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Structure-Activity Relationships for Enhancement of Adenosine A_1 Receptor Binding by 2-Amino-3-benzoylthiophenes

ROBERT F. BRUNS,[1] JAMES H. FERGUS, LINDA L. COUGHENOUR, GENEVA G. COURTLAND, THOMAS A. PUGSLEY, JOHN H. DODD,[2] and FRANCIS J. TINNEY[3]

Departments of Pharmacology (R.F.B., J.H.F., L.L.C., G.G.C., T.A.P.) and Chemistry (J.H.D., F.J.T.), Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan 48105

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SUMMARY

The structural requirements for stimulation of adenosine A_1 agonist binding by 2-amino-3-benzoylthiophenes and related compounds were investigated. Slowing of the dissociation of [^{3}H]N^{6}cyclohexyladenosine binding was used as a specific measure of the allosteric effects of these compounds. The thiophene ring could be replaced with benzene but not with several nitrogen-containing heterocycles. The 2-amino group was required, and at least one hydrogen on the amino group appeared to be necessary for activity. The keto carbonyl was also essential. Alkyl substitution at the 4-position of the thiophene ring increased activity, whereas 5-position substitution appeared to have little effect. Activity was also increased by various substitutions on the phenyl ring, with 3-(trifluoromethyl) showing optimal activity. The phenyl ring could be replaced with cyclohexyl without major loss of activity. 1-Aminofluoren-9-one, a conformationally locked derivative, was active. Based in part in the latter observation, the active conformation is proposed to have an intramolecular hydrogen bond between the amino nitrogen and the carbonyl oxygen. Because the 2-amino-3-benzoylthiophenes showed competitive adenosine antagonism as well as allosteric enhancement, their affinities as competitive inhibitors of [^{3}H]8-cyclopentyl-1,3-dipropylxanthine binding to A_1 receptors were also assessed. Structure-activity relationships for competitive antagonism were distinct from those for allosteric enhancement, with ratios between the two activities varying by more than 1000-fold. Of the analogs tested, (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone (PD 81,723) had the most favorable ratio of enhancement to antagonism.

Several 2-amino-3-benzoylthiophenes have been reported to enhance binding of agonists to the adenosine A_1 receptor (1, 2). Because many analogs were available from a previous study (3) (see Ref. 2 for a history of this series), we had the opportunity to investigate the effects of structural variations in this chemical series. The present study describes the structural requirements for allosteric enhancement of adenosine A_1 receptor binding by 2-amino-3-benzoylthiophenes and related compounds.

Experimental Procedures

Materials. 2-Amino-3-benzoylthiophene derivatives were synthesized at Parke-Davis by the method of Tinney *et al.* (3). Most of these compounds were synthesized between 1968 and 1973 and stored at room temperature. It should be noted that, except for the examples mentioned below, the compounds in the present study were not retested for purity, and it is possible that impurities may have been generated by chemical breakdown processes such as oxidation or hydrolysis. Compounds **2** and **40** were reanalyzed in 1989 by mass spectroscopy, ^{1}H and ^{13}C NMR spectroscopy, and melting point determination and found to still possess the assigned structures. Compounds **4–21**, **23**, **24**, and **26** were synthesized in 1984. The activity of compound **15** (PD 117,975) did not change between January 1984 and September 1988. Compound **49** (PD 81,723) was resynthesized in 1989 and retained its enhancing and antagonist activity.

2-Aminobenzophenones were purchased from Aldrich Chemical (Milwaukee, WI). [^{3}H]CHA (34 Ci/mmol) and [^{3}H]NECA (18 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [^{3}H]CPX (117 Ci/mmol) was synthesized as described (4). DMSO was spectrophotometric grade (Aldrich). Sources of other reagents were as previously described (5).

Competitive binding assays. Binding of [^{3}H]CPX to A_1 receptors in rat forebrain membranes was carried out as described (4). [^{3}H]NECA binding to A_2 receptors in rat striatal membranes was carried out in the presence of 50 nM N^6-cyclopentyladenosine, as described (5).

Assay for allosteric enhancement: effects of test agents on dissociation of [^{3}H]CHA. [^{3}H]CHA was preassociated with the A_1 receptor for 1 hr in the absence of test agent under the same conditions as the standard [^{3}H]CHA binding assay (5). Membranes from 20 mg of original wet weight of rat whole brain (minus cerebellum and brain-

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[1] Present address: Biochemical Pharmacology Research, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285.

[2] Present address: R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ 08869.

[3] Present address: Patent Department, Warner-Lambert/Parke-Davis.

ABBREVIATIONS: CHA, N^6-cyclohexyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; DMSO, dimethyl sulfoxide; NECA, 1-(6-amino-9*H*-purin-9-yl)-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide; (*R*)-PIA, N^6-[(*R*)-1-methyl-2-phenylethyl]adenosine.

stem) that had been preincubated with adenosine deaminase (6) were incubated with 1 nM [^{3}H]CHA in 2 ml of Tris·HCl, pH 7.7, containing 0.1 units/ml adenosine deaminase, at 25°. Nonspecific binding was determined in parallel by addition of 10 μM (*R*)-PIA before the preassociation. After the 1-hr preassociation, dissociation was initiated by addition of N^6-cyclopentyladenosine (final concentration, 100 μM) and test agent, each in 10 μl of DMSO. After an additional 2 hr, the incubations were terminated by filtration through a GF/B filter sheet on a Brandel 48R cell harvester and the filter discs were counted in a scintillation counter (5). At the end of the 2-hr dissociation, control specific binding was reduced to about 15% of the starting value.

