxy-1-methyl-pyrazolopyridine-5-carboxylate	[22][i]	[19][i]	15±2%
17	M	Ethyl 1,3-dimethyl-4-(4-methoxyphenoxy)-1*H*-pyrazolo-[3,4-b]pyridine	9.39±0.95	11.7±2.0	e
18	M	Ethyl 1,3-dimethyl-4-(4-fluorophenoxy)-1*H*-pyrazolo-[3,4-b]pyridine-5-carboxylate	6.95±1.10	9.24±1.04	e
19	M	Ethyl 4-[3,5-dichlorophenoxy] 1,3-dimethyl-1*H*-pyrazolo [3,4-b]pyridine-5-carboxylate	22.8±6.1	e	e
20	C	5-(Hydroxymethyl)-4,5,6,7-(tetrahydro)imidazo-[4,5-c]pyridine-6-carboxylic acid	e	16 ±2%[d]	86.1 ± 3.4
21	C	5-Oxo-(5,6-dihydro)imidazo[1,5-c]pyrimidine-7-carboxylic acid methyl ester	73.6±24.6	24±2%[d]	n.d.
22	C	1-Chloro-5-oxo-(5,6-dihydro)imidazo[1,5-c]pyrimidine-7-carboxylic acid methyl ester	60.6±20.7	85.1±1.4	36.8 ± 1.0
23	C	1-Chloro-5-oxo-(5,6,7,8-tetrahydro)imidazo[1,5-c]pyrimidine-7-carboxylic acid methyl ester	26±3%[d]	14±7%[d]	n.d.
24	C	1,3-Dichloro-5-oxo-(5,6 dihydro)imidazo[1,5-c]pyrimidine-7-carboxylic acid methyl ester	10.3±2.7	16.5±3.0	53.0 ± 13.0
25	C	5-Oxo-(5,6,7,8-tetrahydro)imidazo[1,5-c]pyrimidine	e	22±1%[d]	53.5 ± 15.8
26	C	1,3-Diiodo-5-oxo-(5,6,7,8-tetrahydro)imidazo[1,5-c]pyrimidine-7-carboxylic acid methyl ester	8.48±0.84	35.9±5.9	n.d.
27	C	1-Iodo-5-oxo-(5,6,7,8-tetrahydro)imidazo[1,5-c]pyrimidine-7-carboxylic acid methyl ester	e	27±4%[d]	n.d.

(continued)

Table 1. Continued

28	C	1-Bromo-5-oxo-(5,6,7,8-tetrahydro)imidazo[1,5-c]pyrimidine-7-carboxylic acid methyl ester	11±4%[d]	e	n.d.
29	C	6-t-Butyloxycarbonyl-8-chloro-5-oxo-(5,6,7,8-tetrahydro)imidazo[1,5-c]pyrimidine-7-carboxylic acid methyl ester	e	24±7%	n.d.
30	C	5-Oxo-(5,6,7,8-tetrahydro)imidazo[1,5-c]pyrimidine-7-carboxylic acid benzyl ester	e	35±8%[d]	n.d.
31	C	5-Oxo-(5,6,7,8-tetrahydro)imidazo[1,5-c]pyrimidine-7-carboxylic acid methyl ester	e	e	n.d.
32	C	7-(Methoxycarbonyl)-2-phenylmethyl-5-oxo-(5,6,7,8-tetrahydro)imidazo[1,5-c]pyrimidinium bromide	440	e	n.d.
33	E	4-Amino-5,6,-dimethyl-2-phenyl-7H-pyrrolo-[2,3-*d*]pyrimidine	[1.49][g]	[21.3][l]	15% (10^{-5})
34	E	4-Amino-5,6,-dimethyl-2-phenyl-7H-7-(phenyl)pyrrolo-[2,3-*d*]pyrimidine	[0.036][g]	[14.3][l]	25±2% (10^{-5})
35	M	Ethyl 6-(4-chlorophenyl)-4-methyl pyrazolo[1,5-*a*] pyrimidine 3-carboxylate	12±1%	e	e
36	M	5,7-*bis*(Trifluoromethyl)-3-cyano-2-(methylthio)pyrazolo-[1,5-*a*]pyrimidine	e	e	e
37	G	Anhydro-2-phenyl-6,8-diethyl-5-hydroxy-7-oxo-1,3,4-thiadiazolo[3,2-*a*]pyrimidinium hydroxide	[80][h]	23±6%	55.6 ± 17.1
38	D	2-Phenyl-4,6-dimethyl thiazolopyrimidine-5,7-dione	[79][j]	[130][k]	31±7% (10^{-5})
39	C	8-(Heptafluoropropyl)adenine	e	27±3%[d]	99.5 ± 11.5
40	C	8-(Heptafluoropropyl)guanine	e	24±1%[d]	77.7 ± 27.1
41	R	6-[(1-Methyl-4-nitro-1H-imidazol-5-yl)thio]-1H-purine (Azathioprine)	18± 1%	28± 5%	116±3

(continued)

