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Hybrid Ortho/Allosteric Ligands for the Adenosine A_1 Receptor

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Many G protein-coupled receptors (GPCRs), including the adenosine A_1 receptor (A_1AR), have been shown to be allosterically modulated by small molecule ligands. So far, in the absence of structural information, the exact location of the allosteric site on the A_1AR is not known. We synthesized a series of bivalent ligands (**4**) with an increasing linker length between the orthosteric and allosteric pharmacophores and used these as tools to search for the allosteric site on the A_1AR. The compounds were tested in both equilibrium radioligand displacement and functional assays in the absence and presence of a reference allosteric enhancer, (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone, PD81,723 (**1**). Bivalent ligand N^6-[2-amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]-pyridin-6-yl-9-nonyloxy-4-phenyl]-adenosine **4h** (LUF6258) with a 9 carbon atom spacer did not show significant changes in affinity or potency in the presence of **1**, indicating that this ligand bridged both sites on the receptor. Furthermore, **4h** displayed an increase in efficacy, but not potency, compared to the parent, monovalent agonist **2**. From molecular modeling studies, we speculate that the allosteric site of the A_1AR is located in the proximity of the orthosteric site, possibly within the boundaries of the second extracellular loop of the receptor.

Introduction

G protein-coupled receptors (GPCRs[a]) represent the largest family of cell-surface proteins mediating cellular communication.[1] Consequently, they represent attractive drug targets. Indeed, it is estimated that approximately 30% of the marketed medicines act through members of this protein class.[2] Classically, such drugs are agonists, antagonists, or inverse agonists and interact with the so-called orthosteric ligand binding site. This orthosteric binding site is the natural binding site for endogenous ligands such as hormones, neurotransmitters, and autocrine factors. The nucleoside adenosine is such an endogenous agonist, for which there are four characterized GPCRs (A_1, A_{2A}, A_{2B}, and A_3 receptors).[3] The adenosine A_1 receptor (A_1AR) is responsible for mediating the physiological effects of adenosine in various tissues and cell types such as the brain, heart, kidney, and adipocytes, and signals mainly via G proteins of the $G_{i/o}$ family to inhibit adenylyl cyclase and thus reduce intracellular levels of cyclic adenosine monophosphate (cAMP).[4]

Many GPCRs, including the adenosine A_1 receptor, have been shown to be allosterically modulated by both small-molecule ligands and ions. The term "allosteric" refers to binding sites that are topographically distinct from the orthosteric ligand binding site. The binding of small-molecule allosteric modulators is believed to result in conformational changes of the receptor which in turn affect the binding of the orthosteric ligand and/or receptor activity.[5] An allosteric modulator may have no activity by itself but may potentiate or inhibit activation of the receptor by its natural ligand. In terms of ligand binding affinity, different scenarios are feasible: allosteric modulators are capable of enhancing the binding of the orthosteric ligands, termed as positive cooperativity, of diminishing the ligand binding (negative cooperativity), or of leaving the binding unaffected (neutral cooperativity). Moreover, the nature of allosteric modulation can be greatly dependent on the orthosteric ligand, so-called probe dependency.[6] Despite these complexities, the concept of allosteric modulation of established drug targets such as GPCRs has fueled an intensive search for new classes of lead compounds which are different in their mode of action compared to the classical agonists and antagonists.[5,7,8]

A bivalent ligand is a hybrid molecule in which two pharmacophores are covalently linked with each other via a spacer.[9] The two pharmacophores are the same in the case of homobivalent ligands and different in the case of heterobivalent ligands. Furthermore, heterobivalent ligands may have pharmacophores that bind to different molecular targets or to two distinct sites on the same molecular target, for example, an orthosteric and allosteric binding site, so-called "bitopic" ligands.[9−13] Bivalent ligands have been reported to display enhanced receptor subtype selectivity and/or enhanced affinity for the receptor target.[14−16] In addition, such compounds have proven to be useful tools in efforts to estimate the distance between two receptors or indeed two binding sites within the same target.[14,17−19] Bivalent ligands have been shown to enhance biological activity via the activation of different receptors that mediate the same effect and, in the case of an agonist/antagonist bivalent ligand for the same receptor,

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[a] Abbreviations: cAMP, cyclic adenosine-5′-monophosphate; CCPA, 2-chloro-N^6-cyclopentyladenosine; CPA, N^6-cyclopentyladenosine; CHO-hA_1, Chinese hamster ovary cells expressing human A_1 receptor; DMF, dimethylformamide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GPCR, G protein-coupled receptor; SEM, standard error of the mean.

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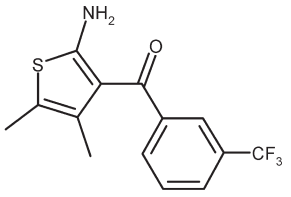
the ability to produce partial agonism.[15,20] Finally, the relatively novel concept of bitopic ligands—those targeting an orthosteric and an allosteric site within the same receptor—has yielded ligands that can have improved receptor selectivity or affinity. Moreover, such ligands can engender functional selectivity in the actions of the orthosteric pharmacophore, highlighting a viable means of further sculpting GPCR ligand responses.[9−13]

Bhattacharya et al. have provided evidence that the recognition site for the reference allosteric enhancer **1**, (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone, PD81,723 (Scheme 1), is on the A_1AR and that this enhancer acts to directly stabilize the receptor in a conformational state capable of coupling with G_i or G_o.[21] However, so far, in the absence of structural information, the exact location of the allosteric site on the A_1AR is not known. Several mutation studies have been performed in order to identify the amino acids involved in the binding site of allosteric modulators on the A_1AR. However, none of these studies to date have provided credible evidence regarding the probable location of the allosteric site and the amino acids involved in the binding of the allosteric ligand.[22−24] It is often speculated that the allosteric site on the A_1AR is in close proximity to the orthosteric site, most recently by Ferguson et al. who synthesized a series of dihydrothieno[3,4-*d*]pyridazines as allosteric modulators.[25]

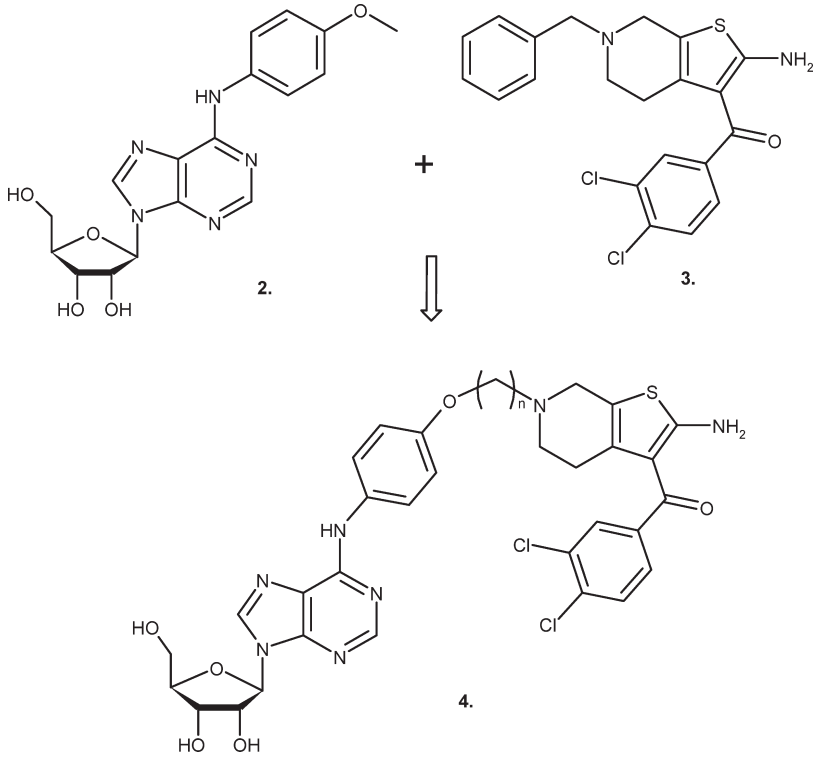
We hypothesized that, when a heterobivalent ligand with a correct spacer length occupies both the allosteric and orthosteric sites of the receptor, it may not show an increase in activity in the further presence of an "external" allosteric enhancer. Therefore, to test this hypothesis we designed and synthesized bivalent ligands for A_1AR linking an allosteric and orthosteric pharmacophore. We decided to couple an orthosteric adenosine-derived agonist **2** (Scheme 2) to an allosteric enhancer using aliphatic chains of varying lengths as spacers. It is important that the agonist and the allosteric enhancer are functionalized at positions not involved in binding to the recognition site. It is known from literature that the introduction of bulky substituents at the N^6 position of adenosine is often well-tolerated and does not influence the agonistic activity significantly.[26,27] Consequently, we decided to introduce the linkers at an N^6 substituent, as in **2**. In the early 1990s, Bruns and co-workers reported on the allosteric modulator PD81,723, capable of enhancing the binding and activity of A_1 receptor agonists.[28,29] They observed that the amino group at the 1-position and carbonyl group at the 2-position on the thiophene ring are vital for allosteric activity and any modification of the amino functionality resulted in the complete loss of activity. Previously, we reported piperidine-derived analogues of PD81,723 (e.g., **3**, see Scheme 2) with improved allosteric enhancing activity.[30] From that series, we observed that the introduction of bulkier substituents at the 4 and 5 positions of the thiophene ring was well-tolerated and did not affect the allosteric activity. Hence, we used that scaffold to link with adenosine-derived agonist **2**. This paper describes the synthesis and pharmacological characterization of a series of bivalent ligands with an

