Allosteric Modulation of the Adenosine Family of Receptors

Zhan-Guo Gao[†], Soo-Kyung Kim[†], Adriaan P. IJzerman[Σ] and Kenneth A. Jacobson[†*]

[†]*Molecular Recognition Section, Laboratory of Bioorganic Chemistry, NIDDK, National Inst. of Health, Department of Health and Human Services, Bethesda, Maryland 20892-0810, USA*

[Σ]*Division of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA, Leiden, The Netherlands*

Abstract: Allosteric modulators for adenosine receptors (ARs) are of an increasing interest and may have potential therapeutic advantage over orthosteric ligands. Benzoylthiophene derivatives (including PD 81,723), 2-aminothiazolium salts, and related allosteric modulators of the A_1 AR have been studied. The benzoylthiophene derivatives were demonstrated to be selective enhancers for the A_1 AR, with little or no effect on other subtypes of ARs. Allosteric modulation of the A_{2A} AR has also been reported. A_3 allosteric enhancers may be predicted to be useful against ischemic conditions. We have recently characterized two classes of A_3 AR allosteric modulators: 3-(2-pyridinyl)isoquinolines (e.g. VUF5455) and 1H-imidazo-[4,5-c]quinolin-4-amines (e.g. DU124183), which selectively decreased the agonist dissociation rate at the human A_3AR but not at A_1 and A_{2A} ARs. DU124183 left-shifted the agonist conc.-response curve for inhibition of forskolin-stimulated cAMP accumulation in intact cells expressing the human A_3AR with up to 30% potentiation of the maximal efficacy. The increased potency of A_3 agonists was evident only in the presence of an A_3 antagonist, since VUF5455 and DU124183 also antagonized, i.e. displaced binding at the orthosteric site, with K_i values of 1.68 and 0.82 μM, respectively. A_3AR mutagenesis studies implicated $F182^{5.43}$ and $N274^{7.45}$ in the action of the enhancers and was interpreted using a rhodopsin-based A_3AR molecular model, suggesting multiple binding modes. Amiloride analogues, SCH-202676 (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5(2H)-ylidene)methanamine), and sodium ions were demonstrated to be common allosteric modulators for at least three subtypes (A_1, A_{2A}, and A_3) of ARs.

INTRODUCTION

Four subtypes of adenosine receptors (ARs) have been cloned, termed A_1, A_{2A}, A_{2B} and A_3 ARs [1]. Activation of A_1 and A_3 ARs induces the inhibition of the enzyme adenylate cyclase, whereas activation of A_{2A} and A_{2B} receptors leads to the stimulation of this enzyme. All four subtypes belong to the largest category, termed Group 1, within the superfamily of G protein-coupled receptors (GPCRs), which possess seven membrane-spanning α-helices [1]. The therapeutic areas for which there is growing interest in these receptors include: immune function and inflammation, CNS disorders, pulmonary and cardiovascular diseases. While there are not yet highly potent and selective agents for any of these receptors in use as therapeutic agents, a means of indirectly modulating the action at these receptors by pharmacological means seems appealing.

It has been suggested that allosteric modulators may provide therapeutic advantages over orthosteric agonists [2,3]. Such advantages may include greater subtype selectivity and fewer side effects. For example, diazepam and other benzodiazepines, which act as allosteric enhancers of the ion channel-coupled $GABA_A$ receptor, have acceptable side effects and are used clinically. In contrast, directly acting $GABA_A$ agonists have widespread side effects and are not used clinically. In the ligand gated ion channel nicotinic receptors, the alkaloid galanthamine acted as an acetylcholinesterase inhibitor as well as an allosteric modulator at nicotinic receptor sites potentiating nicotinic cholinergic neurotransmission. Galanthamine has recently been extensively and successfully used in the clinic and also showed satisfactory therapeutic effects in Alzheimer's disease [4].

Thus, the presence of allosteric site(s) on GPCRs has provided new targets for drug discovery. The effects of an allosteric enhancer on an organ or tissue might be event-specific due to an increase in the local concentration of the endogenous agonist [3,5]. For example, hypoxic conditions increase the local production of cyto-protective adenosine. Compounds that either augment the concentration of adenosine or enhance its action, locally, may have a better therapeutic profile than the agonists. Additionally, neurotransmitter receptors have been reported to be less sensitive to desensitization or down-regulation by allosteric enhancers than by exogenous agonists [3]. Thus, allosteric modulators could offer a control of receptor function not found with competitive agonists.

