Allosteric Enhancers for A_1 Adenosine Receptor

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Abstract: Allosteric enhancers at the adenosine A_1 receptor have received attention as anti-arrhythmic cardiac agents, and, more recently, as anti-lipolytic agents. In addition, allosteric modulators at the adenosine A_1 receptor have therapeutic potential as analgesics and neuroprotective agents. In particular, the compounds with improved potency as enhancers and reduced antagonist properties are mentioned.

Key Words: A_1-adenosine receptor, allosteric modulators, enhancer, cAMP content, neuroprotection.

1. INTRODUCTION

Allostery (derived from Greek, allos, "other", and stereos, "shape") is an approach that hypothesizes that a drug can act at another site, or *allosteric site*, distant from the *orthosteric site* to activate a receptor. Allosteric modulators can enhance or diminish the effects of endogenous agonists. The *allosteric modulation* of the receptor by molecules binding at a second (*allosteric*) site is relatively unexplored yet for G-protein-coupled receptors (GPCRs), but quite common in the family of ion-channel receptors. Allosteric sites have been found on the multimeric ion-channel GABA receptor that provides the basis for the therapeutic action of benzodiazepines [1]. Specific applications of allosteric modulation of GPCR signals have been described over the last years to developing drugs that fight Alzheimer's-related dementia, Parkinson's disease, schizophrenia, cardiovascular disorders, depression, chronic pain, and even increased stomach acid secretion, asthma, migraine and vision problems [2].

Extracellular purines such as ATP and adenosine act through membrane-associated purinergic receptors as autocrine and paracrine substances as well as neurotransmitters [3]. The ubiquitous nature of these ligands, and the abundance of the expression of purinergic receptors, account for purinergic control of diverse effects in many tissues. This large family of purinergic receptors has been subdivided into two classes, P1 and P2, that have preferential affinity for adenosine and ATP, respectively. The P1 receptors belong to the family of GPCRs and have been cloned in several animal species. Adenosine exerts its action interacting with four different P1- receptor subtypes, classified as A_1, A_{2A}, A_{2B}, and A_3, each of which has been cloned and characterized biochemically and pharmacologically. Adenosine A_1 and A_3 receptors are coupled to a G_i protein, thereby inhibiting the production of cAMP *via* adenylate cyclase. In contrast, the A_{2A} and A_{2B} receptors are coupled to a G_s protein, thereby increasing the production of cAMP by the activation of adenylate cyclase. In some cases, the activation of the A_{2B} receptor has been documented occurs through other effectors, specifically mobilization of intracellular calcium, and this may result from interaction with G_q or other G proteins [4]. The primary downstream effects of A_3 activation are inhibition of adenylyl cyclase function and, in some cases, activation of phospholipase C to provide an intracellular calcium signal.

The idea of allosteric modulation of agonist activity is an approach with many potential benefits. Allosteric enhancers (AE) would binds to an allosteric site on the receptor and not activate the receptor by itself. Rather, it would enhance the activation of the receptor from the orthosteric site, enhancing the potency of the endogenous agonist. And because allosteric modulators modify the actions of the endogenous agonist, they should be able to avoid the adverse effects observed by the use of exogenous agonists.

Recently, the new concept of allosteric modulation has emerged also for the adenosine receptors [4,5]. By analogy with the GABA receptor system, allosteric enhancers of adenosine receptor might provide a more selective therapeutic effect than direct-acting adenosine agonists. Such agents might synergize with endogenous adenosine but have minimal effects in the absence of adenosine. Their actions would therefore be limited to time and location at which significant release of adenosine occurred (for example, hypoxic conditions increase the local production of cyto-protective adenosine), so that systemic side effects would largely be avoided.

Allosteric enhancers of the action of adenosine, upon binding, are believed to stabilize a conformation of the A_1 adenosine receptor having a high affinity for agonists. This effect is manifested as a slowing of the rate of dissociation of agonist from the receptor [5]. In addition, an allosteric enhancer appears to stabilize an active conformation of the receptor even in the absence of the agonist.

In the early 1990s Bruns and coworkers, preparing some intermediates for the synthesis of candidate thiazepine anx-

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iolytics [6], reported various 2-amino-3-benzoylthiophene derivatives capable of enhancing the binding and activity of N^6-cyclopentyladenosine (CPA), an A_1-adenosine receptor agonist. Further investigation have shown that this class of molecules are able to act as allosteric enhancers at the adenosine A_1 receptor, both in binding and *in vitro* functional assays [5]. The allosteric nature of the interaction was shown by the slowing of the dissociation of the agonist [^{3}H]CHA. The enhancing effect was specific for the A_1 receptor, because no enhancement was observed for the others adenosine-receptor subtypes. Furthermore, the positive allosteric effect on A_1-receptor was observed only for selective agonists, because the antagonist binding was not enhanced [5,7].

In one of the seminal papers by Bruns and coworkers [5], three compounds were tested in substantial details: PD 81,723 (1), PD 71,605 (2) and PD 117,975 (3) (Fig. **1**).

The 2-amino-3-aroylthiophene allosteric enhancers discovered by Bruns and his colleagues [5,8] have been shown to enhance adenosine binding and the functional activation of the A_1 receptor in heart and cardiovascular tissues, thus they could be useful both as antiarrythmic [9,11] and cardioprotective [7] agents. Furthermore, due to their capability to mitigate allodynia [12] and thus apparently are accessible to the central nervous system, they could be also useful in managing chronic pain.

