

# Allosteric modulation of adenosine receptors

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**Abstract** Allosteric modulators for adenosine receptors may have potential therapeutic advantage over orthosteric ligands. Allosteric enhancers at the adenosine A<sub>1</sub> receptor have been linked to antiarrhythmic and antilipolytic activity. They may also have therapeutic potential as analgesics and neuroprotective agents. A<sub>3</sub> allosteric enhancers are postulated to be useful against ischemic conditions or as antitumor agents. In this review, we address recent developments regarding the medicinal chemistry of such compounds. Most efforts have been and are directed toward adenosine A<sub>1</sub> and A<sub>3</sub> receptors, whereas limited or no information is available for A<sub>2A</sub> and A<sub>2B</sub> receptors. We also discuss some findings, mostly receptor mutation studies, regarding localization of the allosteric binding sites on the receptors.

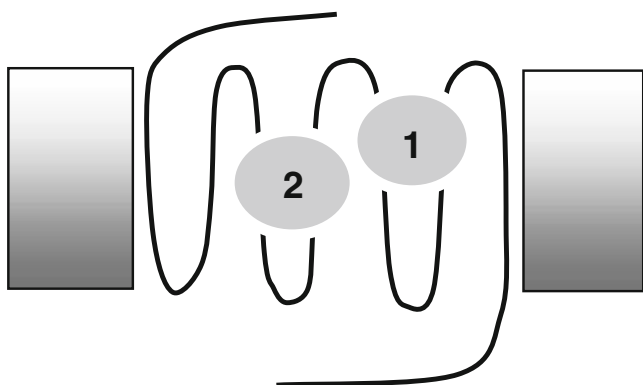
**Keywords** Adenosine receptors · Allosteric modulation · Mutagenesis studies · Chemical classes of allosteric modulators · PD 81 · 723 · LUF6000

## Abbreviations

AR adenosine receptor  
cAMP cyclic adenosine monophosphate  
cDNA complementary deoxyribonucleic acid  
CGS21680 2-[p-(2-carboxyethyl)phenyl-ethylamino]-5'-N-ethylcarboxamidoadenosine  
CGS15943 5-amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-c]quinazoline

CHO Chinese hamster ovary  
CI-IB-MECA 2-chloro-N<sup>6</sup>-(3-iodobenzyl)-5'-N-methylcarboxamidoadenosine  
CP cyclopentyl  
DMA 5-(N,N-dimethyl)amiloride  
DMF dimethylformamide  
DMSO dimethylsulfoxide  
DPCPX 8-cyclopentyl-1,3-dipropylxanthine  
DU124183 2-cyclopentyl-4-phenylamino-1H-imidazo[4,5-c]quinoline  
GPCR G protein-coupled receptor  
GTPγS guanosine 5'-[γ-thio]triphosphate  
h human  
HMA 5-(N,N-hexamethylene)amiloride  
HPLC-MS high-performance liquid chromatography mass spectrometry  
I-ABA N<sup>6</sup>-4-amino-3-iodo-benzyladenosine  
I-AB-MECA N<sup>6</sup>-(4-amino-3-iodobenzyl)-5'-N-methylcarbamoyladenosine  
LUF6000 N-(3,4-dichlorophenyl)-2-cyclohexyl-1H-imidazo[4,5-c]quinolin-4-amine  
MRS1754 8-(4-[(4-cyanophenyl)carbamoylmethyl]oxy]phenyl)-1,3-di(n-propyl)xanthine  
NECA 5'-N-ethylcarboxamidoadenosine  
PD 71,605 (2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-(2-chloro-phenyl)-methanone  
PD 117,975 (2-amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)(4-chloro-phenyl)methanone  
PD 81,723 2-amino-4,5-dimethyl-3-thienyl-[3-(trifluoromethyl)phenyl]methanone  
R-PIA N<sup>6</sup>-[(R)-phenylisopropyl]adenosine  
SAR structure-activity relationships  
SCH-202676 (N-(2,3-diphenyl-[1,2,4]thiadiazole-5(2H)-ylidene)methanamine)

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**Fig. 1** Binding of an allosteric ligand (1) modulates the binding of hormone or neurotransmitter (2)

T-62	(2-amino-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)-(4-chlorophenyl)-methanone transmembrane domain
TM	
VUF5455	4-methoxy-N-[7-methyl-3-(2-pyridinyl)-1-isoquinolinyl]benzamide
VUF8502	4-methyl-N-[3-(2-pyridinyl)-1-isoquinolinyl]benzamide
VUF8504	4-methoxy-N-[3-(2-pyridinyl)-1-isoquinolinyl]benzamide
VUF8507	N-[3-(2-pyridinyl)-1-isoquinolinyl]benzamide
ZM241385	4-{2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-a]1,3,5]triazin-5-yl-amino]ethyl}phenol