Table 1. Continued

42	P	8-Methyl-6-(1-piperidinlyl)-1,2,4-triazolo[4,3-*b*]pyridazine (MDL 257)	[>100]	[0.9][k]	32±6% (10^{-5})
43	P	6- (Morpholinyl)-1,2,4-triazolo [4,3-b]pyridazine (MDL 850)	[22]	11.2±2.5	21±9% (10^{-5})
44	Mu	7,9-Dibenzyl-1-methylxanthinium chloride	20%	23±6%	25±4% (10^{-5})
45	G	Anhydro-1,6-dimethyl-5-hydroxy-6-propyl-1,3,4-triazolo[3,2*a*]pyrimidinium hydroxide	[>>250][h]	e	20±10% (10^{-5})
46	R	1-Methylisoguanosine	n.d.	1.97±0.56	25.7 ± 0.1
		Fused Bicyclics (6:6)			
47	A	Esculin	27±5%[d]	32±5%[d]	101 ± 29
48	F	Disodium chromoglycate	n.d.	n.d.	19%
49	A	8-(β-D-Glucopyranosyloxy)-7-hydroxyl-6-methoxycoumarin	15±7%[d]	e	104±6
50	A	Hesperidine	22±3%[d]	e	263 ± 34
51	R	5,7-Dichloro-4-hydroxy-quinoline-2-carboxylic acid (5,7-Dichlorokynurenic acid)	22±5%	e	38±3%
52	T	1-(5-Chloronaphthalenesulfonyl) piperazine (ML-9)	e	e	96.2 ± 21.0
53	T	1-(5-Iodonaphthalenesulfonyl) piperazine (ML-7)	e	e	119 ± 67
54	T	1-(5-Isoquinolinesulfonyl) piperazine (HA-100)	e	e	24.5±10.0
55	T	1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7)	e	e	50.9 ± 15.2
56	T	1-(5-Isoquinolinesulfonyl)-3-methylpiperazine	e	e	59.0 ± 21.8
57	A	1-(5-Isoquinolinesulfonyl) homopiperazine (HA-1077)	23±9%[d]	e	43.3 ± 13.9
58	T	1-[5-(8-Chloro-isoquinoline)sulfonyl]piperazine (HA-156)	e	e	40.4 ± 12.9

(continued)

Table 1. Continued

59	R	6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX)	25±2%	e	36±9%
60	R	6-Chloro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide (Chlorothiazide)	e	24±5%	34±4%
61	R	7-Benzyl-1-ethyl-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid (Amfonelic acid)	25.2±7.5	e	157±3
62	W	4H-Pyrido[1, 2-a]-pyrimidin-4-one	e	e	48.3 ± 4.8
63	D	1,3-Dipropyllumazine	[20]j	24±7%	46.1±3.8
64	D	1,3-Dimethyl-7-phenyllumazine	32.9±7.1	107±8	20±1% (10^{-5})
65	R	Neopterin	e	e	n.d.
66	A	2, 6-Diamino-6-(hydroxymethyl)-pteridine	14±7%[d]	e	135 ± 21
67	A	2, 4-Diamino-6,7-diisopropyl-pteridine	2.51±0.47	11.9±3.2	71±14%
68	A	4-[N-(2,4-Diamino-6-pteridinylmethyl)amino]benzoic acid	e	e	48.2±7.5
69	A	4-[N-(2,4-Diamino-6-pteridinyl-methyl)-N-methylamino]benzoic acid	27±1%[d]	e	48.5 ± 10.0
70	A	Folic acid	e	20.3%	28.4 ± 9.9
71	A	Dihydrofolic acid	33.8±2.3	e	45.9 ± 18.0
72	A	Dipyridamole	22±17%[d]	54±2%	19.0 ± 0.7
		Fused Bicyclic (>6)			
73	W	1-Aza-8,9-benzcyclo-nonadi-2,7-one	22.5%	e	70.5 ± 0.7
74	Br	7-Methyl-4-propyl-4,5,6,7-tetrahydro-6*H*-imidazo[4,5*e*][1,4]diazepine-5,8-dione	19±2%	16±7%	27±3% (10^{-5})
75	Br	7-Benzyl-1,4-dipropyl-4,5,6,7-tetrahydro-6*H*-imidazo[4,5*e*][1,4]diazepine-5,8-dione	13.4±2.1	2.18±0.12	e (10^{-5})

(continued)

Table 1. Continued

		Cytochalasins (Macrocyclics)			
76	A	Cytochalasin A	1.91±0.43	29±9%	see text
77	A	Cytochalasin B	27.0±3.8	e	47.5 ± 21.8
78	A	Cytochalasin C	e	e	18±2% (10^{-5})
79	A	Cytochalasin D	e	e	53.7 ± 11.8
80	A	Cytochalasin E	e	10±1%	155 ± 56
81	A	Cytochalasin H	e	16±7%	23.0±7.1
82	A	Cytochalasin J	e	19±8%	27.9 ± 11.9
		Fused Tricyclics (5:6:5)			
83	P	1,7-Dihydro-3,5-dimethylbenzo[1,2-c:5,4-c']dipyrazole (MDL 26,020)	[8.0][m]	[25.6][k]	12±2% (10^{-5})
84	P	1,7-Diethyl-1,7-dihydro-3,5-dimethylbenzo[1,2-c:5,4-c']dipyrazole (MDL 26,629)	[27][m]	[56][k]	e (10^{-5})
85	P	1- Hydro-3,6-dimethylbenzo-[1, 2-c: 5, 4-c']dipyrazole (MDL 26,687A)	[74]	[17][k]	55.7 ± 22.4
		Fused Tricyclics (6:5:6)			
86	R	Methyl 6,7-dimethoxy-4-ethyl-β-carboline-3 carboxylate (DMCM)	1.57±0.32	3.33±0.67	49.2 ± 6
87	E	4-Hydroxy-5,6,7,8-tetrahydro-9-phenyl-9*H*-pyrimido[4,5-*b*]indole	[2.07][g]	1.49±0.51	15±1% (10^{-5})
88	W	11-Hydroxytetrahydro-carbazolenine	e	20%	21.9 ± 6.5
89	G	Anhydro-1-cyclopropylmethyl-3-ethyl-2-hydroxy-4-oxo-pyrimido[2,1*a*] benzothiazolium hydroxide	[37][h]	6.15±0.69	53.4 ± 16.1
90	E	4-Hydroxy-9-phenyl-9*H*-pyrimido[4,5-*b*]indole	[0.88][g]	[1.44][l]	18% (10^{-5})