2. ALLOSTERIC ENHANCERS FOR ADENOSINE A_1 RECEPTORS

The evidence of potential clinical uses of allosteric enhancers for A_1 adenosine receptor stimulated several groups to expand the panel of compounds available for more detailed structure-activity analysis.

The initial chemical lead for the enhancement of [^{3}H] CHA binding was the 4,5,6,7-tetrahydrothieno[2,3-c]pyridine derivative PD 117,975 (compound 3, Fig. **1**). By the synthesis of new analogs, it became clear that the tetrahydropyridine ring was not essential, because it can be replaced with a tetrahydrobenzene nucleus or even eliminated entirely without loss of activity. For instance, both PD 81,723 (1) and PD 71,605 (2) showed enhancing activity equal to that of PD 117,975.

Bruns and co-workers reported that 2-amino-3-benzoylthiophene derivatives are capable both of enhancing the binding and activity of reference A_1 receptor agonists, such as N^6-cyclopentyladenosine (CPA), to the A_1-adenosine receptor. They also reported that these compounds were capable of acting as competitive antagonists at the same receptor, usually at higher concentrations [5]. Therefore, the concentration range where these compounds can enhance the effects of agonists is limited. Among the compounds tested by Bruns, PD 81,723 was one of the most potent and effective of the series and represents a specific and selective allosteric enhancer of agonist binding to the A_1 receptor, with the best ratio of enhancement to antagonistic action at this receptor. Since this initial discovery, other researchers have directed significant effort to refining the structure–activity relationships of the 3-, 4- and 5-positions of the 2-aminothiophene moiety [13-17].

2-Amino Group

The lack of the 2-amino group resulted in complete loss of activity, implying that this group is essential for activity [5]. At least one of the amino hydrogen was necessary, and in some cases both hydrogen atoms appeared to be required. *N*-acetyl substitution destroyed activity [5]. Also the functionalization of the 2-amino group as benzamide was negative for enhancement (see compound **13**, Table **5**). [14]

Substitution at the 3-Position of the Thiophene Ring

Structure-activity relationship (SAR) studies on the benzoyl moiety were performed by several groups [13-14 and 16-20], leading to several series of 4,5-dialkyl (**4a**–**h**) and tetrahydrobenzothiophene (**4i**–**r**) derivatives (Table **1**). These molecules were evaluated both as allosteric enhancers and as antagonists on the adenosine A_1 receptor. Among them, compounds **4b**, **4l**, **4m** and **4r**, proved similar or superior to PD 81,723 as enhancing activity, with concomitant diminished antagonistic behavior.

In the series of 4,5-dialkyl derivatives (**4a**–**h**), none was more potent than PD 81,723 as allosteric enhancer, although chloro substitution on either the *meta* or the *para* position of the benzoyl ring led to activities comparable to that of the *meta*-trifluoromethyl counterpart PD 81,723. The same compounds **4a**–**h** were less active as antagonists with respect to PD 81,723, improving the window between enhancing and antagonistic activity. This was particularly true for **4e** and **4g**. Obvious extensions of these series were the "cyclized" PD 81,723 analogues **4i-r**. The "Topliss" approach [15] was applied on this series by varying substituents on the benzoyl ring system. On the *para* position halogen substitution was preferred over nitro (**4p**) or carboxylate (**4q**). Also, methyl and trifluoromethyl substitution on this position yielded compounds (**4n** and **4o**, respectively) with higher enhancing activity than PD 81,723. For those compounds with the same substituents either on the *meta* or the *para* position, it ap-

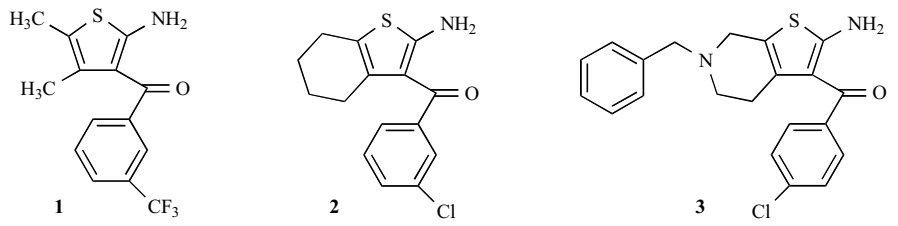
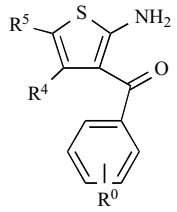


Fig. (1). Representative adenosine A1 receptor allosteric modulators.

Table 1. Adenosine A_1 Receptor Enhancement and Antagonistic Activity of Compounds 4a-r