A_1 ARs

Allosteric enhancers, in theory, are such a means of indirectly enhancing the action of a native agonist such as adenosine. The allosteric modulators act at a site distinct from the agonist binding site, and their effect is evident only in the presence of exogenously added agonist. Bruns and colleagues introduced the first allosteric modulators of the A_1AR in 1990 [6,7]. These initial reports described benzoylthiophene derivatives (Fig. (**1**)), such as PD 81,723 (2-amino-4,5-dimethyl-3-thienyl-[3-(trifluoromethyl)phenyl]-

*Address correspondence to this author at the Molecular Recognition Section, Bldg. 8A, Rm. B1A-19, NIH, NIDDK, LBC, Bethesda, MD 20892-0810, USA; Tel: 301-496-9024; Fax: 301-480-8422; E-mail: kajacobs@helix.nih.gov

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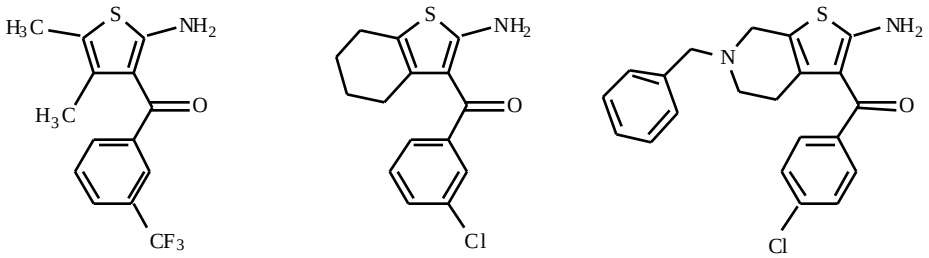


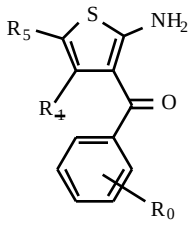
1, PD 81,723 **10,** PD 71,605 **17,** PD 117,975

Fig. (1). Benzoylthiophenes as A_1AR allosteric modulators.

methanone) **1**, as A_1AR allosteric enhancers. These allosteric enhancers were identified while screening chemical libraries in a binding assays at the rat A_1AR. Bruns made the initial discovery of this effect based on small increases (~25%) in the level of agonist binding.

PD 81,723 has now been extensively modified structurally, leading to a variety of substituted derivatives. In one of the seminal papers by Bruns and coworkers [7] three compounds (PD 71,605, PD 81,723 and PD 117,975) were tested in substantial detail (Fig. (**1**)). They have since served

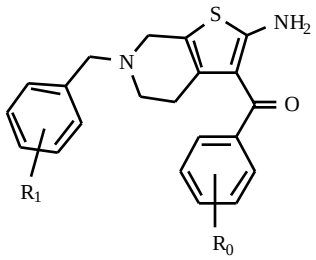
Table 1. Adenosine A_1 Receptor Enhancement and Antagonism by 2-Amino-3-Benzoylthiophenes in Rat Brain Membranes



				Enhancement		Antagonism	
compound	**R_0**	**R_4**	**R_5**	**%***	**EC_{50} (μM)**	**%****	**K_i (μM)**
PD81,723	3-CF_3	CH_3	CH_3	100	15	40	4.7
2	H	CH_3	CH_3	8		14	18
3	3,4-Cl	CH_3	CH_3	116		50	3.2
4	4-tBu	CH_3	CH_3	125		47	4.4
RS74,513	3-CF_3	CH_2CH_3	CH_3	112		29	10
5	H	CH_2CH_3	CH_3	31		13	15
6	3-Cl	CH_2CH_3	CH_3	30		22	13
7	3-CF_3	-$(CH_2)_4$-		122	6.0	32	
8	H	-$(CH_2)_4$-		47		35	5.5
9	2-Cl	-$(CH_2)_4$-		73		35	
PD71,605	3-Cl	-$(CH_2)_4$-		93	11	51	4.2
10	4-Cl	-$(CH_2)_4$-		123	6.8	40	
11	4-CF_3	-$(CH_2)_4$-		131	4.7	57	
12	4-CH_3	-$(CH_2)_4$-		137	35	30	
13	4-NO_2	-$(CH_2)_4$-		34		20	
LUF 5484	3,4-Cl	-$(CH_2)_4$-		151	6.2	35	
14	4-tBu	-$(CH_2)_4$-		137		40	
15	4-Br	-$(CH_2)_4$-		128	16	42	

* Enhancing activity by 10 μM of test compound is expressed as percent decrease in [^{3}H]CCPA dissociation over control (0%) and that of 10 μM PD81,723 (100%). For some compounds EC_{50} values were determined, defined as the ligand concentration causing halfmaximal enhancement ** Antagonistic activity is expressed as percent displacement of 0.4 nM of [^{3}H]DPCPX by 10 μM of test compound. For some compounds K_i values were determined.

Table 2. Adenosine A_1 Receptor Enhancement and Antagonism by 2-amino-3-benzoyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridines in Rat Brain Membranes



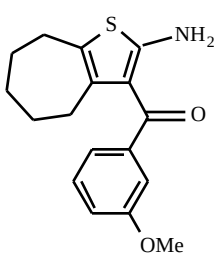
			Enhancement		Antagonism
	R_0	**R_1**	**%***	**EC_{50} (μM)**	**%****
PD117,975	H	H	53		67
	4-Cl	H	132	11.3	61
	3,4-Cl	H	174	9.2	51
	H	3-Cl	106	15.1	80
	H	4-Cl	69		52
	H	3,4-Cl	57		4

*,** Enhancing and antagonistic activity were expressed as described in Table 1.