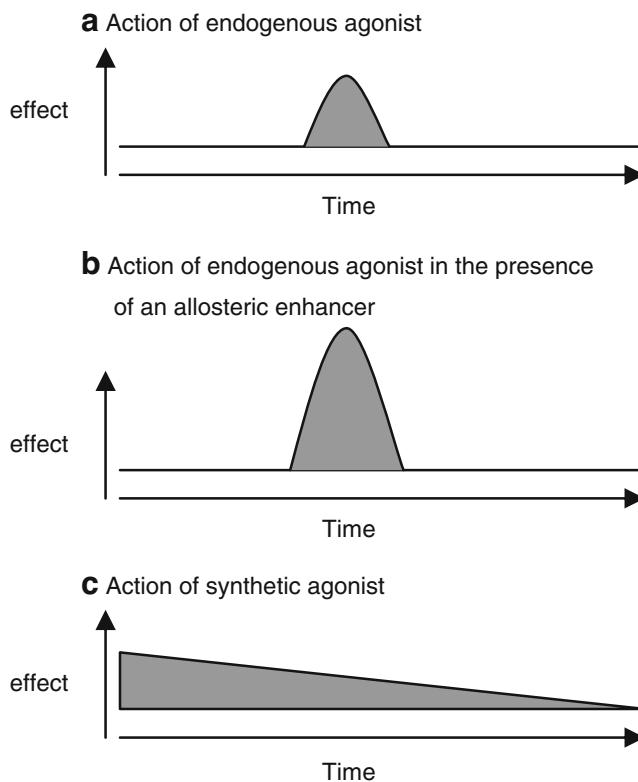
## Introduction

The superfamily of human G protein-coupled receptors (GPCRs) consists of three major classes: A, B, and C. Class A, also termed rhodopsin-like after the prototypic visual pigment, constitutes the largest group of GPCRs. Adenosine receptors belong to this class. Four subtypes of adenosine receptors have been cloned and pharmacologically characterized: A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>. Among the human adenosine receptors, the most similar are A<sub>1</sub> and A<sub>3</sub> (49% sequence similarity) and A<sub>2A</sub> and A<sub>2B</sub> (59% similarity). Activation of A<sub>1</sub> and A<sub>3</sub> receptors induces the inhibition of the enzyme adenylate cyclase mediated by an inhibitory G protein (G<sub>i</sub>). Activation of A<sub>2A</sub> and A<sub>2B</sub> receptors leads to a G<sub>s</sub>-mediated stimulation of this enzyme [1].

Many representatives of class A receptors, among them adenosine receptors, have been shown to be allosterically regulated. Allosteric refers to binding sites that are different from the orthosteric primary ligand-binding site to which ligands such as endogenous adenosine and synthetic derivatives thereof bind. The binding of modulators is supposed to result in conformational changes of the receptor that affect binding of the orthosteric ligand with consequences for receptor activity (see Fig. 1).

Strictly speaking, the allosteric modulator in this scenario has no activity by itself and needs the orthosteric ligand (often the endogenous hormone or neurotransmitter) in order to become activated. The nature of allosteric modulation is therefore very much dependent on this orthosteric ligand. As a consequence, the overall pharmacology invoked by the allosteric modulator resembles physiology more closely than is possible with most of today's drugs, i.e., synthetic orthosteric ligands (see Fig. 2 for the effect of an allosteric enhancer).

The concept of allosteric modulation of established drug targets such as GPCRs has fueled an intensive quest for new classes of lead compounds different from classic agonists and antagonists. This has been the subject of several recent reviews [2–5]. As adenosine receptors were among the first GPCRs found to be allosterically regulated, these receptors have often been reviewed also [6–8]. The aim of this review is to summarize the latest developments in this fascinating field for all four subtypes of adenosine receptors, with a twofold emphasis. First, chemical classes of allosteric ligands, their receptor preference, and structure-



**Fig. 2** Allosteric modulators may either increase or decrease the effect of an agonist or an antagonist. An allosteric enhancer can be a more physiologic alternative to synthetic agonists (b, c). An allosteric enhancer can only act in the presence of an (endogenous) agonist. It mimics the duration and intensity of action of a hormone or neurotransmitter (a) much better than a synthetic agonist does (c). This combined action of two compounds (i.e., agonist and enhancer) might also induce selectivity of drug action

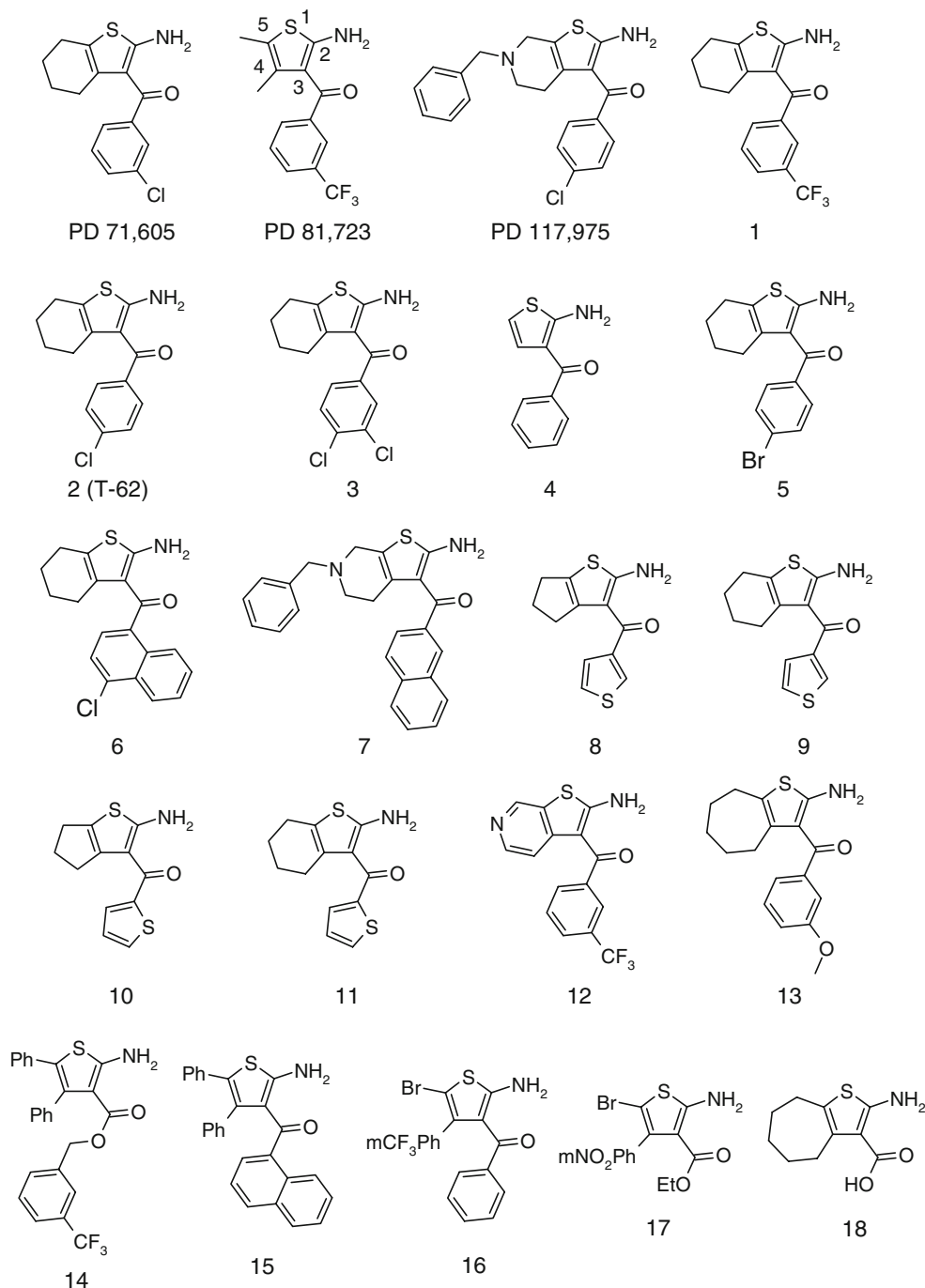
activity relationships (SAR) are discussed. Second, attention is given to the putative binding site of the modulators.