Scheme 1. Chemical Structure of PD81,723

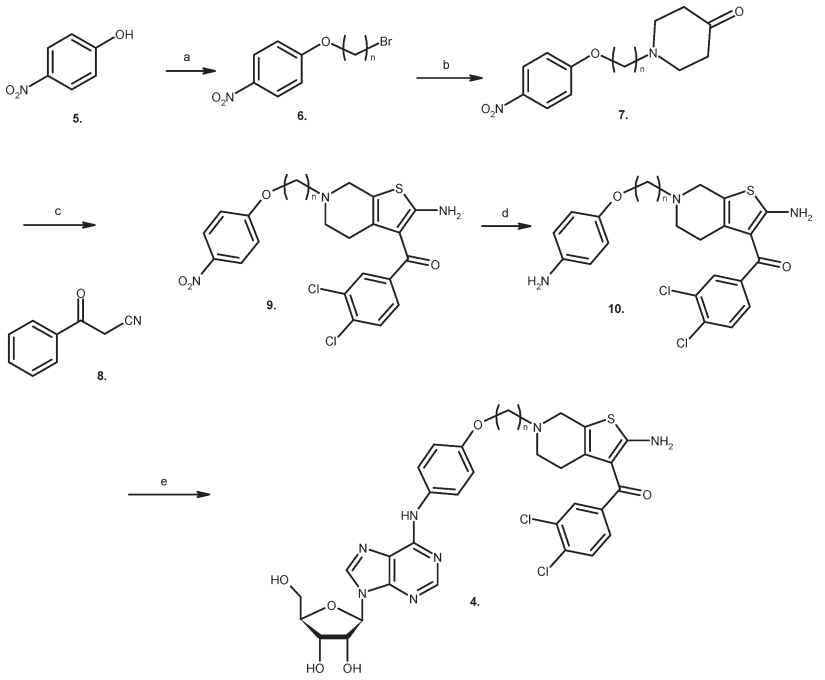


1. PD81,723

Scheme 2. Synthetic Strategy for the Synthesis of Bivalent Ligands **4**



Scheme 3. Synthetic Route to Bivalent Ligands **4**[a]



[a] Reagents and conditions: (a) dibromoalkane, K_2CO_3, acetone, 60−70 °C, 4−8 h, 36−72%; (b) 4-piperidinone hydrochloride monohydrate, K_2CO_3, DMF, RT, 24−48 h, 68−93%; (c) **7**, S, Et_2NH, EtOH, heat, 50 °C, 2−4 h, 17−40%; (d) anhydrous $SnCl_2$, abs. EtOH, 70 °C, 6−12 h, 33−58%; (e) 6-chloropurine 9-β-D-ribofuranoside, Et_3N, EtOH, MW, 120 °C, 2.5 h, 43−65%.

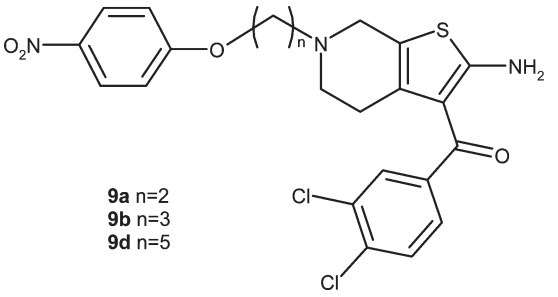
increasing linker length between the orthosteric and allosteric pharmacophores.

Results and Discussion

Chemistry. A series of allosteric/orthosteric bivalent ligands was synthesized as depicted in Scheme 3. The adenosine component was introduced later in the reaction sequence in order to minimize the handling of polar products. The synthesis commenced from 4-nitrophenol **5**. Dibromoalkane was monosubstituted with the phenolic −OH of 5 using anhydrous K_2CO_3 in acetone at 60−70 °C. The N-alkylation of **6** was achieved using piperidinone monohydrate hydrochloride and anhydrous K_2CO_3 in DMF to afford ketone **7**. The intermediate benzoylacetonitrile **8** was synthesized as reported previously in a two-step synthesis from 3,4-dichlorobenzoic acid.[30] The multicomponent Gewald reaction of ketone **7**, benzoylacetonitrile **8**, and sulfur in absolute ethanol gave the aminothiophene **9**. The chemoselective nitro group reduction of **9** was accomplished using anhydrous $SnCl_2$ in absolute ethanol at 70 °C to get amine **10**. The subsequent incorporation of the adenosine component was achieved by the reaction of the amine moiety of compound **10** with commercially available 6-chloropurine riboside using absolute ethanol and triethyl amine under microwave conditions at 120 °C to obtain the bivalent ligands **4a**−**i** with spacer lengths n = 2−10 (Scheme 1, Table 2).

Pharmacology and Structure−activity Relationships. The novel PD81,723 analogues **9a**, **9b**, and **9d**, as representatives of the whole series of **9**, were first tested for their ability to allosterically enhance the binding of the orthosteric radiolabeled agonist [^{3}H]CCPA (2-chloro-N^6-cyclopentyladenosine). All three compounds were more efficacious in their ability to enhance [^{3}H]CCPA binding than PD81,723 (Table 1). Furthermore, as shown in Table 1, the potencies (EC_{50} values)

Table 1. E_{max} and EC_{50} Values for the Ability of Allosteric Modulator PD81,723 and Analogue Compounds to Enhance the Binding of the Orthosteric Agonist Radioligand [^{3}H]CCPA at the Human Adenosine A_1 Receptor



9a n=2
9b n=3
9d n=5

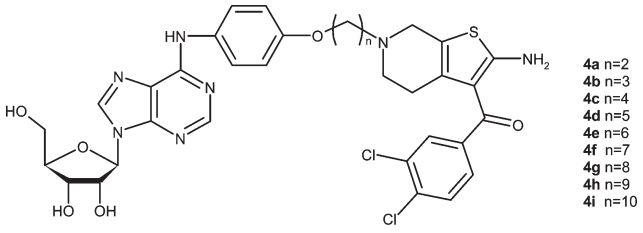
compd.	chain length (n)	pEC$_{50}$ (EC$_{50}$, μM)	E_{max} (%)[a]
9a	2	5.07 ± 0.1 (8.5)	250 ± 6
9b	3	5.43 ± 0.2 (3.7)	204 ± 6
9d	5	5.28 ± 0.1 (5.2)	185 ± 2
PD81,723	−	5.76 ± 0.1 (1.7)	140 ± 12

[a] Specific [^{3}H]CCPA binding in the absence of any modulator set at 100%.

of the compounds for enhancing orthosteric agonist binding are in the low micromolar range, similar to that of PD81,723. This provides further proof that introduction of bulky substituents at the C-4 and C-5 positions of the thiophene ring did not negatively affect the allosteric enhancing ability.