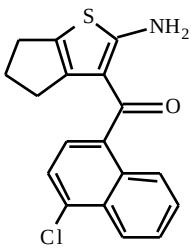
as source of inspiration for further synthetic efforts, leading to a variety of analogs. Their structure-activity relationships will be discussed here. Table **1**, based on the work of Van der Klein *et al.* [8] and Kourounakis *et al.* [9], shows that appropriate substitution of the benzoyl ring is essential for high activity. In the largest series of PD 71,605 analogues the potency order for substitution at the benzoyl ring was 3,4-Cl_2 > 4-CH_3 = 4-*t*-Bu > 4-CF_3 ≥ 4-Br ≥ 4-Cl ≥3-CF_3 > 3-Cl > 2-Cl > H > 4-NO_2, with the latter three substantially less active as enhancers than PD 81,723. All analogues appeared to possess some degree of antagonistic activity, potentially compromising their enhancing effectiveness. However, the SAR (structure activity relationship) for allosteric enhancement and antagonism appeared to be different. In this series, LUF 5484 (2-amino-3-(3,4-dichlorobenzoyl)-4, 5, 6, 7-tetrahydrobenzo[b]thiophene) was 2.4 times more potent than PD 81,723 as an enhancer, while showing comparable antagonistic activity. In the series of 6-benzyl-2-amino-3-benzoyl-4, 5, 6, 7-tetrahydrothieno[2.3-c] pyridines the compound with a 3,4-dichloro substituted benzoyl moiety **18** was again the most potent allosteric enhancer (Table **2**). Substitution of the 6-benzyl group generally lowered enhancing activity, although a 3-chloro substituent **19** was well tolerated. All compounds showed antagonistic activity to varying extents.

In a similar manner, Baraldi *et al.* [10] developed a series of PD 81,723 analogs. The assay used was quite different from the above (cAMP determinations in CHO cells expressing human adenosine A_1 receptors vs. radioligand dissociation experiments on rat brain membranes). However, similar findings were obtained, although none of the compounds synthesized was more active at 10 μM than PD 81,723. Among the more potent derivatives, when tested at 0.1 μM, were three compounds in Table **1**, i.e. **2**, **10** and **15**. In some cases in which compounds with both enhancing and substantial antagonistic activity were tested, no significant effects on cAMP production were noted.

Tranberg *et al.* [11] also performed a systematic survey of the PD 81,723 lead structure. When studying tetrahydrobenzo derivatives such as PD 71,605, they obtained findings similar to the ones in Table **1**, now with a radioligand dissociation assay on human rather than rat adenosine A_1 receptors. Interestingly, extending the



22 from Tranberg *et al*. 2002



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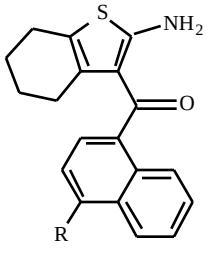


24, R = H, Cl, Br, I

Fig. (2). Extension of the tetrahydrobenzo moiety of analogues of PD 81,723 **1** with methylene groups.

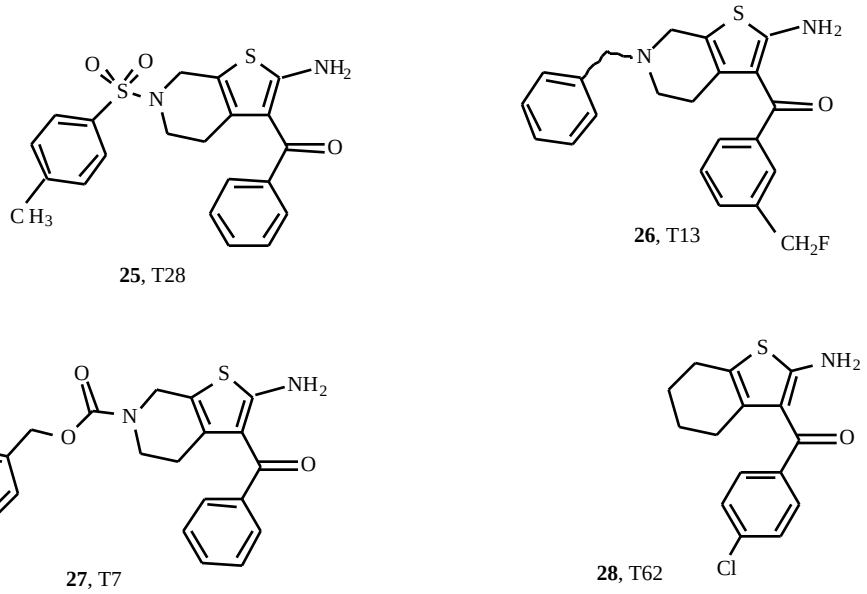


Fig. (3). Representative A_1AR allosteric modulators from a series of recent patents [13-16].

tetrahydrobenzo moiety with one methylene group also yielded potent enhancers (Fig. (**2**)). The 3-OMe derivative **22** had high enhancing activity and relatively low antagonistic potency, 99% and 13% at 100 μM, respectively.

Replacing the aroyl for a naphthoyl moiety also yielded potent enhancers. Baraldi and coworkers [12] identified five compounds more potent than PD 81,723 (e.g. **23**, **24**, see Fig. (**2**)) in increasing radiolabeled agonist binding to both human and rat adenosine A_1 receptors.