### A<sub>1</sub> adenosine receptors

Baraldi et al. recently published an extensive review of allosteric modulators of A<sub>1</sub> receptors [8]. Here, we briefly provide an overview of historical as well as new allosteric

modulators for A<sub>1</sub> adenosine receptors. Bruns and coworkers introduced the first allosteric modulators of the A<sub>1</sub> receptor in 1990 [9, 10]. Three compounds, (2-amino-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)-(2-chloro-phenyl)-methanone (PD 71,605); 2-amino-4,5-dimethyl-3-thienyl-[3-(trifluoromethyl)phenyl]methanone (PD 81,723); and (2-amino-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridin-3-yl)(4-chloro-phenyl) methanone (PD 117,975) were studied in detail (see Fig. 3). These chemical structures were part of the Parke-Davis

**Fig. 3** 2-Amino-3-substituted thiophene derivatives, allosteric modulators for A<sub>1</sub> adenosine receptors



compound library at the time and were originally used as intermediates in the synthesis of benzodiazepine-like compounds [11].

Their allosteric-enhancing nature was identified by accident while screening the library in a radioligand-binding assay at the rat A<sub>1</sub> adenosine receptor. The three compounds increased rather than decreased (the original aim of the screening campaign) the binding of the agonist radioligand [<sup>3</sup>H]N<sup>6</sup>-cyclohexyladenosine to A<sub>1</sub> receptors in rat-brain membranes. The enhancers appeared to slow the dissociation of the agonist [<sup>3</sup>H]N<sup>6</sup>-cyclohexyladenosine from the A<sub>1</sub> receptor, indicating an allosteric mechanism of action. These compounds were also competitive antagonists at the same receptor. Therefore, the concentration range within which these compounds enhance the effects of agonists is limited. Among the compounds tested by Bruns et al., PD 81,723 stood out as one of the more potent compounds with the best ratio of enhancing to antagonistic action. PD 81,723 has been thoroughly and independently studied over the years, and it became the reference allosteric modulator of A<sub>1</sub> receptors. It has been extensively modified structurally [9, 10].

Van der Klein and colleagues synthesized a new series of 2-amino-3-benzoylthiophenes with PD 81,723 as a template. Among these compounds, **1–3** (Fig. 3) proved to be more potent enhancers than PD 81,723, with comparable antagonistic activity. The biological assays were carried out on membranes of rat-brain cortex, and the enhancing activity was measured at 10 μM of test compound. For compound **1**, enhancement was 122%; for compound **2**, 123%; and for compound **3**, 151% compared with that of PD 81,723 (100%).

It was concluded that in this class of 2-amino-3-benzoylthiophene derivatives, lipophilic benzoyl (3-CF<sub>3</sub>, 3,4-Cl, or 4-*t*-butyl) substitution and thiophene 4-alkyl substitution favored allosteric enhancing activity. Thiophene 5-bulky substitution favored antagonistic properties, which rendered the separation of these two activities in these structures possible [12, 13].

