Tetrahydrobenzothiophenone Derivatives as a Novel Class of Adenosine Receptor Antagonists†

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A novel class of non-nitrogen-containing heterocycles, the tetrahydrobenzothiophenones, was found to bind to adenosine receptors as antagonists in the micromolar range. Affinity was determined in radioligand-binding assays at rat brain A_1 and A_{2a} receptors. A structure−activity analysis indicated that a 3-thioether group is favored and affinity at A_{2a}, but not at A_1, receptors is highly dependent on this thioether substituent. A carboxylic acid-derived substituent is required at the 1-position of the thiophene ring, with esters being more potent in binding at A_1 receptors than the corresponding carboxyl hydrazide or carboxylic acid derivatives. The methyl (**15**) and ethyl (**16**) esters are about equipotent at A_1 but not at A_{2a} receptors. A 4-keto group on the saturated ring is favored for receptor affinity. Dimethyl substitution at the 6-position of the saturated ring is allowed. One of the most potent derivatives was the nonselective compound ethyl 3-(benzylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (BTH_4, **7**; Figure 1), which antagonized adenosine agonist-induced inhibition of adenylyl cyclase in rat adipocyte membranes with a K_B value of 1.62 ± 0.73 μM and adenosine agonist-induced stimulation of adenylyl cyclase in pheochromocytoma cell membranes with a K_B value of 9.19 ± 0.98 μM. Displacement of radioligand binding by BTH_4 (**7**) at cloned human A_3 receptors was negligible, but one slightly A_3 selective compound (**11**, 3.9-fold over A_1 and >7.5-fold over A_{2a}) was found. A 1-methylpropyl thioether (**17**) was 29-fold selective for A_1 vs A_{2a} receptors. BTH_4 (**7**) alone, at 10 mg/kg, stimulated locomotor activity in mice but paradoxically acted, under certain circumstances, synergistically with an A_1 selective agonist to depress locomotor activity. A pharmacophore model relating structural features of xanthine and non-xanthine adenosine antagonists to BTH_4 (**7**) suggests a high degree of similarity in electrostatic surfaces, assuming that the thiophene ring superimposes the region of the uracil ring of xanthines.

Introduction

Adenosine receptors are involved in many peripheral and central regulatory mechanisms, including vasodilatation[1] and vasoconstriction in the kidney,[2] inhibition of lipolysis[3] and insulin release,[4] inhibition of neurotransmitter release,[5] and moderation of cerebral ischemia.[6−8] Four subtypes of adenosine receptors (*i.e.*, A_1, A_{2a}, A_{2b}, and A_3) have been identified, both pharmacologically and through cloning techniques.[6,9] The actions of adenosine on A_1 and A_{2a} adenosine receptors are readily antagonized by potent and selective xanthine-based antagonists, but the A_3 adenosine receptor subtype, however, seems to be less susceptible to blockade by these compounds.[10]

A number of classes of non-xanthine adenosine antagonists (Figure 1),[6,11] including triazoloquinazolines (*e.g.*, CGS15943),[12] 9-methyladenines,[13] pyrazolotriazolopyrimidines (*e.g.*, SCH58261),[14] and triazolotriazines (*e.g.*, ZM241385),[15] have been found. Many of the non-xanthine antagonists are relatively nonselective, although selectivity for A_1 receptors[13,16−18] or A_2 receptors[12,14,15] has been achieved. Nearly all of the non-xanthine derivatives previously found to act as adenosine antagonists have been nitrogen-containing heterocycles. There were only two reports of non-nitrogen-containing natural products:[19,20] the protein tyrosine kinase inhibitor genistein and a benzofurancarbaldehyde derivative (Figure 1), which bound to A_1 receptors with K_i values of 5 μM and 17 nM, respectively. In our ongoing effort to provide selective ligands for adenosine receptors, especially those with affinity at A_3 receptors,[21,22] we have screened 110 cyclic compounds for affinity at adenosine receptors,[23] resulting in the identification of previously unknown classes of adenosine ligands.

In the present study we have identified a novel class of non-nitrogen-containing derivatives of tetrahydrobenzothiophenone as non-xanthine inhibitors of binding of agonists to adenosine receptors. Moreover, we have investigated the functional effects of one of the more potent analogues (ethyl 3-(benzylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate, BTH_4, **7**) as an adenosine antagonist and have explored its behavioral properties. Although the compounds bear little structural resemblance to xanthines, using molecular modeling we have discovered a possible electronic basis for molecular recognition of these compounds as adenosine receptor antagonists.

† Abbreviations: [^{125}I]AB-MECA, [^{125}I]-N^6-(4-amino-3-iodobenzyl)-adenosine-5′-*N*-methyluronamide; BTH_4, ethyl 3-(benzylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate; CGS21680, 2-[[[4-(2-carboxyethyl)phenyl]ethyl]amino]-5′-(*N*-ethylcarbamoyl)-adenosine; CHA, N^6-cyclohexyladenosine; CHO cells, Chinese hamster ovary cells; CP66713, 9-chloro-2-phenyl[1,3,4]triazolo[5,1-*c*]quinazolin-5-amine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; K_B, dynamic inhibition constant; K_i equilibrium inhibition constant; NECA, *N*-(ethylcarbamoyl)adenosine; PIQA, 2-phenyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine; (*R*)-PIA, (*R*)-N^6-(phenylisopropyl)adenosine; Tris, tris(hydroxymethyl)aminomethane; XAC, 8-[4-[[[[(2-aminoethyl)amino]-carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine, xanthine amine congener.

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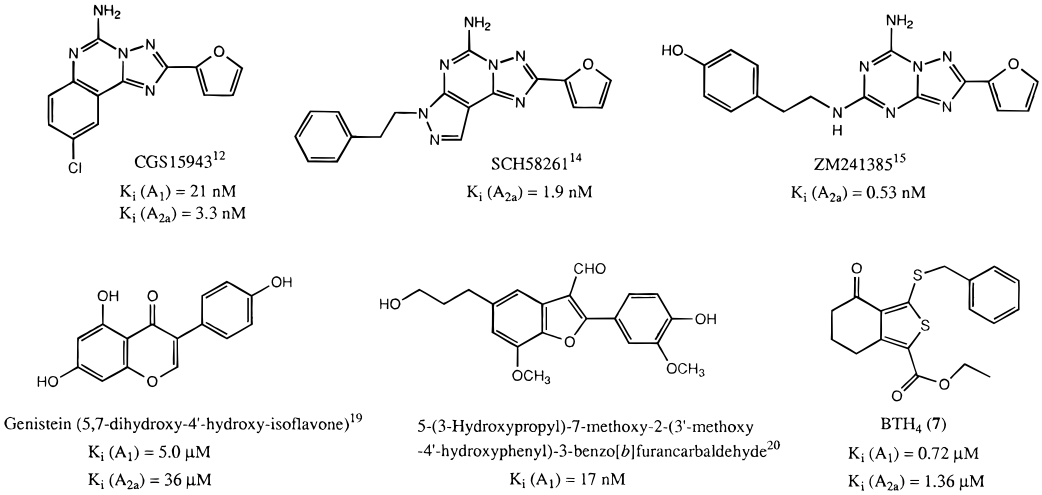


Figure 1. Non-xanthine antagonists of adenosine receptors.