Slightly more exotic derivatives stem from a number of patents on allosteric enhancers, filed by Medco Research, Inc. (presently King Pharmaceuticals) [13-16]. Fig. (**3**) shows the structures of some typical examples (**25** – **28**). T28 proved a potent enhancer of the binding of [^{3}H]CCPA (2-chloro-*N*^{6}-cyclopentyladenosine) to membranes of CHO cells expressing the human A_1 receptor. Its maximum effect (≈ 185% of control values) was reached at a low concentration of 100 nM only. T7 enhanced the binding over a small range of concentrations (50 – 500 nM) with modest efficacy (maximum of 120%), whereas it acted as a receptor antagonist at higher concentrations. T13 also enhanced agonist binding, with a maximum effect (130%) at 500 nM.

Thus, allosteric enhancement of agonist binding and activity on adenosine A_1 receptors has been convincingly demonstrated *in vitro* for a series of 3-substituted 2-aminothiophenes. The activity of the compounds is relatively modest, and is sometimes compromised by a far from negligible antagonism. From the SAR it appears that lipophilicity of the 3-substituent is important for activity, which is a drawback to the solubility of the materials. These are all aspects that need to be addressed in order to arrive at chemical entities with utility in whole animal studies.

A new class of allosteric enhancers for the A_1AR was recently reported [17]. 2-Aminothiazolium salts (Fig. (**4**)), such as the catechol derivative **29**, the acetate ester **30**, and the cyclopentanoate ester **31**, which decreased the agonist dissociation rates from the A_1AR with EC_{50} values of 1.2, 3.8, and 4.5 μM respectively. These enhancers appear to be more chemically stable that the benzoylthiophenes and therefore may prove to be more useful *in vivo*.

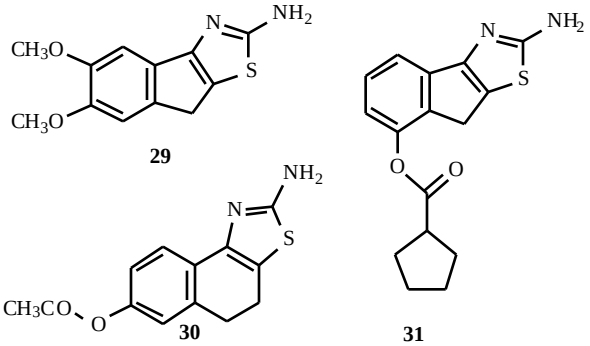


Fig. (4). 2-Aminothiazolium salts as A_1AR allosteric modulators.

The potassium sparing diuretic amiloride has been shown to act as both an antagonist and an allosteric modulator for a number of GPCRs [41-45]. Other allosteric modulators (Fig. (**5**)) for A_1ARs include amiloride derivatives [21], the nonselective modulator of GPCRs SCH-202676 (**35**, *N*-(2,3-diphenyl-1,2,4-thiadiazol-5(2H)-ylidene)methanamine) [20,47], and sodium ions [22]. These are nonselective allosteric modulators, as they also modulate other adenosine receptor subtypes and other GPCRs [3].

The allosteric site(s) on the A_1 AR have not been explored in detail. However, several residues have been demonstrated to be critical to the modulation of either PD 81,723 or sodium ions. The mutation of Thr277 (7.42) to Ala not only decreased agonist affinity but also inhibited the effects of PD 81,723 [23]. Studies of the D55A$^{2.50}$ mutant A_1 AR revealed that Asp55 is responsible for allosteric regulation of binding by sodium because the affinity for [^{3}H]CCPA did not change over broad ranges of sodium concentrations [22].

The unique pharmacological properties of the prototypical A_1 AR allosteric enhancer PD 81,723 **1**, in

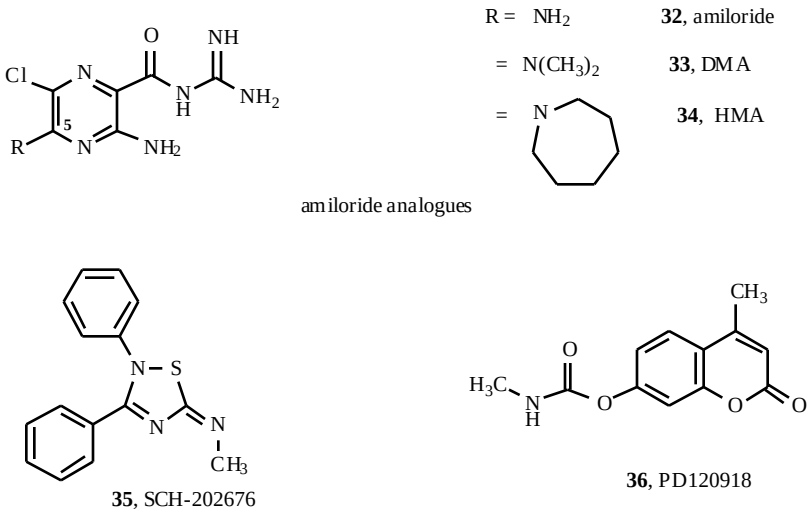


Fig. (5). Amiloride derivatives and other allosteric modulators of A_1 and A_{2A} ARs.

comparison to agonists, have been characterized in additional detailed studies. PD 81,723 is less likely to cause desensitization and down-regulation of receptors than are selective A_1 AR agonists [18]. The behavior of PD 81,723 on functional effects of A_1AR activation in CHO cells was studied [19]. Additionally, it has been demonstrated that PD 81,723 possesses some degree of tissue selectivity, modulating neuronal but not adipocyte adenosine receptors [5].

