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3- and 6-Substituted 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines as A_1 adenosine receptor allosteric modulators and antagonists

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ABSTRACT

A series of 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines were prepared and evaluated as potential allosteric modulators at the A_1 adenosine receptor. The structure–activity relationships of the 3- and 6-positions of a series of 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines were explored. Despite finding that 3- and 6-substituted 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines possess the ability to recognize an allosteric site on the agonist-occupied A_1AR at relatively high concentrations, the structural modifications we have performed on this scaffold favor the expression of orthosteric antagonist properties over allosteric properties. This research has identified 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines as novel class of orthosteric antagonist of the A_1AR and highlighted the close relationship between structural elements governing allosteric modulation and orthosteric antagonism of agonist function at the A_1AR.

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1. Introduction

The initial studies of Bruns et al.[1,2] on the allosteric modulation of the A_1 adenosine receptor (A_1AR), described four compounds of interest, including the prototypical modulator PD 81,723[1] (Fig. 1). Improving the properties of such leads has received much subsequent attention and, in particular, analogues of the related PD 71,605 led to the discovery of two allosteric modulators, LUF 5484 and T-62 with improved potency over PD 81,723.[3–5] Despite selectively potentiating agonist action at the A_1AR via interaction with an allosteric site, however, a characteristic feature of all these modulators is a propensity to also cause antagonism at higher concentrations,[3] which is most commonly interpreted as a lower affinity interaction of the modulator with the A_1AR's orthosteric site.[1–3] Thus, there is an ongoing need to gain a better understanding of the structure–activity relationships that govern the allosteric effects of these compounds, on the one hand, and the possible orthosteric effects, on the other. There have also been additional studies with respect to the leads PD 117,975 and PD 78,416, in which the main focus has been variation of the benzoyl substituent in the 3-position.[4,6–8] Testing of analogs of the two lead compounds PD 117,975 and PD 78,416 conducted by Bruns et al. revealed that the same changes in the 3-position provided comparative enhancement of agonist action, yet, a marked difference was noted if the 6-position was N-methylated, making these compounds virtually inactive.[2] Bruns et al.'s reasoning for this was that

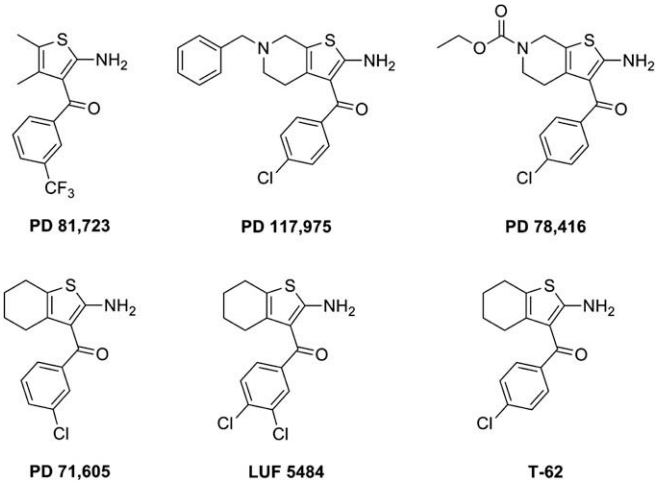

Figure 1. The PD series, discovered as allosteric enhancers of the A_1AR in the study conducted by Bruns et al., and the second generation allosteric enhancers, LUF 5484 and T-62.

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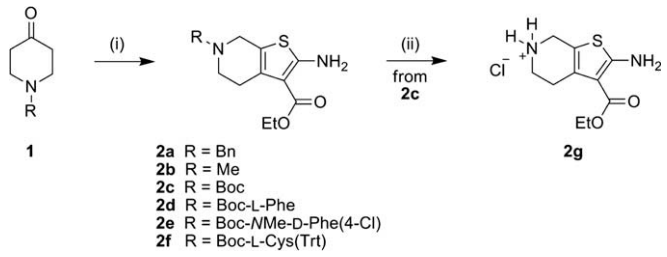
the *N*-methyl derivative is more basic than the *N*-benzyl and therefore protonation causes deleterious effects; this is why the *N*-ethoxycarbonyl series are potent enhancers, an observation confirmed by Baraldi et al.[6]

In a recent study conducted in our group, it was shown that amides in the 3-position of 2-aminothiophenes analogous to PD 71,605 supported allosteric enhancement at the A_1AR.[9] This was the first report in which a detailed SAR study of various carboxamides in the 3-position was performed for the allosteric enhancers at the A_1AR. In the current study, we report on synthesis and biological evaluation of analogues of PD 117,975 and PD 78,416, in which we focus on varying the substituent in the 3- and 6-positions of 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines.