Data analysis. Total binding data from competitive binding assays were fit to a Langmuir isotherm using nonlinear least-squares curve-fitting, as described (5). In this analysis, nonspecific binding was treated as a very high concentration of test agent, thus forcing the final plateau of the concentration-inhibition curve to be approximately equal to nonspecific binding.

Results from the dissociation assay were also fit to a Langmuir isotherm. It should be noted that a few test agents (for instance, compound **59**) did not reach a true plateau at the highest concentration of test agent examined. In these cases, the values for maximum stimulation and EC_{50} will be poorly defined (due to covariance of these two parameters). However, the ratio between the maximum stimulation and the EC_{50} should still be reasonably accurate, because this value depends only on the slope of the initial part of the concentration-response curve.

Results

Independent measurement of allosteric enhancement and competitive antagonism. In addition to enhancing A_1 agonist binding by an allosteric mechanism, the 2-amino-3-benzoylthiophenes also inhibit A_1 binding by acting as competitive antagonists (2). In the standard [^{3}H]CHA binding assay, competitive antagonism by these compounds tends to limit or even reverse the stimulation of binding caused by allosteric enhancement, making it impossible to determine the true enhancing potential of these compounds. To study enhancement in the absence of interference from competitive inhibition, we used a specific assay for allosteric effects based on the ability of test agents to slow the dissociation of [^{3}H]CHA. The agonist [^{3}H]CHA was preassociated with the receptor in the absence of test agent, after which dissociation was initiated by simultaneous addition of test agent and a saturating concentration of unlabeled adenosine agonist. Interference from competitive blockade should not be possible under these conditions, because the unlabeled agonist prevents reassociation of [^{3}H]CHA with the A_1 receptor throughout the entire period in which the test agent is present.

Affinities of test agents for competitive inhibition at the A_1 receptor were determined using binding of the A_1 antagonist [^{3}H]CPX. Because the A_1 enhancers did not appear to have any significant allosteric effects on [^{3}H]CPX binding (2), this assay is probably a reasonably undistorted measure of competitive inhibition.

An important objective of this study was to compare the structure-activity relationships for enhancement of binding with those for competitive inhibition. To aid in this comparison, an enhancement/antagonism ratio was derived as follows. The initial slope of the concentration curve for enhancement is calculated by dividing the maximum enhancement by the EC_{50} for enhancement. This slope is then divided by the absolute value of the initial slope of the competitive inhibition curve (the maximum inhibition is assumed to be 100%), as summarized in Eq. 1.

$$\text{Ratio} = \frac{\text{maximum enhancement}/EC_{50}}{100/K_i} \qquad (1)$$

Pharmacophore for binding enhancement. The initial chemical lead for enhancement of [^{3}H]CHA binding was the 4,5,6,7-tetrahydrothieno[2,3-c]pyridine derivative PD 117,975 (compound **15** in Table 1) (2). Our first step in investigating the structure-activity relationships of this lead was to determine which parts of this rather complex structure were essential for activity. With results from a few analogs, it became clear that the tetrahydropyridine ring was not essential, because it could be replaced with tetrahydrobenzene (Table 2) or even eliminated entirely (Table 3) without loss of activity. For instance, PD 71,605 (compound **29**, Table 2) and PD 81,723 (compound **49**, Table 3) both showed enhancing activity equal to that of PD 117,975. Other structural variations revealed that the 2-amino group, the ketone, and the exocyclic phenyl ring were required for activity (see below). The minimum active structure, or pharmacophore, was therefore 2-amino-3-benzoylthiophene (compound **44** in Table 3).

Thiophene ring. Activity was retained when the thiophene ring of the 2-amino-3-benzoylthiophenes was replaced with benzene (Table 4). 2-Aminobenzophenone (compound **55**, Table 4) was roughly half as active as 2-amino-3-benzoylthiophene (compound **44**, Table 3), with 34 and 59% enhancement at 100 μM, respectively. Several other 2-aminobenzophenones (compounds **57**, **59**, and **60**, Table 4) showed enhancement of 100–150% at 100 μM. Although a comparison of Tables 3 and 4 leaves one with the overall impression that the benzophenones are less active than the corresponding thiophene analogs, this impression could not be confirmed directly, because comparable analogs were not available from both series.

Replacement of the thiophene ring with other aromatic heterocycles, including pyrazole (compounds **75–79** and **86**, Table 5), isoxazole (**85**), imidazole (**87**), and uracil (**88**), resulted in loss of activity.

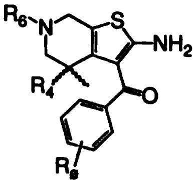
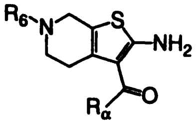
2-Amino group. Omission of the 2-amino group resulted in complete loss of activity (see compounds **54** and **67**, Tables 3 and 4), implying that this group is essential for activity in both the thiophene and benzophenone series. At least one of the amino hydrogens was necessary, and in some cases both hydrogens appeared to be required. In the benzophenone series, monomethyl substitution of the 2-amino group (compound **72**, Table 4) resulted in an inactive compound. Replacement of the amino group with a hydroxyl, which contains only one hydrogen, also eliminated activity compounds **70** and **71**, Table 4). In the thiophene series, *N*-acetyl substitution (compound **39**, Table 2) destroyed activity. However, the 2-iodoacetyl derivative **40** showed considerable activity (Table 2), as did several other 2-haloacetyl analogs (data not shown). Reanalysis of compound **40** indicated that it had not hydrolyzed on storage, eliminating the possibility that the activity was due to the presence of the deacylated derivative **27** in the sample of **40**. However, the possibility that **40** may have hydrolyzed to **27** during the assay cannot be ruled out.