Baraldi et al. also developed a series of PD 81,723 analogues. For their pharmacological evaluation, cyclic adenosine monophosphate (cAMP) determinations were carried out in Chinese hamster ovary (CHO) cells expressing human A<sub>1</sub> receptors (CHO:hA1) vs. radioligand dissociation experiments on rat-brain membranes. In their studies, compounds **2**, **4**, and **5** (Fig. 3) proved to be nearly as efficacious (effect on E<sub>max</sub>) as and more potent (effect on EC<sub>50</sub>) than PD 81,723 and caused a significant reduction of the cAMP content of CHO:hA1 cells at a concentration of 0.1 μM [14]. In a later and more extensive study, the same authors investigated a large number of 2-amino-3-naphthoylthiophenes [15]. Among these compounds, **6** was the most active, increasing agonist binding to CHO:hA1,

human-brain, and rat-cortex membranes by 149%, 43%, and 27% (51%, 15%, and 22% for PD 81,723). In the series of 4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine derivatives, the only compound that showed significant allosteric enhancer activity at any concentration was compound **7**. A series of 2-amino-3-heteroarylthiophenes have also been synthesized. The same functional cAMP assay also revealed that compounds **8–11** are allosteric enhancers at the human A<sub>1</sub> receptor. Replacement of the phenyl ring at the 3 position of the thiophene ring with a 2- or 3-thienyl moiety maintained the enhancing activity of PD 81,723 [16]. Thieno[2,3-*c*]pyridine derivatives were accessed by the Baraldi group by means of microwave-assisted synthesis. Among the prepared compounds, **12** showed activity comparable with that of PD 81,723 (Fig. 3) [17].

Tranberg et al. also performed a systematic survey of the PD 81,723 lead structure. The 3-OMe derivative **13** had high enhancing activity and relatively low antagonistic potency: 99% and 13%, respectively, at 100 μM [18]. The same research group showed that chemical structures more different from the prototypic PD 81,723 were allosteric enhancers also [19]. The allosteric enhancing activity score of PD 81,723 was 19% and served as a standard for comparison. For compound **14**, it was 48%; and for the 3-(1-naphthoyl substituted compound **15**, it was 52%. Compounds **16** and **17** seemed to be more potent, with scores of 91% and 80%, respectively (Fig. 3). In this study assessment of allosteric enhancing activity was done in a somewhat different way and consisted of three phases: (1) binding to equilibrium of the agonist N<sup>6</sup>-4-amino-3-iodobenzyladenosine (<sup>125</sup>I-ABA) to the A<sub>1</sub> adenosine receptor–G protein ternary complex, (2) stabilization of that complex by the allosteric enhancer, and (3) dissociation of the complex by adding a combination of an A<sub>1</sub> receptor antagonist and guanosine 5'-[γ-thio]triphosphate (GTPγS), to accelerate agonist radioligand dissociation. A score of 100% means no dissociation and a score of zero complete dissociation. Similarly, Nikolakopoulos et al. studied 2-aminothiophene-3-carboxylates and carboxamides as adenosine A<sub>1</sub> receptor allosteric enhancers. Compound **18** was notably more potent and efficacious than PD 81,723 (Fig. 3) [20]. Interestingly, 2-aminoselenophene-3-carboxylates also proved to be allosteric enhancers of A<sub>1</sub> receptors [21].

In 2006, the first radiolabeled adenosine A<sub>1</sub> allosteric enhancer, the tritiated form of compound **2**, was prepared (Fig. 3). The biological investigation of this compound, 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)-(4-chlorophenyl)-methanone ([<sup>3</sup>H]T-62), suggested the presence of an allosteric binding site on the adenosine A<sub>1</sub> receptor distinct from the orthosteric binding site for agonists and antagonists [22].

Another chemical class of allosteric enhancers of agonist binding at cloned hA<sub>1</sub> adenosine receptors are the 2-

aminothiazoles. The most potent and efficacious enhancer of the series reported is compound **19** (Fig. 4). The potency of this 2-aminothiazole derivative was reported to be 127 times that of PD 81,723 (EC<sub>50</sub> values 0.3 μM and 38 μM, respectively). The intrinsic activity of compound **19** was about twice that of PD 81,723. The enhancing effect at A<sub>2A</sub> and A<sub>3</sub> receptors by this compound was found to be weak [23].

We also prepared a number of 2-aminothiazoles and their amide derivatives. The 2-aminothiazoles (the free bases) were tested as allosteric enhancers of agonist binding to human A<sub>1</sub> receptors. In a variety of experimental setups, the compounds showed no such effect, in contrast to the above findings of Chordia et al. [23]. Some of the amides emerged as moderately active antagonists on human A<sub>1</sub> and/or A<sub>2A</sub> receptors, with lower or negligible potency at A<sub>3</sub> receptors [24].

Chordia et al. next investigated these contrasting findings in more detail and concluded that the free bases of this type of compound in fresh solutions are only weakly active as allosteric enhancers. However, the allosteric enhancing activity in solutions of these compounds in dimethylformamide (DMF), dimethylsulfoxide (DMSO), or acetonitrile increased after 4 h and finally led to a constant activity equivalent to that of the hydriodide salts after 12 h [25].

Fawzi et al. reported SCH-202676 (N-(2,3-diphenyl-[1,2,4]thiadiazole-5(2H)-ylidene)methanamine) to act as a more promiscuous agent (Fig. 4). It appeared to modulate the activity of many GPCRs, including opioid, muscarinic, adrenergic, and dopaminergic receptors [26]. We studied its activity on adenosine receptors. We also synthesized a number of 2,3,5-substituted [1,2,4]thiadiazole analogues of SCH-202676 and tested these as potential allosteric modulators of adenosine receptors. SCH-202676 displayed a mixed behavior on adenosine receptors. It displaced agonist binding to A<sub>1</sub> receptors in a reversible manner and both agonist and antagonist binding to A<sub>2A</sub> receptors. The displacement curves were very steep, dissimilar to normal competitive displacement curves. On A<sub>1</sub> receptors, compound **20** was shown to be an allosteric enhancer of antagonist 8-cyclopentyl-1,3-dipropylxanthine ([<sup>3</sup>H]DPCPX) binding (Fig. 4) [27].

