2-Aminothienopyridazines as Novel Adenosine A_1 Receptor Allosteric Modulators and Antagonists

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A pharmacophore-based screen identified 32 compounds including ethyl 5-amino-3-(4-*tert*-butylphenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (**8**) as a new allosteric modulator of the adenosine A_1 receptor (A_1AR). On the basis of this lead, various derivatives were prepared and evaluated for activity at the human A_1AR. A number of the test compounds allosterically stabilized agonist-receptor-G protein ternary complexes in dissociation kinetic assays, but were found to be more potent as antagonists in subsequent functional assays of ERK1/2 phosphorylation. Additional experiments on the most potent antagonist, **13b**, investigating A_1AR-mediated [^{35}S]GTPγS binding and [^{3}H]CCPA equilibrium binding confirmed its antagonistic mode of action and also identified inverse agonism. This study has thus identified a new class of A_1AR antagonists that can also recognize the receptor's allosteric site with lower potency.

Introduction

Adenosine receptors (ARs)[a] are members of the guanine nucleotide binding protein (G protein)-coupled receptor (GPCR) superfamily.[1] Four subtypes of human AR have been cloned and characterized, denoted A_1, A_{2A}, A_{2B}, and A_3ARs.[2] A_1 and A_3ARs are preferentially coupled to G_i proteins and can mediate inhibition of adenylyl cyclase, while A_{2A} and A_{2B} ARs preferentially couple to G_s proteins to stimulate adenylyl cyclase. Additional pathways have also been identified for these receptors, including the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2).[3] Selective adenosine receptor ligands have the potential for numerous therapeutic applications including treatment of cardiovascular, inflammatory and neurodegenerative diseases.[4] Although a number of potent and selective adenosine agonists have been reported, their development as therapeutic agents has been limited by side-effects associated with indiscriminate adenosine receptor activation and their propensity to cause receptor desensitization upon prolonged exposure. A promising alternative area of research is the development of allosteric modulators of ARs which, by binding to a subtype-specific and topographically distinct site away from the orthosteric pocket, can either enhance or inhibit the action of endogenous adenosine and thus act more selectively to tune AR signaling in both a site- and event-specific fashion.[5]

Bruns *et al.* first discovered A_1AR allosteric enhancers (AEs) in 1990.[6,7] These compounds were mainly 2-amino-3-benzoylthiophenes and 2-amino-3-benzoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines, which were found to mediate increases in orthosteric agonist, but not antagonist, affinity at the A_1AR due to their ability to retard the dissociation of agonists from the orthosteric binding site by allosterically promoting a high affinity receptor-agonist-G protein ternary complex.[8,9] Interestingly, all of these compounds also show inhibition of orthosteric ligands of the A_1AR at higher concentrations, which has most commonly been interpreted as reflecting a recognition of the orthosteric site on the receptor in addition to an allosteric site.[6,7] PD 81,723 (**1**) (Figure 1) was one of the most potent and effective enhancers in the original series, with the most favorable ratio of allosteric enhancement to antagonism,[7] and is thus used as the benchmark compound for further studies of A_1AR AEs.

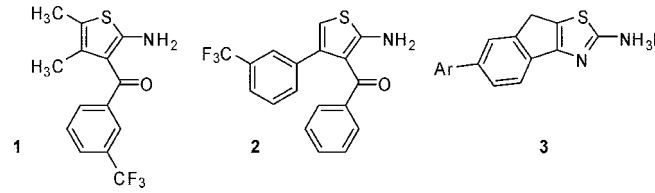

Figure 1. Current allosteric enhancers.

The structure−activity relationships of 2-aminothiophene AEs have been studied extensively[8−17] since the original report from Bruns and co-workers. It has been confirmed that the 2-amino group is essential for activity while various carbonyl containing substituents (including ketones, ester and amides) in the 3-position support allosteric activity. A range of alkyl and aryl substituents in the 4- and 5-positions have also been found to improve allosteric activity. This list includes fused cycloalkyl and heterocyclic (tetrahydropyridyl) substituents. A number of promising compounds, such as [2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl]phenyl methanone (**2**),[17] have been identified during these studies. However, the 2-aminothiophene class of enhancers generally only possess modest efficacy and potency and are known to act as A_1AR antagonists at higher concentrations. In some cases, compound stability has also been a problem. To date, 2-aminothiazoles (e.g., **3**, Figure 1) are the only alternative class of compounds that have been reported to be allosteric enhancers at the A_1AR.[18,19]

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[a] Abbreviations. AE: allosteric enhancer, AR: adenosine receptor, GPCR: G protein-coupled receptor, ERK1/2: extracellular signal-regulated kinases 1 and 2.

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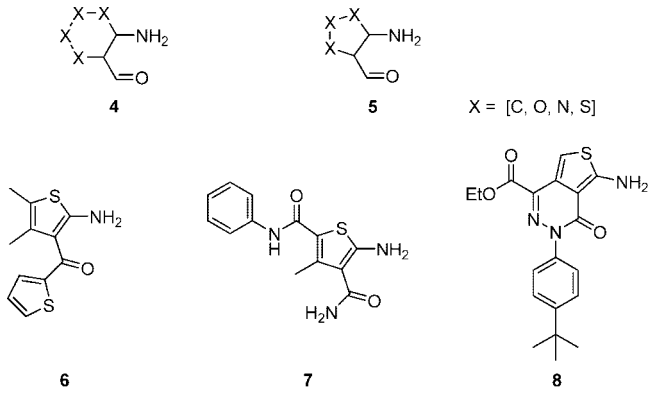


Figure 2. Template structures (**4**, **5**) used for pharmacophore searching and the resulting lead compounds that were identified (**6**−**8**).

Given that all known classes of A_1AR AEs have to date also been reported to act as antagonists at higher concentrations, the current study was undertaken to identify new classes of A_1AR AEs beyond the currently described series and to further probe the structural relationships that govern allosteric modulation versus orthosteric antagonism.

Results and Discussion

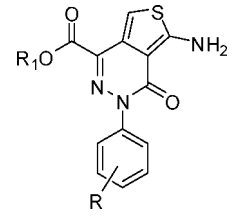
Pharmacophore Searching. We note that a carbonyl and adjacent amino group are important features of the A_1AR allosteric enhancers reported to date. Accordingly, we searched the Maybridge library of screening compounds for variations on templates **4** and **5** (Figure 2). The resulting compounds were manually inspected to limit the number of chemically similar derivatives and a final set of 32 compounds was selected for assay. From these, three compounds were identified (Figure 2) that caused significantly greater percent retardation of orthosteric radioligand dissociation ("K-score" for "kinetic score"; see Experimental Section) than the benchmark compound **1**, which itself has a K-score of 28% (Table 1). This pharmacophore analysis thus identified ethyl 5-amino-3-(4-*tert*-butylphenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (**8**) as a new lead compound (K-score 59%, Table 1). 2-Aminothienopyridazines have not been previously assayed as A_1AR allosteric modulators or ligands; therefore, structural analogues of **8** were synthesized with the aim of further exploring their mode of action. Our initial focus was to investigate the effect of the aromatic substituent in the 3-position, as the corresponding position in the 2-aminothiophene class is already known to play an important role in conferring allosteric activity.

Synthesis of Novel 2-Aminothienopyridazines. Analogues of the lead compound were synthesized (Scheme 1) from commercially available anilines **9a**−**h** which were transformed into aryl diazonium salts and reacted with ethyl acetoacetate to form intermediate phenyl hydrazones **11a**−**h**, as a mixture of *E*/*Z* isomers. Subsequent Knoevenagel condensation type reactions were performed using the synthesized hydrazones, ethyl cyanoacetate and 4-aminobutyric acid under solvent-free conditions at high temperature, which allowed ring closure to form the corresponding pyridazines **12a**−**h**. The pyridazines were then subjected to the Gewald synthesis[20] to produce the target compounds **13a**−**13h**. In the Gewald synthesis, it was found that under conventional methods reflux for between 6 to 12 h was required. However, when microwave heating at 150 °C was employed reaction times were reduced to 10 min and clean products were obtained in comparable yields.