Results and Discussion

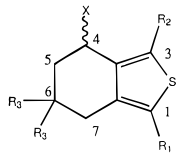
Affinity at Adenosine Receptors. The tetrahydrobenzothiophenone 3-thioether derivatives **1**−**20** bound to rat A_1 and A_{2a} adenosine receptors in the micromolar range (Table 1A). BTH_4 (**7**) had one of the highest affinities among the analogues at both A_1 and A_{2a} subtypes. Compound **18** had an affinity at A_1 receptors that was comparable to that of BTH_4 (**7**) but showed a considerably lower affinity at A_{2a} receptors. BTH_4 (**7**) was nonselective, with K_i values at A_1 and A_{2a} receptors in brain membranes (determined with [^{3}H]-(*R*)-PIA and [^{3}H]CGS21680, respectively, to facilitate comparison with earlier work[24,25]) of 0.72 and 1.4 μM, respectively. K_i values for compounds **1**, **6**−**8**, **11**, **14**, and **19** at rat or human A_3 adenosine receptors were determined by displacement of [^{125}I]AB-MECA from either rat A_3 receptors expressed in CHO cells or human A_3 receptors expressed in HEK-293 cells (Table 1B).[26,39,40] Contrary to earlier findings with xanthine-based antagonists, displaying a higher affinity for human than for rat A_3 receptors, BTH_4 derivatives were 2−3-fold more potent at rat A_3 receptors than at human A_3 receptors.[39] In general there was a tendency toward A_1 selectivity, in particular for compounds **17** and **18**, with A_1 vs A_{2a} selectivities of 29- and >17-fold (estimated), respectively. Out of 20 compounds determined, there was only one compound that showed any degree of A_{2a} selectivity (**14**, 3.3-fold over A_1 and 2.8-fold over A_3) and one compound that showed some degree of A_3 selectivity (**11**, 3.9-fold over A_1 and >7.5-fold over A_{2a}). Compound **11** was structurally unique in this series having a double bond between positions 4 and 5 of the benzo ring as shown in Table 1.

The effects of substitutions of the thiophene ring on receptor binding affinity were studied. A 1-carboxyl group appears necessary for affinity, as evidenced by the inactivity in binding of the 1-unsubstituted aryl-H analogue **12**. Furthermore, substitution of an alkyl ester group with a 1-carboxylic acid hydrazide decreased affinity at both A_1 and A_{2a} receptors (**8** vs **10**). Among the esters, the methyl ester **15** was nearly equipotent with the ethyl ester **16** in binding at A_1 and A_{2a} receptors, whereas the ethyl ester **16** showed a preference for A_1 receptors (8.9-fold). At A_1 receptors, a nonpolar derivative of the carboxylic acid was preferred, since conversion of the ethyl 1-carboxylate (**7**) to a 1-carboxylic acid (**14**) yielded a 70-fold decrease in affinity at A_1 receptors but only an 11-fold decrease in affinity at A_{2a} receptors, resulting in the only A_{2a} selective compound in this series (**14**). The other 1-carboxylic acid in this series (**13**) had a very low affinity and was slightly A_1 selective (vs A_{2a}). Among the derivatives examined, a thioether appeared to be favored at the 3-position, since the 3-sulfone derivative **19** displayed a much lower affinity at A_1 receptors and was nearly inactive at A_{2a} receptors. The 3-hydrazino derivative **20** was less potent than the benzylthio compound **18** at A_1 receptors (31-fold) but more potent than compound **18** at A_{2a} receptors, resulting in loss of selectivity of the compound. The affinities of some *S*-alkyl, *S*-alkenyl, and *S*-benzyl 3-thioethers were compared. A_1 receptor binding affinity was largely insensitive to alkyl (both saturated and unsaturated) or aryl variation at the 3-thio position (**1**−**7**). A_{2a} receptor affinity was more dependent on the 3-substituent, and aromatic substitution enhanced the affinity slightly, as in the *S*-benzyl analogue **7**.

The effects of substitutions of the tetrahydrobenzo ring on receptor binding affinity were also studied. Alkyl substitution at the 6-position of the saturated ring was allowed. The 6,6-dimethyl substitution was tolerated and, when combined with the *S*-benzyl 3-thioether, favored A_1 selectivity (**18** vs **7**) by reducing the affinity for the A_{2a} receptor. For both A_1 and A_{2a} receptors, a 4-keto group was necessary for high affinity. When the keto group was reduced to a hydroxyl group (**8** and **10**) or was absent, as in the case of the unsaturated hydrocarbon (**11**), the affinity was ca. 10-fold lower at both A_1 and A_{2a} receptors. However, affinity for the rat A_3 receptor was retained (Table 1B), resulting in the only A_3 selective compound of the series. When the keto group was converted to a methoxyimino group (**9**), affinity at A_1 and A_{2a} receptor subtypes was decreased by an even greater degree.