4 a-r

Compound	R^0	R^4	R^5	% Enhancement[a]	% Antagonism[b]	Ki (μM)[c]
PD 81,723	3-CF_3	CH_3	CH_3	100	39 ± 4	4.7 ± 0.8
4a	H	CH_3	CH_3	8 ± 5	14 ± 3	18 ± 1
4b	3-Cl	CH_3	CH_3	80 ± 19	19 ± 4	
4c	4-Cl	CH_3	CH_3	93 ± 32	41 ± 6	
4d	H	CH_2CH_3	CH_3	31 ± 4	13 ± 3	15 ± 2
4e	3-CF_3	CH_2CH_3	CH_3	112 ± 10	5 ± 11	10 ± 0
4f	3-Cl	CH_2CH_3	CH_3	30 ± 7	22 ± 2	13 ± 5
4g	4-Cl	CH_2CH_3	CH_3	97 ± 25	20 ± 12	
4h	H	$CH_3CH_2CH_2$	CH_3CH_2	69 ± 19	17 ± 6	10 ± 4
4i	H	-$(CH_2)_4$-		47.2 ± 5	34.7 ± 6	5.5 ± 0.1
4j	2-Cl	-$(CH_2)_4$-		73 ± 19	35 ± 3	
4k	3-Cl	-$(CH_2)_4$-		93 ± 6	51 ± 5	4.2 ± 1.2
4l	4-Cl	-$(CH_2)_4$-		123 ± 15	40 ± 5	
4m	3-CF_3	-$(CH_2)_4$-		122 ± 19	32 ± 8	4.0 ± 1.1
4n	4-CF_3	-$(CH_2)_4$-		131 ± 11	57 ± 4	
4o	4-CH_3	-$(CH_2)_4$-		137 ± 21	30 ± 3	
4p	4-NO_2	-$(CH_2)_4$-		34 ± 22	19.5 ± 2	
4q	4-CO_2H	-$(CH_2)_4$-		29 ± 3	nd	
4r	3,4-Cl	-$(CH_2)_4$-		151 ± 24	35 ± 4	

[a] Enhancing activity (at 10 μM of test compound) is expressed as % decrease (±SEM) in [^{3}H]CCPA dissociation over control (0%) and that of PD 81,723 (100.0%, n=3).

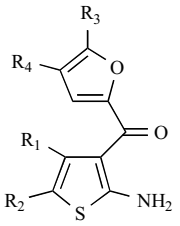
[b] Antagonistic activity is expressed as % displacement (±SEM) of 0.4 nM of [^{3}H]DPCPX by 10 μM of test compound.

[c] As affinity constants (Ki values), determined for some compounds.

peared that the *para* substitution was better (**4l** *vs.* **4k**). Interestingly, combining *meta*/*para* substitution, as in the dichloro compound **4r**, seemed to have an additive effect, appearing more active than either the *meta* or the *para* substituted chloro derivatives. Moreover, the close PD 81,723 analogues **4b** and **4e** were equipotent to PD 81,723 in their enhancing activity, but had less antagonistic properties, while compounds **4l**, **4m**, and **4r** proved more potent enhancers than PD 81,723 with comparable antagonistic activity. Lipophilic *meta* and/or *para* substituents such as halogen are preferred for enhancing activity, whereas more hydrophilic groups such as nitro (**4p**) and carboxylate (**4q**) are not favorable.

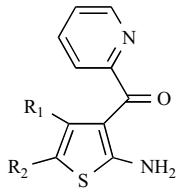
By the synthesis of a series of compounds with general formulae **5-8** (Fig. **2**), we have evaluated the allosteric enhancer effect due to the replacement of the phenyl at the 3-position of the thiophene with a heterocycle or benzoheterocycle.

The heterocycles were selected to possess heteroatoms capable of forming hydrogen bonds within the binding domain (2-furanyl and 2-pyridyl moieties in compounds with general formulae **5** and **6**, respectively). In addition, especially with pyridine (compound **6**), the hydrophilic properties of the molecules were increased, since low-solubility is one of the major limitations of the 2-amino-3-benzoylthiophene derivative **5**. Unfortunately, the derivatives of general formu-



5

$R_1 = R_2$ where R_1, R_2 = H, CH_3
R_1, R_2 = -$(CH_2)_n$- with n = 3 or 4
R_3, R_4 = -(CH=CH)$_2$-
$R_3 = R_4$ = H
If R_4 is H, R_3 is halogen, alkyl, substituted alkyl, aryl, amino, trifluoromethyl, nitro or cyano



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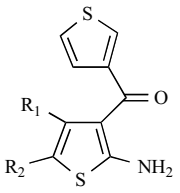
$R_1 = R_2$ where R_1, R_2 = H, CH_3
R_1, R_2 = -$(CH_2)_n$- with n = 3 or 4



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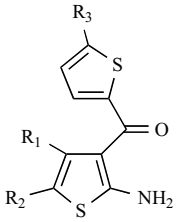
$R_1 = R_2$ where R_1, R_2 = H, CH_3
R_1, R_2 = -$(CH_2)_n$- with n = 3 or 4



8

$R_1 = R_2$ where R_1, R_2 = H, CH_3
R_1, R_2 = -$(CH_2)_n$- with n = 3 or 4
R_3 = halogen, alkyl, substituted alkyl, aryl, amino, trifluoromethyl, nitro or cyano

Fig. (2). General structures (**5-8**) of 2-amino-3-heteroaryl thiophenes.

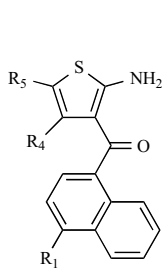
lae **5** and **6**, which potentially could form a hydrogen bond with the allosteric site of the A_1 adenosine receptor, were significantly less potent as allosteric enhancers than the reference compound PD 81,723.

The replacement of the phenyl at the 3-position with an isosteric/isoelectronic thienyl was generally well-tolerated. Compounds of general formulae **7** and **8**, characterized by the presence of a 3- or 2-thienyl, respectively, maintained allosteric enhancer activity, but increased competitive antagonist profile. The position at which the thiophene is connected to carbonyl does not seem important, since 3-thienyl derivatives with general formula **7** possessed the same activity as the 2-thienyl isomer **8**.