(continued)

Table 1. Continued

		Fused Tricyclics (6:6:5)			
91	-	*lin*-Benzohypoxanthine	21.2±4.9	0.992	26±8%
92	Pf	1,3-Dimethyl-7-phenyl-6H-imidazo-(4,5-g)lumazine	e	e	n.d.
93	P	2,11-Dihydro-11-(4-morpholinyl)-6H-pyrimido [2,1-b]quinazolin-6-one (MDL 43400A)	[>100]	[160][k]	12±1% (10^{-5})
94	Pf	1,3,6-Trimethyl-7-(3,4-dichlorophenyl)-imidazo-(4,5-g)lumazine	n.d	n.d.	15% (10^{-5})
95	M	Ethyl 5-chloropyrazolo[1,5-a]quinazoline-3-carboxylate	5.36±0.36	4.06±0.50	e
		Fused Tricyclics (6:6:6)			
96	A	Alloxazine[n]	n.d.	n.d.	32±3%
97	T	Roseoflavin	28.9±4.0	e	84±8
98	B	Riboflavin	12.7±2.9	e	see text
99	S	Flavin adenine dinucleotide (FAD)	33.8±12.0	18.3±2.5	e
100	S	Fluorescein	76.1±6.8	34±5%	37±2%
101	A	Carminic acid	21±9%[d]	e	117 ± 22
		Fused Tricyclics (>6)			
102	T	Doxepin	66.4±16.8	e	72±14%
103	W	*R,S*-6-Hydroxy-7,8,9,10-tetrahydro-6H-cyclohept-[b]indole	e	n.d.	68.2 ± 23.8
104	W	5,6,8,9,10,11-Hexahydro-7H-cyclohepta[c]-quinolin-6-one	45±7%	e	n.d.
		Fused Rings (Misc.)			
105	M	Cedrol	28± 2%	34±10%	50 ± 0%
106	M	Cedrene	18± 3%	e	26± 1%
107	A	Reserpine	e	25.2%	n.d.

(continued)

Table 1. Continued

108	T	Podophyllotoxin	31±8%[d]	e	79±11%
109	T	4'-Dimethylepipodophyllotoxin	18±5%[d]	e	70±15%
110	D	Mitragynine[o]	>100	75.6±28.0	55±2%

n.d. : not determined.

a) Displacement of specific [^{3}H]PIA binding, unless noted, in rat brain membranes expressed as $K_i \pm$ S.E.M. in μM (n = 3-5).

b) Displacement of specific [^{3}H]CGS 21680 binding, unless noted, in rat striatal membranes, expressed as $K_i \pm$ S.E.M. in μM (n = 3-6).

c) Displacement of specific [^{125}I]AB-MECA binding, unless noted, in membranes of CHO cells stably transfected with the rat A_3-cDNA, expressed as $K_i \pm$ S.E.M. in μM (n = 3-5).

d) A percent value indicates the percent displacement of radioligand at the concentration (M) given in parentheses or at 10^{-4}M, if none specified.

e) ≤10% displacement of radioligand.

f) A = Aldrich (Milwaukee, WI); B = BioRad; Bo, Boehringer-Ingelheim (Germany); Br = Prof. Peter Bridson (Univ. Memphis); C = Dr. Louis Cohen (NIH), D = Dr. John W. Daly (NIH); E = Prof. Kurt Eger (Univ. Tübingen, Germany); F = Dr. John Fozard (Sandoz, Geneva); G = Prof. Richard Glennon (Medical College of Virginia, Richmond); M = Maybridge (Trevillett, UK); Mu = Dr. Christa Müller (Univ. Tübingen); P = Dr. Norton Peet (Marion Merrell Dow, Cincinnati OH); Pf = Prof. Wolfgang Pfleiderer (Univ. Konstanz); R = RBI (Natick MA); S = Sigma (St. Louis MO), T = Toronto Research Chemicals (Toronto); W = Dr. B. Witkop (NIH).

g) ref. 33.

h) ref. 34.

i) ref. 12.

j) ref. 8.

k) K_B versus NECA-stimulation of adenylate cyclase in human platlets.

l) K_i versus [^{3}H]NECA in rat striatal membranes.

m) ref. 35.

n) ref. 40.

o) ref. 38.