Next, the affinities of the bivalent compounds **4** and the parent compound **2** were determined for the human A_1AR by competitive displacement of the radiolabeled inverse agonist [^{3}H]DPCPX. In the control condition, the biphasic curve displayed by parent compound **2** was best fit with a

Table 2. Affinity of Bivalent Ligands **4** (K_i Values) at the Human Adenosine A_1 Receptor Determined by Their Ability to Displace the Radioligand [^{3}H]DPCPX[a]



compd.	spacer length (n)	pK_i (K_i, nM)	p$K_{i,+PD\ 81}$ (K_i, nM)	increase in affinity (fold)	max. displacement, % specific [^{3}H]DPCPX binding
4a	2	6.13 ± 0.06 (751)	6.33 ± 0.03 (469)*	1.6	100 ± 5
4b	3	6.38 ± 0.08 (439)	6.61 ± 0.11 (268)	1.9	100 ± 4
4c	4	6.21 ± 0.04 (618)	6.51 ± 0.03 (313)*	1.9	100 ± 5
4d	5	6.67 ± 0.06 (219)	6.96 ± 0.08 (114)*	1.9	100 ± 5
4e	6	6.32 ± 0.06 (419)	6.56 ± 0.07 (288)*	1.7	103 ± 2
4f	7	6.35 ± 0.06 (450)	6.46 ± 0.01 (347)	1.3	91 ± 2
4g	8	6.11 ± 0.12 (836)	6.34 ± 0.20 (591)	1.4	65 ± 2
4h	9	6.18 ± 0.08 (679)	6.19 ± 0.05 (651)	1.07	83 ± 1
4i	10	5.90 ± 0.20 (1470)	5.89 ± 0.11 (1360)	1.07	55 ± 3
2	–	6.08 ± 0.06 (842)	6.72 ± 0.06 (195)**	4.3	100 ± 6
2K$_{i,high}$		7.37 ± 0.3 (71)			
2K$_{i,low}$		5.73 ± 0.1 (1990)			

[a] Values of pK_i were compared between control and + 10 μM PD81,723 conditions using a Student's t-test. Significant differences were annotated as follows: $p < 0.05$ - *, $p < 0.01$ - **, $p < 0.001$ - ***. Values of pK_i obtained for the compounds in the control condition were compared using a one-way ANOVA with a Bonferroni post-hoc test. Significant differences were observed as follows: 4d > 4a, 4c, 4g, 4h, 4i, $p < 0.05$ - *,.

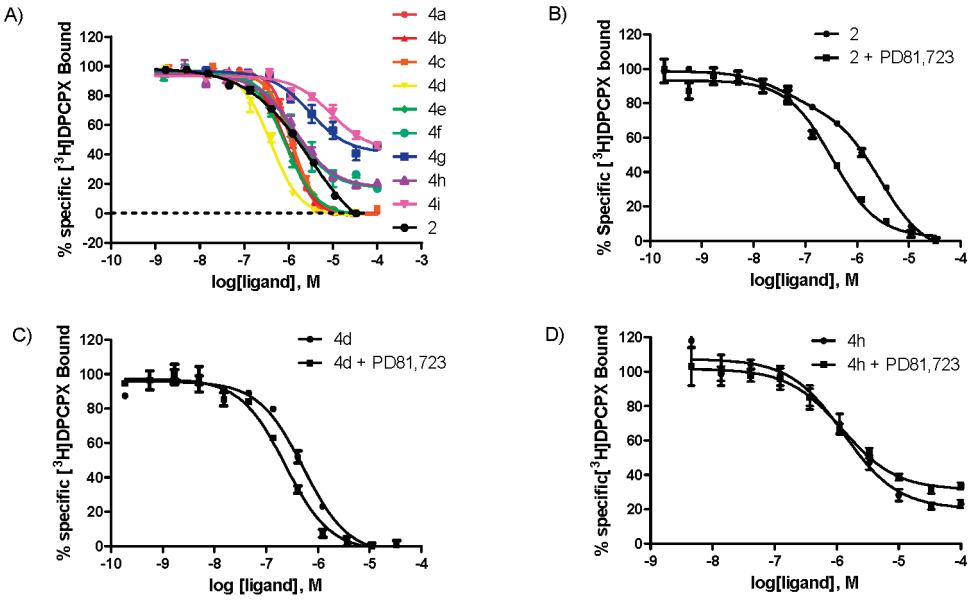


Figure 1. Displacement of specific [^{3}H]DPCPX binding by increasing concentrations of compounds **2**, **4a**−**i** (A). Displacement of specific [^{3}H]DPCPX binding by increasing concentrations of compounds **2** (B), **4d** (C), and **4h** (D) in the absence or presence of 10 μM PD81,723.

$K_{i,high}$ of 71 ± 48 nM and a $K_{i,low}$ of 1.99 ± 0.5 μM. In the presence of the allosteric enhancer PD81,723 (10 μM), the curve was monophasic ($K_{i,+PD81,723}$ = 195 nM) (Figure 1B, Table 2). Conversely, the displacement of [^{3}H] DPCPX by all bivalent ligands (**4**) was best characterized by monophasic isotherms. The pentyl-linked bivalent ligand **4d** (Figure 1C, Table 2) was found to have the highest affinity for the A_1AR. When tested in the presence of allosteric enhancer PD81,723 (10 μM), bivalent ligands with a spacer length of 6 or less showed a significant increase in affinity (2-fold), although this was less than that observed for the orthosteric ligand **2** (4-fold, Table 2). Smaller, nonsignificant 1.3−1.4-fold shifts were observed in the cases of **4f** and **4g**, respectively. For compounds **4h** (see also Figure 1D) and **4i**, the increase in affinity was negligible.

It was important to characterize the bivalent ligands in terms of their ability to modulate agonist binding. Bound [^{3}H]CCPA was completely displaced by the orthosteric agonist **2** with a K_i value of 48 nM (Figure 2, Table 3). This value is similar to the $K_{i,high}$ of compound **2** observed in the [^{3}H]DPCPX displacement assay, consistent with displacement of [^{3}H]CCPA bound to the high affinity G protein-coupled

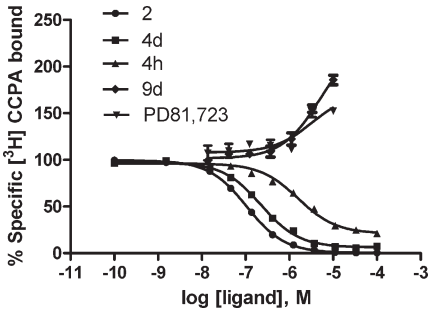


Figure 2. Ability of increasing concentrations of compounds PD81,723, **9d**, **2**, **4d**, and **4h** to displace or enhance the binding of the radiolabeled agonist [^{3}H]CCPA to CHO membranes expressing the A_1AR.