Displacement of the 2-amino group to the 3- or 4-position (compounds **68** and **69**, Table 4) also destroyed activity in the

TABLE 1

Adenosine A_1 receptor enhancement and antagonism by 2-amino-3-benzoyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridines

Most values are from single determinations. Variability of data for PD 117,975 (entry **15**) was as follows: enhancement at 100 μM, 285 ± 102% (mean ± SD), six experiments; maximum enhancement, 362 ± 110%, three experiments; EC_{50}, 15.6 ± 5.8 μM, three experiments; [^{3}H]CPX K_i, 3.65 ± 0.77 μM (mean ± SD), three experiments.

Compound		Structure		Enhancement [^{3}H]CHA dissociation[a] 100 μM[d]	Enhancement [^{3}H]CHA dissociation[a] Concentration-response[e] Maximum	 EC_{50}	Antagonism [^{3}H]CPX competition[b] K_i	Enhancement/ antagonism ratio[c]
	R_4	R_6	R_ϕ	%	%	μM	μM	
								
1	H	Methyl	H	14			82.6	
2	H	Acetyl	H	−3				
3	H	Ethoxycarbonyl	H	112			0.530	0.0178[f]
4	H	Ethoxycarbonyl	4-Amino	20			10.3	
5	H	Ethoxycarbonyl	3-Chloro	222			0.178	
6	H	Ethoxycarbonyl	4-Chloro	227			0.805	
7	H	Ethoxycarbonyl	3,4-Dichloro	356	348	15.9	1.33	0.291
8	H	Ethoxycarbonyl	4-Dimethylamino	114			1.14	
9	H	Ethoxycarbonyl	4-Methoxy	151			2.23	
10	H	Ethoxycarbonyl	2-Methyl	122			1.61	
11	H	Ethoxycarbonyl	4-Methyl	178			0.635	
12	H	Ethoxycarbonyl	4-Nitro	82			3.60	
13	H	Benzyl	H	102			1.41	
14	H	Benzyl	3-Chloro	201	384	70.3	1.28	0.0699
15	H	Benzyl	4-Chloro	285	362	15.6	3.65	0.847
16	H	Benzyl	4-Isopropyloxy	136	192	40.3	16.2	0.772
17	H	Benzyl	4-Methoxy	146			4.21	
18	H	Benzyl	2-Methyl	180	223	42.6	2.65	0.139
19	H	Benzyl	4-Methyl	257	377	45.6	1.14	0.0942
20	Methyl	Benzyl	H	224	291	28.0	7.77	0.808
								
		R_6	R_α					
21		Methyl	Amino	6			>100	
22		Methyl	Ethoxy	7			38.1	
23		Ethoxycarbonyl	Amino	11			>100	
24		Ethoxycarbonyl	2-Thienyl	40			0.246	0.0030[g]
25		Benzyl	Ethoxy	66			0.570	
26		Benzyl	2-Thienyl	58			0.921	

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[a] A_1-enhancing activity was determined using the dissociation procedure as follows. [^{3}H]CHA (1 nM) was preassociated with rat brain membranes for 1 hr at 25° in 2 ml of assay buffer, after which test agent and (*R*)-PIA (final concentration, 10 μM) were added simultaneously in a 20-μl volume of DMSO. Samples were filtered after an additional 2-hr incubation at 25°.

[b] Affinities of test agents as competitive antagonists were determined using [^{3}H]CPX binding in rat brain membranes, as previously described (4).

[c] The "enhancement/antagonism ratio" is described in Eq. 1 (see Results). A higher ratio denotes a greater degree of enhancing activity relative to antagonism.

[d] Screening experiments measured enhancement at a single concentration of test agent (100 μM). Enhancement was expressed relative to a control dissociation with (*R*)-PIA but without test agent. None of the compounds affected nonspecific binding.

[e] Concentration-response experiments employed seven concentrations from 1 to 100 μM. Maximum enhancement and EC_{50} were derived from nonlinear least-squares curve-fitting (5). The EC_{50} is the concentration causing half-maximum enhancement.

[f] Because concentration-response data for compound **3** (PD 78,416) were not available, a rough estimate of the enhancement/antagonism ratio for this compound was calculated as follows. Given that other weak enhancers in this series had EC_{50} values of 40–70 μM, a "reasonable" EC_{50} of 50 μM was assigned. This assumed EC_{50} was then used to derive a maximum enhancement of 168%, based on the observed value of 112% enhancement at 100 μM. The values for maximum enhancement and EC_{50} were then substituted into Eq. 1.

[g] The enhancement/antagonism ratio for compound **24** was calculated based on the assumption of an EC_{50} of 50 μM and a maximum enhancement of 60%.

benzophenone series, implying that the position of the amino group relative to the benzoyl moiety is important.