Effects of BTH_4 (7) on A_1 and A_{2a} Receptor-Coupled Adenylyl Cyclase. BTH_4 (**7**) antagonized the agonist-elicited ((*R*)-PIA) inhibition of isoproterenol-stimulated adenylyl cyclase assay in rat adipocytes expressing A_1 adenosine receptors,[11,27] shifting the dose−response curve of (*R*)-PIA to the right (Figure 2A). A K_B value of 1.62 ± 0.73 μM (n = 3) was calculated using the Schild equation K_B = C/(CR − 1), where C denotes the concentration of the competitor BTH_4 and CR is the ratio of the EC_{50} values in the presence and absence of competitor, respectively. BTH_4 (**7**) also

Table 1



and R_3 = H, unless noted[h]

A. Affinities of Tetrahydrobenzothiophenone Derivatives in Radioligand-Binding Assays at A_1 and A_{2a} Adenosine Receptors

				K_i (μM) or percent inhibition[c]		
compd	R_1	R_2	X	A_1[a]	A_{2a}[b]	A_{2a}/A_1
1	$CO_2CH_2CH_3$	SCH_3	=O	1.93 ± 0.21	3.66 ± 1.19	1.9
2	$CO_2CH_2CH_3$	$S(CH_2)_2CH_3$	=O	1.26 ± 0.30	4.69 ± 1.09	3.7
3	$CO_2CH_2CH_3$	$SCH(CH_3)_2$	=O	1.79 ± 0.50	4.63 ± 0.15	2.6
4	$CO_2CH_2CH_3$	$SCH_2CH{=}CH_2$	=O	1.06 ± 0.12	6.04 ± 0.44	5.7
5	$CO_2CH_2CH_3$	$S(CH_2)_3CH_3$	=O	1.42 ± 0.27	15.6 ± 2.3	11.0
6	$CO_2CH_2CH_3$	$SCH_2CO_2CH_2CH_3$	=O	2.22 ± 0.39	25.6 ± 6.8	11.5
7	$CO_2CH_2CH_3$	SCH_2Ph	=O	0.715 ± 0.116	1.36 ± 0.25	1.9
8	$CO_2CH_2CH_3$	SCH_3	OH, H	18.3 ± 1.9	33.5 ± 7.0	1.8
9	$CO_2CH_2CH_3$	SCH_3	$=NOCH_3$	69.8 ± 9.4	22 ± 5% (10^{-4})	>1.4
10	$CONHNH_2$	SCH_3	OH, H	71.2 ± 7.5	28.9% (10^{-4})	>1.4
11	$CONHNH_2$	SCH_3	H[d]	51.6 ± 6.8	*c* (10^{-4})	>1.9
12[e]	H	SCH_3	=O	28 ± 7% (10^{-4})	*c* (10^{-4})	>1
13[e]	COOH	SCH_3	=O	69.4 ± 10.7	15 ± 5% (10^{-4})	>1.4
14	COOH	SCH_2Ph	=O	50.0 ± 2.3	15.2 ± 5.6	0.3
15[e]	CO_2CH_3	SCH_2CH_3	=O	3.34 ± 0.70	5.64 ± 0.85	1.7
16[e]	$CO_2CH_2CH_3$	SCH_2CH_3	=O	2.69 ± 0.72	23.9 ± 2.6	8.9
17[e]	$CO_2CH_2CH_3$	$SCH(CH_3)CH_2CH_3$	=O	3.64 ± 0.84	106 ± 23	29.1
18[e]	$CO_2CH_2CH_3$	SCH_2Ph	=O	0.567 ± 0.139	34 ± 2% (10^{-5})	>17.6
19[e]	$CO_2CH_2CH_3$	SO_2CH_3	=O	32.7 ± 2.3	13% (10^{-4})	>3.1
20[e]	$CO_2CH_2CH_3$	$NHNH_2$	=O	17.5 ± 2.2	36.1 ± 5.1	2.1

B. Affinities of Selected Tetrahydrobenzothiophenone Derivatives in Radioligand-Binding Assays at Human and Rat A_3 Adenosine Receptors[f]

compd	human A_3	rat A_3	human A_3/rat A_3	rat A_3/rat A_1
1	nd	15.2 ± 4.9		7.88
6	nd	10.2 ± 1.5		4.59
7	24%[g]	nd		
8	35.8 ± 0.5	15.4 ± 7.0	2.32	0.84
11	nd	13.1 ± 3.0		0.25
14	117 ± 13	42.0 ± 12.5	2.79	0.84
19	nd	51.8 ± 16.4		1.58

[a] Displacement of specific [^{3}H]PIA binding in rat brain membranes, expressed as K_i ± SEM in μM (n = 3−5). [b] Displacement of specific [^{3}H]CGS21680 binding in rat striatal membranes, expressed as K_i ± SEM in μM (n = 3−6). [c] Displacement <10% of specific binding at concentration indicated. [d] C=C double bond at positions 4 and 5. [e] $R_3 = CH_3$. [f] Displacement of specific [^{125}I]AB-MECA binding in CHO cell membranes transfected with rat A_3 cDNA or HEK-293 cell membranes transfected with human A_3 cDNA. Values are expressed as K_i ± SEM in μM (n = 3). [g] Percentage displacement of specific binding at 10^{-4} M. [h] Systematic name differs from numbering scheme for compounds **11** and **12**. nd = not determined.

attenuated agonist-elicited (NECA) stimulation of adenylyl cyclase activity in pheochromocytoma cells, PC12 cells, expressing A_{2a} adenosine receptors (Figure 2B). The K_B value calculated from the Schild equation was 9.19 ± 0.98 μM (n = 3). These experiments demonstrate that the tetrahydrobenzothiophenones are functional antagonists at both A_1 and A_{2a} adenosine receptors.

Effect of BTH_4 (7) on Locomotor Activity. Open field locomotor effects in mice were studied in a computerized monitor by standard methods.[28] The effects of the nonselective compound **7** (BTH_4) alone or in combination with N^6-cyclohexyladenosine (CHA) were examined (Figure 3). The potent and A_1 selective adenosine agonist was chosen to maintain consistency throughout a series of publications.[27,28] For comparison with BTH_4 (**7**), the weak, nonselective adenosine antagonist caffeine was used.[29] The behavioral effects of BTH_4 (**7**) were subtle, with a modest stimulation of locomotor activity observed (Figure 3A). BTH_4 (**7**) affected locomotor activity in mice only when administered intraperitoneally at 10 mg/kg (1.4-fold increase; $p < 0.02$), indicating a narrow pharmacological bandwidth. At the highest dose tested (30 mg/kg), BTH_4 (**7**) had no effect on locomotor activity. A similar dose−response relationship, although over a broader dose range, is known for caffeine,[29] which at high doses (≥50 mg/kg) in mice either does not stimulate or even depresses locomotor activity. At 10 mg/kg, caffeine causes a marked locomotor stimulation (Figure 3A).

The stimulation by caffeine was evident even when it was coadministered with the A_1 selective agonist CHA (Figure 3B,C). Unexpectedly, a dose of 10 mg/kg BTH_4 (**7**), unlike caffeine or the selective A_1 antagonist 1,3-dipropyl-8-cyclopentylxanthine,[28] not only failed to block the locomotor depression elicited by 100 μg/kg CHA but rather caused a slight statistically significant potentiation of this locomotor depression ($p < 0.02$; Figure 3B).