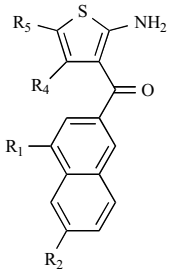
Our group has also reported the allosteric enhancer activity of a new series of PD 81,723 derivatives, characterized by the replacement of the benzoyl at the 3-position with a naphthoyl moiety (Table **2**) [20]. In these compounds, various modifications on the naphthalene ring were studied to establish the structural requirements and the structure-activity relationships for enhancement of the action of an agonist at the human A_1-adenosine receptor [22]. The receptor environment neighboring the benzoyl binding site is lipophilic, and for this reason the substituents at the 4-position on the naphthalene were varied from H to groups with a different degree of lipophilicity (CH_3, CH_3O and Cl). For the substituents at the 4- and 5-position of the thiophene, we have examined the methyl (obtaining 4,5-dimethyl thiophene derivatives) and an alkyl chain between the 4- and 5-position, which varied from three to four methylene units, to yield the 4,5,6,7-tetrahydrobenzo[b]thiophene and 5,6-dihydro-4H-cyclopentadien[b]thiophene derivatives, respectively. Biological assays for the allosteric enhancement activity were performed using Chinese hamster ovary (CHO) cells stably transfected to express the recombinant human A1-adenosine receptors. Activation of these receptors causes an inhibition of the activity of adenylyl cyclase and a reduction of cAMP content of CHO cells. Allosteric enhancement was measured as the ability of the new compounds at four different concentrations (0.01, 0.1, 1 and 10 μM) to reduce the cAMP content on CHO:hA_1 cells. The results are shown in Table **2**.

In the series of derivatives characterized by the presence of the unsubstituted 1-naphthoyl moiety at the 3-position of the thiophene ring (**9a, 9e, 9i**) and which differ in the R_4- and R_5-substituents, it appeared that the most potent compounds had a three- or four-carbon methylene linkage between the 4- and 5-position of the thiophene (compounds **9a** and **9i**, respectively). The presence of a methyl group in both of these positions (compound **9a**) decreased slightly the potency at 10 μM concentration of the tested compound.

Table 2. Percentage Change of cAMP Content in Presence of Compounds 9a-l and 10 a-i



9a-l



10a-i

					Change in cAMP Content from Control (mean±SEM)[a] Concentration of compounds		
Compound	**R_1**	**R_2**	**R_4**	**R_5**	**0.1 μM**	**1μM**	**10 μM**
PD81,723					-7±2	-13±1	-50±1
9a	H		CH_3	CH_3	-3±3	6±5	-45±2
9b	CH_3		CH_3	CH_3	-8±3	-32±2	-51±4
9c	OCH_3		CH_3	CH_3	3±10	-5±9	-38±9
9d	Cl		CH_3	CH_3	-18±5	-47±6	-56±2
9e	H		$-(CH_2)_3-$		-6±1	-29±4	-60±1
9f	CH_3		$-(CH_2)_3-$		4±3	-17±4	-48±2
9g	OCH_3		$-(CH_2)_3-$		-3±4	-8±4	-35±3
9h	Cl		$-(CH_2)_3-$		-15±4	-27±2	-55±2
9i	H		$-(CH_2)_4-$		-15±4	-22±3	-52±3
9j	CH_3		$-(CH_2)_4-$		13±5	-21±1	-45±5
9k	OCH_3		$-(CH_2)_4-$		-5±4	-3±3	-17±1
9l	Cl		$-(CH_2)_4-$		3±3	-25±4	-44±2
10a	H	H	CH_3	CH_3	-4±3	-8±3	-28±3
10b	CH_3	H	CH_3	CH_3	-3±5	-9±6	-22±2
10c	CH_3	Cl	CH_3	CH_3	0±5	-18±4	-24±6
10d	H	H	$-(CH_2)_3-$		-14±4	-15±3	-51±3
10e	CH_3	H	$-(CH_2)_3-$		-7±4	-13±3	-34±1
10f	CH_3	Cl	$-(CH_2)_3-$		-14±3	-6±2	-17±1
10g	H	H	$-(CH_2)_4-$		-24±3	-28±2	-42±3
10h	CH_3	H	$-(CH_2)_4-$		-9±5	-8±4	-20±5
10i	CH_3	Cl	$-(CH_2)_4-$		1±3	-11±5	-13±2

[a] The results are the average of six experiments at each of four concentrations of tested compound.

Several chemically different substituents at the 4-position of the 1-naphthoyl moiety were investigated. These modifications will alter the electronic, steric and lipophilic features of this residue. Generally, by the introduction of a lipophilic and electron-releasing methyl group at the 4- position of the naphthoyl ring, the allosteric enhancing activity was increased for compound **9b**, but decreased for **9f** and **9j** with respect to unsubstituted derivatives **9e** and **9i**, respectively. However, compound **9b** was more active both than PD 81,723 and **9a** at any concentration.

The replacement of the methyl with a less lipophilic and more electron-releasing moiety (a methoxy group), reduced allosteric enhancer activity. For this latter compound, the reduction in activity may be attributed both to steric and electronic factors, in fact, the methoxy group has an angularity component, i.e. part of the methoxy group can extend significantly above or below the plane of the naphthalene ring, thus introducing a unique steric parameter. Although the introduction of a chlorine at the 4-position of the 1-naphthoyl moiety (compounds **9d**, **9h** and **9l**) did not have a profound effect on activity relative to the 4-unsubstituted derivatives, the introduction of a lipophilic halogen or methyl group at the 4-position of the naphthalene ring was preferred for enhancing activity, whereas a more hydrophilic group, such as the methoxy, was not favorable._The results indicate a significant qualitative direct correlation between the allosteric enhancer activity and the lipophilicity of the substituent at the 4-position of the 1-naphthoyl moiety, with the compounds characterized by the presence of moieties with higher lipophilic character (e.g., Cl) among the most potent as A_1 adenosine allosteric enhancers.