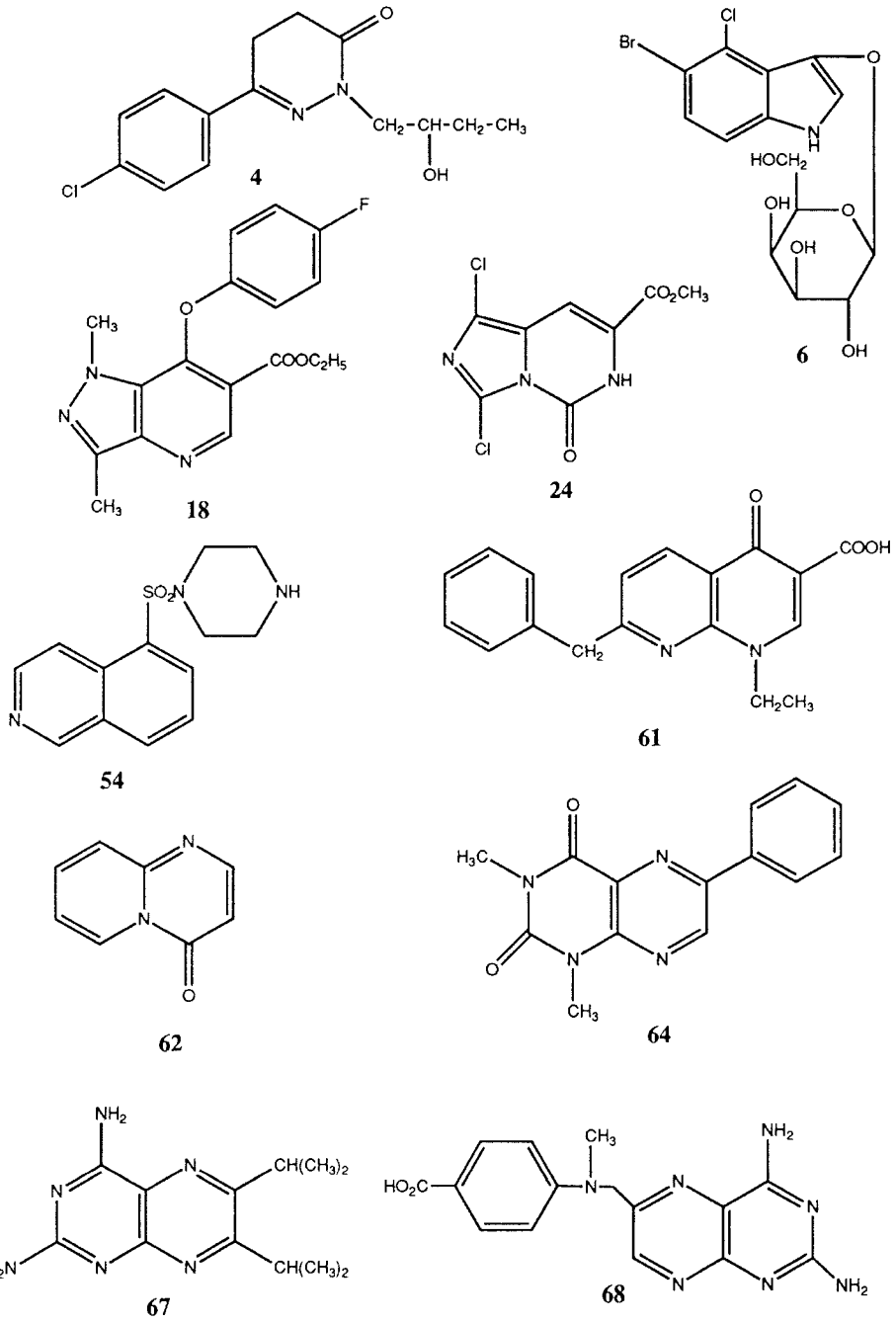

Fig. 1. Structures of selected adenosine receptor ligands.