The cardiovascular effects of PD 81,723 have been studied extensively [24-30]. The slowing effects of PD 81,723 on nodal conduction mimicked those of adenosine acting at the A_1 AR. Adenosine is also known to be a cardioprotective mediator, in case of ischemia, which has been demonstrated through the application of exogenous A_1 AR selective agonists. PD 81,723 also has cardioprotective properties *in vivo* [28].

Activation of A_1 ARs *in vivo* produces anti-nociception [31] and also reduces hypersensitivity in models of inflammatory and nerve-injury pain [32]. The allosteric enhancer T62 (**28**, Fig. (**3**)) was injected intrathecally to produce a similar anti-nociceptive effect in a model of spinal nerve ligation in the rat. This anti-nociceptive effect was blocked using a selective A_1 AR antagonist, thus supporting the interpretation that enhanced activation of the A_1 receptor in the brain in the presence of T62 reduces pain. Similar conclusions were reached in a spinal model of neuropathic pain [32]. Positive allosteric modulation of the A_1 AR by T62 reduced hypersensitivity, suggesting of the use of such modulators in the treatment of chronic pain associated with hyperalgesia and allodynia.

A_{2A} and A_{2B} ARs

Isolated examples of allosteric modulation of the A_{2A}AR have been described [20,33,34]. PD 120,918 (**36**, 4-methyl-7-([methylamino]carbonyl)oxy)-2H-1-benzopyran-2-one)was reported to enhance agonist radioligand binding to the rat striatal A_{2A}AR, but functional enhancement was not demonstrated [33]. PD 120,918 also appeared to slow the dissociation of agonist from the rat brain A_1AR, but had no effect on A_1AR responses in FRTL-5 thyroid cells.

Other substances appeared to have allosteric effects on antagonist binding to the A_{2A}AR without selectivity for this subtype. Both amiloride analogues [21,34] and SCH-202676 [20,47] increased the dissociation rate of the antagonist [^{3}H]ZM241385 from the A_{2A}AR, while they did not show any effect on the dissociation rate of the agonist [^{3}H]CGS21680. The effects of these compound classes on dissociation kinetics were more pronounced at the A_{2A}AR than at other AR subtypes.

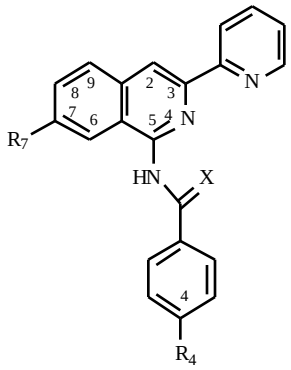
In A_{2A} AR mutagenesis studies, amiloride displayed different binding characteristics compared with the competitive antagonists [36], suggesting different modes of binding. At V84L$^{3.32}$ mutant receptors, AR antagonists CPX (8-cyclopentyl-1,3-dipropylxanthine) and XAC (8-(2-aminoethyl)aminocarbonylmethyloxy-1,3-dipropylxanthine) had reduced affinity (4-6-fold) compared with wild type receptor. However, amiloride displayed wild type affinity for V84L$^{3.32}$ mutant receptors. At H250N$^{6.52}$ mutant receptors, competitive antagonists displayed slightly decreased or approximately wild type affinity, whereas amiloride displayed a 4-fold gain in affinity [36]. A E13Q$^{1.39}$ mutation, which decreased agonist binding, influenced the affinity of neither the classic A_{2A} receptor antagonists nor amiloride [37]. The E13Q$^{1.39}$ and H278Y$^{7.43}$ mutations did not significantly influence the effect of GTP on agonist binding but reduced the effects of sodium ions. This suggested these two residues are partly responsible for the allosteric regulation by sodium ions [35,37].

There are no known allosteric modulators of the A_{2B} AR. However, it is speculated that amiloride analogues, SCH-202676, and sodium ions may be allosteric modulators for A_{2B} receptors from the fact that they are allosteric modulators for the other three subtypes of ARs and several other GPCRs.

A_3ARs

In a series of recent studies, the first allosteric modulators of the human A_3 ARs have been characterized [21,38,39]. The first chemical series shown to act in this manner consisted of derivatives of 3-(2-pyridinyl)isoquinoline. These derivatives, originally synthesized as A_3 AR antagonists by

Table 3. Effects of Pyridyl Isoquinoline Derivatives on Kinetic and Binding Parameters at the Human A_3AR