To further investigate this phenomenon, we reversed the order of administration of CHA and BTH_4 (**7**; Figure 3C). When BTH_4 (**7**) was administered 10 min prior to a single dose of 100 μg/kg CHA, the potentiation was observed for all three doses of BTH_4 (**7**; $p < 0.02$). However, when BTH_4 (**7**) was administered 10 min after CHA, potentiation of locomotor depression by CHA was observed only at 30 mg/kg BTH_4 (**7**; $p < 0.02$). Furthermore, reversal of the order of administration did not

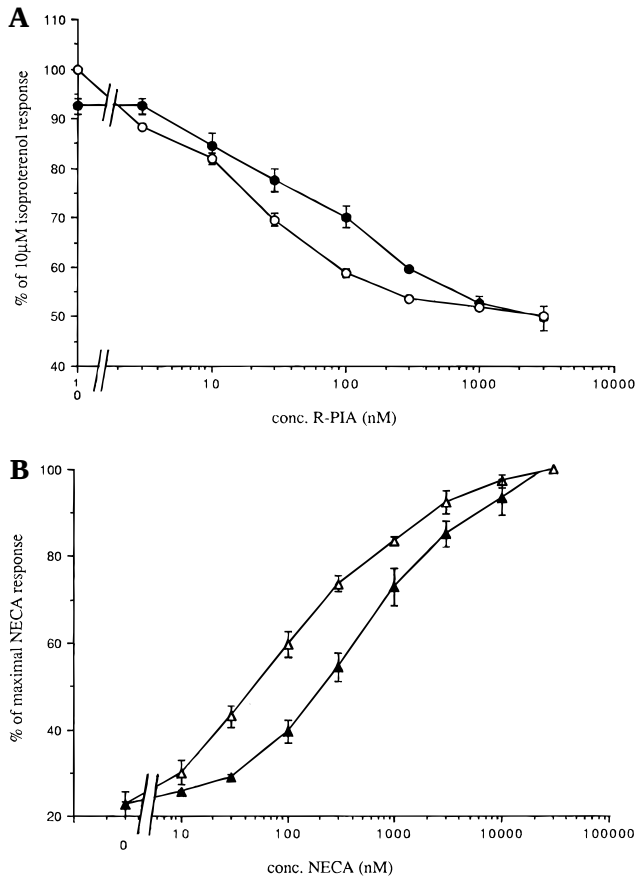


Figure 2. (A) Effects of BTH_4 (**7**) on agonist-elicited inhibition of adenylyl cyclase via rat A_1 receptors in adipocyte membranes (mean ± SEM for $n = 3$): (○) (*R*)-PIA alone and (●) (*R*)-PIA + 3 μM BTH_4 (**7**). (B) Effects of BTH_4 (**7**) on agonist-elicited stimulation of adenylyl cyclase via A_{2a} receptors in PC12 cell membranes (mean ± SEM for $n = 3$): (△) NECA alone and (▲) NECA + 30 μM BTH_4 (**7**).

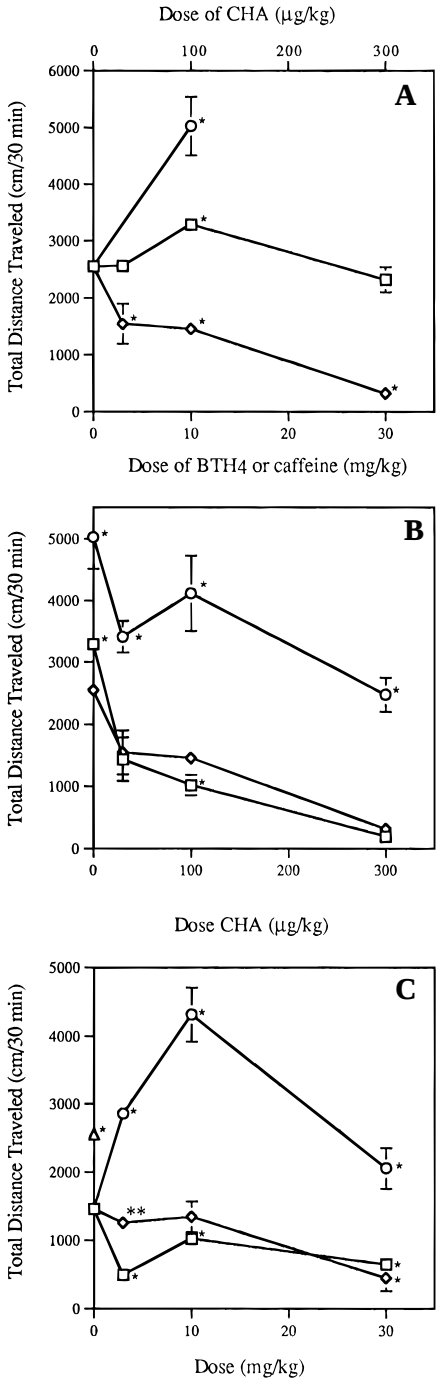


Figure 3. (A) Locomotor effects of BTH_4 (**7**), caffeine, and CHA: (□) BTH_4 (**7**), (○) caffeine, and (◇) CHA (an asterisk indicates significantly different from vehicle at $p < 0.02$; mean ± SEM of at least 4 and maximally 14 experiments). (B) Locomotor effects of 10 mg/kg BTH_4 (**7**) and 10 mg/kg caffeine administered 10 min prior to varying doses of CHA: (□) BTH_4 (**7**), (○) caffeine, (◇) CHA alone (an asterisk indicates significantly different from CHA alone at $p < 0.02$; mean ± SEM of at least 4 and maximally 13 experiments). (C) Locomotor effects of BTH_4 (**7**) and caffeine coadministered with 100 μg/kg CHA: (△) vehicle, (□) BTH_4 (**7**) 10 min prior to CHA, (◇) CHA 10 min prior to BTH_4 (**7**), and (○) CHA 10 min prior to caffeine (an asterisk indicates significantly different from a single dose of 100 μg/kg CHA at $p < 0.02$, and double asterisks indicate significantly different from reverse order of administration at $p < 0.02$; mean ± SEM of at least 3 and maximally 14 experiments).

yield a significant change in the locomotor activity of a dose of 10 mg/kg caffeine ($p < 0.02$; Figure 3B,C).

The differences between the results of the various protocols suggest that rapid metabolism may play a role in the *in vivo* effects of BTH_4 (**7**). Although the observed potentiation is statistically significant, considering the extent of the potentiation and the possible involvement of metabolic processes therein, the biological significance of this effect is unclear. Apart from the potentiation under specific circumstances, the characteristics of BTH_4 (**7**) are consistent with those of a typical nonselective antagonist and may therefore be mediated by either the A_1 or A_{2a} adenosine receptor subtype, or a combination thereof.