By examining regioisomers of various compounds with the same substituents, it has been possible to further elucidate the role of lipophilicity in the structure-activity relationships associated with these molecules. This is exemplified by two groups of regioisomers, composed of thiophene derivatives substituted in 3-position with the 1-naphthoyl or 2-naphthoyl moieties (compounds **9a-l** and **10a-i**, respectively, Table **2**).

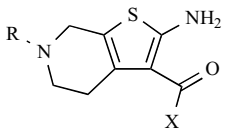
By comparing the activities of derivatives bearing the same substituents on the 4- and 5-position of the thiophene ring, it may be concluded that the 1-naphthoyl derivatives (general formula **9**) are generally more potent than the corresponding analogues with the 2-naphthoyl substituent (general formula **10**). Therefore, because molecules with equivalent lipophilicity may have very different levels of activity, we can conclude that lipophilicity is a necessary condition but not the only determining parameter for the allosteric enhancer activity.

In the series of derivatives which possess the 4-methyl-2-naphthoyl moiety (**10a**, **10e**, **10h**), the introduction of a lipophilic chloro atom at the 6-position of the naphthalene moiety did not improve the allosteric enhancer potency, which indicates an unfavorable steric interaction originating from the presence of 6-chloro substitution in compounds **10c**, **10f**, **10i**.

This large panel of compounds confirmed the previous studies by Bruns [8], suggesting that the addition of a fused ring on the thiophene improved allosteric enhancer activity. In particular, cycloalkylthiophenes tended to be more potent then 4,5-dimethyl analogues, and in the series of cycloalkylthiophenes, tetrahydrobenzo[*b*]thiophene derivatives appeared to be more potent than the dihydrocyclopentadien[*b*]thiophene counterparts.

In the benzophenone series previously described by Bruns [5], removing the keto carbonyl resulted in complete loss of activity (data not shown). The initial study by Bruns included two 2-amino-3-ethoxycarbonyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines [5,8]. Whilst these compounds maintained some enhancing ability, they proved to be less potent than the corresponding 3-benzoyl substituted 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines (see Table **3**).

Table 3. 3-Ethoxycarbonyl and 3-Benzoyl Substituted Allosteric Enhancers Reported by Bruns *et al.* [5,8]



R	X	Enhancement (%)[a]
Me	OE	6
Me	Ph	14
CH_2Ph	OEt	66
CH_2Ph	Ph	102

[a] Allosteric enhancement was evaluated using an assay that measured the % increase over control in specific [^{3}H]N6-cyclohexyladenosine ([^{3}H]CHA) binding to rat brain membranes after addition of 100 lM R-PIA for 2h.

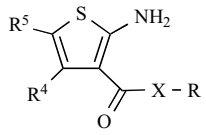
Since relatively few 2-aminothiophenes with esters or amides in the 3-position have been tested as allosteric enhancers, recently Scammels *et al.* [23] described some novel 2-aminothiophene-3-carboxylates and carboxamides to explore the effect of these substituents on AE activity (Table **4**). Allosteric enhancement was evaluated using an *in vitro* assay which measures their ability to stabilize the agonist–A_1AR–G protein ternary complex [24].

In general, the benzyl 2-aminothiophene-3-carboxamides proved to be more efficacious than the corresponding benzyl 2-aminothiophene-3-carboxylates (compare compounds **11h-j** with **11a-d**). The benzyl 2-aminothiophene-3-carboxamides were also more active than the corresponding phenyl 2-aminothiophene-3-carboxylates (compare **11i** with **11e**). Interestingly, high efficacy and unusually high potency was observed for 2-amino- 5,6,7,8-tetrahydrocyclohepta[b]thiophene-3-carboxylic acid (compound **11g**). Differently, the ethyl ester derivative **14**, described by Kourounakis [13], resulted completely inactive as allosteric enhancer, while the antagonistic properties were not influenced (compare compound **14** in Table **5** with **4i** in Table **1**).

Substitution at the 4- and 5-Positions of the Thiophene Ring

To study the role of various substitutions and the importance of the 4,5-dimethyl group on the thienyl ring, van der Klein [13] and Kourounakis [14] have described the synthesis and biological evaluation of 4,5-dialkyl-2-amino-3-benzoyl thiophenes (**12a-l,** Table **5**). Upon comparison of the antagonistic activity of structures **12a-k** with that of **4a-h**, previously depicted in Table **1**, it seemed that bulky 5-alkyl substituents (such as cyclopentyl, cyclohexyl, or phenyl) increased 10-fold antagonistic properties. In contrast, substitution on the 4-position of the thiophene ring seemed to favor allosteric enhancer activity, decreasing competitive antagonistic potency. In this respect, increasing the length of

Table 4. Allosteric Enhancer Activity of 2-aminothiophene-3-carboxylates and Carboxamides from Scammels *et al.* [23]