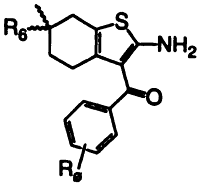
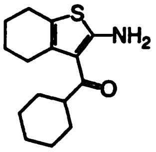
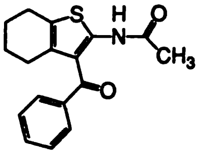
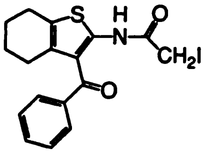
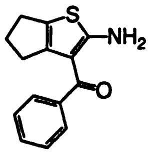
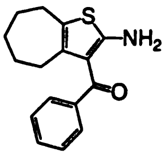
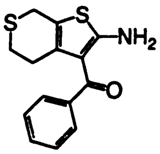
Keto group. In the benzophenone series, omission of the keto carbonyl resulted in complete loss of activity (compound **66**, Table 4).

Substitution at the 4- and 5-positions of the thiophene ring. Although the unsubstituted 2-amino-3-benzoylthiophene (**44**, Table 3) was active (59% enhancement at 100 μM), activity could be increased considerably by substitution at the 4- and 5-positions of the thiophene ring. An analysis of the separate effects of these two positions indicated that most or all of the increased activity could be attributed to the effects of 4-position modification. 4-Methyl substitution doubled enhancing activity (compare 110% enhancement by **46** with 59% enhancement by **44**), and the lengthening of the 4-position alkyl group from methyl to ethyl imparted about an additional doubling of activ-

TABLE 2

Adenosine A_1 receptor enhancement and antagonism by 4,5,6,7-tetrahydrobenzo[*b*]thiophenes and homologs

Allosteric enhancement and competitive inhibition were measured as described in Table 1.

Compound	Structure	Enhancement			Antagonism	Enhancement/ antagonism ratio
		[^{3}H]CHA dissociation			[^{3}H]CPX competition	
		100 μM	Concentration-response			
			Maximum	EC_{50}	K_i	
R_6	R_9	%	%	μM	μM	
						
27 H	H	135				
28 H	2-Chloro	224	260	37.6	6.42	0.444
29 H	3-Chloro	363	470	29.0	1.89	0.306
30 H	4-Chloro	319	323	15.3	1.98	0.418
31 H	2-Fluoro	111				
32 H	2-Methoxy	75				
33 H	3-Methoxy	197			3.88	
34 H	4-Methoxy	84	75	14.6		
35 H	2-Methyl	240	286	32.2		
36 H	3-Trifluoromethyl	296	326	10.2	4.02	1.28
37 Methyl	H	189			2.25	
 38	 39	 40	 41		 42	 43
38		83				
39		22				
40		192				
41		81	68	7.42		
42		165			2.38	
43		145			3.33	

ity (compare 216% enhancement by **50** with 131% enhancement by **48**). Phenyl substitution at the 4-position caused a greater than 3-fold increase in activity (201% enhancement by **53** compared with 59% enhancement by **44**). However, cyclopropyl (compound **52**, 135%) appeared to be little better than methyl (**46**, 110%).

In contrast to the positive effects of 4-substitution, substitution at the 5-position appeared to have little effect on allosteric enhancing activity. Substitution with chloro or methyl (compare **47** and **48** with **46**) or phenyl (compare **45** with **44**) did not result in any significant improvement. However, substitution of the 5-position of 2-aminobenzophenone (roughly equivalent to the 5-position of the thiophenes) resulted in a 2- to 4-fold improvement in enhancing activity (compare **58**, **60**, and **61** with **55**).

Bridging of the 4- and 5-positions with methylene chains of various lengths (compounds **27**, **41**, and **42**) also gave good activity. Activity increased steadily as the size of the fused ring increased from five (compound **41**, 81% enhancement, Table 2) to six (**27**, 135% enhancement) to seven members (**42**, 165% enhancement). However, these cyclized analogs were less active than the 4-ethyl-5-methyl derivative (**50**, 216% enhancement), indicating that joining of the 4- and 5-position alkyl groups was disadvantageous.

Heteroatoms could be inserted into the ring bridging the 4- and 5-positions. Substitution of sulfur for CH_2 had little effect on enhancing activity (compound **43**, 145% enhancement, Table 2; compared with 135% for **27**). Many aza-substituted analogs were available (Table 1). The *N*-methyl derivative **1** was inactive. The inactivity of this compound may have been due to deleterious effects of a positive charge at neutral pH, because the *N*-(ethoxycarbonyl) derivative **3**, in which the positive charge is suppressed, showed substantial enhancing activity (112% enhancement).[4] The *N*-benzyl derivative **13** was also active (102% enhancement). The greater activity of the *N*-benzyl compared with the *N*-methyl derivative could have been due to a lower pK_a of the amine, because benzyldimethylamine has a pK_a about 0.8 units lower than trimethylamine (8.93 versus 9.76) (7). Binding of the extra phenyl ring to an accessory hydrophobic site could also play a role.

Methyl substitution at the 4-position of the tetrahydropyridine ring increased enhancement from 102 to 224% (compare **20** to **13**).

Phenyl ring. Replacement of the benzoyl group with carbamyl (compound **23**, Table 1) or acetyl (compound **73**, Table

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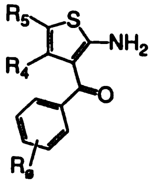
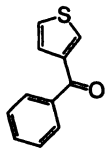
[4] The lack of activity of the *N*-acetyl derivative **2** is puzzling, because the difference in size and hydrophobicity between an acetyl group and an ethoxycarbonyl group is not great.