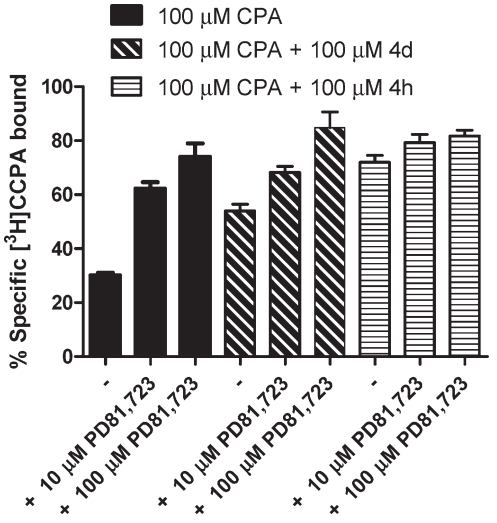


Figure 3. Ability of compounds **4d** and **4h** to decrease the rate of dissociation in a single-point kinetic assay. At time = 0, dissociation was initiated with the addition of 100 μM CPA with or without modulator compounds PD81,723, **4h**, or **4d** or a combination. Reactions were stopped after 30 min and bound [^{3}H]CCPA was determined. Results are presented as a percentage of specific [^{3}H]CCPA bound at time = 0.

Table 3. Affinity of Bivalent Ligands **4** (K_i values) at the Human Adenosine A_1 Receptor Determined by Their Ability to Displace the Radioligand [^{3}H]CCPA[a]

compd.	spacer length (n)	pK_i (K_i, nM)	maximal displacement, % of specific [^{3}H]CCPA binding
4d	5	6.99 ± 0.05 (103)	97 ± 0.9
4h	9	6.16 ± 0.12 (738)	79 ± 0.3
2	–	7.32 ± 0.06 (48)	100 ± 0.9

[a] Values of pK_i obtained for the compounds were compared using a one-way ANOVA with a Bonferroni post-hoc test. Significant differences were observed as follows: 2 > 4d > 4h. $p < 0.05$.

population of A_1AR. The values of K_i observed for compounds **4d** and **4h** for the displacement of [^{3}H]CCPA (103 and 738 nM, respectively) are within the same order of magnitude to values of K_i obtained for the displacement of [^{3}H]DPCPX. However, while compound **2** and compound **4d** show complete displacement of [^{3}H]CCPA, compound **4h** displaces bound [^{3}H]CCPA to a maximal value of approximately 80% (Table 3, Figure 2). Finally, it was important to show that the functionality of the allosteric pharmacophore was retained in the bivalent ligands. A single-point kinetic assay revealed that with the addition of 10 μM or 100 μM of PD81,723, **4d**, or **4h** caused an increase in measured specific [^{3}H]CCPA binding compared to the control (100 μM CPA only) condition (Figure 3). Therefore, the allosteric pharmacophore of both bivalent ligands was functional.

To demonstrate that the bivalent ligands retained their ability as agonists to activate the receptor, we tested two selected bivalent ligands, **4d** and **4h**, using a [^{35}S]GTPγS binding assay. Both compounds **4d** and **4h** acted as agonists and compared to the monovalent agonist compound **2** showed a significant increase in efficacy (Table 4, Figure 4A). It is interesting to note that the binding affinities and rank order of affinity observed for these three compounds in the [^{3}H]CCPA competition assays were similar to the potencies obtained in this assay. Indeed, **4d** and **4h** were 2-fold and 3-fold less potent than the monovalent compound **2**. A differential effect on the potencies of the three compounds was observed with the addition of 10 μM PD81,723. In the case of both the monovalent compound **2** and the bivalent compound **4d**, a 5-fold increase in potency was observed with the addition of PD81,723. In contrast, a smaller 2-fold shift in potency was observed for compound **4h**. Similarly, a significant decrease in the maximal effect of both compound **2** and **4d** but not compound **4h** was observed with the addition of PD81,723. Intriguingly, the Hill slopes for the bivalent ligands **4d** and, in particular, **4h** were significantly higher than unity (1.3 and 1.5, respectively), whereas the Hill slope of the monovalent agonist **2** was close to unity (0.8). Hill slopes higher than unity are signs of positive cooperativity, suggesting a simultaneous sensing and synergy of the orthosteric and allosteric site on the receptor. With addition of 10 μM PD81,723 the Hill slopes of **4d** and **4h** no longer differed significantly from unity.

It was of further interest to determine the functional effect of the bivalent ligands using a whole-cell assay, in this case the measurement of ERK 1/2 phosphorylation. In this assay, the monovalent compound **2** had the highest potency followed by **4d** and **4h**, the same rank order observed for the [^{35}S]GTPγS data (Figure 4B, Table 5). When compared to values obtained in the [^{35}S]GTPγS assay, compounds **2** and **4d** showed a 2-fold and 7-fold increase in potency for ERK 1/2 phosphorylation, while a 2.5-fold decrease in potency was observed for compound **4h**. The presence of 10 μM PD81,723 caused a 4-fold increase in potency for compound **2**, a 2-fold increase in potency for compound **4d**, and no significant increase in potency for compound **4h**. Upon addition of 10 μM PD81,723 a significant 30% increase in maximal efficacy (E_{max}) was observed for compound **2**. Compounds **4d** and **4h** had a significantly higher E_{max} as compared to compound **2** in the control condition. However, only the E_{max} of compound **4h** was unaffected by the addition of PD81,723.

The linking of the parent A_1AR agonist **2** to an A_1AR allosteric modulator has resulted in the generation of bivalent ligands with novel properties. Initial characterization of the bivalent ligands using competition experiments with [^{3}H]DPCPX suggested that the ligands had a similar (**4h**) or slightly improved (**4d**) affinity for the receptor. However, subsequent experiments demonstrated that these ligands had a lower affinity for receptors labeled with the agonist [^{3}H]CCPA and a lower potency in functional assays. Linking such an agonist to an allosteric enhancer might be expected to result in an increase in affinity or potency. Indeed, one such study demonstrated an increased affinity of a bitopic for

Table 4. Ability of **2**, **4d**, and **4h** to Activate the Human Adenosine A_1 Receptor Stably Expressed in CHO Cell Membranes Measured Using a [^{35}S]GTPγS Binding Assay[a]

	pEC_{50} (EC_{50}, nM)			E_{max}, %		Hill slope	
compd.	control	+ PD81,723	fold shift	control	+ PD81,723	control	+ PD81,723
4d	6.57 ± 0.04 (273)	7.29 ± 0.06** (52)	5.2	110 ± 3	97 ± 2*	1.29 ± 0.09	0.89 ± 0.07 *
4h	6.41 ± 0.03 (390)	6.75 ± 0.04 ** (181)	2.2	110 ± 2	105 ± 3	1.45 ± 0.03	0.88 ± 0.09 **
2	6.91 ± 0.05 (142)	7.52 ± 0.03*** (29)	4.9	100 ± 2	91 ± 3*	0.84 ± 0.04	0.98 ± 0.13

[a] Values are displayed for the control condition or in the presence of 10 μM PD81,723. E_{max} values are displayed as a percentage of maximal stimulation of compound **2**. For all compounds, values of pEC_{50}, E_{max}, and Hill slope were compared between control and + PD81,723 conditions using a Students *t*-test. Significant differences were annotated as follows: $p < 0.05$ - *, $p < 0.01$ - **, $p < 0.001$ - ***. Values of pEC_{50} and E_{max} obtained for the compounds were compared using a one-way ANOVA with a Bonferroni post-hoc test. Rank orders were as follows: E_{max} 4d = 4h > 2, $p < 0.05$; pEC_{50} 2 > 4d = 4h, $p < 0.05$.