Compound	X	R^4/R^5	R	ScoreMax[a]	ED_{50} (μM)	% Inhibition[b]
PD 81, 723				28 ± 1.1%	13.6 ± 2.1	18.8 ± 2.46
11a	O	$-(CH_2)_4-$	$PhCH_2-$	71.4 ± 1.2%	10.4 ± 1.2	15.2 ± 1.89
11b	O	$-(CH_2)_4-$	$3-CF_3PhCH_2-$	31 ± 6.5%	> 20	
11c	O	$-(CH_2)_5-$	$PhCH_2-$	88.0 ± 1%	> 20	
11d	O	$-(CH_2)_5-$	$3-CF_3PhCH_2-$	59.0 ± 6%	> 20	
11e	NH	$-(CH_2)_4-$	$3-CF_3Ph-$	32.5 ± 3.5%	> 20	
11f	O	$-(CH_2)_4-$	H	49.5 ± 3%	> 20	
11g	O	$-(CH_2)_5-$	H	79.5 ± 1.5%	1.35 ± 0.17	9.23 ± 1.20
11h	NH	$-(CH_2)_4-$	$PhCH_2-$	79.5 ± 1.5%	10.4 ± 0.9	6.53 ± 1.10
11i	NH	$-(CH_2)_4-$	$3-CF_3PhCH_2-$	55.5 ± 3.5%	3.0 ± 0.7	9.47 ± 1.13
11j	NH	$-(CH_2)_5-$	$PhCH_2-$	68.5 ± 7.5%	> 20	

[a] For compounds with an ED_{50} < 20 μM this value is based on curve fitting relating AE score to AE concentration using the equation Score = ScoreMax * [AE]/(ED_{50} + [AE]). Data points are means± SEM, n = 2–3. For compounds with ED_{50} values > 20 μM the score at 50 μM is recorded.
[b] % inhibition of specific [^{3}H]CPX binding by 10 μM allosteric enhancer, n = 3

the alkyl chain at the 4-position (from methyl to propyl), we have observed a relatively higher enhancement activity for the corresponding structures (**4a** *vs.* **4d** and **4e** or **4h** *vs.* **12d**).

A further investigation of the effects of the 5-substitution of the thiophene system was performed by Olsson *et al.* [18], which reported the syntheses of two kinds of 2-amino-thiophenes namely, 2-amino 3-benzoyl-4,5-diphenylthiophenes, **15a-g**, and 2-amino-5- bromo-3-benzoyl-4-phenylthiophenes, **15h-n** (Table **6**). The AE activity was measured by an *in vitro* assay employing the A_1AR agonist [^{125}I]ABA and membranes from CHO-K1 cells stably expressing the hA_1AR.

Compounds **15a-g** are 2-amino-3-aroyl- 4,5-diphenyl-phenylthiophenes having modifications of either the 3- and/or 4-substituent. The uniformly modest activity of the other members of this series offers clues of the unfavored 5-substitution with bulky group for positive A_1 enhancement.

It is noteworthy to note that the series of 5-bromothiophenes derivatives (**15h-n)** showed an allosteric enhancer activity comparable (compounds **15i** and **15l**) or superior (**15h**, **15j-k** and **15m-n**) to that of PD 81,273. Derivatives **15h**, **15j** and **15k** have in common a strong electron-withdrawing group in either the *meta* or *para* position of the 4-phenyl group. That activity owes to electronic effects is uncertain, because compounds with strong electron-withdrawing groups on the 4-phenyl (derivatives**15i** and **15l**) have modest allosteric enhancer activity. Also the presence of sterically hindered substituents on the 4-phenyl (compounds **15m** and **15n)** resulted favorable for a positive allosteric enhancement. That activity is reminiscent of the enhanced activity conferred by large 3-aroyl groups previously shown [17] and raises the possibility that bulky substituents at the 3- or 4-position of thiophene ring bind to a common hydrophobic pocket of the receptor.

van der Klein *et al*. [13] has also claimed a series of compounds structurally related to the tetrahydrobenzothiophene derivatives **4i-r** (Table **1**) previously described, in which the C-6 methylene group was replaced with a nitrogen atom to give the corresponding tetrahydrothieno [2,3-c]pyridines with general structure **16** and **17** (Table **7**).

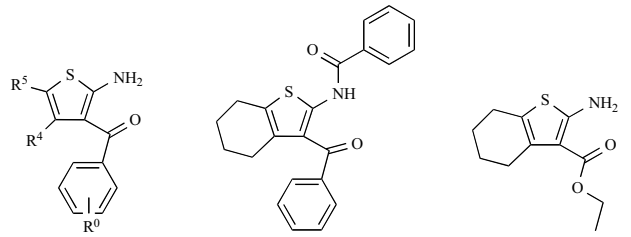
In the series of compounds with general structure **16**, several novel potent allosteric enhancers were identified (derivatives **16a-g**, Table **7**). By varying substituents on the benzoyl moiety, the 3,4-dichloro substitution was most favored (**16g** *vs.* **16a** and **16e**). The benzyl moiety was best substituted with a *meta* chloro substituent (**16b** *vs.* **16a**, **16c** and **16d**).

It has been reported by Bruns [8] that a positive charge on the nitrogen reduces enhancing activity. This is substantiated by the N-benzoyl derivative **17**, which provided only little enhancing activity and, on contrary, high antagonism comparable to that of theophylline, an A_1-adenosine receptor antagonist.

Substitution of the Thiophene Ring

Since the initial discovery of allosteric enhancers for the adenosine A_1 receptor, it was evident the crucial importance of the thiophene ring for activity. When the thiophene was replaced with a benzene, the resulting 2-aminobenzophenone was less active than the corresponding thiophene analogue [5].