TABLE 3

Adenosine A_1 receptor enhancement and antagonism by 2-amino-3-benzoylthiophenes

Allosteric enhancement and competitive inhibition were measured as described in Table 1.

Compound		Structure		Enhancement			Antagonism	Enhancement/antagonism ratio
				$[^3H]$CHA dissociation			$[^3H]$CPX competition	
				100 μM	Concentration-response			
					Maximum	EC_{50}	K_i	
	R_4	R_5	R_6	%	%	μM	μM	
								
44	H	H	H	59				
45	H	Phenyl	H	77				
46	Methyl	H	H	110				
47	Methyl	Chloro	H	90				
48	Methyl	Methyl	H	131				
49	Methyl	Methyl	3-Trifluoromethyl	321	492	9.83	11.3	5.66
50	Ethyl	Methyl	H	216	217	30.7	19.2	1.36
51	Propyl	Ethyl	H	193	268	18.6	3.75	0.540
52	Cyclopropyl	H	H	135				
53	Phenyl	H	H	201				
54				 1				

4) resulted in complete loss of activity. Surprisingly, substitution of ethoxycarbonyl for the benzoyl function (altering the ketone to an ester) did not appear to destroy activity (compound **25**, 66% enhancement, Table 1). A cyclohexyl ring could be substituted for the phenyl ring with only moderate loss of activity (compare **38**, 83% enhancement, with **27**, 135% enhancement). The latter result suggests that a moderately bulky hydrophobic group (not necessarily planar or aromatic) at this position contributes to the affinity of the enhancer-receptor complex. Replacement of the phenyl ring with its 2-thienyl isostere halved activity (compare **24** and **26** with **3** and **13**, respectively).

Appropriate substitution of the phenyl ring markedly increased enhancing activity. In the tetrahydrobenzo[*b*]thiophene series (Table 2), the order of potency for substitution at different positions of the phenyl ring was *meta* > *para* ≥ *ortho*, regardless of whether the substituting moiety was chloro (compounds **28–30**) or methoxy (compounds **32–34**). Among the *meta* derivatives, trifluoromethyl (**36**) appeared to be slightly superior to chloro (**29**), based on a lower EC_{50} for the former, whereas methoxy (**33**) was intermediate between chloro and hydrogen (**27**). The superior activity of the 3-(trifluoromethyl) substitution was also seen in the simple thiophene series (compound **49**, Table 3).

Interestingly, the potency order 3-chloro > 4-chloro did not apply to the tetrahydrothieno[2,3-*c*]pyridine series (Table 1). In this series, the 4-chloro derivative was equal (compounds **4** and **5**) or superior (compounds **14** and **15**) to the 3-chloro. Substitution with polar groups such as 4-amino (compound **4**) or 4-nitro (compound **12**) resulted in loss of activity, suggesting that the receptor environment neighboring the phenyl binding site is probably hydrophobic.

Conformationally rigid analogs. 1-Aminofluoren-9-one (compound **64**, Table 4) is locked in a planar conformation by a bond from the *ortho* position of one phenyl to the 6-position of the other. This compound was more than 3 times as active as the corresponding uncyclized compound **55** (122% enhancement versus 34%), implying that the conformation adopted by **64** is allowed and possibly even preferred. 1-Aminoanthraquinone (**65**), which is structurally similar to **64** except that the two phenyl rings are bridged by a ketone, was inactive, presumably because the bridging carbonyl projects into a receptor domain that does not tolerate hydrophilic groups.

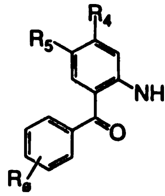
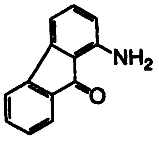
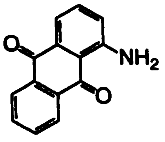
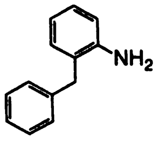
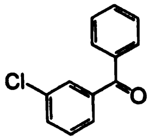
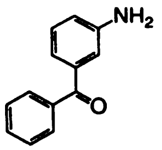
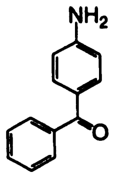
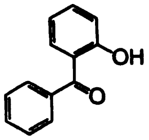
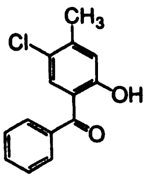
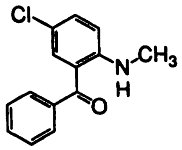
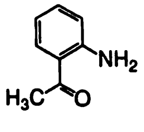
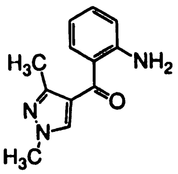
Thienodiazepines. CI-718, the thienodiazepine derived from cyclization of compound **27** (3), was essentially inactive (compound **80**, Table 5), but the corresponding 3-chloro derivative **81** showed significant activity (83% stimulation at 100 μM).

Structure-activity relationships for competitive inhibition: comparison with allosteric enhancement. The usefulness of the adenosine enhancers as pharmacological tools will depend in part on whether analogs that are free of interfering adenosine antagonist activity can be found. For this reason, affinities of selected compounds for inhibition of binding of the A_1 antagonist $[^3H]$CPX were determined and were compared with enhancing activities by means of an enhancement/antagonism ratio. Structure-activity relationships for antagonism were also of some interest in their own right, although the compounds in the present study are not very impressive compared with potent A_1 blockers such as CPX or xanthine amine congener (4, 8).