Compounds in this series included phenyl, halophenyl, trifluoromethylphenyl, methoxyphenyl and nitrophenyl derivatives, which were previously reported to confer high allosteric activity.[16] The majority of aromatic substituent variations were introduced in the first step of the synthesis with the formation of aryl diazonium salts from corresponding anilines. It was found that isolation of the tetrafluoroborate salt of the 4-aminophenol was problematic, possibly as a result of complexation of the phenol with boron. Therefore, the corresponding aryl diazonium chloride (**10d**) was formed as an alternative. This diazonium chloride was converted to the desired thienopyridazine (**13d**). From the 4-hydroxyphenylthienopyridazine (**13d**) a number of modifications were applied at the hydroxyl group to further explore this region in the allosteric binding site. Simple acylation of **13d** with acetic anhydride afforded the corresponding 4-acetoxyphenylthienopyridazine (**13i**), while the reaction with *N*-phenyltriflimide under microwave irradiation[21] gave the corresponding triflate **13j**.

In order to increase water solubility, potentially increase bioavailability, and further explore the binding requirements, compounds **13a** and **13e** were saponified to produce the corresponding carboxylic acids **14a** and **14e** (Scheme 2).

Table 1. Allosteric and Antagonist Activity

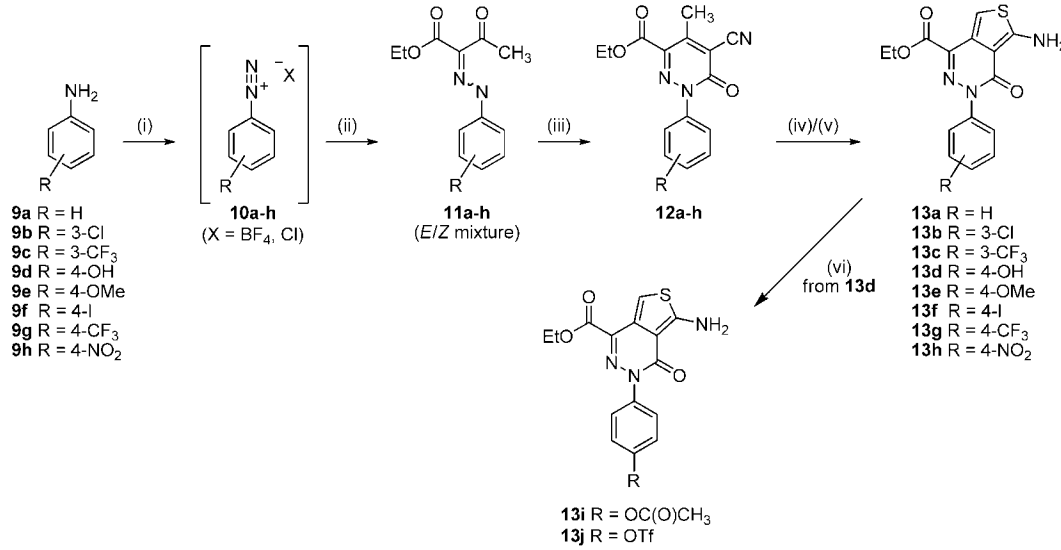


no.	R	R^1	K-score (%)[a]	EC_{50} (μM)[b]	% inhibition[c]	pA_2[d]
1			28 ± 1.1	13.6 ± 2.1	18.8 ± 2.5	ia[e]
6			33.2 ± 2.3	11.2 ± 1.2	50.3 ± 0.7	ia
7			80.3 ± 6.4	3.95 ± 1.1	32.3 ± 0.9	ia
8	4-*t*-Bu	Et	59.0 ± 3.0	9.75 ± 0.4	23.0 ± 0.5	ia
13a	H	Et	60.4 ± 1.0	13.6 ± 1.0	22.8 ± 4.2	7.8
13b	3-Cl	Et	69.2 ± 8.4	5.7 ± 1.2	100 ± 0.8	8.7
13c	3-CF_3	Et	66.1 ± 5.3	2.7 ± 1.0	32.0 ± 2.4	ia
13d	4-OH	Et	75.9 ± 4.6	7.1 ± 1.1	65.8 ± 1.1	7.4
13e	4-OMe	Et	68.6 ± 2.9	4.3 ± 1.5	92.5 ± 0.3	6.7
13f	4-I	Et	65.4 ± 11.9	7.1 ± 1.8	100 ± 0.2	7.1
13g	4-CF_3	Et	81.0 ± 6.0	8.4 ± 1.0	77.2 ± 0.6	ia
13h	4-NO_2	Et	19.6 ± 0.6[f]	13.0 ± 0.9[f]	73.4 ± 1.8	ia
13i	4-OAc	Et	66.6 ± 5.0	2.5 ± 1.2	48.9 ± 4.2	7.4
13j	4-OTf	Et	68.5 ± 5.3	4.9 ± 0.9	21.5 ± 4.4	ia
14a	H	H	76.2 ± 0.9[f]	9.8 ± 1.2[f]	51.8 ± 3.2	ia
14e	4-OMe	H	55.8 ± 6.0	2.6 ± 0.8	29.5 ± 4.3	ia

[a] Percentage of specific binding remaining after 10 min of dissociation of agonist, [^{125}I]-ABA, binding to A_1AR-G protein ternary complex in CHO-K1 cells stably expressing the hA_1AR ($n = 3$) followed by pretreatment with the candidate AE (50 μM). [b] Concentration of modulator producing half-maximal allosteric effect on [^{125}I]-ABA dissociation. [c] Percent inhibition of specific [^{3}H]CPX equilibrium binding by 50 μM test compound ($n = 3$). [d] Negative logarithm of the molar concentration of antagonist that makes it necessary to double the concentration of agonist (*R*-PIA) in order to achieve the same response level as that seen in the absence of antagonist; a measure of antagonist potency. [e] Inactive. [f] $n = 2$.

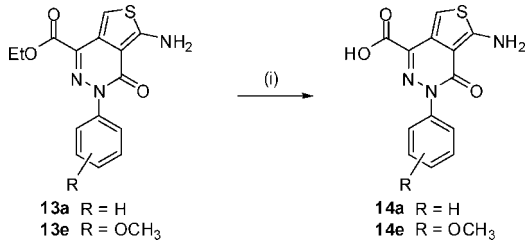
A_1AR Allosteric Modulation of Radioligand Binding. All candidate A_1AR modulators were initially screened using a rapid in vitro dissociation kinetic binding assay that measures the ability of each compound to stabilize an orthosteric agonist-A_1AR-G protein ternary complex.[22] The resulting K-score is thus a value ranging from 0 to 100 as the percentage of the ternary complex remaining after 10 min of radioligand dissociation. The K-scores for the target thienopyridazines **13a**−**j** and **14a**,**e** are shown in Table 1. All but one of the test compounds slowed radiolabeled agonist dissociation to a greater extent than **1** (Table 1), suggesting that they all have the capacity

Scheme 1[a]



[a] Reagents and conditions: (i) X = BF_4; HBF_4, $NaNO_2$, 67−100%, X = Cl; isoamyl nitrite, 92%; (ii) NaOAc, ethyl acetoacetate, EtOH, 58−100%; (iii) 4-aminobutyric acid, ethyl cyanoacetate, 42−95%; (iv) S_8, morpholine, EtOH, reflux, 55−100%; (v) S_8, morpholine, MW, 150 °C, 10−20 min, 79−100%; (vi) for **13i**: acetic anhydride, NEt_3, DMAP, CH_2Cl_2, 84%, for **13j**: *N*-phenyltriflimide, K_2CO_3, MW: 120 °C, 6 min, 100%.