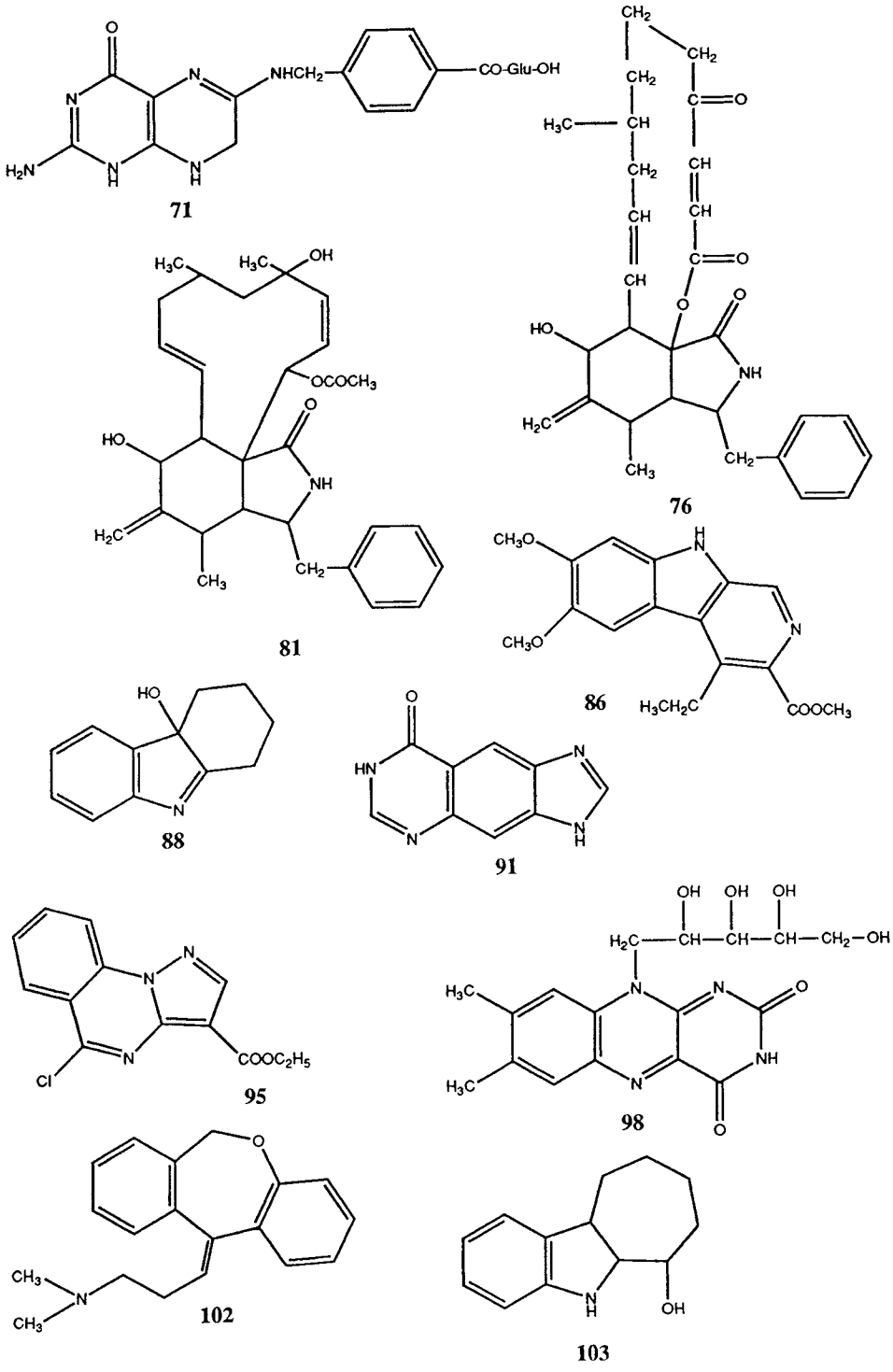

Fig. 1. Continued

Table 2. Antagonism of A_1 receptor-mediated inhibition of adenylyl cyclase in rat adipocyte membranes.[a]

Compound[b]	$K_B \pm$ SEM (μM)
4	600
18	17.1 ± 3.6
24	10.8 ± 3.0
26	600
61	600
64	46.7 ± 11.9
67	3.94 ± 0.79
77	81
86	1.36 ± 0.48
95	12.7 ± 2.95

a) Calculated from shift in dose-response curve to *R*-PIA (see Figure 3), using the Schild equation[8] (n=3-5, or for single determination).

b) Compound **71** (50 μM) caused a slight shift of the *R*-PIA dose-respose curve to the left. Compound **91** (50 μM) antagonized the effects of *R*-PIA only weakly, thus a K_B could not be determined.

μM of either **6** or **47**. In preliminary experiments, the amino-substituted pteroic acid derivative **68** alone inhibited forskolin-stimulated adenylyl cyclase in A3 transfected CHO cells, indicating possible agonist properties.

Numerous fused ring compounds and a few nonfused ring compounds were examined in binding assays in the present study. Among nonfused ring compounds examined, only compounds **3** and **4** were weak competitors at A_1 receptors. A number of fused 5:6 heterocyclic ring compounds showed moderate receptor affinity. The effects of certain pyrazolopyridines[12], compounds **17** - **19**, in binding were studied. They had K_i values around 10 μM at both A_1 and A_{2a} receptors. No binding activity was detected at A3 receptors. Related pyrazolopyridines, such as tracazolate, have been reported to have

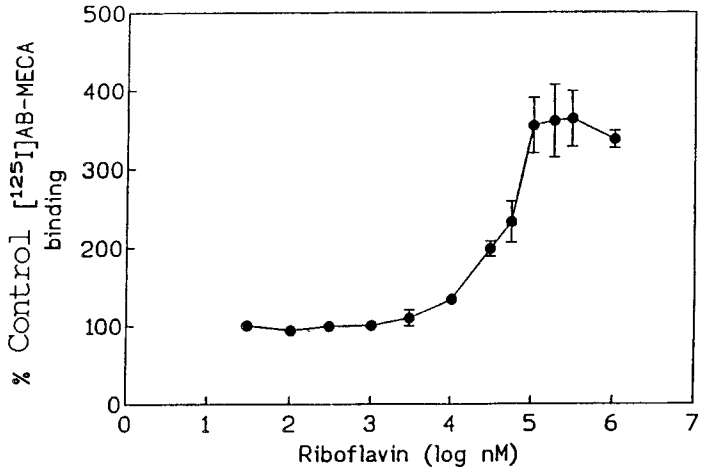

Figure 2. Enhancement of radioligand (0.4 nM) binding in A3-transfected CHO cell membranes by riboflavin, **98**.

anxiolytic activity, and the A_1 and A_{2a} receptor antagonist activity of a large series of pyrazolopyridines has been reported.[12]

Imidazopyrimidine-7-carboxylic acid derivatives, compounds **24** and **26**, have K_i values of 10 and 8 μM, respectively at A_1 receptors. This class of histidine derivatives has been prepared in connection with potential antimalarial activity.[13] Compound **42** is a triazolopyridazine bronchodilator[36] that did not bind appreciably to adenosine receptors.