TABLE 4

Adenosine A_1 receptor enhancement and antagonism by benzophenones

Allosteric enhancement and competitive inhibition were measured as described in Table 1.

Compound		Structure		[^{3}H]CHA dissociation			Antagonism	Enhancement/ antagonism ratio
				100 μM	Concentration-response		[^{3}H]CPX competition	
					Maximum	EC_{50}	K_i	
	R_4	R_5	R_6	%	%	μM	μM	
								
55	H	H	H	34				
56	H	H	4-Chloro	93			5.25	
57	H	H	4-Methyl	148			26.3	
58	H	Chloro	H	75				
59	H	Chloro	4-Chloro	136	910[a]	264	4.99	0.172
60	H	Nitro	H	128			1.49	
61	H	Phenyl	H	70				
62	Methyl	H	H	27				
63	Methyl	Fluoro	H	0				
 64		 65	 66	 67		 68	 69	
 70		 71	 72	 73		 74		
64				122			9.8	
65				−27				
66				−3				
67				8				
68				7				
69				1				
70				4				
71				−1				
72				0				
73				−1				
74				28				

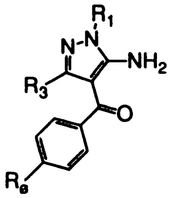
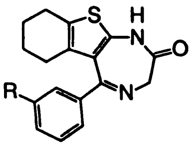
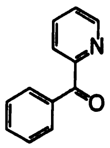
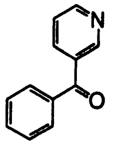
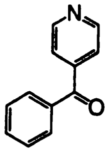
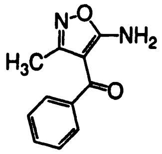
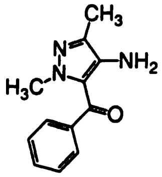
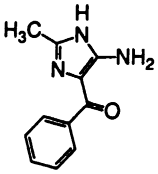
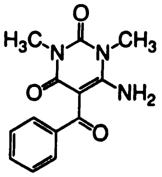
[a] The EC_{50} and maximum enhancement values for this compound were calculated by extrapolation from concentrations of 1–100 μM and, therefore, should not be considered accurate. However, the ratio between maximum enhancement and EC_{50} may still be meaningful, because it depends mainly on data from the lower part of the concentration curve.

There were some broad similarities between structure-activity relationships for antagonism and those for enhancement. For instance, analogs in which the thiophene ring was replaced with benzene (Table 4) retained antagonist activity as well as enhancing activity. Conversely, replacement of the benzoyl moiety with a carbamyl group (compound **23**, Table 1) abolished both blocking and enhancing activity. However, on closer examination several modifications appeared to have differential effects on antagonism compared with enhancement. For example, replacement of the benzoyl group with an ethoxycarbonyl moiety (compound **25**, Table 1) lowered enhancing activity and increased antagonist affinity (compare with compound **13**). Compound **25** might be a lead toward obtaining more potent and soluble adenosine antagonists from this series. Replacement of the phenyl ring with a 2-thienyl group also lowered enhancing activity and improved antagonist activity, resulting in an overall drop of 6-fold in the enhancement/antagonism ratio (compare **24** with **3**, Table 1). Compound **24** had the lowest enhancement/antagonism ratio (about 0.003) of the compounds in this study.

TABLE 5

Adenosine A_1 receptor enhancement and antagonism by other benzoyl-substituted aromatics

Allosteric enhancement and competitive inhibition were measured as described in Table 1.

Compound		Structure		$[^3H]$CHA dissociation enhancement at 100 μM
	R_1	R_3	R_4	%
				
75	Methyl	Methyl	H	−8
76	Methyl	Methyl	Chloro	27
77	Methyl	Propyl	H	7
78	Propyl	Methyl	Chloro	2
79	Phenyl	Methyl	H	−32
				
		R		
80		H		23
81		Chloro		83
				
82	83	84		85
				
86		87		88
82				0
83				0
84				2
85				−10
86				−2
87				−9
88				−20

Several modifications at the 4- and 5-positions of the thiophene increased enhancing activity relative to antagonism. In the tetrahydropyridine series (Table 1), the N^6-benzyl derivatives were consistently about 3-fold less potent as antagonists than the corresponding N^6-ethoxycarbonyl compounds but were about equal to the latter in enhancing activity (compare compounds **13**, **14**, **15**, **17**, **18**, and **19** with compounds **3**, **5**, **6**, **9**, **10**, and **11**, respectively). Addition of a methyl group at the 4-position of the tetrahydropyridine ring (compound **20**) markedly improved enhancing activity while reducing antagonist activity about 5-fold, compared with **13**. Replacement of the *N*-benzyltetrahydropyridine ring with a tetrahydrobenzene ring increased antagonist activity without any effect on enhancement, resulting in a roughly 2-fold decrease in the enhancement/antagonism ratio (for instance, compare **30** in Table 2 with **15** in Table 1). However, replacement of the tetrahydrobenzene ring with a simple 4,5-dimethyl substitution lowered antagonist activity by 3-fold, while causing a small increase in enhancing activity (compare compound **49** in Table 3 with compound **36** in Table 2). Larger alkyl groups at the 4- and 5-positions increased enhancing activity (compare **51** with **48**) but also increased antagonism (compare **51** with **50**). With the available data, independent assessment of the effects of 4-position and 5-position substitution was not possible.