Scheme 2[a]



[a] Reagents and conditions: (i) NaOH, EtOH, reflux, then conc. HCl, 31−78%.

to recognize an allosteric site on the A_1AR when the latter is prelabeled with an orthosteric agonist. Ethyl 5-amino-3-phenyl-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (**13a**) recorded a K-score of 60%. Substitution of the 3-phenyl group in the *meta*- and *para*-positions generally produced a slight increase in ternary complex stabilization, with the K-score of the monosubstituted 3-phenyl analogues **13b**−**g** ranging from 65 to 81%. The *para*-nitro substituted analogue **13h** was an exception with a relatively poor K-score of 20%. Derivatization of the phenol moiety of the 4-hydroxyphenylthienopyridazine (**13d**) reduced allosteric activity slightly; the K-score of the parent phenol **13d** was 76% compared with 67% for the corresponding acetyl derivative (**13i**) and 69% for the triflate (**13j**). Saponification of the ethyl ester group of the 3-phenylthienopyridazine **13a** to give the corresponding acid **14a** resulted in a slight increase in allosteric efficacy (K-score increased from 58% to 76%). In contrast, saponification of the ethyl ester of the 4-methoxyphenyl analogue **13e** to give **14e** resulted in the K-score decreasing from 69% to 56%. The potency (EC_{50}) range for all the compounds to mediate their half-maximal effects on radioligand dissociation ranged from 2.5 ± 1.2 μM (**13i**) to 13.6 ± 1.0 μM (**13a**). These latter values provide a semiquantitative estimate of the apparent affinity of the modulators for the allosteric site on the agonist-occupied receptor.

A_1AR Antagonism of Radioligand Binding. The potential for antagonist activity of the test compounds was initially assessed with respect to the ability of a 50 μM concentration to inhibit the binding of the orthosteric antagonist, [^{3}H]CPX, at equilibrium. All of the thienopyridazines were found to inhibit [^{3}H]CPX binding more potently than **1**, as inferred by their ability to promote greater percent inhibition of binding at equivalent concentrations (Table 1). The 3-chlorophenyl and 4-iodophenyl substitution of the 3-position (compounds **13b** and **13f**, respectively) showed the greatest antagonist activity in this assay, inhibiting 100% of [^{3}H]CPX specific binding at 50 μM.

Assessment of Functional Activity. The results of the radioligand binding screens indicated that the compounds were able to recognize an allosteric site on the agonist-occupied A_1AR, but that they also inhibited the binding of an antagonist at equilibrium, which may be indicative of either a dual allosteric/orthosteric mode of binding, or an allosteric interaction characterized by positive cooperativity for agonists and negative cooperativity for antagonists. To further investigate the mechanism of action of these compounds, functional assays were performed as such assays are more likely to reflect the physiologically relevant mode of interaction between the compounds and the A_1AR.

In the first instance, all compounds were screened using an AlphaScreen plate-based assay of A_1AR-mediated phosphorylation of ERK1/2 (pERK1/2) in intact CHO cells.[23] As described in the Experimental Section, these assays involved the determination of complete concentration−response curves to the orthosteric agonist, R-PIA, in the absence or presence of increasing concentrations of test compound. Interestingly, all compounds tested were either inactive against R-PIA-mediated pERK1/2, or else inhibited agonist function, which was manifested as parallel rightward shifts of the R-PIA pERK1/2 concentration−response curve. In this latter instance, analysis of these data according to a simple model of antagonism[24] yielded pA_2 values (Table 1), which are empirical measures of antagonist potency. For a competitive antagonist, the pA_2 is an estimate of the pK_B, i.e., the negative logarithm of the antagonist dissociation constant; for a noncompetitive antagonist, the pA_2 is an index of the (negative log) molar concentration of antagonist that makes it necessary to double the concentration of agonist in order to achieve the same response level as that seen in the absence of antagonist.[25] The use of pA_2 or pK_B values is preferable, whenever possible, over the use of EC_{50}

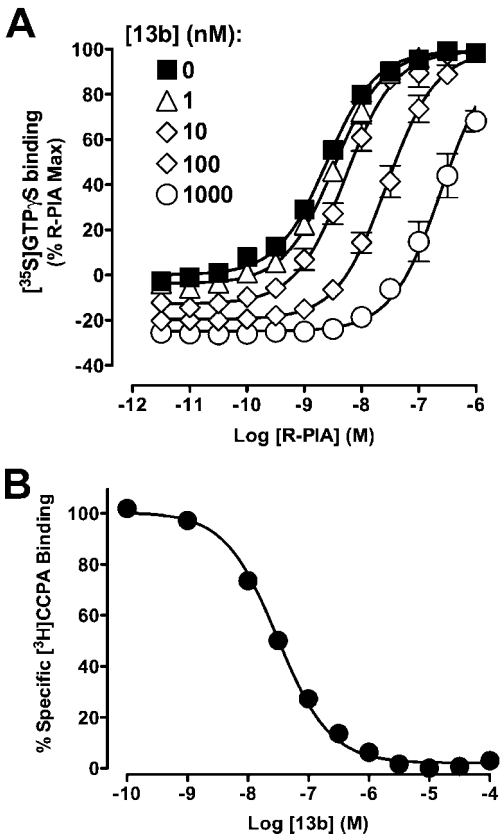


Figure 3. Effect of **13b** on (A) R-PIA mediated binding of [^{35}S]GTPγS (100 pM) to activated G proteins and (B) inhibition of specific [^{3}H]CCPA (2 nM) binding in membranes from CHO FlpIn cells stably expressing the human A_1AR. Data points represent the mean ± SEM of three experiments conducted in duplicate.

or IC_{50} values to quantify antagonist effects, as the former two reflect the true affinity of an antagonist for its binding site, while the latter two are purely empirical and can reflect multiple system factors in addition to antagonist affinity.

Several results from the assay of the thienopyridazine derivatives were of interest. The first, as mentioned above, was the lack of allosteric enhancement of agonist function, with either no activity or antagonism being noted instead. Second, it appears that substitution of the aromatic ring is not necessary for antagonism of hA_1AR, as **13a** is a potent antagonist (pA_2 = 7.8). In contrast, saponification of the pyridazine ester **13a** to the corresponding carboxylic acid **14a** significantly reduced antagonism of hA_1AR, as the compound was inactive in the pERK1/2 assay. This finding was further confirmed with saponification of the pyridazine ester **13e** (pA_2 = 6.7) to the corresponding carboxylic acid **14e** (no activity). A third finding is the structure activity relationship that can be seen when changing the aromatic substituent from 4-OH (**13d**), 4-OMe (**13e**), 4-OC(O)CH_3 (**13i**) and 4-OTf (**13j**). Both the 4-hydroxy (**13d**) and 4-acetoxy (**13i**) were potent antagonists (pA_2 = 7.4), while changing to 4-methoxy (**13e**) (pA_2 = 6.7) or 4-trifluorosulfonate (**13j**) (no activity) resulted in reduced antagonistic potency at the A_1AR.

The most potent degree of antagonism in this series of functional assays was seen with the 3-chlorophenylthienopyridazine (**13b**), with a pA_2 of 8.7 indicating activity in the low nanomolar range. As a consequence, this compound was subjected to additional experiments to confirm its antagonistic mode of action. Figure 3A shows that **13b** inhibited R-PIA mediated [^{35}S]GTPγS binding to activated G proteins with a similar potency (pA_2 = 8.1 ± 0.2; n = 3) to that for inhibiting pERK1/2. An additional finding was that **13b** is actually an *inverse* agonist of the A_1AR, because the receptor displayed constitutive activity under our [^{35}S]GTPγS binding assay conditions and **13b** reduced this activity in a concentration-dependent manner (Figure 3A), as well as inhibiting agonist-mediated activation; this was not evident in our pERK1/2 assays, as the constitutive activity was too low to be detected.