Affinity at Other Binding Sites. Since BTH_4 (**7**) was one of the more potent compounds of the series (both at A_1 and A_{2a} receptors) and since it displayed unusual behavioral properties, its affinity at non-adenosine receptor binding sites was examined in a battery of radioligand-binding assays (NovaScreen, Div. of Oceanix Biosciences, Hanover, MD).[30] At a concentration of 10^{-5} M, there was no significant (0 ± 25%) displacement of radioligand from adrenergic (α_1, α_2, and β), cholinergic (nicotinic and muscarinic M_1, M_2, M_3, M_4, and M_5), dopaminergic (D_1 and D_2), serotoninergic (5-HT_1 and 5-HT_2), central benzodiazepine (RO 151788), $GABA_A$ (muscimol), $GABA_B$ (baclofen), NMDA, kainate, quisqualate, glycine (strychnine sensitive and insensitive), σ (MK-801), angiotensin (AT-II), substance P (NK1), substance K (NK2), vasopressin V_1, neuropeptide Y, cholecystokinin (central and peripheral), neurotensin,

somatostatin, ANF1, and EGF receptors. There was also no significant displacement of binding of radioligand from second-messenger sites (forskolin, phorbol ester, and inositol trisphosphate), ion channels (N-, T-, and L-type calcium channels, chloride channels, and low-conductance potassium channels), and uptake sites (dopamine, norepinephrine, serotonin, choline, and adenosine). The observation that significant affinity was not demonstrated at any of these receptors or sites, including the (NBTI sensitive) adenosine transporter, emphasizes the selectivity of BTH_4 (**7**) for adenosine receptors.

Pharmacophore Modeling. The present series of tetrahydrobenzothiophenones was selected from a commercial library for screening at adenosine receptor binding, based on the occurrence of fused 5:6-membered rings, as are present in xanthines.[23] However, in xanthines, as in nearly all non-xanthine adenosine antagonists discovered to date, there are multiple nitrogen atoms present in the heterocyclic rings. The tetrahydrobenzothiophenones bind to A_1, A_{2a}, and A_3 adenosine receptors, in spite of the lack of nitrogen atoms. Therefore, it was of interest to determine a hypothesis for a common mode of binding of the tetrahydrobenzothiophenones and xanthines in the receptor-binding site based on molecular modeling. Models for the binding of antagonists to A_1 adenosine receptors have been proposed earlier by Francis *et al.*,[31] van Galen *et al.*,[32,33] Jacobson *et al.*,[6] and more recently by van der Wenden *et al.*[34] By modeling the electrostatic potential functions of the previously unknown class of tetrahydrobenzothiophenone adenosine antagonists and comparing these with adenosine antagonists previously described in the literature,[6,32−34] we were able to offer a rationale for the unexpected antagonistic properties of these compounds.

The steric and electronic properties of xanthines, the non-xanthine adenosine antagonists CP66713 and 2-phenyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (PIQA), and BTH_4 (**7**) were calculated using the MOPAC semiempirical program and the MNDO parametrization.[6,32,33] Calculations for caffeine, BTH_4 (**7**), CP66713, and PIQA were also performed *ab initio* with the MC-311G basis set, which was specifically developed for second-row atoms. Although slightly different numerical results were obtained with the MNDO semiempirical and the MC-311G *ab initio* methods, for the purpose of qualitatively comparing electrostatic surfaces the results were identical. An examination of the van der Waals surfaces of xanthines, such as the A_1 selective high-affinity xanthine-based antagonist XAC (xanthine amine congener), and the low-affinity nonselective xanthine-based antagonist caffeine, in comparison with the surfaces of a relatively active member of the tetrahydrobenzothiophenone class (BTH_4, **7**), failed to reveal any similarity other than the fused 5:6-membered ring system (Figure 4, top). It has been argued earlier that A_1 adenosine receptor agonists and antagonists alike bind to the receptor due to a common electrostatic potential profile.[6,32−34] We, therefore, calculated the electrostatic contours of the potent and A_1 selective xanthine-derived antagonist XAC ($K_i(A_1) = 1.2$ nM; $K_i(A_{2a}) = 60$ nM),[6] the moderately potent and A_{2a} selective non-xanthine antagonist CP66713 ($K_i(A_1) = 270$ nM; $K_i(A_{2a}) = 21$ nM),[6] the moderately potent and A_1 selective non-xanthine antagonist PIQA ($K_i(A_1) = 34$ nM; $K_i(A_{2a}) = 290$ nM),[33] and the novel nonselective non-xanthine antagonist BTH_4 (**7**; $K_i(A_1) = 715$ nM; $K_i(A_{2a}) = 1360$ nM). Electrostatic contours were calculated for the 1, 5, and 10 kcal/mol significance levels. Increasing the cutoff potential led to contraction, and a more narrow allocation to the points indicated in Figure 5, of the isoelectric surfaces. To emphasize the overall similarity, rather than exact atomic matches, and to compare our results with those of others,[6,32−34] we chose the 5 kcal/mol significance level for this study. The electrostatic contours of all compounds bore a general semblance to each other, as detailed in the next section.

The molecular electrostatic potential map (Figure 4, bottom) identifies five points that show a high degree of similarity in all structures, and XAC and BTH_4 (**7**) more specifically. The first two points (marked a and b in Figure 5) are the carbonyl oxygens at positions 2 and 6 in XAC, respectively. The second set of two points (marked c and d) designate the 1- and 3-propyl alkyl regions of XAC, respectively. The fifth point (marked e) identifies the center of the aromatic substituent at the 8-position in XAC. Equivalent positions in BTH_4 (**7**), CP66713, and PIQA are indicated in Figure 5. The sixth point (marked f), which designates a favorable additional substitution site in XAC and is absent in BTH_4 (**7**), its analogues, and the reference compounds CP66713 and PIQA, is the primary amine at the terminal of the 8-substituent. A semblance in electronic properties was demonstrated earlier for various other non-xanthine adenosine antagonists[6,32,34] and led to the development of new classes of antagonists.[31,33] We propose here that the novel antagonist BTH_4 (**7**) also fits this model.