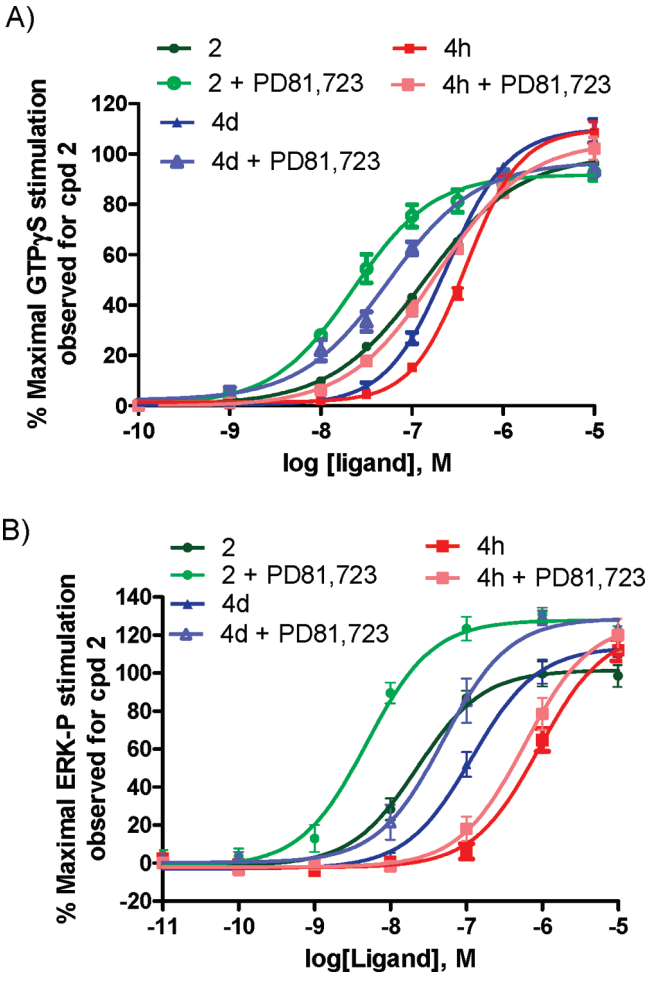


Figure 4. Ability of increasing concentrations of compounds **2**, **4d**, and **4h**, both in the absence and in the presence of PD81,723, to (A) stimulate [^{35}S]GTPγS binding in CHO cell membranes expressing the A_1AR or (B) to stimulate A_1AR-mediated ERK1/2 phosphorylation in intact CHO cells. Value of 100% is equivalent to a stimulation of 5-fold and 7.2-fold over basal of [^{35}S]GTPγS and ERK1/2 phosphorylation data, respectively.

the target receptor compared to the binding affinities of the relevant individual pharmacophores.[11] However, it has been demonstrated that the combination of a negative allosteric modulator pharmacophore with an agonist pharmacophore resulted in a decrease in affinity of the bitopic ligand as compared to the parent orthosteric compound.[9,12] Therefore, gain or loss of affinity of a bitopic ligand compared to the parent ligand is greatly dependent on the allosteric pharmacophore partner. Several reports have demonstrated that PD81,723 acts to increase the fractional coupling of A_1ARs to G proteins rather than to increase the affinity of the receptor to agonists; indeed, PD81,723 has been shown to be an allosteric agonist.[33−35] Therefore, both the leftward shift observed upon addition of PD81,723 in [^{3}H]DPCPX displacement experiments and the increase in potency and efficacy observed in functional assays can be attributed to an increase in receptor coupling rather than an increase in affinity for the G protein-coupled population of receptors. Consequently, the linking of an agonist to a PD81,723 analogue pharmacophore should not necessarily be expected to increase ligand affinity. Conversely, an increase in ligand efficacy compared to the parent compound **2** might be expected, and indeed, this is observed for both compounds **4d** and **4h**.

The original hypothesis of this research was that if a bivalent ligand was generated in which the allosteric and orthosteric pharmacophores occupy the receptor simultaneously then this compound would be insensitive to the addition of an additional allosteric enhancer. Indeed, for compounds with a spacer length of greater than 7 carbon atoms no significant affinity or potency shift was observed with the addition of 10 μM PD81,723. Furthermore, no increase in efficacy was observed upon the addition of excess PD81,723. The bivalent ligands have a number of different modes of binding. Therefore, interpretation of this result is complex due to the reversibility of binding and the distribution of the ligands across a number of receptor species. These would include species in which the ligand is bound only to the allosteric site, only to the orthosteric site, or if the linker is long enough, simultaneously to both sites. Furthermore, one receptor can be occupied by two bivalent ligands, one binding allosterically and one binding orthosterically. Upon addition of extra monovalent allosteric modulator, two scenarios are possible: first, that the bivalent ligand will fail to bind resulting in a loss of potency, or second, that the bivalent ligand still binds orthosterically. In this case, the change in potency will depend on the interaction with the allosteric ligand and the nature and efficacy of this allosteric ligand compared to that of the allosteric pharmacophore of the bivalent ligand. In the present study, the allosteric pharmacophore is structurally related to and has a similar efficacy to that of the added monovalent modulator PD81,723. Therefore, if the linker is of sufficient length to allow simultaneous occupation of the allosteric and orthosteric binding sites, the addition of excess PD81,723 will have a minimal effect on the potency of the bivalent compounds as was observed for compounds **4f**−**i**. Following the same hypothesis, if the linker length is insufficient to occupy both sites, a bivalent ligand can occupy either the orthosteric or allosteric site. In this situation, the occupancy of the allosteric site will be related to the concentration of the bivalent

Table 5. Ability of **2**, **4d**, and **4h** to Stimulate ERK1/2 Phosphorylation in Intact CHO Cells Stably Expressing the Human Adenosine A_1 Receptor[a]

	pEC_{50} (EC_{50}, nM)			E_{max}, %	
compd.	control	+ PD81,723	fold shift	control	+ PD81,723
4d	6.82 ± 0.07 (156)	7.05 ± 0.00* (89)	1.8	114 ± 5	131 ± 2*
4h	6.02 ± 0.12 (1071)	6.16 ± 0.14 (813)	1.3	123 ± 6	122 ± 8
2	7.68. ± 0.06 (22)	8.34 ± 0.11** (5)	4.4	100 ± 1	131 ± 3***

[a] The data shown are the mean ± SEM of at least 3 separate experiments. Values are displayed for the control condition or in the presence of 10 μM PD81,723. E_{max} values are displayed as a percentage of maximal stimulation of compound **2**. For all compounds, values of pEC_{50}, E_{max} were compared between control and + PD81,723 conditions using a Student's *t*-test. Significant differences were annotated as follows: $p < 0.05$ -*, $p < 0.01$ - **, $p < 0.001$ - ***. Values of pEC_{50} and E_{max} obtained for the compounds were compared using a one-way ANOVA with a Bonferroni post-hoc test. Rank orders were as follows: E_{max} 4h > 2 = 4d, $p < 0.05$; pEC_{50} 2 > 4d > 4h, $p < 0.05$.

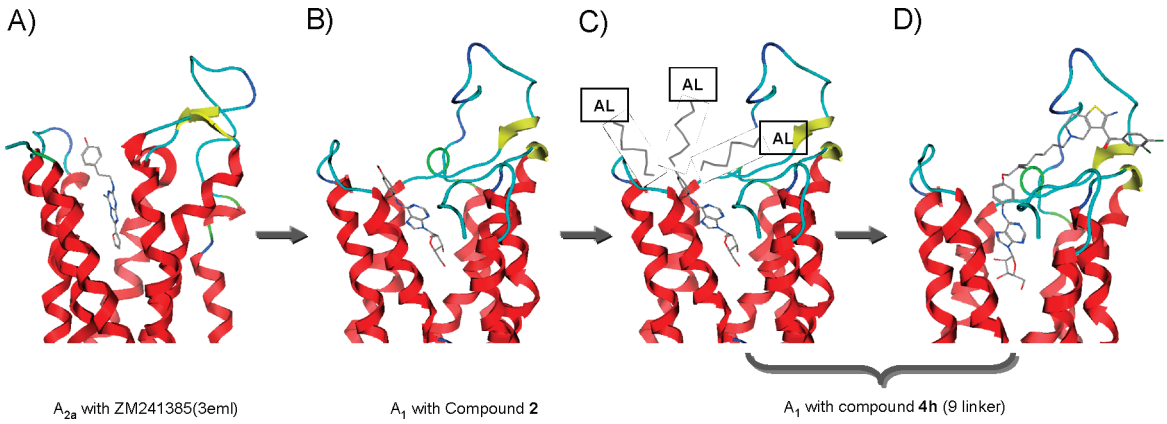


Figure 5. Molecular docking studies to demonstrate potential binding orientation of **4h**, the bitopic ligand with a 9 carbon atom linker. A homology model of the human A_1 receptor was generated (B−D) using the adenosine A_{2A} receptor crystal structure as a template (A). Using this model, docking analysis was performed for compounds **2** (B) and **4h** (C,D). The potential orientations of the allosteric pharmacophore are demonstrated (C), including one orientation in which the allosteric pharmacophore makes significant interactions with extracellular loop 2 (D).