Because the potency of **13b** to antagonize agonist function was higher than its potency to stabilize agonist-receptor-G protein complexes in the dissociation kinetic assays (Table 1), we performed an additional equilibrium binding assay using the orthosteric agonist, [^{3}H]CCPA, tested against **13b** to confirm that the predominant pharmacological effect of this compound would be an antagonism of agonist binding (in addition to function) at equilibrium, rather than an allosteric potentiation. As shown in Figure 3B, **13b** completely reduced the specific equilibrium binding of the radiolabeled agonist, with an estimated pK_I value of 8.24 ± 0.04 (n = 3). This value is in excellent agreement with the pA_2 values obtained in the pERK1/2 and [^{35}S]GTPγS functional assays, and all three assays are thus consistent with a potent and preferentially orthosteric mode of antagonism for this compound at the A_1AR. Indeed, given that where both antagonism and allosteric stabilization were noted, compounds displayed antagonist activity or were inactive in this functional assay (see pA_2 values in Table 1) than allosteric modulators (EC_{50} values in Table 1), it is likely that orthosteric antagonism is a general property of this novel series of compounds.

Conclusions

A pharmacophore-based screening approach identified ethyl 5-amino-3-(4-*tert*-butylphenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]-pyridazine-1-carboxylate (**8**) as an interesting lead compound in the search for new classes of allosteric modulators of the A_1AR. Accordingly, a series of dihydrothieno[3,4-*d*]pyridazines related to **8** were prepared to further explore the pharmacology of this class of compound. Interestingly, despite the finding that all of the dihydrothieno[3,4-*d*]pyridazines recognized an allosteric binding site on the A_1AR as revealed by agonist radioligand dissociation kinetic assays, the predominant form of pharmacological activity was more consistent with potent orthosteric antagonism. This antagonism was observed in both equilibrium binding and two different functional assays. These findings have a number of important implications. First, we have identified a novel class of potent, small molecule, antagonists/inverse agonists of the A_1AR that can be used as useful tools to further probe receptor function. Second, our results highlight the fact that changes in orthosteric ligand dissociation kinetics by a test compound, although a useful screen of the propensity of a compound to bind allosterically to a receptor-orthosteric ligand complex, cannot guarantee that the predominant pharmacological effect will be allosteric potentiation. Third, given the close structural relationships between compounds that preferentially act either allosterically or orthosterically at the A_1AR, it is tempting to speculate that the allosteric site on the A_1AR is in closer proximity to the orthosteric site than is the case in other Family A GPCRs or, alternatively, that the allosteric interactions thus far identified for the A_1AR actually represent modulation across multiple orthosteric sites on an A_1AR dimer/oligomer complex.

Experimental Section

Computational Chemistry. Molecular modeling studies used Sybyl versions 7.1 and 7.3. Substructure searching was preformed with the Tripos Unity database (Tripos Ltd. St Louis MO, USA).

Chemistry. Melting points were determined on an Electrothermal 9100 capillary melting-point apparatus and are uncorrected. All microwave reactions took place in a Biotage Initiator Microwave synthesizer. All NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer at ^{1}H and ^{13}C NMR spectra were recorded at 300.13 and 75.4 MHz, respectively. Low resolution electrospray mass spectra (MS) using electrospray ionization (ESI) were obtained on a Micromass Platform II. Unless otherwise stated, cone voltage was 20 eV. High resolution electrospray mass spectra (HRMS) utilized electrospray ionization (ESI) and were obtained on a Water LCT Premier mass spectrometer. Thin layer chromatography was conducted on 0.2 mm plates using Merck silica gel 60 F_{254}. Column chromatography was achieved using Merck silica gel 60 (particle size 0.063−0.200 μm, 70−230 mesh).

General Procedure for 2-Phenylhyrazones. HBF_4 (48 wt % solution in H_2O, 11.00 mL) was added to cold aryl aniline (2.00 mL, 2.04 g, 21.93 mmol). After stirring of the sample for 1 h, $NaNO_2$ (2.00 g in 4 mL H_2O) was added dropwise to the reaction mixture, followed by 10 min of stirring at room temperature. The precipitate was collected by filtration, and washed with ethanol (2 × 10 mL) and diethyl ether (30 mL) to afford the aryldiazonium tetrafluoroborate salt. To a solution of ethyl acetoacetate (2.00 mL, 15.70 mmol) in ethanol (20 mL), anhydrous NaOAc (3.92 g, 47.10 mmol) was added. The reaction mixture was stirred at room temperature for 1 h followed by addition of aryldiazonium tetrafluoroborate (2.82 g, 14.70 mmol) in ethanol (10 mL). After 1 h, the reaction mixture was diluted with water and extraction with ether (3 × 20 mL). Organic extracts were combined, dried ($MgSO_4$), and filtered and solvent was removed under reduced pressure to afford the target compound. ^{1}H NMR shows that target compounds are formed as a mixture of *E* and *Z* isomers.

Ethyl 2,3-Dioxobutanoate 2-Phenylhydrazone (11a). Yield 70%. mp 72−73 °C. ^{1}H NMR ($CDCl_3$) δ *(E/Z* mixture) 12.82 (br s, NH), 7.50−7.39, 7.26−7.19 (m, 10H, 2 × ArH), 4.44, 4.41 (q, 4H, J = 7.2 Hz, 2 × CH_2CH$_3$), 2.65, 2.55 (s, 6H, 2 × CH_3), 1.46 (t, 6H, J = 7.2 Hz, 2 × CH$_2$CH_3). MS *m/z* 235.2 (M + H$^+$).

Ethyl 2,3-Dioxobutanoate 2-(3-Chlorophenyl)hydrazone (11b). Yield 85%. mp 83−85 °C.^{1}H NMR ($CDCl_3$) δ *(E/Z* mixture) 12.65 (br s, 2H, 2 × NH), 7.47−7.12 (m, 8H, 2 × ArH), 4.38, 4.36 (q, 4H, J = 7.1 Hz, 2 × CH_2CH$_3$), 2.60, 2.50 (s, 6H, 2 × CH_3), 1.42, 1.40 (t, 6H, J = 7.1 Hz, 2 × CH$_2$CH_3). MS *m/z* 269.2 (M + H$^+$).

Ethyl 2,3-Dioxobutanoate 2-[3-(Trifluoromethyl)phenyl]hydrazone (11c). Yield 95%. mp 79.3−79.4 °C. ^{1}H NMR ($CDCl_3$) δ *(E/Z* mixture) 12.74 (br s, 2H, 2 × NH), 7.68−7.37 (m, 8H, 2 × ArH), 4.39, 4.36 (q, 4H, J = 7.1 Hz, 2 × CH_2CH$_3$), 2.61, 2.51 (s, 6H, 2 × CH_3), 1.42, 1.41 (t, 6H, J = 7.1 Hz, 2 × CH$_2$CH_3). MS *m/z* 303.2 (M + H$^+$).