Table 5. Adenosine A_1 Receptor Enhancement and Antagonism by Compounds 12a-l, 13 and 14



12a-l **13** **14**

Compound	R^0	R^4	R^5	% Enhancement[a]	% Antagonism[b]	Ki (μM)[c]
PD 81,723	3-CF_3	CH_3	CH_3	100	39 ± 4	4.7 ± 0.8
12a	3-CF_3	H	$CH_3CH_2CH_2$	88 ± 8	52 ± 3	7.3 ± 2.9
12b	3-Cl	H	$CH_3CH_2CH_2$	67 ± 18	54 ± 5	3.5 ± 0.4
12c	H	H	$CH_3CH_2CH_2$	0 ± 30	50 ± 7	4.4 ± 0.4
12d	3-CF_3	H	C_5H_9	99 ± 25	49 ± 2	3.6 ± 0.8
12e	3-Cl	H	C_5H_9	52 ± 12	64 ± 1	2.7 ± 0.1
12f	3-Cl	H	C_6H_{11}	57 ± 2	64 ± 3	3.1 ± 0.4
12g	H	H	C_6H_5	21 ± 5	75 ± 2	1.2 ± 0.2
12h	3-Cl	H	C_6H_5	38 ± 6	80 ± 1	1.0 ± 0.2
12i	3-CF_3	H	C_6H_5	42 ± 7	58 ± 3	2.4 ± 0.4
12j	H	H	$(CH_3)_2CHCH_2$	-7 ± 14	47 ± 5	4.1 ± 0.6
12k	H	CH_3	CH_3CH_2	13 ± 17	27 ± 7	15 ± 4
12l	3,4-Cl	CH_3	CH_3	116 ± 7	50 ± 1	3.2 ± 0.3
13				31 ± 13	28 ± 1	9.9 ± 0.8
14				2 ± 7	47 ± 2	3.9 ± 0.7

[a]Enhancing activity (at 10 μM of test compound) is expressed as % decrease (±SEM) in [^{3}H]CCPA dissociation over control (0%) and that of PD 81,723 (100.0%, n=3).

[b]Antagonistic activity is expressed as % displacement (±SEM) of 0.4 nM of [^{3}H]DPCPX by 10 μM of test compound.

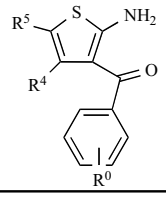
[c]As affinity constants (Ki values).

Despite such evidence, 2-aminothiophenes may not be ideal drug candidates due to their instability. A new class of allosteric enhancers for adenosine A_1 receptor, characterized by a 2-aminothiazole core, was identified by Chordia *et al.* [26,27] These compounds lack the 3-aroyl moiety thought to be necessary for allosteric enhancer activity of 2-aminothiophenes. The only structural features that 2-aminothiazoles appear to share with the 2-aminothiophenes is a five-membered aromatic ring containing sulphur and an exocyclic amine (general formula **18** and **19**, Fig. **3**).

Electron-donating phenyl substituents, especially in the *para* position, supported activity, and conversely, electron-withdrawing substituents reduced the allosteric enhancer activity. A large aryl substituent neither promoted nor diminished allosteric enhancer activity. There is some evidence that the 2-aminoindenothiazoles and the 2-amino-3-aroylthiophenes might have similar docking modes in the allosteric site. Whereas the allosteric enhancer activity of the 2-aminothiophenes depended on both the 2-amino and the 3-aroyl groups, these aminothiazoles lack a substituent that corresponds to the aroyl group. However, superimposing the two heterocycles suggests that the thiazole nitrogen could act as a surrogate for the benzoyl, acting, for example, as a hydrogen-bond acceptor. The aryl group of the 2-amino-3-aroylthiophenes can occupy a position near, but not overlapping, the benzene moiety of the indenothiazole. Finally, Bruns has suggested [5] that the allosteric enhancer activity of a 2-amino-3-aroylthiophene is due to the planarity of the molecule, conferred by a hydrogen bond between the 2-amino and the aroyl carbonyl groups. By analogy with another tricyclic planar molecule, the 9*H*fluorene [28], also the indeno[1,2-*d*]thiazoles nucleus should be probably planar, and thus would support the Bruns model.

The structure-activity profile of the 2-aminothiazoles indicated that bicyclic 2-aminothiazole derivatives have less allosteric enhancer activity than tricyclic compounds.

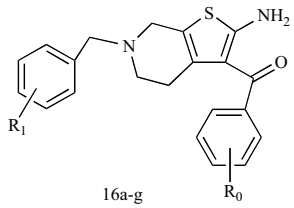
Table 6. Enhancing Activity of Derivatives 15a-n



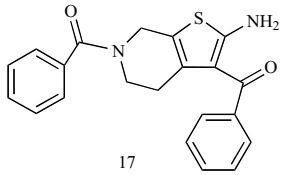
Compound	R^0	R^4	R^5	Activity (%)[a]
PD 81,723				19 ± 2.9
15a	H	Ph	Ph	10.7 ± 2.0
15b	3-Cl	Ph	Ph	9.4 ± 1.2
15c	4-Cl	Ph	Ph	10.8 ± 2.0
15d	H	4-CH_3 Ph	Ph	13.4 ± 1.9
15e	3-Cl	4-CH_3 Ph	Ph	8.1 ± 4.2
15f	4-Cl	4-CH_3 Ph	Ph	8.4 ± 0.5
15g	H	4-Cl Ph	Ph	5.6 ± 2.5
15h	H	3-CF_3 Ph	Br	91.0 ± 5.6
15i	H	3-NO_2 Ph	Br	21.8 ± 3.3
15j	H	4-CF_3 Ph	Br	80.4 ± 2.0
15k	H	4-NO_2 Ph	Br	68.3 ± 1.5
15l	H	4-CN Ph	Br	20.3 ± 3.7
15m	H	4-Ph Ph	Br	42.7 ± 7.0
15n	H	2-naphth	Br	49.7 ± 5.4