ligand added and the affinity of the bivalent ligand for the allosteric site. In the case of compound **4d**, the affinity of the ligand for the orthosteric binding site as indicated by the [^{3}H]DPCPX displacement assay is approximately 10-fold higher than the potency of the allosteric pharmacophore (Table 1). Therefore, the allosteric site will not be fully occupied. Hence, for this compound a shift in affinity is observed upon addition of 10 μM PD81,723 as the occupancy of the allosteric site increases.

It is interesting to note that bivalent ligands with a linker length of seven or greater failed to completely displace bound [^{3}H]DPCPX or [^{3}H]CCPA as compared to the maximal displacement observed for the monovalent agonist **2**. This inability of bivalent compounds with a linker length greater than seven to displace the competing radioligand fully also coincided with their observed insensitivity to PD81,723 in both binding and functional assays. Therefore, this incomplete displacement could be associated with their ability to simultaneously bind to both the allosteric and orthosteric binding sites. Incomplete displacement has been observed in a previous study characterizing a bitopic ligand generated by linking orthosteric ligand and allosteric enhancer pharmacophores.[12] Such behavior was reconciled with the idea that such a bitopic ligand would produce an effect that was intermediate between that of a positive allosteric modulator and that of a simple competitive orthosteric inhibitor.[12] One might argue that incomplete displacement of radioligand by compounds **4h** and **4i** is explained by insolubility of these compounds. However, the curves have a Hill slope of unity and begin to plateau at low concentrations of approximately 1 μM. This is not consistent with dissolution issues.

The relatively short length of linker (≥7 carbon atoms) required to observe potential bitopic ligand binding suggests that the allosteric site is located in close proximity of the orthosteric site. We therefore performed homology modeling and docking studies using the A_{2A} adenosine receptor crystal structure as a template (Figure 5A), yielding a structure for the A_1AR complexed with agonist 2 (Figure 5B). As shown by the 3-D model (Figure 5C), the best solutions for compound **4h** (9 carbon atom linker) demonstrate that the linker and allosteric pharmacophore extend out of the binding pocket into the extracellular space. Once such solution (Figure 5D) shows the allosteric pharmacophore interacting with extracellular loop 2, a region that has been suggested as the binding site for allosteric modulators at other GPCRs.[36] However, due to the inherent flexibility of both the linker region of the molecule and the extracellular loop it is inappropriate to implicate specific amino acid residues in this interaction without validation with mutagenesis studies.[37] In agreement with the extracellular orientation of the linker and allosteric pharmacophore, it has been observed that large substituents are tolerated at the N^6 position. Indeed, bivalent ligands with adenosine A_1 receptor and β_2-adrenergic receptor pharmacophores have been successfully generated using this strategy.[31]

The data from the present study show that linking orthosteric and allosteric pharmacophores for the A_1AR into a single ligand provides clues about the location of the allosteric site. This advocates that a minimum chain length is needed to connect both sites on the receptor. Similarly, work by Portoghese et al. demonstrated that an optimal linker length was required for the generation of bivalent ligands that specifically targeted $\delta-\kappa$ opioid receptor heterodimers.[17,32] In this case, a

spacer length of 21 atoms (maximal molecule length 90 Å) was required to allow the simultaneous binding of the κ-opioid selective pharmacophore and the δ-opioid selective pharmacophore to their respective orthosteric sites on each monomer of the $\delta-\kappa$ opioid receptor heterodimer. This is much greater than the 9 carbon atom spacer length (maximal molecule length 49 Å) of compound **4h** in this study. It is interesting to note that other bitopic ligands, simultaneously binding to orthosteric and allosteric sites within the same receptor, have similar spacer lengths. Three separate studies characterized distinct bitopic ligands for the muscarinic M_2 receptor, with molecule lengths between 17 and 45 Å.[10−12] Therefore, the length of spacer used for these bitopic ligands, and indeed the bitopic ligands described in this study, is considerably smaller than that required for the simultaneous occupation of two orthosteric sites within a receptor heterodimer as determined by Portoghese and co-workers.[17,32]

Conclusion

A series of bivalent ligands linking both the agonist and allosteric pharmacophores of the A_1AR was synthesized and used as a tool to locate the allosteric site on the A_1AR. These ligands were tested in radioligand competition assays in the absence and presence of an allosteric enhancer. Bivalent ligands **4f−i** did not show a significant affinity change in the presence of an allosteric enhancer, indicating that they bridge both sites on the receptor. The data suggest that the allosteric site of A_1AR is in close proximity to the orthosteric site and that both sites can be simultaneously occupied by one bivalent ligand. Homology modeling and molecular docking studies using the bivalent ligands suggest that the allosteric pharmacophore may interact with extracellular loop 2 of the same A_1AR. Thus, bivalent ligands may be used as a tool to determine the distance between two sites and a strategy similar to ours could be used to determine the location of allosteric sites on other GPCRs.

Experimental Section

Chemistry: Materials and Methods. All reagents were obtained from commercial sources and all solvents were of analytical grade. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 400 (^{1}H NMR, 400 MHz; ^{13}C NMR, 100 MHz) with tetramethylsilane as an internal standard. Chemical shifts are reported in δ (ppm). Reactions were routinely monitored by TLC using Merck silica gel F254 plates. Melting points were measured on a Büchi melting point apparatus and are uncorrected. Microwave reactions were performed on an Emrys Optimizer (Biotage AB). Wattage was automatically adjusted to maintain the desired temperature. Purity of all final products was determined by analytical HPLC to be ≥95%. HPLC purity of compounds was measured with a reverse-phase column (RP18, 125 × 4 mm, 5 μm, 254 nm and diode array, 210−360 nm) with two diverse solvent systems. A: Water, 10% acetonitrile, 10 mM HOAc, 5 mM SDS. B: Water, 90% acetonitrile, 10 mM HOAc, 5 mM SDS. Samples were eluted by a gradient between 100% A and 100% B at a flow rate of 0.6 mL/min.

General Procedure for Microwave-Assisted Amination of 6-Chloropurine 9-β-D-Ribofuranoside. To a solution of aniline **10** (1 equiv) in ethanol, 6-chloropurine 9-β-D-ribofuranoside (1 equiv), triethylamine (1.5 equiv) was added and the resulting mixture was heated in microwave at 120 °C for 2.5 h. Upon completion (TLC), the reaction mixture was diluted with chloroform (20 mL) and evaporated in vacuo. The crude compound was purified by flash column chromatography ($MeOH/CH_2Cl_2$, 1:9) to afford the desired product as yellow colored solid.

***N*^{6}-[2-Amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-2-ethyloxy-4-phenyl]-adenosine (4a).** Yield: 45 mg, 47%. Mp 158−160 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.35 (s, 1H), 8.17 (s, 1H), 7.65 (d, J = 8.8 Hz, 2H), 7.57−7.51 (m, 2H), 7.31 (dd, J = 8.0 Hz, J = 1.0 Hz, 1H), 6.96 (d, J = 8.8 Hz, 2H), 5.93 (d, J = 6.8 Hz, 1H), 4.81 (t, J = 6.0 Hz, 1H), 4.37 (d, J = 4.4 Hz, 1H), 4.21 (t, J = 5.6 Hz, 2H), 3.96 (d, J = 12.4 Hz, 1H), 3.77 (d, J = 12.4 Hz, 1H), 3.70 (s, 2H), 3.06 (t, J = 6.0 Hz, 2H), 2.78 (t, J = 6.0 Hz, 2H), 2.02 (t, J = 6.4 Hz, 2H) ppm.