Ethyl 2,3-Dioxobutanoate 2-(4-Hydroxyphenyl)hydrazone (11d). 4-Aminophenol (2.00 g, 18.35 mmol) was dissolved in an absolute ethanol solution of HCl (1.67 mL conc HCl in 30 mL) at 0 °C. After stirring of the sample for 30 min, isoamyl nitrite (2.70 mL, 20.10 mmol) was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred for 3 h. Ether was added until precipitate developed. The precipitate was collected by filtration under reduced pressure to afford 4-hydroxybenzenediazonium chloride (2.64 g, 16.87 mmol, 92%). 4-Hydroxybenzenediazonium chloride (1.09 g, 6.93 mmol) was added to a solution of ethyl acetoacetate (0.82 mL, 6.40 mmol), NaOAc (1.63 g, 19.63 mmol), in DMSO (4 mL) and water (5 mL). After 1 h, the reaction mixture was diluted with water and extraction with EtOAc (3 × 20 mL). Organic extracts were combined, dried ($MgSO_4$), filtered and solvent removed under reduced pressure to afford the titled compound (1.60 g, 6.40 mmol, 100%). mp 160−163 °C. ^{1}H NMR (DMSO) δ *(E/Z* mixture) 11.83 (br s, 2H, 2 × NH), 7.36−7.28, 6.83−6.78 (m, 8H, 2 × ArH), 4.27, 4.22 (q, 4H, J = 7.1 Hz, 2 × CH_2CH$_3$), 2.46, 2.34 (s, 6H, 2 × *CH$_3$*), 1.29, 1.26 (t, 6H, J = 7.1 Hz, 2 × CH$_2$CH_3). MS *m/z* 251.2 (M + H$^+$).

Ethyl 2,3-Dioxobutanoate 2-(4-Methoxyphenyl)hydrazone (11e). Yield 86%. mp 65−67 °C. ^{1}H NMR ($CDCl_3$) δ *(E/Z* mixture) 13.00 (br s, 2H, 2 × NH), 7.40−7.28, 6.96−6.90 (m, 8H, 2 × ArH), 4.36, 4.32 (q, 4H, J = 7.1 Hz, 2 × CH_2CH$_3$), 3.82, 3.81 (s, 6H, 2 × OCH_3), 2.58, 2.47 (s, 6H, 2 × CH_3), 1.39, 1.38 (t, 6H, J = 7.1 Hz, 2 × CH$_2$CH_3). MS *m/z* 265.2 (M + H$^+$).

Ethyl 2,3-Dioxobutanoate 2-(4-iodophenyl)hydrazone (11f). Yield 88%. mp 101−105 °C. ^{1}H NMR ($CDCl_3$) δ *(E/Z* mixture) 12.71 (br s, 2H, 2 × NH), 7.68−7.10 (m, 8H, 2 × ArH), 4.41−4.30 (m, 4H, 2 × CH_2CH$_3$), 2.59, 2.49 (s, 6H, 2 × CH_3), 1.42−1.37 (m, 6H, 2 × CH$_2$CH_3). MS *m/z* 361.1 (M + H$^+$).

Ethyl 2,3-Dioxobutanoate 2-[4-(Trifluoromethyl)phenyl]hydrazone (11g). Yield 72%. mp 45−48 °C. ^{1}H NMR ($CDCl_3$) δ *(E/Z* mixture) 12.69 (br s, 2H, 2 × NH), 7.70−7.41 (m, 8H, 2 × ArH), 4.40, 4.37 (q, 4H, J = 7.1 Hz, 2 × CH_2CH$_3$), 2.61, 2.52 (s, 6H, 2 × CH_3), 1.42, 1.41 (t, 6H, J =7.1 Hz, 2 × CH$_2$CH_3). MS *m/z* 303.2 (M + H$^+$).

Ethyl 2,3-Dioxobutanoate 2-(4-Nitrophenyl)hydrazone (11h). Yield 98%. mp 127.5−128 °C. ^{1}H NMR ($CDCl_3$) δ *(E/Z mixture)* 12.71 (br s, 2H, 2 × NH), 8.31−7.42 (m, 8H, 2 × ArH), 4.44−4.34 (m, 4H, 2 × CH_2CH$_3$), 2.62, 2.53 (s, 6H, 2 × CH_3), 1.42, 1.41 (t, 6H, J = 7.1 Hz, 2 × CH$_2$CH_3). MS *m/z* 280.2 (M + H$^+$).

General Procedure for 1,6-Dihydropyridazine-3-carboxylates. A mixture of 2-phenylhydrazone (1.47 g, 6.29 mmol), ethyl cyanoacetate (1 mL, 9.44 mmol) and 4-aminobutyric acid (1.30 g, 12.58 mmol) was heated to 160 °C with stirring for 2.5 h and the reaction mixture was allowed to cool to room temperature. With the exception of **12e**, the crude product was purified by column chromatography using a short silica column eluted with hexane/EtOAc (1:1). In the case of **12e**, saturated sodium bicarbonate was added and the resultant mixture was stirred vigorously for 3 h. The solid was collected by filtration, washed with water, air-dried and recrystallized from ethanol.

Ethyl 5-Cyano-4-methyl-6-oxo-1-phenyl-1,6-dihydropyridazine-3-carboxylate (12a). Yield 66%. mp 162−164 °C (Lit. 150−162 °C) ^{1}H NMR ($CDCl_3$) δ 7.64−7.62 (m, 2H, ArH), 7.53−7.44 (m, 3H, ArH), 4.43 (q, 2H, J = 7.1 Hz, CH$_3$CH_2), 2.82 (s, 3H, CH$_3$), 1.46 (t, 3H, J = 7.1 Hz, CH$_3$). MS *m/z* 284.2 (M + H$^+$).

Ethyl 1-(3-Chlorophenyl)-5-cyano-4-methyl-6-oxo-1,6-dihydropyridazine-3-carboxylate (12b). Yield 63%. mp 109−110 °C. ^{1}H NMR ($CDCl_3$) δ 7.67 (br s, 1H, ArH), 7.60−7.56 (m, 1H, ArH), 7.44−7.42 (m, 2H, ArH), 4.44 (q, 2H, J = 7.1 Hz, CH$_2$), 2.76 (s, 3H, CH$_3$), 1.41 (t, 3H, J = 7.1 Hz, CH$_3$). MS *m/z* 318.2 (M + H$^+$).

Ethyl 5-Cyano-4-methyl-6-oxo-1-(3-(trifluoromethyl)phenyl)-1,6-dihydropyridazine-3-carboxylate (12c). Yield 52%. mp 106−110 °C. ^{1}H NMR ($CDCl_3$) δ 7.95 (br s, 1H, ArH), 7.90 (d, 1H, J = 8.0 Hz, ArH), 7.73−7.62 (m, 2H, ArH), 4.44 (q, 2H, J = 7.1 Hz, CH$_2$), 2.77 (s, 3H, CH$_3$), 1.41 (t, 3H, J = 7.1 Hz, CH$_3$). MS *m/z* 352.1 (M + H$^+$).

Ethyl 5-Cyano-1-(4-hydroxyphenyl)-4-methyl-6-oxo-1,6-dihydropyridazine-3-carboxylate (12d). Yield 95%. mp 220−223 °C. ^{1}H NMR ($CDCl_3$), δ 9.95 (br s, 1H, OH), 7.35 (d, 2H, J = 8.8 Hz, ArH), 6.89 (d, 2H, J = 8.8 Hz, ArH), 4.33 (q, 2H, J = 7.1 Hz, CH$_2$), 2.62 (s, 3H, CH$_3$), 1.29 (t, 3H, J = 7.1 Hz, CH$_3$). MS *m/z* 300.2 (M + H$^+$).

Ethyl 5-Cyano-1-(4-methoxyphenyl)-4-methyl-6-oxo-1,6-dihydropyridazine-3-carboxylate (12e). Yield 81%. mp 164.0−164.5 °C (Lit. 153−155 °C). ^{1}H NMR ($CDCl_3$) δ 7.54 (d, 2H, J = 8.9 Hz, ArH), 6.97 (d, 2H, J = 8.9 Hz, ArH), 4.41 (q, 2H, J = 7.1 Hz, CH$_2$), 3.84 (s, 3H, COCH$_3$), 2.73 (s, 3H, CH$_3$), 1.39 (t, 3H, J = 7.1 Hz, CH$_2$CH_3). ^{13}C NMR ($CDCl_3$) δ 162.1, 160.2, 150.3, 137.0, 133.1, 126.3, 115.5, 114.2, 112.6, 62.7, 55.7, 19.2, 14.1. MS *m/z* 314.1 (M + H$^+$).