A high degree of similarity in the electrostatic surfaces of XAC and BTH_4 (**7**) assumes that the thiophene ring roughly superimposes on the region of the uracil ring of xanthines rather than a superpositioning of the 5-membered thiophene and imidazole rings. There are regions of partial negative charge surrounding the two carbonyl groups of BTH_4 (**7**), which correspond spatially to isolated regions of partial negative charge around the two carbonyls of xanthines.[6,32−34] Thus, the keto group of the tetrahydrobenzo ring would occupy the same position as the 6-position carbonyl group of xanthines. The carbonyl of the 1-carboxylic acid ester group, although it does not occur in a ring, occupies the same position as the 2-position carbonyl group of xanthines. To accommodate the presence of the tetrahydrobenzo ring, the 6,6-dimethyl groups present in some analogues, and the ester function into a generalized model, we selected the high affinity A_1 selective antagonist XAC, which is substituted with 1,3-dipropyl and 8-aryl groups, for further examination. A positively charged region around the 3-propyl group of XAC[6,32−34] overlays the 1-ethyl ester group of BTH_4 (**7**); thus, the alkoxy moiety of this ester substituent appears to correspond to the 3-alkyl group of xanthines. The conformationally constrained $(CH_2)_3$ portion of the tetrahydrobenzo ring may mimic the flexible 1-propyl group of XAC. In xanthines, a variety of alkyl and aryl substitutions at the 1- and 3-positions are tolerated.[6,32−34] The corresponding groups of BTH_4 (**7**) can be similarly substituted without loss of affinity. Consequently, the 1-ester group may consist of either a methyl (**15**) or an ethyl

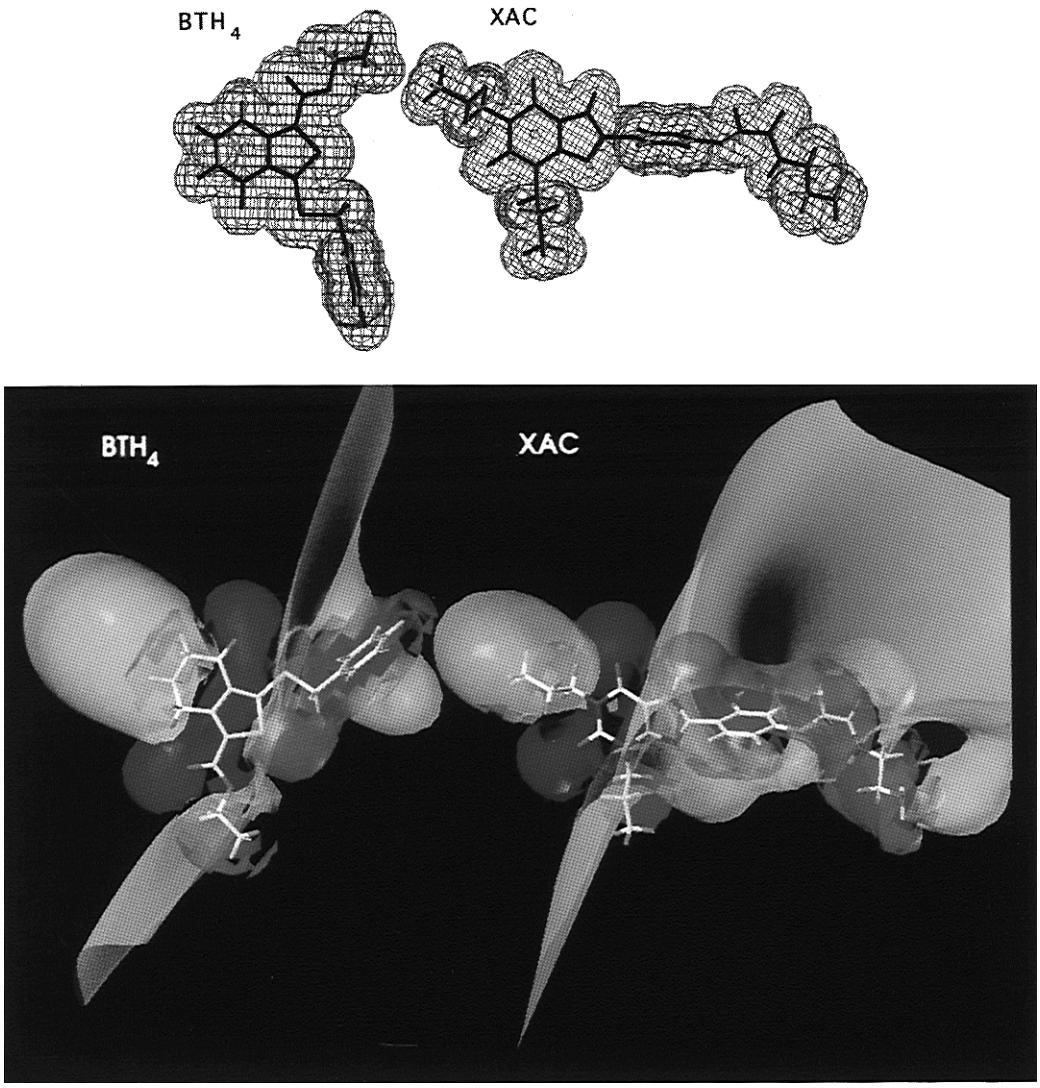
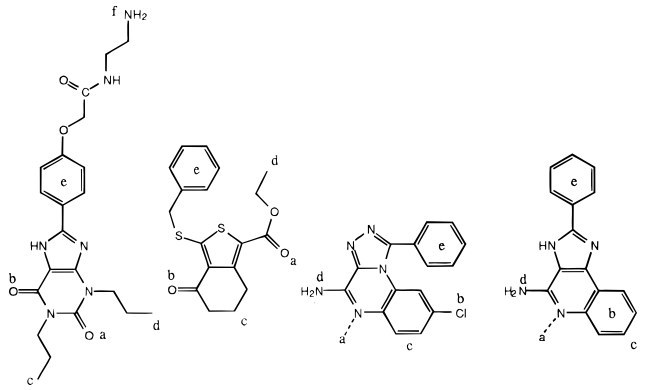


Figure 4. (Top) van der Waals volumes of BTH_4 (**7**) and XAC. (Bottom) Isopotential surfaces of BTH_4 (**7**) and XAC (red = 5 kcal/mol, yellow = 0, and blue = −5 kcal/mol).

(**16**) ester, and the 6-position of the tetrahydrobenzo ring may be branched (**18**−**20**).