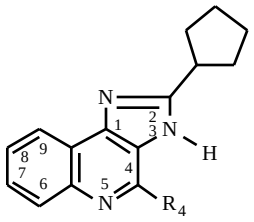


R_4 =	R_7 =	X =	Compound	%Change[a] in k_{-1}	K_i (binding, μM)
37 O-CH_3	CH_3	O	VUF5455	-43%	1.68
38 H	H	NH	VUF8501	N.E.	0.74
39 O-H	H	O	VUF8502	-43%	0.096
40 O-H	H	NH	VUF8503	N.E.	0.58
41 O-CH_3	H	O	VUF8504	-48%	0.017
42 O-CH_3	H	NH	VUF8505	N.E.	0.31
43 H	H	O	VUF8507	-50%	0.20

a) in presence of 10 μM
N.E. no effect

IJzerman and colleagues, were investigated as allosteric enhancers [38] by examining their effects on the dissociation of a high affinity A_3 AR agonist radioligand, [^{125}I]*N*^{6}-(4-amino-3-iodobenzyl)-5'-*N*-methyl-carboxamidoadenosine (I-AB-MECA). Several 3-(2-pyridinyl)isoquinoline derivatives (Table **3**), including VUF5455, VUF8502, VUF8504, and VUF8507, slowed the dissociation of the agonist radioligand [^{125}I]I-AB-MECA in a concentration-dependent manner, suggesting an allosteric interaction. An EC_{50} of ~ 10 μM was observed for this allosteric effect of VUF5455 **37**, which displayed a relatively low degree of antagonism. These compounds had no effect on the dissociation of the radiolabeled antagonist [^{3}H]PSB-11 (8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2.1-i]purin-5-one) [40] from the A_3 AR, suggesting a selective enhancement of agonist binding. By comparison, compounds of similar structure (VUF8501, VUF8503, VUF8505), the classical AR antagonist CGS15943 (5-amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-c]quinazoline) and the A_1 receptor allosteric enhancer PD 81,723 did not significantly influence the dissociation rate of [^{125}I]I-AB-MECA.

Table 4. Effects of Imidazoquinoline Derivatives on Kinetic and Binding Parameters at the Human A_3AR



R_4 =	Compound	%Change[a] in k_{-1}	K_i (binding, μM)
44 O-Ph	DU124182	-45%	0.31
45 NH-Ph	DU124183	-46%	0.82
46 NH-Cp	DU124184	-25%	0.32

a) in presence of 10 μM

Functional effects of these allosteric enhancers were also studied. The effect of the A_3 AR agonist Cl-IB-MECA (2-chloro-N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide) on forskolin-induced cAMP production was significantly enhanced by VUF5455. The functional enhancement by VUF5455 was only observed in the presence of an extremely high concentration of A_3 receptor antagonist to overwhelm its antagonistic activity. When the subtype-selectivity of the allosteric enhancement was tested the compounds had no effect on the dissociation of either the agonist [^{3}H]N^6-[(*R*)-phenylisopropyl]adenosine ([^{3}H]R-PIA) from the A_1 AR or the agonist [^{3}H]CGS21680 from the A_{2A} AR.

Probing of SAR suggested that an amide carbonyl group is essential for allosterism but preferred only for competitive antagonism. The presence of a 7-methyl group decreased the competitive binding affinity without a major loss of the allosteric enhancing activity, suggesting that the structural requirements for allosteric enhancement might be distinct from those for competitive antagonism.

A second series of A_3 AR allosteric modulators was a group of 1H-imidazo-[4,5-*c*]quinolines [39] (Table **4**), which acted as selective allosteric enhancers of human A_3 ARs. Similar to the 3-(2-pyridinyl)isoquinoline derivatives [38], several of these compounds selectively decreased the dissociation of the agonist [^{125}I]I-AB-MECA from human A_3 ARs. There was no effect on the dissociation of the antagonist [^{3}H]PSB-11 from the A_3 AR, as well as [^{3}H]PIA from rat brain A_1 AR and [^{3}H]CGS21680 from rat striatal A_{2A} AR, suggesting the selective enhancement of agonist binding at A_3 ARs. The analogs were tested as antagonists of competitive binding at the human A_3 AR, and K_i values ranging from 120 nM to 101 μM were observed; as for many allosteric modulators of GPCRs, an orthosteric effect was also present. Some members of this series also bound competitively at the A_1 AR. The most promising leads from the present set of analogs seem to be the 2-cyclopentyl-1H-imidazo[4,5-*c*]quinoline derivatives, of which the 4-phenylamino analog DU124183 **45** had the most favorable degree of allosteric modulation versus receptor antagonism.

Functional effects of the imidazo-[4,5-*c*]quinolines were also studied [39]. The inhibition of forskolin-stimulated cyclic AMP accumulation in intact cells that express the human A_3 AR was employed as a functional index of A_3 AR activation. The enhancer DU124183 caused a marked leftward shift of the concentration-response curve of the A_3 AR agonists in the presence of antagonist and, surprisingly, a potentiation of the maximum agonist efficacy by approximately 30%. The functional potentiation of A_3 agonists was evident only in the presence of the A_3 antagonist MRS 1220 or upon stimulation of the receptor by 100 μM 2-chloroadenosine, an extremely high agonist concentration. Thus, we have identified a novel structural lead for developing allosteric enhancers of A_3 ARs. Based on studies of agonists as cited in a recent review [1], such enhancers may be useful for treating brain ischemia and other hypoxic conditions.

Recently, it was shown that amiloride and amiloride analogues (Fig. (**5**)) are allosteric modulators for the A_{2A} AR [34]. In a subsequent study it was demonstrated that amiloride analogues are allosteric modulators for agonist binding at A_3 but not A_1 and A_{2A} ARs and that they are allosteric inhibitors for antagonist binding to A_1, A_{2A} and A_3 ARs [21]. The binding modes of the amiloride analogues at agonist-occupied and antagonist-occupied AR subtypes are markedly different [21].