***N*^{6}-[2-Amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-3-propyloxy-4-phenyl]-adenosine (4b).** Yield: 135 mg, 65%. Mp 143−145 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.35 (s, 1H), 8.17 (s, 1H), 7.62 (d, J = 8.8 Hz, 2H), 7.56−7.51 (m, 2H), 7.31 (dd, J = 8.4 Hz, J = 2.8 Hz, 1H), 6.93 (d, J = 8.8 Hz, 2H), 5.93 (d, J = 6.8 Hz, 1H), 4.80 (t, J = 6.4 Hz, 1H), 4.37 (dd, J = 5.2 Hz, J = 1.6 Hz, 1H), 4.27 (d, J = 1.6 Hz, 1H), 4.05 (t, J = 6.0 Hz, 2H), 3.96 (dd, J = 12.6 Hz, J = 1.8 Hz, 1H), 3.77 (dd, J = 12.6 Hz, J = 1.4 Hz, 1H), 3.52 (s, 2H), 2.74 (t, J = 8.0 Hz, 2H), 2.62 (t, J = 5.4 Hz, 2H), 2.09−1.96 (m, 4H) ppm.

***N*^{6}-[2-Amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-4-butyloxy-4-phenyl]-adenosine (4c).** Yield: 40 mg, 52%. Mp 140−142 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.34 (s, 1H), 8.21 (s, 1H), 7.62−7.52 (m, 4H), 7.32 (dd, J = 8.4 Hz, J = 2.8 Hz, 1H), 6.93 (d, J = 8.8 Hz, 2H), 5.94 (d, J = 6.4 Hz, 1H), 4.80 (t, J = 6.8 Hz, 1H), 4.37 (d, J = 2.8 Hz, 1H), 4.26 (s, 1H), 4.02 (t, J = 6.0 Hz, 2H), 3.96 (d, J = 12.4 Hz, 1H), 3.77 (d, J = 12.4 Hz, 1H), 3.56 (s, 2H), 2.71−2.62 (m, 4H), 2.02 (t, J = 6.4 Hz, 2H), 1.80−1.65 (m, 4H) ppm.

***N*^{6}-[2-Amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-5-pentyloxy-4-phenyl]-adenosine (4d).** Yield: 120 mg, 61%. 123−125 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.36 (s, 1H), 8.09 (s, 1H), 7.63 (td, J = 8.8 Hz, J = 2.8 Hz, 2H), 7.54 (d, J = 2.0 Hz, 1H), 7.51 (d, J = 8.4 Hz, 1H), 7.30 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H), 6.93 (td, J = 8.8 Hz, J = 2.8 Hz, 2H), 5.89 (d, J = 6.8 Hz, 1H), 4.82 (t, J = 6.8 Hz, 1H), 4.37 (dd, J = 5.2 Hz, J = 1.6 Hz, 1H), 4.28 (d, J = 1.6 Hz, 1H), 4.03−3.95 (m, 3H), 3.77 (dd, J = 12.6 Hz, J = 1.8 Hz, 1H), 3.48 (s, 2H), 2.63−2.52 (m, 4H), 1.84 (t, J = 6.8 Hz, 2H), 1.74−1.58 (m, 2H), 1.67−1.60 (m, 2H), 1.55−1.47 (m, 2H) ppm.

***N*^{6}-[2-Amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-6-hexyloxy-4-phenyl]-adenosine (4e).** Yield: 70 mg, 43%. Mp 133−135 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.34 (s, 1H), 8.16 (s, 1H), 7.60 (dd, J = 9.2 Hz, J = 3.2 Hz, 2H), 7.56−7.51 (m, 2H), 7.30 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H), 6.93 (td, J = 8.8 Hz, J = 2.8 Hz, 2H), 5.92 (d, J = 6.8 Hz, 1H), 4.81 (t, J = 6.8 Hz, 1H), 4.37 (dd, J = 6.0 Hz, J = 2.0 Hz, 1H), 4.27 (d, J = 2.0 Hz, 1H), 4.02−3.94 (m, 3H), 3.77 (dd, J = 12.8 Hz, J = 2.0 Hz, 1H), 3.43 (s, 2H), 2.57−2.47 (m, 4H), 1.94 (t, J = 6.8 Hz, 2H), 1.85−1.72 (m, 2H), 1.63−1.48 (m, 4H), 1.46−1.37 (m, 2H) ppm.

***N*^{6}-[2-Amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-7-heptyloxy-4-phenyl]-adenosine (4f).** Yield: 60 mg, 54%. Mp 125−126 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.35 (s, 1H), 8.15 (s, 1H), 7.61 (d, J = 8.4 Hz, 2H), 7.52 (d, J = 9.6 Hz, 2H), 7.30 (d, J = 8.0 Hz, 1H), 6.93 (d, J = 8.8 Hz, 2H), 5.92 (d, J = 6.4 Hz, 1H), 4.81 (t, J = 5.8 Hz, 1H), 4.37 (d, J = 6.0 Hz, 1H), 4.27 (d, J = 2.0 Hz, 1H), 4.02−3.94 (m, 3H), 3.77 (d, J = 12.4 Hz, 1H), 3.43 (s, 2H), 2.55−2.46 (m, 4H), 1.94 (t, J = 5.8 Hz, 2H), 1.85−1.74 (m, 2H), 1.61−1.47 (m, 4H), 1.44−1.33 (m, 4H) ppm.

***N*^{6}-[2-Amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-8-octyloxy-4-phenyl]-adenosine (4g).** Yield: 35 mg, 47%. Mp 128−129 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.36 (s, 1H), 8.06 (s, 1H), 7.61 (d, J = 8.8 Hz, 2H), 7.52 (d, J = 9.2 Hz, 2H), 7.30 (d, J = 8.4 Hz, 1H), 6.94 (d, J = 8.8 Hz, 2H), 5.88 (d, J = 6.8 Hz, 1H), 4.83 (t, J = 6.0 Hz, 1H), 4.35 (d, J = 5.2 Hz, 1H), 4.27 (d, J = 1.2 Hz, 1H), 4.01−3.94 (m, 3H), 3.77 (d, J = 12.4 Hz, 1H), 3.43 (s, 2H), 2.53−2.44 (m, 4H), 1.94 (t, J = 5.8 Hz,

2H), 1.82−1.75 (m, 2H), 1.58−1.44 (m, 4H), 1.42−1.29 (m, 6H) ppm.

N^6-[2-Amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-9-nonyloxy-4-phenyl]-adenosine (4h). Yield: 22 mg, 43%. Mp 124−126 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.35 (s, 1H), 8.09 (s, 1H), 7.62 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 9.6 Hz, 2H), 7.31 (d, J = 8.4 Hz, 1H), 6.93 (d, J = 8.8 Hz, 2H), 5.89 (d, J = 6.8 Hz, 1H), 4.82 (t, J = 6.0 Hz, 1H), 4.36 (d, J = 4.4 Hz, 1H), 4.27 (d, J = 1.6 Hz, 1H), 4.02−3.93 (m, 3H), 3.77 (d, J = 12.8 Hz, 1H), 3.66 (s, 2H), 2.84−2.67 (m, 4H), 2.03 (t, J = 5.8 Hz, 2H), 1.84−1.74 (m, 2H), 1.69−1.59 (m, 2H), 1.51−1.42 (m, 2H), 1.41−1.31 (m, 8H) ppm.