We have determined the K_i value of a nucleoside analogue, 1-methylisoguanosine, **46**, at A_{2a} and A_3 receptors to be 2.0 μM and 25 μM, respectively. This compound was reported previously to bind to adenosine receptors as an agonist.[14]

The sulfonylpiperazine derivatives **52-58** displayed no binding activity at A_1 and A_{2a} receptors. However, they did displace radioligand binding weakly at A_3 receptors. These compounds are members of a well-known class of inhibitors of protein kinase C activity, acting in the concentration range of 0.1 - 1 μM.[15]

A number of fused 6:6 bicyclic heterocycles displayed affinity at adenosine receptors. The K_i value of amfonelic acid, **61**, at the A_1 receptor was 25 μM. Amfonelic acid has been reported to induce seizures,[16] and at that time it was not realized that the same compound binds to adenosine receptors with an affinity similar to that of caffeine, another proconvulsant agent. 4H-Pyrido[1,2-a]pyrimidin-one, **62**, bound selectively to rat A_3 receptors with a K_i value of 48 μM. A phenyllumazine derivative, **64**, which could be considered an analogue of the A_1/A_2 antagonist 8-phenyltheophylline,[1] bound to A_1 receptors with a K_i value of 33 μM.

Various pteridine derivatives were shown to act as antagonists at adenosine receptors.[8] We have found that the presence of chained alkyl groups (*e.g.* isopropyl) at the 6- and 7- positions enhance the potency of binding to A_1 receptors in this series. Thus, compound **67** has a K_i value of 2.5 μM. Pteridine derivatives **68** and **69**, related to the anticancer and antiinflammatory drug methotrexate, displaced radioligand from A_3 receptors with K_i values of 48 μM. Another related heterocycle, dihydrofolic acid, **71**, bound to A_1 receptors with a K_i value of 34 μM, while curiously the more planar folic acid, **70**, itself, was inactive at A_1 or A_{2a} receptors. The adenosine uptake blocker dipyridamole, **72**, bound to A_3 receptors with a K_i value of 19 μM.

Surprisingly, several of the cytochalasins showed considerable affinity at adenosine receptors. Cytochalasin A, **76**, was the most potent of the series with a K_i value of 1.9 μM at A_1 receptors. Cytochalasin A caused an increase in the amount of [^{125}I]AB-MECA bound in membranes of A_3-transfected CHO cells (150±16% of control at 300 μM, n = 4). In the presence of 100 μM NECA, the level of radioligand binding was 59±8% of control, thus the additional binding likely occurs at a non-A3 receptor site. Cytochalasin B, **77**, displayed a K_i value of 27 μM at A_1 receptors. The other cytochalasins, **78-82**, were totally inactive in binding at A_1 receptors. None of the cytochalasins bound appreciably at A_{2a} receptors. Given the pharmacological difference

between cytochalasins A and B and their subtle differences in molecular structure, the region of the molecule which is responsible for binding to or modulating binding to the adenosine receptors is not apparent. The cytochalasins are macrocyclic compounds that are fungal metabolites, used as tools in cytological research and in characterization of polymerization properties of actin.[17]

A number of benzodipyrazole derivatives, **83** and **84**, studied previously at adenosine receptors[35] lacked affinity at A_3 receptors. A related compound, **85**, had a K_i of 56 μM at A_3 receptors.

Methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), **86**, a β-carboline derivative, displayed considerable affinity for adenosine receptors, with K_i values of 1.6 and 3.3 μM at A_1 and A_{2a} receptors, respectively. DMCM acts as a proconvulsant, by virtue of its activity as a benzodiazepine inverse agonist.[18] Previously β-carboline itself and several derivatives[8] were shown to have weak affinity at the A_1 receptor and to act as antagonists at that receptor, and this was proposed to be related to their proconvulsant activity. Since the potency of DMCM is even greater than other β-carbolines examined,[8] it becomes an even more relevant aspect of the biological effects. DMCM was used to induce seizures in a study of the anticonvulsant effect of adenosine agonists,[19] apparently without knowledge of its potency in adenosine antagonism. Another carbazole derivative, 11-hydroxytetrahydrocarbazolenine, **88**,[22] was A_3 selective in binding with a K_i value of 22 μM.

A series of *lin*-benzoxanthine derivatives was shown to have adenosine antagonist activity.[20] *lin*-Benzohypoxanthine, **91**,[21] formalistically an elongated derivative of unsubstituted hypoxanthine, showed considerable affinity at adenosine receptors, although it was nonselective. An imidazoquinazolinone derivative, **93**,[37] lacked affinity

at adenosine receptors. Another 6:6:5 fused tricyclic, compound **95**, a pyrazoloquinazoline derivative, showed an affinity of 4-5 μM at A_1 and A_{2a} receptors, with no displacement of radioligand from A_3 receptors.