In our next work, we investigated a new series of 2,3,5-substituted [1,2,4]thiadiazoles. Results of receptor-ligand-

binding experiments and stability studies by high-performance liquid chromatography (HPLC) and HPLC mass spectrometry (MS) showed that the compounds are highly reactive sulfhydryl-modifying agents rather than allosteric inhibitors. They appear to be reduced into their thiourea precursors by thiol groups of, e.g., cysteine residues in proteins. This general feature most probably explains their overall lack of selectivity [28].

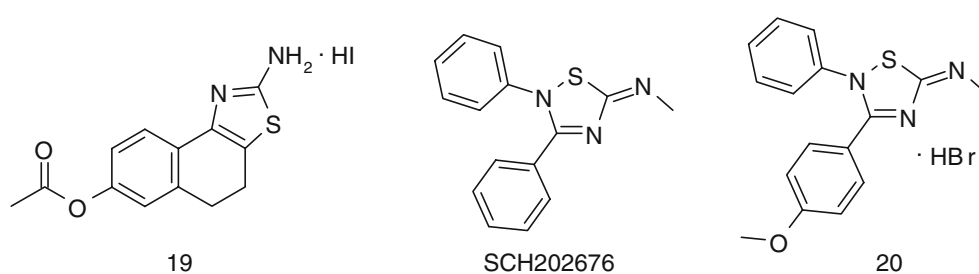
Amiloride (a diuretic) also acts as a promiscuous agent, which had been reported before to interact with many GPCRs such as α<sub>2</sub>-adrenergic receptors [29]. Results by Garritsen et al. suggest that amiloride interacts with the A<sub>1</sub> receptor at a site distinct from the ligand-binding site. Furthermore, amiloride appeared to be an A<sub>1</sub> receptor antagonist with binding characteristics that differ from the classic A<sub>1</sub> receptor antagonists such as DPCPX [30–31].

Allosteric modulation of A<sub>1</sub> receptors has been demonstrated not only in vitro but also in vivo in various species, including humans. Local administration of PD 81,723 in rats caused a concentration-dependent increase of extracellular acetylcholine levels of approximately 40%. However, the antagonistic actions of the allosteric enhancer PD 81,723 on the adenosine A<sub>1</sub> receptor canceled the allosteric actions [32]. Treating dogs with PD 81,723 reduced the time required for ischemia to produce preconditioning [33]. Compound **2** (T-62) has been in preclinical development for the treatment of chronic pain [34, 35] and is now in phase II clinical trials (King Pharmaceuticals).

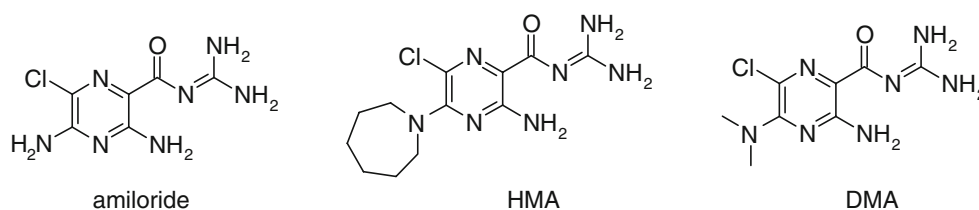
### A<sub>2A</sub> adenosine receptors

Although an allosteric enhancer for the A<sub>2A</sub> receptors has not yet been developed, amiloride and analogues were demonstrated to be allosteric inhibitors also for the A<sub>2A</sub> receptor. Among the derivatives tested, 5-(N,N-hexamethylene) amiloride (HMA) proved to be the most potent allosteric inhibitor (Fig. 5). Amiloride analogues increased the dissociation rate of the antagonist 4-{2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-a]1,3,5]triazin-5-yl-amino}ethyl} phenol ([<sup>3</sup>H]ZM241385) from the A<sub>2A</sub> receptor. However they did not show any effect on the dissociation rate of the agonist 2-[p-(2-carboxyethyl)phenyl-ethylamino]-5'-N-ethyl-carboxamidoadenosine ([<sup>3</sup>H]CGS21680) [36]. In contrast to

**Fig. 4** 2-Aminothiazoles and [1,2,4]thiadiazole analogues, questionable allosteric modulators for A<sub>1</sub> adenosine receptors



**Fig. 5** Amiloride derivatives, allosteric modulators of adenosine receptors



the effects of amiloride analogues on the  $A_{2A}$  receptor, sodium ions decreased the dissociation rate of the antagonist [ $^3$ H]ZM241385 from the  $A_{2A}$  receptor in a concentration-dependent manner.

Gao and colleagues later showed that amiloride analogues are also allosteric inhibitors of antagonist binding at  $A_1$  and  $A_3$  receptor subtypes [37]. Amiloride and 5-(N,N-dimethyl)amiloride (DMA) were more potent at  $A_1$  receptors than at  $A_3$  receptors, whereas HMA was more potent at  $A_3$  receptors (Fig. 5).

### $A_{2B}$ adenosine receptors

Allosteric modulators for  $A_{2B}$  receptors have not yet been described. Research on the  $A_{2B}$  receptor subtype has been hampered by the lack of suitable, commercially available radioligands. However, with the advent of the radiolabeled antagonist 8-(4-[(4-cyanophenyl)carbamoylmethyl]oxy)phenyl)-1,3-di(n-propyl)xanthine ([ $^3$ H]MRS1754) and other radioligands, such studies are now feasible. Still, a radiolabeled agonist is not yet available, which is hindering the identification of allosteric enhancers of agonist binding.