The enhancement/antagonism ratio could also be improved by appropriate substitution of the phenyl ring. In the tetrahydrothieno[2,3-*c*]pyridine series (Table 1), a 4-chloro substituent is optimal, greatly improving enhancing activity while slightly reducing antagonism (compare compounds **6** and **15** with **3** and **13**, respectively). In contrast, a 3-(trifluoromethyl) substituent appears to be optimal for the tetrahydrobenzo[*b*]thiophene series (compound **36**). Among the limited number of analogs available in the 2-aminobenzophenone series, 4′-methyl substitution gave the best ratio of enhancement to antagonism (compound **57**, Table 4).

In the end, by combining various substitutions that increase the enhancement/antagonism ratio, it is possible to attain a ratio of over 5 (compound **49**, Table 3). Conversely, compound **24** in Table 1 combines several variations that decrease the enhancement/antagonism ratio, resulting in a ratio of only 0.003. The difference of almost 2000-fold in the enhancement/antagonism ratios of these two compounds clearly shows that enhancing activity can be separated from antagonist activity by appropriate structural modification.

Structure-activity relationships for A_2 blockade. Because the compounds in the present study do not enhance A_2 agonist binding (2), valid affinities for competitive A_2 blockade could be determined in the standard A_2 binding assay, which uses the agonist ligand [^{3}H]NECA in rat striatal membranes. A_2 affinities were determined for the initial chemical leads **3** and **15** and for several other members of the tetrahydrothieno[2,3-*c*]pyridine series. These compounds were 3- to 40-fold weaker at the A_2 receptor than at the A_1 (Table 6).

Discussion

The results of this study show that there are distinct structural requirements for allosteric enhancement of adenosine A_1 agonist binding and that these requirements differ from the requirements for competitive A_1 antagonist activity.

Two features that are important for allosteric enhancing activity are an unsubstituted 2-amino group and an adjacent ketone. These two moieties, which are the only hydrophilic groups in the molecule, may provide clues regarding the conformation involved in binding to the A_1 receptor. In particular, a hydrogen bond between the 2-amino group and the ketone may create an additional ring coplanar with the thiophene ring (Fig. 1). Such an intramolecular hydrogen bond has been reported to be necessary for leukotriene antagonist activity in a series of 2-hydroxyacetophenones (9). In compound **64**, the amino and the ketone are locked into a conformation in which the two groups are adjacent and coplanar, virtually forcing the formation of a hydrogen bond between the two (Fig. 1). Com-

TABLE 6

Affinities of 2-amino-3-benzoyl-4,5,6,7-tetrahydrothieno[2,3-c] pyridines for inhibition of adenosine A_2 receptor binding

A_2 affinities were determined in [^{3}H]NECA binding to rat striatal membranes in the presence of 50 nM N^6-cyclopentyladenosine, as previously described (5). A_1 data are the same as in Table 1.

Compound	K_i		A_2/A_1 ratio
	A_1, [^{3}H]CPX	A_2, [^{3}H]NECA	
	μM		
2		47	
3	0.53	16.1	30
4	10.3	48	4.7
5	0.178	7.3	41
6	0.80	12.4	15.5
7	1.33	8.2	6.2
9	2.2	29	13.2
10	1.61	18.6	11.6
11	0.64	12.3	19.2
12	3.6	43	11.9
13	1.41	14.6	10.4
14	1.28	32	25
15	3.6	36	10.0
17	4.2	11.5	2.7
19	1.14	35	31
20	7.8	46	5.9
23	>100	>100	
25	0.57	6.0	10.5

pound **64** shows good enhancing activity, confirming that this conformation is compatible with binding to the allosteric site.

The presence of an extra ring formed by the amino, ketone, and intramolecular hydrogen bond also explains the otherwise puzzling observation that this series possesses adenosine antagonist activity. Adenosine antagonists are almost invariably flat, fused-ring, nitrogen heterocycles (10–12). Without the intramolecular hydrogen bond, the 2-amino-3-benzoylthiophenes share none of these properties (although thiophene is technically a heterocycle, its lack of hydrogen-bonding ability makes it functionally similar to a phenyl ring). However, the extra ring formed by hydrogen bonding (Fig. 1) converts the molecule into a flat, fused-ring, nitrogen-containing heterocycle.

Although the hydrogen bond-containing ring of the 2-amino-3-benzoylthiophenes is coplanar with the thiophene ring, the phenyl ring should not normally be coplanar with these other two rings, because of steric repulsion from the substituent at the 4-position of the thiophene ring (see Fig. 1). Even an unsubstituted 4-position should cause considerable steric hindrance. In this respect, the conformationally locked analog **64** differs from the other active compounds in this study, because the phenyl ring in **64** is coplanar with the other two rings. Obviously, both torsion angles of the phenyl ring must be acceptable for binding to the A_1 receptor (this applies to both the allosteric site and the antagonist-binding site).