[a] The assay employed membranes from CHO-K1 cells stably expressing the human A_1 adenosine receptor. Concentration of allosteric enhancer was 100 μM. All data are mean ± SEM (n =3) [17]

Table 7. Enhancing and Antagonistic Activity of Compounds 16a-g, 17 and theophylline



16a-g

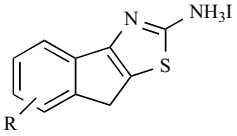


17

Compound	R^0	R^1	% Enhancement[a]	% Antagonism[b]
PD 81,723			100	39.5 ± 4.3
16a	H	H	52.8 ± 36.6	67.1 ± 5.5
16b	H	3-Cl	105.7 ± 2.3	80.1 ± 1.2
16c	H	4-Cl	69.0 ± 23.5	52.2 ± 2.5
16d	H	3,4-Cl	57.0 ± 35.8	4.0 ± 2.0
16e	4-Cl	H	132.4 ± 21.0	60.9 ± 0.1
16f	4-Cl	3,4-Cl	106.5 ± 30.6	45.7 ± 2.1
16g	3,4-Cl	H	173.6 ± 37.5	51.0 ± 0.0
17			13.8 ± 27.3	71.8 ± 2.1
theophylline			14.9 ± 7.5	56.2 ± 5.1

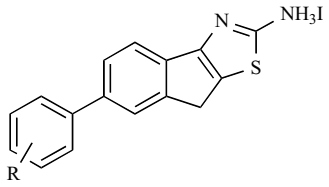
[a] Enhancing activity by 10 μM of test compound is expressed as a percentage decrease (± SEM) in [^{3}H]CCPA dissociation over control (0%) that of 10 μM PD 81,723 (100.0%, n=3).

[b]Antagonistic activity is expressed as percent displacement (±SEM, n=3-5) of 0.4 nM of [^{3}H]DPCPX by 10 μM of test compound.



18

R = halogen, alkyl, hydroxyalkyl group at different positions.



19

R = halogen, alkyl, trifluoromethyl, thienyl, nitro, hydroxyl group at different positions.

Fig. (3). General structures **18** and **19** of 2-aminothiazoles .

Thus planarity of the overall skeleton and the dihedral angle between the thiazole ring and the aromatic ring might be important. Exchanging the positions of nitrogen and sulphur had a marked effect on enhancer activity, suggesting that the disposition of the nitrogen is important for molecular recognition. An electron donor group on the aromatic ring improved allosteric enhancer activity.

Nevertheless, 2-aminothiazoles derivatives do not appear to behave always as pure allosteric enhancer for the A_1 adenosine receptor, in fact they often show a strong antagonistic activity and discrepancy of results among the different research groups.

Göblyös *et al*. [29] were not able to confirm the allosteric enhancing capacity of the 2- aminothiazoles. Some of the compounds proved to be antagonists for the adenosine receptors and showed affinity in the higher nanomolar range. In fact, in Globyos' experiments 2-aminothiazoles of general formula **18** did not behave as allosteric enhancers of agonist binding to adenosine A_1 receptors as suggested by Chordia *et al*. [26]. Amides derived from the 2-aminothiazoles of general formula **18** were also tested in radioligand binding studies and some of them proved to have modest affinity for adenosine A_1 and/or adenosine A_{2A} receptors. They were classified as antagonists based on the results obtained in a functional assay based on cAMP production.

For this reason, up to now 2-amino-3-aroylthiophene derivatives have been proved the best positive allosteric modulators for the A_1 adenosine receptors.

CONCLUSIONS

Allostery is now being employed where traditional methods have failed. Sometimes candidate molecules acting at the orthosteric binding site may not be the safest and effective options for a given condition. Agonist-based compounds may not be selective enough for the receptor subtype that produces a desired therapeutic effect. In addition, high doses of an agonist-based drug might result in a level of receptor activation that causes dangerous side effects.

A wide range of allosteric enhancers based on the structure of PD 81,723 have been synthesized by different research group over the years. A disadvantage of this approach is that the current allosteric modulators for adenosine A_1 receptors have both an allosteric effect and apparent antagonistic activity, with consequent opposing effects on receptor activation. For this reason, researchers have directed significant effort to refining the structure-activity relationships of the 2-, 3-, 4- and 5-positions of the 2-amino-thiophene core, as well as the disclosure of new lead compounds (e.g., 2-aminothiazoles), in order to obtain ideal drug candidates with more potent enhancing effect but without antagonistic activity.

The compounds are claimed for uses including protection against hypoxia, ischaemia, induced injury and treatment of adenosine-sensitive cardiac arrhythmias.

The derivative (2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-(4-chloro-phenyl)-methanone (**4l**, also known by its abbreviation T62) normalized hyperexcited sensory nerve functions in a model of neuropathic pain [30]. This effect is consistent with the action of a drug that increases adenosine binding to A_1 receptors, and suggest that this compound could be useful as therapeutic agent in treating neuropatic pain. The combined action of a selective adenosine A_1 allosteric enhancers (PD 81,723) and a selective A_1 agonist (CPA) was claimed to induce angiogenesis at a desired location for treating conditions in which increased angiogenesis is desired (ischemic tissues and peripheral vascular disease) and theoretically may have broader impact, although efficacy has yet to be shown [31].

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