Ethyl 5-Cyano-1-(4-iodophenyl)-4-methyl-6-oxo-1,6-dihydropyridazine-3-carboxylate (12f). Yield 50%. mp 181−183 °C. ^{1}H NMR ($CDCl_3$) δ 7.83 (m, = 2H, ArH), 7.41 (m, 2H, ArH), 4.42 (q, 2H, J = 7.1 Hz, CH$_2$), 2.75 (s, 3H, CH$_3$), 1.40 (t, 3H, J = 7.1 Hz, CH$_3$). ^{13}C NMR ($CDCl_3$) δ 161.9, 155.6, 150.7, 139.7, 138.2, 137.5, 126.6, 116.0, 112.3, 95.0, 62.8, 19.2, 14.1. MS *m/z* 410.1 (M + H$^+$).

Ethyl 5-cyano-4-methyl-6-oxo-1-(4-(trifluoromethyl)phenyl)-1,6-dihydropyridazine-3-carboxylate (12g). Yield 40%. ^{1}H NMR ($CDCl_3$) δ 7.80 (m, 4H, ArH), 4.44 (q, 2H, $J = 7.1$ Hz, CH_2), 2.77 (s, 3H, CH_3), 1.41 (t, 3H, $J = 7.1$ Hz, CH_3). MS *m/z* 352.2 (M + H^+).

Ethyl 5-cyano-4-methyl-1-(4-nitrophenyl)-6-oxo-1,6-dihydropyridazine-3-carboxylate (12h). Yield 42%. mp 173−175 °C. ^{1}H NMR ($CDCl_3$) δ 8.36 (dt, 2H, $J = 9.1$ Hz, $J = 2.0$, 2.8 Hz, ArH), 7.94 (dt, 2H, $J = 9.1$ Hz, $J = 2.0$, 2.8 Hz, ArH), 4.45 (q, 2H, $J = 7.1$ Hz, CH_3), 2.77 (s, 3H, CH_3), 1.41 (t, 3H, $J = 7.1$ Hz, CH_3). MS *m/z* 329.2 (M + H^+).

General Procedure (A) for Thienopyridazines. To a suspension of 1,6-dihydropyridazine-3-carboxylate (1.69 g, 5.65 mmol) and elemental sulfur (0.28 g, 8.77 mmol) in ethanol (5 mL), morpholine (0.97 mL, 11.12 mmol) was added. The reaction mixture was stirred and heated at reflux temperature (90 °C) for 6 h. The reaction mixture was allowed to cool to room temperature and water was added until a precipitate developed. The precipitate was collected by filtration under reduced pressure, washed with water and the crude product was recrystallized from ethanol to afford the target compound.

General Procedure (B) for Thienopyridazines. To a suspension of 1,6-dihydropyridazine-3-carboxylate (181 mg, 0.58 mmol) and elemental sulfur (27 mg, 0.85 mmol) in ethanol (2 mL), morpholine (0.1 mL, 1.14 mmol) was added. The reaction vessel was sealed and placed in a microwave reactor, prestirred (1 min) and reacted at 150 °C for 10 min. A precipitate developed and the reaction mixture was diluted with water. The precipitate was collected by filtration under reduced pressure, air-dried and recrystallized from ethanol to afford the target compound.

Ethyl 5-Amino-4-oxo-3-phenyl-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (13a). Procedure A. Yield 55%. mp 196−198 °C. ^{1}H NMR (DMSO-d_6) δ 7.61 (br s, 2H, NH_2), 7.52−7.45 (m, 4H, ArH), 7.40−7.38 (m, 1H, ArH), 7.11 (s, 1H, CH), 4.33 (q, 2H, $J = 7.1$ Hz, CH_2), 1.30 (t, 3H, $J = 7.1$ Hz, CH_3). ^{13}C NMR (DMSO-d_6) δ 163.2, 162.4, 158.3, 140.7, 132.8, 128. 5, 127.3, 126.3, 126.0. 103.9, 103.3, 61.3, 14.0. HRMS (ESI^+) calcd for $C_{15}H_{13}N_3NaO_3S^+$ 338.0570, found 338.0562. Anal. ($C_{15}H_{13}N_3O_3S$) C, H, N.

Ethyl 5-amino-3-(3-chlorophenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (13b). Procedure B. Yield 94%. mp 164−166 °C. ^{1}H NMR (DMSO-d_6) δ 7.65 (br s, 3H, NH_2 and ArH), 7.55−7.51 (m, 2H, ArH), 7.48−7.43(m, 1H, ArH), 7.12(s, 1H, CH), 4.34 (q, 2H, $J = 7.1$ Hz, CH_2), 1.31 (t, 3H, $J = 7.1$ Hz, CH_3). ^{13}C NMR (DMSO-d_6) δ 163.4, 162.3, 158.1, 141.9, 133.3, 132.5, 130.1, 127.2, 126.0, 125.9, 124.7, 103.8, 103.7, 61.4, 14.0. HRMS (ESI^+) calcd for $C_{15}H_{12}ClN_3NaO_3S^+$ 372.0180, found 372.0173. Anal. ($C_{15}H_{12}ClN_3O_3S$) C, H, N.

Ethyl 5-Amino-4-oxo-3-(3-(trifluoromethyl)phenyl)-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (13c). Procedure B. Yield 100%. mp 176−178 °C. ^{1}H NMR (DMSO-d_6) δ 7.96 (br s, 1H, ArH), 7.88−7.86 (m, 1H, ArH), 7.75−7.73 (m, 2H, ArH), 7.68 (br s, 2H, NH_2), 7.13 (br s, 1H, CH), 4.34 (q, 2H, $J = 7.1$ Hz, CH_2), 1.31 (t, 3H, $J = 7.1$ Hz, CH_3). ^{13}C NMR (DMSO-d_6) δ 162.9, 162.0, 159.4, 141.2, 134.4, 131.6 (q, $J = 32.8$ Hz), 129.3, 129.1, 124.2 (q, $J = 3.7$ Hz), 123.9 (q, $J = 272$ Hz), 122.9 (q, $J = 3.9$ Hz), 106.6, 105.3, 62.2, 14.3. HRMS (ESI^+) calcd for $C_{16}H_{12}F_3N_3NaO_3S^+$ 406.0444, found 406.0453. Anal. ($C_{16}H_{12}F_3N_3O_3S$) C, H, N.

Ethyl 5-Amino-3-(4-hydroxyphenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (13d). Procedure A. Yield 80%. mp 253−256 °C. ^{1}H NMR (DMSO-d_6) δ 9.64 (s, 1H, OH), 7.56 (br s, 2H, NH_2), 7.25 (d, 2H, $J = 8.7$ Hz, ArH), 7.07 (s, 1H, CH), 6.83 (d, 2H, J =8.7 Hz, ArH), 4.31 (q, 2H, $J = 7.1$ Hz, CH_2), 1.29 (t, 3H, $J = 7.1$ Hz, CH_3). HRMS (ESI^+) calcd for $C_{15}H_{13}N_3NaO_4S^+$ 354.0519, found 354.0526. Anal. ($C_{15}H_{13}N_3O_4S$) C, H, N.