The amino and ketone groups together form a hydrophilic "core" of the 2-amino-3-benzoylthiophene structure. Because this core is essential for activity, it may be involved in hydrogen bonding to a complementary domain of the receptor, which can be envisioned as surrounding these groups in a small semicircular array. If both NH_2 hydrogens are necessary for activity, the one that is not involved in intramolecular hydrogen bonding may form an essential hydrogen bond with the receptor. In addition to accepting a hydrogen from the 2-amino group, the

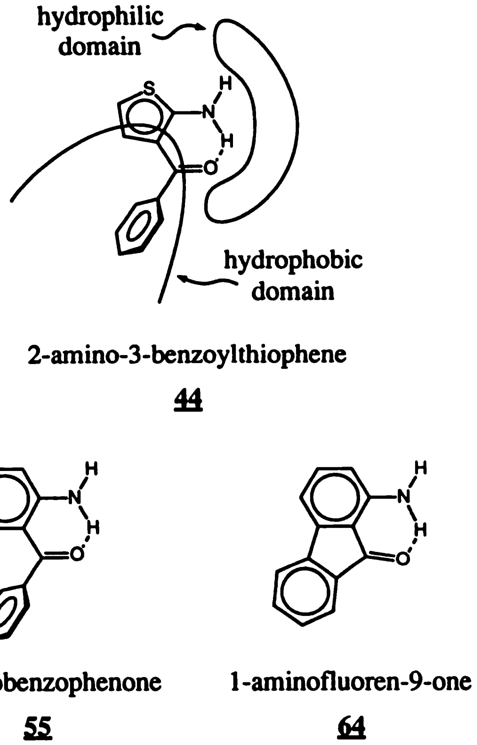


Fig. 1. Active conformation of allosteric enhancers. Important features are an intramolecular hydrogen bond (in all three compounds) and a phenyl ring rotated out of the plane of the rest of the molecule (in compounds **44** and **55** only). The two receptor domains are shown with compound **44**.

carbonyl oxygen may also accept a second hydrogen from the receptor.

The remainder of the molecule is composed of hydrophobic groups. The thiophene and phenyl rings are both necessary for allosteric enhancement, although the phenyl ring can be replaced with an alkyl or cycloalkyl group (compounds **25** and **38**). This suggests that a hydrophobic domain of the receptor may lie under the thiophene and phenyl rings. Because substitution of the phenyl ring can greatly increase allosteric enhancing activity, the hydrophobic domain must extend beyond the area covered by the unsubstituted phenyl ring. Similarly, the effects of thiophene ring substitutions can be used to map the extent of the hydrophobic domain surrounding the thiophene ring. Substitutions at the thiophene 5-position have little effect on allosteric enhancing activity, implying that this position may not be in contact with the receptor. Substitution at the 4-position of 2-aminobenzophenone (roughly equivalent in position to the sulfur in thiophene) also has little effect. In contrast, alkyl substitution of the thiophene 4-position increases activity, with maximal activity being seen with propyl or phenyl (or larger). This suggests that the hydrophobic domain of the receptor may extend out from the thiophene 4-position for a considerable distance. Because the 4-position of the thiophene ring is adjacent to the exocyclic phenyl ring, these two portions of the hydrophobic domain may actually form a single continuous hydrophobic surface that extends out from the 3- and 4-positions of the thiophene ring. The improvement in activity in compound **20** compared with **13** also supports this possibility, because the extra methyl group in **20** projects from the distal 4-position of thiophene toward the 3-position benzoyl. The cyclic compound **64** bridges the thiophene and phenyl domains, implying that the two domains are continuous. In contrast, the deterioration in activity from cyclization of 4,5-dialkyl groups (compare **40** with **50**) confirms that the hydrophobic domain does not extend to the area between the 4- and 5-positions. Because an N^6-benzyl group is tolerated and appears to increase affinity in the tetrahydrothieno[2,3-*c*]pyridine series, the hydrophobic domain may extend a considerable distance from the thiophene ring.

From the above discussion, it is clear that the potency of enhancement is not governed by the simple presence of hydrophobic groups, regardless of location, but rather by the presence of specific groups at specific locations. For example, 4-position substitution of the thiophene ring increases activity, whereas 5-position substitution appears to be irrelevant. The same substituent added to the phenyl ring can increase activity to different degrees depending on whether its position is *ortho*, *meta*, or *para*. These position-dependent effects imply that the enhancer compounds bind to a well defined surface (presumably some part of the surface of the A_1 receptor protein) with distinctive features that are complementary to the enhancer molecule, rather than simply partitioning into a relatively featureless solvent-like domain, such as the hydrophobic core of the membrane bilayer.

In the present study, all of the compounds that showed allosteric enhancement also showed some degree of competitive adenosine antagonism. However, the ratio of enhancement to antagonism varied by more than 1000-fold, showing very clearly that these effects are separable. This result has two important implications. Mechanistically, this rules out the possibility that the compounds might be producing their enhancing effects by binding to the antagonist site[5] of the receptor. On a practical level, it holds out the promise that the usefulness of this series might be increased by selectively improving allosteric enhancing activity relative to competitive antagonism.

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We thank Gina Lu for determination of A_2 binding affinities, James McKee for assistance in the synthesis of compounds **4–21**, **23**, **24**, and **26**, Sandra Lobbestael for identification of analogs through substructure searches, David W. Robertson and James Fritz for resynthesis of compound **49**, and B. K. Trivedi, Walter Moos, and Alex Bridges for helpful comments.

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Send reprint requests to: Robert F. Bruns, Biochemical Pharmacology Research, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285.

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[5] Whether agonists and antagonists bind to the same site has not been definitely resolved, but several intriguing parallels between structure-activity relationships for the two sets of compounds suggest that they may share a common binding site (12).