A small, positively charged region around the imidazole NH of XAC, which is likely a determinant of its high affinity,[6,32−34] has an equivalent in BTH_4 (**7**), *i.e.*, a region of partial positive charge around the thioether sulfur atom. Even the 8-aryl group of the high-affinity xanthines, such as XAC, corresponds roughly to the benzyl ring of BTH_4 (**7**). However, the presence of a benzyl group enhances A_1 receptor affinity of BTH_4 (**7**) by, at most, only 2-fold, while 8-aryl substituents greatly enhance affinity for A_1 receptors.[6,32]

To compare the binding modes of XAC, BTH_4(**7**), CP66713, and PIQA, we determined distances, in-plane angles, and dihedral angles in the energy-minimized structures (Figure 5). Analysis of the distances A (ab), B (bc), C (ac), D (ad), and E (be), the nonbonded in-plane angles ∠dab and ∠cbe, and the nonbonded dihedral angle ∠dabe reveals that although there is some difference between the values for the various compounds, there is sufficient ground to accept the validity of this pharmacophore model[6,32−34] for BTH_4 (**7**). It suggests that a ring structure at the position of the 5-membered ring in xanthines, CP66713, or PIQA is not strictly required and that, inversely, the tetrahydrobenzo ring structure of BTH_4 (**7**) can substitute for the linear propyl group in XAC. The distinct difference in the distances A might be one of the major factors determining the large difference in affinity observed for the compounds. The formation of hydrogen bonds to the xanthine carbonyl groups is limited to a certain range of distances, bond angles, and dihedral angles. If these criteria are not exactly met, a sharply decreased affinity, such as that which occurs in the tetrahydrobenzothiophenones, may be observed. The dependency of the affinity on the distances B and D, both about 4.6 Å, was already shown for XAC[6,10] and seems to hold true for BTH_4 (**7**) as well. Also significant is the distance E between the carbonyl oxygen marked b and the center of the aromatic substituent marked e. In BTH_4 (**7**; 715 nM at A_1), this distance is 6.3 Å, and in XAC (1.2 nM at A_1), it is 6.9 Å. A similar effect is observed for CP66713 (270 nM), which is only about 2.6-fold more potent than BTH_4 (**7**; 715 nM) at A_1 adenosine receptors, where the distance E is only 5.5 Å. The distance E in PIQA is 7.8 Å, and this is reflected in a considerably higher affinity at A_1 adenosine receptors (34 nM). The rigidity of either XAC, CP66713, or PIQA (one rotatable bond between the heterocycle and the phenyl ring) allows for the occupation of less conformational space than the three corresponding rotatable bonds in BTH_4 (**7**), as reflected in the dihedral angle ∠dabe (14.5°, −6°, 11.3°, and



	XAC	BTH_4	CP66713	PIQA
Distances in Å				
ab = A	4.625	6.585	6.736	2.821
bc = B	4.588	3.556	4.633	2.489
ac = C	4.510	4.473	4.033	4.565
ad = D	4.689	4.149	2.698	2.333
be = E	6.948	6.236	5.535	7.818
ef = F	8.952			
Angles in °				
dab =	117.04	123.08	111.23	154.64
cbe =	149.42	140.08	117.85	170.15
dabe =	14.5	-15.8	-6.0	11.3
K_i (nM)				
A_1	1.2[34]	715	270[34]	34[33]
A_{2a}	60[34]	1360	21[34]	290[33]

Figure 5. Pharmacophore models for XAC, BTH_4 (**7**), CP66713, and PIQA, showing distances, in-plane angles, and dihedral angles. Point e is defined as the center of the aromatic ring; all other positions are defined by the atomic coordinates.

−15.8° for XAC, CP66713, PIQA, and BTH_4 (**7**), respectively). More narrowly defined are the nonbonded in-plane angles ∠dab and ∠cbe (117° and 123° for ∠dab and 149° and 140° for ∠cbe for XAC and BTH_4 (**7**), respectively). All differences between the base structures summed, plus the additional binding enhancing site f in XAC, may account for the difference in affinity (715 nM for BTH_4 (**7**) and 1.2 nM for XAC[6]) at A_1 adenosine receptors. The correspondence of pharmacophores is novel and not immediately obvious without the aid of a computer model.

Conclusions

It was discovered that heterocyclic compounds that do not contain nitrogen can act as antagonists at A_1, A_{2a}, and A_3 adenosine receptors. The completely new class of ligands described here shows a general selectivity toward A_1 adenosine receptors, but minor modifications can readily reverse this selectivity in favor of A_{2a} adenosine receptors. The compound that displayed a relatively high affinity at both A_1 and A_{2a} adenosine receptors was shown to be an antagonist in functional studies of inhibition (A_1) and stimulation (A_{2a}) of adenylyl cyclase activity and to increase locomotor activity in mice under certain circumstances. The slight, readily reversible A_1 selectivity of some BTH_4 analogues (1.9-fold A_1/A_{2a} selectivity for **7**, 3.3-fold A_{2a}/A_1 selectivity for **14**, and 3.9-fold A_3/A_1 selectivity for **11**) and the apparent absence of affinity for sites other than adenosine receptors, including the (NBTI sensitive) nucleoside transporter, make this novel class of ligands an excellent starting point for the development of new and selective non-xanthine antagonists.

Experimental Section

Materials. Ethyl 3-(methylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**1**), ethyl 3-(propylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**2**), ethyl 3-(2-propylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**3**), ethyl 3-(2-propargylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**4**), ethyl 3-(butylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**5**), ethyl 3-[[(ethoxycarbonyl)methyl]thio]-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**6**), ethyl 3-(benzylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**7**), ethyl 4-hydroxy-3-(methylthio)-4,5,6,7-tetrahydrobenzo[*c*]-thiophene-1-carboxylate (**8**), ethyl 4-(methoxyimino)-3-(methylthio)-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**9**), 4-hydroxy-3-(methylthio)-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylic acid hydrazide (**10**), 4,5-dihydro-1-(methylthio)-benzo[*c*]thiophene-3-carboxylic acid hydrazide (**11**), 5,5-dimethyl-1-(methylthio)-4,5,6,7-tetrahydrobenzo[*c*]thiophen-7-one (**12**), 3-(methylthio)-6,6-dimethyl-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]-thiophene-1-carboxylic acid (**13**), methyl 3-(ethylthio)-6,6-dimethyl-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**15**), ethyl 3-(ethylthio)-6,6-dimethyl-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**16**), ethyl 3-(2-butylthio)-6,6-dimethyl-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene1-carboxylate (**17**), ethyl 3-(benzylthio)-6,6-dimethyl-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**18**), ethyl 3-(methylsulfonyl)-6,6-dimethyl-4-oxo-4,5,6,7-tetrahydrobenzo-[*c*]thiophene-1-carboxylate (**19**), and ethyl 3-hydrazino-6,6-dimethyl-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**20**) were purchased from Maybridge (Trevillett, U.K.). HEK-293 cell membranes transfected with human A_3 cDNA were obtained from Receptor Biology, Inc. (Baltimore, MD). CHO cell membranes transfected with rat A_3 cDNA, provided by Drs. M. Olah and G. Stiles, were prepared as described earlier.[26] All other materials were obtained from commercial sources as described previously.[10,24−27,35,36]

Synthesis. Proton nuclear magnetic resonance spectroscopy was performed on a Varian GEMINI-300 spectrometer, and spectra were taken in DMSO-d_6. Electron-impact mass spectrometry was performed with a VG7070F mass spectrometer at 6 kV. Elemental analysis was performed by Atlantic Microlab Inc. (Norcross, GA).