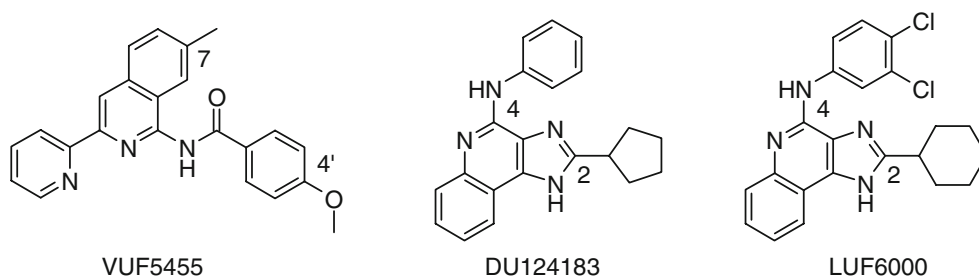
### $A_3$ adenosine receptors

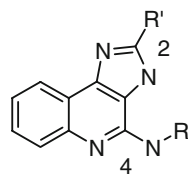
Gao et al. reported the first selective allosteric enhancers of agonist binding at human  $A_3$  receptors [38]. The effects of the reference  $A_3$  receptor agonist 2-chloro- $N^6$ -(3-iodobenzyl)-5'-N-methylcarboxamidoadenosine (Cl-IB-MECA) on forskolin-induced cAMP formation were significantly enhanced by several 3-(2-pyridinyl)isoquinoline derivatives. Most compounds, however, also displayed orthosteric binding affinity to the human  $A_3$  receptor. Their results indicated that 4-methoxy-N-[7-methyl-3-(2-pyridinyl)-1-isoquinolinyl]benzamide (VUF5455) (see Fig. 6) is selective for the agonistic state of the  $A_3$  receptor.

In competitive binding studies on cloned human  $A_3$  receptors VUF5455 is rather weak as an orthosteric ligand ( $K_i=1680$  nM). Replacement of the 7-methyl group of VUF5455 by H [4-methoxy-N-[3-(2-pyridinyl)-1-isoquinolinyl]benzamide (VUF8504)] had no influence on the allosteric activity but increased the  $A_3$  receptor affinity nearly 100-fold ( $K_i=17.3$  nM). Exchanging the 4'-methoxy group of VUF8504 by methyl [4-methyl-N-[3-(2-pyridinyl)-1-isoquinolinyl]benzamide (VUF8502)] or H [N-[3-(2-pyridinyl)-1-isoquinolinyl]benzamide (VUF8507)] lowered the  $A_3$  receptor affinity ( $K_i$  values of 96 nM and 204 nM, respectively) without affecting the allosteric activity. The corresponding imino instead of carboxamido analogues displayed moderate  $A_3$  affinity ( $K_i$  values 300–700 nM) but were devoid of allosteric properties. Thus, although VUF5455 is not devoid of  $A_3$  receptor antagonistic activity, the compound might be used as a lead for the design of pure allosteric enhancers of  $A_3$  receptors.

Van Galen et al. identified 1*H*-imidazo[4,5-*c*]quinolines as nonxanthine adenosine receptor antagonists [39]. Later, these types of compounds were shown to represent a second series of allosteric modulators for the  $A_3$  receptor. Among these compounds, 2-cyclopentyl-4-phenylamino-1*H*-imidazo[4,5-*c*]quinoline (DU124183) (see Fig. 6) selectively enhanced agonist binding and function at  $A_3$  receptors, similar to VUF5455. However, in contrast to VUF5455, DU124183 also increased the maximum efficacy of  $A_3$  receptor agonists in the cAMP assay with cloned human  $A_3$  receptors. For DU124183, there was a marked leftward shift of the concentration-response curve of the  $A_3$  receptor agonists in the presence of an orthosteric antagonist and a potentiation of the maximum agonist efficacy by approximately 30%. In competitive binding studies, DU124183 also displayed some orthosteric activity, as it bound with moderate affinity ( $K_i=820$  nM) to human  $A_3$

**Fig. 6** Structures of allosteric modulators of  $A_3$  receptors



**Table 1** Potency of 1H-imidazo[4,5-c]quinoline-4-amine derivatives in binding assays at human A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> adenosine receptors expressed in CHO cells and allosteric effects at the human A<sub>3</sub> adenosine receptors<sup>a</sup>

No.	R	R'	K <sub>i</sub> (hA <sub>1</sub> AR) nM <sup>a</sup> or % displ. at 10 μM	K <sub>i</sub> (hA <sub>2A</sub> AR) nM <sup>a</sup> or % displ. at 10 μM	hA <sub>2B</sub> AR % inhib. at 10 μM <sup>b</sup>	K <sub>i</sub> (hA <sub>3</sub> AR) nM <sup>a</sup> or % displ. at 10 μM	hA <sub>3</sub> AR ag. Diss. (%) <sup>c</sup> at 10 μM	Relative efficacy (%) at hA <sub>3</sub> AR <sup>d</sup> at 10 μM
	Ph (DU124183)	CP	3420 ± 230	3150 ± 210	-6.8%	786 ± 67 (90%)	174±5	138±8
21	4-Cl-Ph	CP	>10,000 (22%)	>10,000 (17%)	-10.2%	1610 ± 550 (82%)	159±5	136 ±3
22		CP	21%	8%	-10.7%	69%	108±4	111 ±5
23		CP	56%	68%	5.0%	67%	109±3	96 ±2
24	PhCH <sub>2</sub>	CP	28%	77%	-3.4%	86%	145±10	147±8
25	Ph(CH <sub>2</sub> ) <sub>2</sub>	CP	52%	91%	-11.6%	84%	154±7	137 ±4
26	3,4-Cl <sub>2</sub> -Ph	Cheptyl	-4%	-2%	-7.3%	68%	130±2	115 ±7
27	3,4-Cl <sub>2</sub> -Ph		-4%	70%	9.0%	78%	98±3	95 ±4
	3,4-Cl <sub>2</sub> -Ph LUF6000	Chexyl	-2%	-1%	-5.6%	45%	173±5	145±7
28	3,4-Cl <sub>2</sub> -Ph	Cbutyl	-5%	0.4%	-5.5%	52%	116±3	126 ±3
29	3,4-Cl <sub>2</sub> -Ph	H	1.8%	-0.8%	10.2%	39%	91±7	92 ±4
30	3,4-Cl <sub>2</sub> -Ph	<i>n</i> -pentyl	43.9%	-1%	-9.3%	84%	116±5	102±5