Specifically, amiloride analogues (Fig. (**5**)) increased the dissociation rates of the antagonist radioligands, [^{3}H]DPCPX and [^{3}H]PSB-11, from the human A_1 and A_3 ARs, respectively. Amiloride (32, 3,5-diamino-*N*-(aminoiminomethyl)-6-chloro-pyrazinecarboxamide hydrochloride) and 5-(*N*,*N*-dimethyl)amiloride (33, DMA) were more potent in competitive binding at the A_1 AR than at the A_3 AR, while 5-(*N*,*N*-hexamethylene)amiloride (34, HMA) was more potent in binding at the A_3 AR. In contrast to their effects on antagonist-occupied receptors, amiloride analogues did not affect the dissociation rates of the A_1 agonist [^{3}H]R-PIA from the A_1 AR or the A_{2A} agonist [^{3}H]2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5'-*N*-ethylcarboxamidoadenosine ([^{3}H]CGS21680) from the human A_{2A} AR. The dissociation rate of the A_3 agonist radioligand [^{125}I]I-AB-MECA from the human A_3 receptor was significantly decreased by amiloride analogues. Thus, amiloride analogues are allosteric inhibitors of antagonist binding at A_1, A_{2A} and A_3 AR subtypes. The binding modes of amiloride analogues at agonist-occupied and antagonist-occupied receptors differed markedly, which was demonstrated in all three subtypes of ARs tested in this study. The effects of the amiloride analogues on the action of the A_3 AR agonist were further explored using a cyclic AMP functional assay in intact CHO cells expressing the human A_3 AR. Both binding and functional assays support the allosteric interactions of amiloride analogues with A_3 ARs.

Curiously, the nonselective GPCR allosteric modulator SCH-202676 **35** [20,47] increased the dissociation rate of the agonist [^{125}I]I-AB-MECA from the human A_3 AR, while it did not show any effect on the dissociation rate of a selective A_3 AR antagonist.

A_3 AR Mutagenesis Studies

The possible location of allosteric site(s) on the A_3 AR was explored using site-directed mutagenesis [46]. D58$^{2.50}$ and D107$^{3.49}$ were each mutated to an uncharged asparagine, and other residues in transmembrane domains (TMs) 1, 2, 3, 5, 6 and 7 were mutated to alanine. We first examined the effects of various allosteric modulators on the dissociation rates of the agonist radioligand, [^{125}I]I-AB-MECA, from wild-type (WT) and mutant A_3ARs. The N30A$^{1.50}$ and D58N$^{2.50}$ mutations abolished the effects of the imidazoquinoline DU124183 **45** and the pyridinylisoquinoline VUF5455 **37**, but not the amiloride analogue HMA, on the dissociation rate of [^{125}I]I-AB-MECA. In contrast, the D107N$^{3.49}$ mutation abolished the effect of DU124183, but not HMA or VUF5455. The N274A$^{7.45}$ mutation eliminated the effects of all of these allosteric modulators. The F182A$^{5.43}$ mutation eliminated the effects of DU124183, but had no effect on the binding of A_3AR agonist or antagonist. The T94A$^{3.36}$, H95A$^{3.37}$, K152A^{EL2}, W243A$^{6.48}$, L244A$^{6.49}$ and S247A$^{6.52}$ mutations did not significantly influence the effects of any of the allosteric modulators tested. The M177A$^{5.38}$, V178A$^{5.39}$, S271A$^{7.42}$ and H272A$^{7.43}$ mutations lost both agonist and antagonist high affinity binding, and could not be studied further.

We next examined the effects of sodium ions in mutant A_3ARs on slowing the dissociation rate of the antagonist radioligand, [^{3}H]PSB-11. The D58N$^{2.50}$, but not L244A$^{6.49}$ or S247A$^{6.52}$ mutations abolished this effect of sodium ions. We further examined the effects of sodium ions on the equilibrium binding of the agonist, [^{125}I]I-AB-MECA. Sodium ions (100 mM) caused an approx. 80% inhibition of [^{125}I]I-AB-MECA binding in WT. The D58N$^{2.50}$, D107N$^{3.49}$ and F182A$^{5.43}$ mutant receptors were completely insensitive to 100 mM sodium ions. In contrast, 100 mM sodium ions induced a modest but significant increase of agonist binding in N30A$^{1.50}$ and N274A$^{7.45}$ mutant receptors. Previous studies have implicated the aspartic acid in TM2 in the sodium modulatory effect [48]. At the A_3AR, mutation of residues other than (D58) clearly had major effects on the ability of sodium ions to influence ligand recognition allosterically.

Thus, nonequivalent sets of amino acid residues were found to be involved in the three actions at the human A_3 AR: allosteric modulation by heterocyclic derivatives, competitive binding at A_3 AR of the same derivatives (which resembles closely the pattern previously discerned for pure antagonists, such as MRS 1220), and the allosteric modulation of A_3 AR agonist binding by 100 mM sodium ions.