N^6-[2-Amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-10-decyloxy-4-phenyl]-adenosine (4i). Yield: 75 mg, 52%. Mp 119 121 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.35 (s, 1H), 8.11 (s, 1H), 7.62 (d, J = 7.2 Hz, 2H), 7.53 (d, J = 9.6 Hz, 2H), 7.30 (d, J = 7.2 Hz, 1H), 6.94 (d, J = 7.6 Hz, 2H), 5.91 (d, J = 5.2 Hz, 1H), 4.82 (t, J = 6.0 Hz, 1H), 4.36 (d, J = 4.8 Hz, 1H), 4.28 (d, J = 1.6 Hz, 1H), 4.03−3.93 (m, 3H), 3.77 (d, J = 12.4 Hz, 1H), 3.42 (s, 2H), 2.58−2.41 (m, 4H), 1.93 (t, J = 5.4 Hz, 2H), 1.85−1.73 (m, 2H), 1.61−1.41 (m, 4H), 1.41−1.31 (m, 10H) ppm.

Biology. Binding Studies. [^{3}H]DPCPX was purchased from Amersham Biosciences (NL), [^{3}H]CCPA was purchased from Perkin-Elmer (NL). CHO cells expressing the human adenosine A_1 receptor (CHO−hA_1) were provided by Dr. Andrea Townsend-Nicholson, University College London, U.K., and Dr. Stephen Hill, University of Nottingham, U.K. For [^{3}H]DPCPX displacement experiments, reactions were performed in a total volume of 100 μL, 50 mM Tris·HCl pH 7.4, adenosine deaminase 0.8 IU/mL, 1.6 nM [^{3}H]DPCPX, and increasing concentrations of ligand with and without the presence of 10 μM PD81,723. CHO-hA_1 membranes containing 10 μg of protein (B_{max} = 12.8 pmol.mg^{-1} determined by [^{3}H]DPCPX binding) were added and the reaction incubated for 1 h at 25 °C in a shaking water bath before termination by fast-flow filtration over GF/B filters under reduced pressure with a Brandel harvester. Nonspecific binding was determined with the addition of 10 μM CPA. Filters were washed three times with ice-cold 50 mM Tris·HCl pH 7.4, placed in vials, and counted on a liquid scintillation counter (Tri-Carb 2900 TR, Perkin-Elmer). [^{3}H]CCPA binding experiments were performed as described for [^{3}H]DPCPX binding experiments, but with the following differences. Twenty micrograms of CHO-hA_1 membrane protein was added to a final reaction volume of 100 μL containing 2 nM [^{3}H]CCPA and increasing concentrations of modulator and incubated for 2 h at 25 °C before filtration and scintillation counting as described above. For single point kinetics assays, experiments were performed as above, but after two hours incubation at 25 °C, dissociation was initiated by the addition of 100 μM CPA with or without the presence of modulator compounds **2**, **4d**, and **4h**. Reactions were stopped after 30 min by fast-flow filtration as described above.

A_1 Adenosine Receptor-Mediated [^{35}S]GTPγS Binding. CHO-hA_1 membrane homogenates (5 μg, B_{max} = 4.1 pmol.mg^{-1} determined by [^{3}H]DPCPX binding) were equilibrated in a 90 μL total volume of assay buffer (50 mM Tris, 100 mM NaCl, 10 mM MgCl, pH 7.4) containing 3 μM GDP and a range of concentrations of either the monovalent agonist **2** or the bivalent ligands **4d** and **4h** at 25 °C for 30 min. After this, 10 μL of [^{35}S]GTPγS (final concentration 0.3 nM) were added and incubation continued for 45 min at 25 °C. Incubation was terminated by rapid filtration through a 96 well Unifilter (PerkinElmer, NL) using a Filtermate Unifilter 96-well harvester (Perkin-Elmer). Filters were washed three times with ice-cold assay buffer before drying. Twenty-five microliters of Microscint scintillation cocktail was added to each well, and plates were counted in a 1450 Trilux Microbeta liquid scintillation and luminescence counter (Perkin-Elmer). Typically a 5-fold stimulation above basal was observed.

A_1 Receptor Mediated ERK1/2 Phosphorylation. Experiments were performed using an ERK Surefire Alphascreen kit (Perkin-Elmer, Groningen, NL) as per the vendor's instructions. Briefly, Chinese hamster ovary (CHO) cells stably transfected with the human A_1AR were grown to 90% confluence and maintained in Dulbecco's modified eagle serum (DMEM) containing 10% newborn calf serum (NCBS), streptomycin (50 mg/mL), penicillin (50 IU/ml), and G418(0.2 mg/mL) at 37 °C in 5% CO_2. Cells were then harvested using phosphate-buffered saline (PBS) + 0.48 mM EDTA followed by centrifugation (300 g, 5 min). Cells were then seeded into 96 well plates at a density of 40 000 cells/well in DMEM/NCBS. After 6 h, plates were washed twice with PBS and then maintained in DMEM for 14 h. Prior to agonist stimulation, cells were pretreated for 30 min with 0.8 IU/mL adenosine deaminase (ADA) in phenol-red-free DMEM. Stimulation was then initiated with the addition of test compound with or without PD81,723. Stimulation was allowed to proceed for 5 min before the reaction was terminated by the removal of media and the addition of SureFire lysis buffer (100 μL to each well). The plate was then agitated for 2 min before 4 μL of lysate was added to a white opaque 384-well proxiplate. Seven microliters of a 1:1:10:60 mix of Alphascreen acceptor beads, Alphascreen donor beads, activation buffer, and reaction buffer was added to each well. The plate was then incubated in the dark at room temperature for at least 3 h before reading on an EnVision plate reader (Perkin-Elmer, Groningen, NL) using standard alphascreen settings. All data was expressed as a percentage of the maximal stimulation observed for compound **2** minus the basal signal. Typically, an 8-fold stimulation above basal was observed.

Data Analysis. Data were analyzed using nonlinear regression analysis software available in GraphPad *Prism 5.0*. Differences in obtained K_i, IC_{50}, and EC_{50} values were assessed for statistical significance using a paired Student's t-test or one-way ANOVA with a Bonferroni posthoc test where appropriate.

Homology Modeling and Docking Studies. A homology model of the A_1AR was generated by using the "protein modeling" module provided in MOE.[38] The crystal structure of the adenosine A_{2A} receptor (PDB code: 3EML) was used as the template. The sequence of the A_1AR (accession code: p30542) was aligned on the A_{2A} receptor using "sequence editor", which allows organization and modification of amino acids at the chain and residue levels. Amber99,[39] parametrized particularly for proteins and nucleic acids, was used as the force field. By default, ten independent intermediate models were built based on permutational selection of different loop candidates and side chain rotamers. The best scoring model according to the chosen scoring function (GB/VI)[40] was selected for further analysis and was subjected to energy minimization. Finally, the minimized model was inspected for unusual or geometrically incorrect stereochemistry by using an updated Ramachandran $\varphi-\psi$ dihedral plot with refined reference data set.[41] The outliers were subsequently subjected to fine grain minimization to obtain more typical $\varphi-\psi$ angles. The final model was used for docking analysis of compounds **2** and **4h** using the "docking module" in MOE. The homology model of A_1AR was prepared for docking by adding hydrogens and partial charges using the protonate 3D application given in the program. The active site was defined by selecting amino acids, namely, Phe171, Asn254, Lys265, Thr270 and His278, corresponding to amino acids of the active site of the A_{2A} receptor for the co-crystallized ZM241385 in 3EML. Ligands were placed in the active site by using the "Triangle matcher" algorithm where poses are generated by superposition of ligand atom triplets and triplets of receptor site points in a systematic way. The docked poses were first energy minimized in the active site and than ranked according to MM/GBVI binding free energy estimation.

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Supporting Information Available: Experimental details of the synthesis of the compounds described in this paper, their ^{1}H NMR and ^{13}C NMR spectroscopic data, HRMS data and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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