<sup>a</sup> All experiments were performed using adherent CHO cells stably transfected with cDNA encoding the human adenosine receptors. Binding at human A<sub>1</sub>, A<sub>2A</sub> and A<sub>3</sub> adenosine receptors in this study was carried out as described in Experimental Procedures using [<sup>3</sup>H]R-PIA, [<sup>3</sup>H]CGS 20080 or [<sup>125</sup>I]I-AB-MECA as a radioligand. Values from the present study are expressed as mean ± s.e.m., n=3–5. Percentage inhibition at A<sub>1</sub>, A<sub>2A</sub>, or A<sub>3</sub> receptors is expressed as the mean value from 2–4 separate experiments with similar results performed in duplicate.

<sup>b</sup> A<sub>2B</sub> adenosine receptor: effect of compounds at 10 μM on NECA (150 nM)-induced cAMP accumulation from one experiment performed in triplicate, CGS15943 (10 μM) = 100%.

<sup>c</sup> dissociation: % decrease of [<sup>125</sup>I]I-AB-MECA dissociation at 30 min (control=100%)

<sup>d</sup> increase of efficacy: compared to maximal effect by 2-Cl-IB-MECA alone (control=100%)

receptors. Some members of this series also had a competitive interaction with the A<sub>1</sub> adenosine receptor [39, 40].

The aim of a subsequent study was the optimization of DU124183. Structural modifications were made at the 4-amino and 2 positions of the molecule, partly using the Topliss scheme [41]. The compounds were tested in binding and functional assays (Table 1). SARs for the allosteric enhancing effects showed that at the 4-amino position, a phenyl ring or substituted monocyclic phenyl ring (compounds DU124183, **21**) was more enhancing than bicyclic aryl rings (compounds **22**, **25**). A phenyl group (DU124183) was also better in enhancing than the corresponding benzyl and phenylethyl groups (**24**, **25**). As noted previously, substitution at the 2-position was also necessary for allosteric enhancement (compound **29**). At this position, medium-sized cycloalkyl substituents (cyclopentyl and cyclohexyl) were most favorable for enhancement. Analogues bearing smaller or larger rings (compounds **28**, **26**) or an acyclic alkyl group (compound **30**) were considerably less enhancing, and similarly an aromatic five-membered ring (compound **27**) was not conducive to enhancement. Two compounds, 2-cyclopentyl-4-benzylamino (**24**) and 2-cyclohexyl-4-(3,4-dichlorophenyl)amino (LUF6000, see Fig. 6) analogues potentiated the maximum efficacy of the agonist Cl-IB-MECA by 45–50% (Table 1). Moreover, LUF6000 enhanced agonist efficacy in a functional assay and decreased the agonist dissociation rate without influencing agonist potency. Most allosteric enhancers in this study only modestly inhibited orthosteric ligand binding, if at all. Apparently, structural requirements for allosteric enhancement at the A<sub>3</sub> receptor are distinct from those for the inhibition of equilibrium radioligand binding. This suggests even greater selectivity is feasible in a future lead optimization program. Allosteric enhancers of A<sub>3</sub> receptor activation are predicted to be useful against a number of disorders, including ischemic conditions and

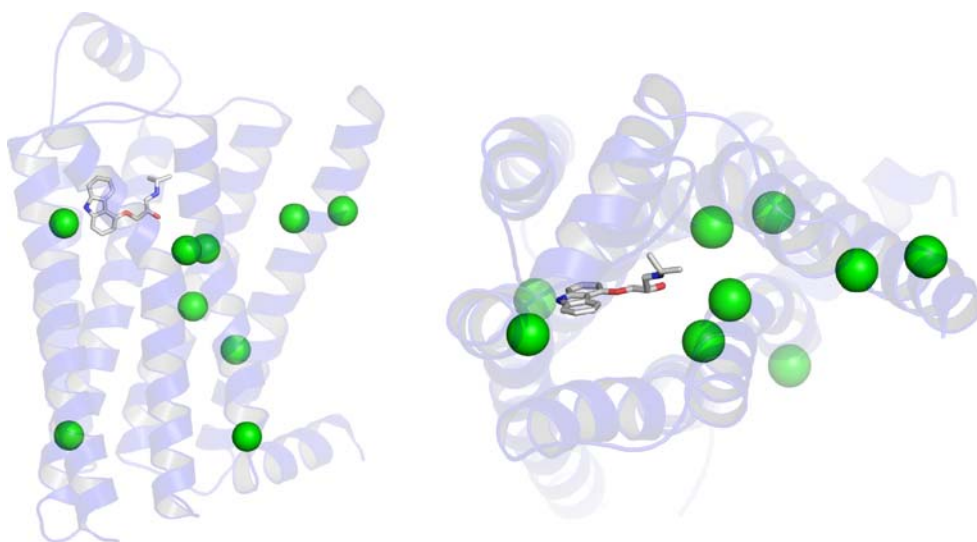
cancer. LUF6000 showed no effect on the basal receptor activity yet showed a significant enhancement of agonist action. This result suggests that such enhancers could be safer drugs and would be particularly effective to enhance agonist efficacy under pathophysiological conditions such as brain and heart ischemia, where adenosine and inosine concentrations are greatly increased [42; Gao et al., manuscript submitted 2008].