Molecular Modeling of A_3 AR

The results were interpreted using a rhodopsin-based A_3AR molecular model, suggesting multiple binding modes of the allosteric modulators [46]. First a minimized family of conformations of VUF 5455 **37** was calculated using a semi-empirical PM3 method, and similar calculations were carried out for MRS 1220 and the other allosteric modulators. Calculations aimed at defining two separate pharmacophores, at the putative orthosteric and allosteric sites on the receptor, were conducted using the Sybyl® (Tripos Associates) module "DISCO", which includes distance correlation. In comparison among the allosteric modulators alone using DU124183 **45** as reference, VUF5455 displayed a high predicted overlap. In a separate comparison, the overlay of the allosteric modulator VUF5455 and the antagonist MRS 1220 suggested commonality of binding features, supporting the hypothesis of binding of the modulator at the orthosteric site, in addition to an allosteric site. Docking of the modulator molecules separately in the unoccupied and agonist-occupied receptor supported this view. A favorable binding mode identified for the modulator molecules in the agonist-occupied A_3 AR (Cl-IB-MECA) suggested that a possible allosteric site in the upper (towards the cytoplasmic loops) regions of TM7 (Fig. (**6**)). Thus, the agonist and the allosteric modulator would bind in adjacent regions, involving mainly different amino acid residues, but be within distance to make direct contact between the two molecules possible. Thus, according to the model, docking of the allosteric modulator VUF5455 to the unoccupied receptor might take place either at the orthosteric ligand binding site (involving TMs 3, 5, 6, and 7) or on a putative allosteric binding site at the extracellular end of TM7. A network of H-bonds in the TM regions proximal to the cytoplasmic side involving residues known to affect the receptor activation and modulation by sodium and/or

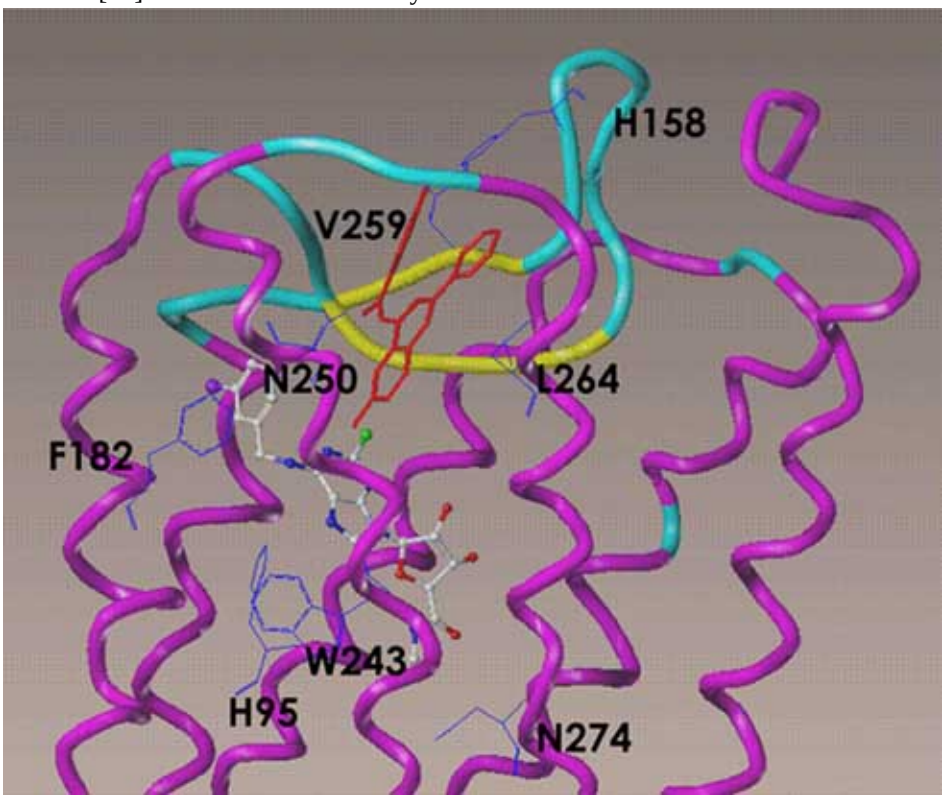


Fig. (6). The putative binding site of the A_3AR with Cl-IB-MECA as an A_3-selective agonist represented by atom type color with ball and stick model and VUF5455 **37** as an allosteric modulator in dark shading with capped sticks. The side chains of amino acids in the binding site were shown in line model. The backbone of A_3AR was displayed by tube. The amino acids in the putative allosteric binding site were S155, H158, Q167, S170 in EL2, L246, I249, N250, I253 in TM6, V259, P260 in EL3, and V263, L264 in TM7.

VUF5455 was characterized. Many of these H-bonds are common features within the Group 1 GPCR family, but others (e.g. those involving $Glu^{1.39}$, $Ser^{3.39}$, $His^{7.43}$, $Asn^{7.45}$, and $Ser^{7.46}$) were unique to the ARs.

SUMMARY

A disadvantage of this approach is that the current allosteric modulators for A_1 and A_3 ARs have both an allosteric effect and apparent antagonistic activity. Thus, when administered *in vivo* they would be expected to have opposing effects on receptor activation. Through structure-activity studies, compounds with more potent enhancing effectbut with lower or no antagonistic activity might be obtained. No AR-specific, or subtype-selective, A_{2A} allosteric modulators have been reported. The mutagenesis and modeling studies might help the rational design of more effective allosteric enhancers.

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