Fused 6:6:6 tricyclic derivatives were also examined. Alloxazine, **96**, has been found to be 9-fold selective for A_{2b} (K_B 2.3 μM) vs A_{2a} receptors.[40] The naturally occurring flavins contain the same ring structure as alloxazine with an appended carbohydrate mioety. Riboflavin,[23] **98**, a vitamin and enzyme cofactor having an attached open chain ribose moiety, and roseoflavin, **97**, the homologous derivative with a shorter carbohydrate chain (C3), bound to A_1 receptors with K_i values of 13 and 29 μM, respectively, with no detectable binding at A_{2a} receptors. Unexpectedly, the binding of the A_3 receptor radioligand was dramatically enhanced (Figure 3), with 355±35% of control binding in membranes of A_3-transfected CHO cells at 100 μM riboflavin. Since the additional binding occurred also in the presence of 100 μM NECA (280±40% of control binding), it does not represent selective binding enhancement at A_3 receptors, as has been shown for benzoylthiophene derivatives (allosteric enhancers) at A_1 receptors.[24] Instead, riboflavin apparently causes enhanced binding of [^{125}I]AB-MECA to a nonreceptor site on the membranes. The nature of this site has not been explored, but perhaps it is related to an enzyme at which riboflavin acts as a cofactor. The adenosine conjugate FAD, **99**, was somewhat weaker than riboflavin in binding to A_1 receptors, yet bound with increased affinity at A_{2a} receptors. Fluorescein dye, **100**, also bound weakly to adenosine receptors with a K_i value of 76 μM at the A_1 subtype.

The antidepressant drug doxepin,[25] **102**, is a dibenzoxepin derivative. This compound showed a K_i value of 66 μM at A_1 receptors. Other psychotropic drugs, such as barbiturates, were previously shown to bind weakly to adenosine receptors.[26]

Functional assays at rat adipocyte A_1 receptors were carried out (Table 2, and Figures 3 and 4). The compounds that bound with highest affinity were examined for the

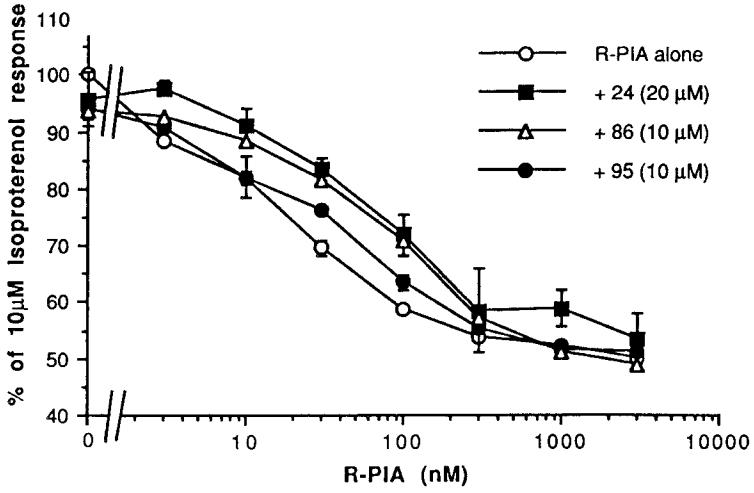

Fig. 3. Effects on A1-agonist-induced inhibition of adenylyl cyclase in rat adipocyte membranes.

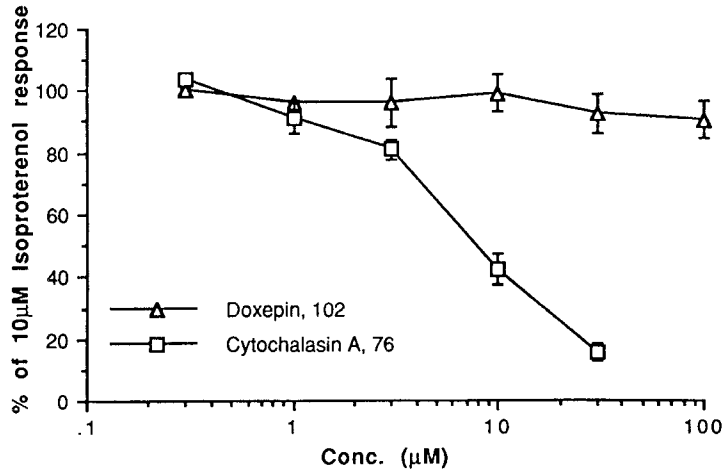

Fig. 4. Inhibition of adenylyl cyclase in rat adipocyte membranes.

ability to antagonize the inhibition of adenylyl cyclase elicited by *R*-PIA. It was found that DMCM, **86**, and the pteridine derivative **67**, were the most potent antagonists, with micromolar K_B values. The cyclized histidine derivative (a 5-oxoimidazopyrimidine) **24**, the pyrazoloquinazoline **95**, the pyrazolopyridine **18**, and the phenyllumazine derivative **64** were also weak antagonists. *lin*-Benzohypoxanthine, **91**, slightly antagonized the effects of *R*-PIA. Cytochalasin A, **76** (10 μM), shifted the *R*-PIA dose response curves to the left and alone inhibited adenylyl cyclase in the adipocyte membranes with an IC_{50} of 6 μM (Figure 4). However, the inhibition by cytochalasin A was not reversed in the presence of the selective A_1-antagonist 1,3-dipropyl-8-cyclopentylxanthine, 1 μM (data not shown). Thus, cytochalasin A appears to have a direct inhibitory effect not mediated through A_1 receptors. Doxepin, **102** (50 μM), clearly shifted the *R*-PIA dose response curve to the left, however alone did not inhibit adenylyl cyclase. Thus doxepin is not an agonist at A_1 receptors, but it may act to enhance agonist-induced effects. If so, it is conceivable that this may occur at an allosteric site. Dihydrofolic acid, **71**, produced a slight left shift of the *R*-PIA dose response curve, but **71** alone had no effect on adenylyl cyclase in adipocyte membranes.