Ethyl 5-Amino-3-(4-methoxyphenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (13e). Procedure A. Yield 81%. mp 173−175 °C. ^{1}H NMR (DMSO-d_6), δ 7.58 (br s, 2H, NH_2), 7.41 (d, 2H, $J = 8.6$ Hz, ArH), 7.09 (s, 1H, CH), 7.01 (d, 2H, $J = 8.6$ Hz, ArH), 4.31 (q, 2H, $J = 7.0$ Hz, CH_2), 3.80 (s, 3H, $COCH_3$), 1.29 (t, 3H, $J = 7.0$ Hz, CH_3). ^{13}C NMR (DMSO-d_6), δ 163.0, 162.4, 158.4, 158.3, 133.7, 132.4, 127.5, 126.2, 113.6, 104.1, 103.0, 61.20, 55.3, 14.0. HRMS (ESI^+) calcd for $C_{16}H_{16}N_3O_4S^+$ 346.0856, found 346.0877. Anal. ($C_{16}H_{15}N_3O_4S$) C, H, N.

Ethyl 5-amino-3-(4-iodophenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (13f). Procedure A. Yield 100%. mp 196−198 °C. ^{1}H NMR (DMSO-d_6) δ 7.83 (d, 2H, $J = 8.6$ Hz, ArH), 7.63 (br s, 2H, NH_2), 7.35 (d, 2H, $J = 8.6$ Hz, ArH), 7.11 (s, 1H, CH), 4.33 (q, 2H, $J = 7.1$ Hz, CH_2), 1.30 (t, 3H, $J = 7.1$ Hz, CH_3). ^{13}C NMR (DMSO-d_6), δ 163.3, 162.3, 160.3, 158.1, 140.5, 137.2, 133.1, 128.1, 125.9, 103.7, 92.7, 61.3, 14.0. HRMS (ESI^+) calcd for $C_{15}H_{12}IN_3NaO_3S^+$ 463.9536, found 463.9529. Anal. ($C_{15}H_{12}IN_3O_3S$) C, H, N.

Ethyl 5-Amino-4-oxo-3-(4-(trifluoromethyl)phenyl)-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (13g). Procedure A. Yield 99%. mp 180−184 °C. ^{1}H NMR (DMSO-d_6) δ 7.82 (br s, 4H, ArH), 7.68 (br s, 2H, NH_2), 7.13 (br s, 1H, CH), 4.33 (q, 2H, $J = 6.8$, 6.9 Hz, CH_2), 1.31 (t, 3H, $J = 6.8$, 6.9 Hz, CH_3). ^{13}C NMR (DMSO-d_6) δ 163.6, 162.2, 158.1, 144.0, 133.6, 127.2 (q, $J = 32.1$ Hz), 125.7, 125.5 (q, $J = 3.8$ Hz), 124.0 (q, $J = 272.2$ Hz), 104.0, 103.7, 61.4, 14.0. HRMS (ESI^+) calcd for $C_{16}H_{12}F_3N_3NaO_3S^+$ 406.0444, found 406.0452. Anal. ($C_{16}H_{12}F_3O_3S$) C, H, N.

Ethyl 5-Amino-3-(4-nitrophenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (13h). Procedure A. Yield 87%. mp 259−261 °C. ^{1}H NMR (DMSO-d_6) δ 8.33 (dt, 2H, $J = 2.1$, 2.9, 9.2 Hz, ArH), 7.90 (dt, 2H, $J = 2.1$, 2.9, 9.2 Hz, ArH), 7.75 (br s, 2H, NH_2), 7.15 (s, 1H, CH), 4.35 (q, 2H, $J = 7.1$ Hz, CH_2), 1.32 (t, 3H, $J = 7.1$ Hz, CH_3). ^{13}C NMR (DMSO-d_6), δ 164.0, 162.1, 158.0, 145.9, 145.2, 134.0, 125.9, 125.5, 123.8, 104.6, 103.3, 61.5, 14.0. MS *m/z* 361.0 (M + H+). Anal. ($C_{15}H_{12}N_4O_5S$) C, H, N.

Ethyl 3-(4-Acetoxyphenyl)-5-amino-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (13i). To a suspension of **13d** (100 mg, 0.30 mmol), triethylamine (56 μL, 0.40 mmol) and 4-dimethylaminopyridine (26 mg, 0.22 mmol) in CH_2Cl_2 (10 mL), acetic anhydride (38 μL, 0.41 mmol) was added. The reaction mixture was stirred at room temperature for 5.5 h. After this period TLC (hexane/EtOAc, 4:6, $R_f = 0.44$) showed complete conversion. The reaction mixture was diluted with H_2O (20 mL) and extracted with EtOAc (2 × 20 mL). The organic extracts were combined, washed with 1 M HCl (10 mL), saturated $NaHCO_3$ (20 mL) and H_2O (20 mL). The organic layer was dried ($MgSO_4$), filtered and evaporated to afford the product as a yellow solid (0.10 g, 0.25 mmol, 84%). ^{1}H NMR (DMSO-d_6) δ 7.61 (br s, 2H, NH_2), 7.54 (d, 2H, $J = 8.8$ Hz, ArH), 7.23 (d, 2H, $J = 8.8$ Hz, ArH), 7.12 (s, 1H, CH), 4.33 (q, 2H, $J = 7.1$ Hz, CH_2), 2.30 (s, 3H, $OCOCH_3$), 1.30 (t, 3H, $J = 7.1$ Hz, CH_3). ^{13}C NMR (DMSO-d_6) δ 169.1, 163.2, 162.3, 158.2, 149.2, 138.1, 132.9, 127.3, 126.0, 121.8, 103.8, 103.5, 61.3, 26.8, 14.0. MS *m/z* 374.2 (M + H^+). Anal. ($C_{17}H_{15}N_3O_5S$) C, H, N.

Ethyl 5-amino-4-oxo-3-(4-(trifluoromethylsulfonyloxy)phenyl)-3,4-dihydro-thieno[3,4-d]pyridazine-1-carboxylate (13j). A solution of **13d** (205 mg, 0.62 mmol), *N*-phenyltriflimide (0.22 g, 0.62 mmol) and K_2CO_3 (251 mg, 1.82 mmol) in THF (1.5 mL) was placed in a sealed vessel and into microwave reactor. The reaction was prestirred (1 min) and then heated at 120 °C for 6 min, followed by rapid cooling. The reaction mixture was diluted with water and extracted with ethyl acetate. The organic extracts were combined, dried ($MgSO_4$), filtered and evaporated to afford the title compound (28 mg, 100%). mp 153−155 °C. ^{1}H NMR (DMSO-d_6) δ 7.75 (dt, 2H, $J = 2.2$, 3.2, 9.1 Hz, ArH), 7.66−7.61 (m, 4H, ArH and NH_2), 7.14 (s, 1H, CH), 4.34 (q, 2H, $J = 7.1$ Hz, CH_2), 1.31 (t, 3H, $J = 7.1$ Hz, CH_3). ^{13}C NMR (DMSO-d_6) δ 162.9, 162.1, 159.3, 148.1, 140.5, 134.4, 127.5, 126.9, 121.6, 118.9 (q, $J = 321$ Hz), 106.5, 105.3, 62.2, 14.3. HRMS (ESI^+) calcd for $C_{16}H_{12}F_3N_3NaO_6S_2^+$ 486.0012, found 486.0017. Anal. ($C_{16}H_{12}F_3N_3O_6S_2$) C, H, N.

General Procedure for Thienopyridazine Carboxylic Acids. To a solution of the ethyl thieno[3,4-*d*]pyridazine-1-carboxylate (0.38 mmol) in ethanol, was added NaOH (16 mg, 0.40 mmol). The reaction mixture was stirred and heated at reflux for 3 h. The reaction mixture was allowed to cool to room temperature, diluted with H_2O followed by the addition of concentrated HCl until a precipitate developed (pH 3). The precipitate was collected by

filtration under reduced pressure and washed with H_2O, followed by freeze-drying overnight to afford the target compound.