### Mutagenesis studies, binding sites for allosteric modulators

The aim of several publications was to determine where the binding sites are for allosteric modulators on adenosine receptors. Theoretically, it might be possible that PD 81,723 binds to another protein, such as a G-protein subunit, and that this complex allosterically regulates the A<sub>1</sub> receptor. However, Bhattacharya et al. suggested that PD 81,723 binds to the A<sub>1</sub> receptor itself and that it stabilizes the high-affinity conformational state of the A<sub>1</sub> receptor–G-protein complex [43].

Site-directed mutagenesis is a useful technique for elucidating ligand–receptor interactions by testing the role of several amino acids within the receptor. Mutant A<sub>1</sub> receptors have been studied, where glycine at position 14 had been changed into threonine (G14T). The G14T mutant renders the receptor constitutively active with (very) high agonist affinity, upon which PD 81,723 loses its enhancing activity. It appears that the G14T receptor is “locked” in an active state and as such does not reveal the site of PD 81,723 binding [44]. Studies on wild-type and mutant (T277A) A<sub>1</sub> receptors gave analogous results. Mutation of a threonine residue to alanine at position 277 in the human A<sub>1</sub> receptor decreased agonist binding to the receptor but also abolished the effects of PD 81,723 [45]. Again, it would be premature to conclude that T277 is part of the PD 81,723 binding site.

**Fig. 7** The location of amino acid residues involved in allosteric modulation of A<sub>1</sub>, A<sub>2A</sub>, and A<sub>3</sub> receptors as displayed in a receptor homology model of the adenosine receptor (side and top view) based on the structure of the β<sub>2</sub>-adrenergic receptor cocrystallized with its ligand carazolol (PDB coordinates: 2rh1). C-α atoms at these locations are shown as spheres. The position of D55 in the A<sub>1</sub> receptor is equivalent to D58 in the A<sub>3</sub> receptor





Barbhaiya et al. mutated a largely conserved aspartic acid residue in TM2 of the A<sub>1</sub> receptor to alanine (D55A). This residue was found to be responsible for allosteric regulation of ligand-A<sub>1</sub> receptor binding by sodium ions [46]. Gao and colleagues performed mutagenesis experiments on the A<sub>2A</sub> receptor, where a glutamic acid residue in TM1 and a histidine residue in TM7 were mutated to glutamine (E13Q) and tyrosine (H278Y), respectively. Results suggest that the two closely linked residues Glu13 and His278 in the A<sub>2A</sub> receptor are the most important for agonist recognition and partly responsible for the allosteric regulation by sodium ions [36, 47]. Furthermore Gao et al. identified residues involved in the allosteric modulation of the human A<sub>3</sub> receptor. A number of residues in the transmembrane helical domains were shown to be critically involved in the recognition of the allosteric modulators. The F182A (TM5) and N274A (TM7) mutations abolished the allosteric effects of the two modulators DU124183 and VUF5455 but had hardly any effect on agonist binding, as was the case for N30A (TM1) and D58N (TM2). The D107N (TM3) mutation eliminated the effects of DU124183 but not of VUF5455 [48].

To summarize, site-directed mutagenesis studies may shed light on amino acids involved in allosteric ligand-receptor interactions. Experiments to that end have been carried out on A<sub>1</sub>, A<sub>2A</sub>, and A<sub>3</sub> receptors, whereas A<sub>2B</sub> receptors have not yet been studied. For A<sub>1</sub> receptors, Asp55 in TM2 is probably responsible for allosteric regulation of binding by sodium ions and amilorides, whereas upon G14T (TM1) and T277A (TM7) mutagenesis, PD 81,723 loses its enhancing activity with respect to carboxypeptidase A (CPA). For A<sub>2A</sub> receptors, Glu13 in TM1 and His278 in TM7 have been shown to be linked to allosteric regulation by sodium ions. For A<sub>3</sub> receptors, F182A (TM5) and N274A (TM7) mutations abolished the allosteric effects of the two modulators DU124183 and VUF5455. The D107N (TM3) mutation eliminated the effects of DU124183 but not of VUF5455. It is fair to assume that the residues studied so far only yield a fragmented view of the amino acids involved in the allosteric regulation of adenosine receptors. The location of the residues discussed here is shown in a receptor homology model of the adenosine receptor based on the recently reported crystal structure of the β<sub>2</sub>-adrenergic receptor [49] (Fig. 7).

## Conclusions

Allosteric modulation is a new way of intervening with receptor function. Because of the ability of allosteric modulators to modulate receptor conformations in the presence of orthosteric ligand, allosteric modulators can “fine tune” classical pharmacological responses. Although an

allosteric modulator may not possess efficacy by itself, it can provide a powerful therapeutic advantage over orthosteric ligands, namely, the ability to selectively influence tissue responses only when the endogenous agonist is present.

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