3-(Benzylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]-thiophene-1-carboxylic Acid (14). Ethyl 3-(benzylthio)-4-oxo-4,5,6,7-tetrahydrobenzo[*c*]thiophene-1-carboxylate (**7**; 100 mg, 0.288 mmol) was dissolved in 5 mL of DMF. To the solution was added 500 μL of 4 M NaOH, and the solution was heated at 70 °C for 45 min, at which time the hydrolysis was shown to be complete by TLC (silica gel 60, ethyl acetate: petroleum ether = 10:90). To the reaction mixture was added 15 mL of water. The basic water phase was then extracted three times with 10 mL of ethyl acetate. The water phase was acidified (pH < 3) with concentrated hydrochloric acid until the product precipitated as an amorphous coagulate. The product was twice extracted with 50 mL of ethyl acetate and the ethyl acetate fraction dried over Na_2SO_4 and evaporated to dryness. The extract was shown to be pure by TLC. The product was dissolved in and crystallized from chloroform. Yield: 79 mg (86%). ^{1}H NMR (ppm relative to TMS): 1.99 (H-6a,b, 2H, q), 2.51 (H-7a,b, coincides partially with DMSO peak), 3.14 (H-5a,b, 2H, t), 4.41 (SCH_2, 2H, s), 7.32−7.53 (phenyl, 5H, m), 13.18 (COOH, 1H, br). Molecular mass: calcd, 318.0384; found, *m/z* = 318.0381. Mp: 226 °C dec. Anal. ($C_{16}H_{14}O_3S_2$) C,H,S.

Pharmacology. Radioligand-Binding Studies. Binding of [^{125}I]-N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]AB-MECA) to cloned rat A_3 receptors expressed on CHO cell membranes or cloned human A_3 receptors expressed on HEK-293 cells was performed as described previously.[10,26,39,40] Nonspecific binding was determined in the presence of 200 μM *N*-(ethylcarbamoyladenosine (NECA).

Binding of [^{3}H]-(*R*)-N^6-(phenylisopropyl)adenosine ([^{3}H]-(*R*)-PIA) to A_1 receptors from rat cerebral cortex membranes and

of [^{3}H]CGS21680 to A_{2a} receptors from rat striatal membranes was performed as described previously.[24,25] Adenosine deaminase (3 U/mL) was present during the preparation of the brain membranes, a preincubation of 30 min at 30 °C, and the incubation with the radioligands.

All nonradioactive compounds were initially dissolved in DMSO and diluted with buffer to the final concentration, where the amount of DMSO never exceeded 1%. At least six different concentrations of antagonist, spanning 3 orders of magnitude adjusted appropriately for the IC_{50} of each compound, were used. IC_{50} values, computer-generated using the nonlinear regression method implemented in the InPlot program (Graph-PAD, San Diego, CA), were converted to apparent K_i values using K_d values of 1.0, 14, 1.5, and 0.6 nM for [^{3}H]-(*R*)-PIA (rat A_1), [^{3}H]CGS21680 (rat A_{2a}), [^{125}I]AB-MECA (rat A_3), and [^{125}I]AB-MECA (human A_3) binding, respectively,[24,25,40] applying the Cheng−Prusoff equation.[38]

The affinity at selected additional receptor- and second-messenger-binding sites was tested by NovaScreen (Div. of Oceanix Biosciences, Hanover, MD), using a variety of binding assays.[30]

A_1 and A_{2a} Receptor-Coupled Adenylyl Cyclase Activity. Adenylyl cyclase assays were carried out, essentially as described previously, for A_1 receptors using rat adipocyte membranes[27] and for A_{2a} receptors using rat pheochromocytoma PC12 cell membranes.[37]

Locomotor Activity. Adult male mice of the NIH (Swiss) strain weighing 25−30 g were housed in groups of 10 animals/cage with a light−dark cycle of 12:12 h. The animals were given free access to standard pellet food and water and were acclimatized to laboratory conditions for 24 h prior to testing. Each animal was only used once in the activity monitor. Locomotor activity of individual animals was studied in an open field using a Digiscan activity monitor (Omnitech Electronics Inc., Columbus, OH) equipped with an IBM-compatible computer. The computer-tabulated measurements represent multivariate locomotor analysis with specific measures, such as simultaneous measurements of ambulatory, rearing, stereotypical, and rotational behavior. Data were collected in the morning, for three consecutive intervals of 10 min each, and analyzed separately and as a group. Statistical analysis was performed using the Student's *t*-test. The results are reported as mean ± standard error for each point. Compound **7** was dissolved initially in DMSO and diluted in at least 20 vol of vehicle, a 20:80 (v/v) mixture of Alkamuls EL-620 (Rhône-Poulenc, Cranbury, NJ) and phosphate-buffered saline. Drugs were administered intraperitoneally in a volume corresponding to 5 mL/kg of body weight.[28]

Molecular Modeling. Structures were drawn in the Sybyl molecular modeling package (Tripos Associates Inc., St. Louis, MO; version 6.04) running on an Iris Indigo XZ4000 workstation (Silicon Graphics Inc., Mountain View, CA, MIPS R4000 CPU). Energy minimizations were subsequently performed in the MOPAC program (Quantum Chemistry Program Exchange, version 6.0), using the MNDO Hamiltonian and the keywords PULAY, PRECISE, and MMOK, and in the Gaussian program (Gaussian Inc., Pittsburgh, PA; version 92, revision F4), using the MC-311G basis set with closed shell RHF. Both the MOPAC and the Gaussian-92 program were run on a Convex C3830 system (Convex Computer Corp., Richardson, TX). After completion of the runs the data were converted back into the Sybyl format using the Babel (University of Arizona; version 1.1, babel@mercury.aichem.arizona.edu) conversion program. van der Waals and electrostatic contours were then generated using standard procedures within Sybyl.

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