5-Amino-3,4-dihydro-4-oxo-3-phenyl-thieno[3,4-d]pyridazine-1-carboxylic acid (14a). Yield 78%. mp 230−232 °C. ^{1}H NMR (DMSO-d_6) δ 12.24 (s, 1H, OH), 8.01 (s, 1H, NH_2), 7.70−7.37 (m, 5H, ArH), 7.11 (s, 1H, CH). ^{13}C NMR (DMSO-d_6) δ 164.5, 163.6, 141.2, 134.0, 128.9, 128.7, 127.7, 126.7, 125.9, 104.5, 104.0. MS m/z 310.1 (M + H+). Anal. ($C_{13}H_9N_3O_3S$) C, H, N.

5-Amino-3,4-dihydro-3-(4-methoxyphenyl)-4-oxo-thieno[3,4-d]pyridazine-1-carboxylic acid (14e). Yield 31%. mp 182.4−182.6 °C. ^{1}H NMR (DMSO-d_6) δ 7.41 (d, 2H, J = 9.0 Hz, ArH), 7.10 (s, 1H, CH), 7.00 (d, 2H, J = 9.0 Hz, ArH), 3.80 (s, 3H, $COCH_3$). ^{13}C NMR (DMSO-d_6), δ 164.0, 162.9, 158.5, 158.2, 133.7, 133.2, 127.5, 126.4, 113.6, 104.2, 103.2, 55.4. MS m/z 318.2 ($M + H^+$). Anal. ($C_{14}H_{11}N_3O_3S$) C, H, N.

Pharmacology. Dissociation Kinetic Assays.[6,7,24] Determination of allosteric modulation of orthosteric radioligand dissociation kinetics consisted of three phases: (1) binding to equilibrium of the agonist, [^{125}I]-ABA, to the A_1AR-G protein ternary complex; (2) stabilization of that complex by adding vehicle or increasing concentrations of allosteric modulator for 30 min, and (3) dissociation of the complex by adding a combination of an A_1AR orthosteric antagonist, 100 μM BW-1433, and 25 μM GTPγS for 10 min. A score was determined at each point between 0% (no different than vehicle) and 100% (complete prevention of [^{125}I]-ABA dissociation). The data was fitted to a variable slope sigmoidal curve and the max and Ec_{50} values extrapolated form the curve. The assay employed membranes from CHO-K1 cells stably expressing the human A_1AR. For agonist binding to equilibrium (phase 1) the buffer consisted of 10 mM HEPES, pH 7.2, containing 0.5 mM $MgCl_2$, 1 U/mL adenosine deaminase, 0.5 nM [^{125}I]-ABA and 10 μg of membrane protein in a final volume of 100 μL applied to 96 well Millipore GF/C glass fiber filter plates. After 90 min at room temperature the addition of 50 μL of test modulator (0.1−50 μM, final) initiated stabilization of the ternary complex (phase 2). After 30 min, a solution containing BW-1433 and GTPγS (50 μL) was added to initiate the dissociation of the ternary complex. Ten minutes later membranes were filtered, washed, dried, and counted for residual [^{125}I]-ABA. The percentage of specifically bound agonist remaining after 10 min of dissociation served as an index of modulator activity, and was calculated as follows:

$$\text{K-score} = 100 \times (B - B_o)/(B_{eq} - B_o)$$

where B = residual binding (cpm) bound at the end of 10 min of dissociation in the presence of a modulator, B_o = residual binding (cpm) at the end of 10 min of dissociation in the absence of a modulator, and B_{eq} = cpm bound at the end of 90 min of equilibrium binding. The percentage of specific binding remaining after 10 min of dissociation constitutes an index of modulator activity for ranking candidate compounds. A score of 100% means no dissociation and a score of zero means complete dissociation. Note, we have previously referred to the K-score as the AE score, but this implies that a retardation of dissociation kinetics will always result in allosteric enhancement at equilibrium, which is not always the case.

A_1AR-Mediated ERK 1/2 Phosphorylation. Chinese hamster ovary (CHO) FlpIn cells, stably transfected with the human A_1AR (FlpIn-CHO A_1 cells), were grown to 90% confluence and maintained in Dulbecco's modified eagle medium (DMEM) containing 20 mM HEPES, 5% fetal bovine serum (FBS) and 200 μG/mL of hygromycin at 37 °C in a humidified incubator containing 5% CO_2:95% O_2. Cells were then harvested by trypsinization followed by centrifugation (300 g, 5 min). Cells were then seeded into 96-well plates at a density of 50 000 cells/well. After 4 h, the cells were washed twice with phosphate-buffered saline (PBS) and then maintained in DMEM containing 20 mM HEPES for at least 4 h. Prior to agonist stimulation, cells were pretreated for 30 min with 1 u/mL adenosine deaminase (ADA) and then for 30 min with test compound at 37 °C. R-PIA was then added and stimulation allowed to proceed for 5 min before the reaction was terminated by the removal of media and the addition of 100 μL of *SureFire* lysis buffer to each well. The plate was then agitated for 1−2 min. A 4:1 v/v dilution of lysate:*SureFire* activation buffer was made in a total volume of 50 μL. A 1:100:120 v/v dilution of AlphaScreen beads:activated lysate mixture:*SureFire* reaction buffer in a 11 μL total volume was then transferred to a white opaque 384-well Proxiplate in diminished light. This plate was then incubated in the dark at 37 °C for 1.5 h after which time the fluorescence signal was measured by a Fusion-α plate reader (PerkinElmer), using standard AlphaScreen settings. All data was expressed as a percentage of the ERK1/2 phosphorylation mediated after a 6 min exposure to DMEM containing 3% FBS.

A_1AR-Mediated [^{35}S]GTPγS Binding. Membrane homogenates (15 μg) were equilibrated in a 450 μL total volume of [^{35}S]GTPγS assay buffer (10 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$; pH 7.4 at 30 °C) containing 10 μM GDP and a range of concentrations of R-PIA (0.01 nM − 1 μM) in the absence or presence of increasing concentrations of **13b** at 30 °C for 60 min. After this time, 50 μL of [^{35}S]GTPγS (100 pM) was added and incubation continued for another 30 min at 30 °C. Incubation was terminated by rapid filtration through Whatman GF/B filters using a Brandell cell harvester (Gaithersburg, MD). Filters were washed three times with 3 mL aliquots of ice-cold 0.9% NaCl buffer and dried before the addition of 4 mL of scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT). Vials were then left to stand until the filters became uniformly translucent before radioactivity was determined using scintillation counting.

Radioligand Equilibrium Binding Assays. For [^{3}H]CPX binding assays, CHO-K1 membranes expressing human adenosine A_1 receptors were resuspended at 400 μg/mL in HE buffer containing 1 U/mL adenosine deaminase. A membrane solution (50 μL) was added to 50 μL of HE buffer containing [^{3}H]CPX (2 nM) and then 100 μL of HE buffer with either vehicle, modulator or NECA (to define nonspecific binding) was added. The final drug concentrations were 50 μM for modulator and 100 μM for NECA. Samples were incubated for 90 min at room temperature, filtered, and counted on a liquid scintillation counter. Binding was performed in triplicate and expressed as % inhibition as compared to control binding.

For [^{3}H]CCPA binding assays, membrane homogenates (15 μg) were incubated in a 500 μL total volume of assay buffer containing [^{3}H]CCPA (2 nM) and a range of concentrations of R-PIA or **13b** at 37 °C for 60 min. Nonspecific binding was defined using 10 μM R-PIA. Incubation was terminated by rapid filtration through Whatman GF/B filters using a Brandell cell harvester (Gaithersburg, MD). Filters were washed three times with 3 mL aliquots of ice-cold 0.9% NaCl buffer and dried before the addition of 4 mL of scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT). Vials were then left to stand until the filters became uniformly translucent before radioactivity was determined in DPM using scintillation counting.

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Supporting Information Available: Elemental analysis details for compounds **13a**−**j** and **14a**,**e** and K-scores for the screened compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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