Conclusions

A selective antagonist at the rat A_3 receptor is lacking. Several xanthine analogs that act as potent antagonists at rat, rabbit, and human A_1 and A_2 receptors only weakly displaced the binding of radioligand from cloned rat A_3 receptors.[27] The present study has not identified any effective A_3 antagonist in the rat, however, it has provided leads for future structural modification.

Sulfonylpiperazines, **52-58**, and cytochalasin H and J, **81** and **82**, respectively, bind to A_3 receptors without binding to the other subtypes. Moreover, compounds **2**, **6**, **25**, **62**, **68**, **70**, **72**, and **88** also were somewhat selective in binding to A_3 receptors.

The pteridine derivative, **67**, was identified as a slightly A_1 selective (binding) antagonist. Compounds **26**, **76**, **77**, **97**, and **98** were also weakly A_1 selective. Compounds **11**, **43**, **75**, and **89** were identified as slightly A_{2a} selective ligands.

The most significant new findings are the discovery of several new classes of adenosine antagonists (*e.g.* 5-oxoimidazopyrimidines and a pyrazoloquinazoline), and that nonpurine heterocycles (*e.g.* **68** at A_3 receptors and **76** at A_1 receptors) inhibit adenylyl cyclase, possibly through activation of adenosine receptors. It will be necessary to explore the mechanism of the inhibition of adenylyl cyclase, since action at the P site would also have this effect.[39] Previously it was observed[1] that only purine nucleosides were known to activate adenosine receptors.

Experimental

Compound **91** was synthesized as described by Leonard and coworkers.[21]

Cell culture and radioligand binding

CHO cells stably expressing the A_3 receptor[9,28] were grown in F-12 medium containing 10% FBS and penicillin/streptomycin (100 U/mL and 100 μg/mL respectively) at 37 °C in a 5% CO_2 atmosphere, and membrane homogenates were prepared as reported.[28]

Binding of [^{125}I]4-amino-3-iodobenzyladenosine-5'-N-methyluronamide ([^{125}I]-AB-MECA) to the CHO cell membranes was performed as described.[29] Assays were performed in 50/10/1 buffer in glass tubes and contained 100 μL of the membrane suspension, 50 μL of [^{125}I]AB-MECA (final concentration 0.3 nM), and 50 μL of inhibitor. Inhibitors were routinely dissolved in DMSO and were then diluted with buffer;

final DMSO concentrations never exceeded 1%. Incubations were carried out in duplicate for 1 hour at 37 °C, and were terminated by rapid filtration over Whatman GF/B filters, using a Brandell cell harvester (Brandell, Gaithersburg, MD). Tubes were washed three times with 3 mL of buffer. Radioactivity was determined in a Beckman gamma 5500B counter. Nonspecific binding was determined in the presence of 40 μM *R*-PIA. K_i-values were calculated according to Cheng-Prusoff,[30] assuming a K_d for [^{125}I]AB-MECA of 1.55 nM.[29]

Binding of [^{3}H]PIA (Amersham, Arlington Heights, IL) to A_1 receptors from rat brain membranes and of [^{3}H]CGS 21680 (DuPont NEN, Boston MA) to A_{2a} receptors from rat striatal membranes was performed as described previously.[31, 32] Adenosine deaminase (3 U/mL) was present during the preparation of brain membranes, in which an incubation at 30°C for 30 min was carried out, and during the incubation with radioligand. At least six different concentrations spanning three orders of magnitude, adjusted appropriately for the IC_{50} of each compound, were used. The IC_{50} values that were computer-generated using a nonlinear regression formula on the InPlot program (GraphPAD, San Diego CA), were converted to apparent K_i values using K_d values of 1.0 and 14 nM for [^{3}H]PIA and [^{3}H]CGS 21680 binding, respectively, and the Cheng-Prusoff equation.[30]

Adenylyl cyclase measurements in rat adipocyte membranes and in A_3-transfected CHO cells were carried out as described.[8,28]

Acknowledgments:

We thank the Cystic Fibrosis Foundation (N.M.), Gilead Sciences of Foster City, CA (M.G.) for financial support. We thank Mary Pound for technical assistance. G.L.S. is supported by NHLBI Grant RO1HL35134 from the National Institutes of Health. In addition, we thank Dr. Norton Peet (Marion Merrell Dow Research Inst., Cincinnati OH),

Professor W. Pfleiderer (University of Konstanz, Germany), Professor Peter Bridson (University of Memphis, TN), Professor Richard Glennon (Medical College of Virginia, Richmond), Dr. Christa Müller and Prof. Kurt Eger (Universität Tübingen, Germany), and Dr. Bernhard Witkop (NIH) for the gift of compounds.

Abbreviations

AB-MECA, N^6-(4-amino-3-iodobenzyl)adenosine-5'-N-methyluronamide;

CGS 21680, 2-[4-[(2-carboxyethyl)phenyl]ethyl8amino]-5'-N-ethylcarboxamido-adenosine;

CHO, Chinese hamster ovary;

DMCM, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate;

DMSO, dimethylsulfoxide;

PIA, *R*-N^6-phenylisopropyladenosine;

Tris, tris(hydroxymethyl)aminomethane.

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