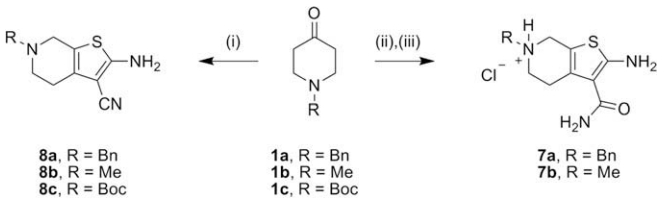
2. Results and discussion

The series of compounds listed in Table 1 were synthesised by the methods depicted in Schemes 1–3. Target 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines with different 6-substitutents were prepared by the Gewald synthesis.[10] Ethyl cyanoacetate was reacted with the commercially available N-substituted piperidones **1a–c** and elemental sulfur providing 2-aminothiophenes 2a–**c** (Scheme 1). In the case of **2d–f**, the appropriate N-substituted piperidone was first prepared from piperidone via a BOP or TBTU mediated coupled with an *N*-Boc protected amino acid. In the subsequent Gewald reaction, thiophenes **2d** and **2e** precipitated during the course of the reaction whereas **2f** was isolated following an aqueous work-up. The 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine analog with no substituent in the 6-position (**2g**), was prepared by treating **2c** with 6 M HCl to cleave the Boc group.

Compounds **2a–c** were subjected to sodium hydroxide mediated hydrolysis providing the amino acids **3a–c** which were initially used as intermediates in the synthesis of the amides **6a–i**. Attempted recrystallisation of **3a** and **3b** caused degradation and



Scheme 1. Reagents: (i) S_8, $EtOCOCH_2CN$, morpholine, EtOH; (ii) 6 M HCl, dioxane.

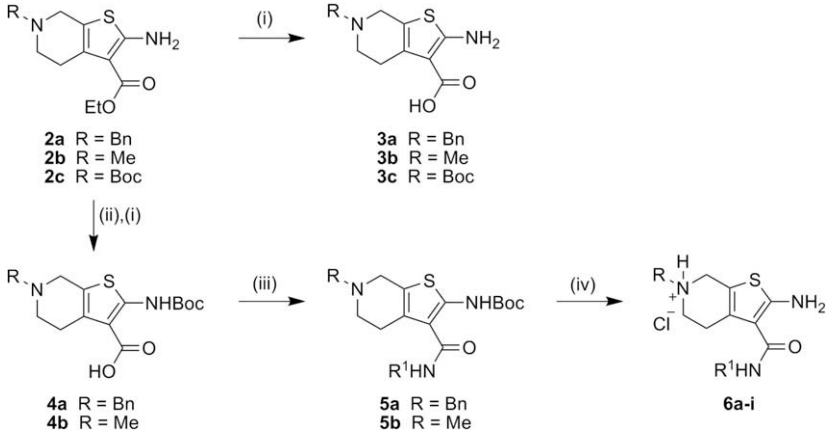


Scheme 3. Reagents: (i) R = Bn, Me, Boc, EtOH, morpholine, S_8 $NCCH_2CN$; (ii) R = Bn, Me, EtOH, morpholine, S_8, H_2NCOCH_2CN, then 2 M ethanolic HCl.

therefore these intermediates were used in their crude form. Amidations of amino acids **3a** and **3b** provided the amides **6**, yet varying degrees of by-products and diminished yields were apparent and it was more expedient to Boc-protect the 2-amino group. Saponification of the ethyl ester proceeded smoothly, provided the acids **4a** and **4b**, which were far more stable than the amino acids **3a** and **3b**. Amidation with the common peptide coupling reagents, BOP or TBTU, afforded good yields of amides **5** that were chromatographed on silica gel. The purified carbamates were treated with excess 6 M HCl to remove the Boc group providing the amides **6a–i** as hydrochloride salts.

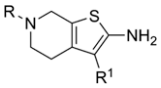
The synthesis of 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamides **7a** and **7b** (Scheme 3) could be achieved by directly amidating the corresponding 3-carboxylic acids. However, a more direct approach was pursued by performing the Gewald synthesis with 2-cyanoacetamide. The isolated 3-carboxamides were converted to their hydrochloride salts which greatly improved their solubility in water. The corresponding 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carbonitriles **8a–c** were also prepared directly via a Gewald reaction between malononitrile and the appropriate N-substituted 4-piperidone.

The potential of the compounds listed in Table 1 to interact allosterically with the A_1AR was initially screened by evaluating their ability to stabilize an orthosteric agonist–A_1AR–G protein ternary complex in an in vitro dissociation kinetic binding assay, using ^{125}I-ABA as the orthosteric radioligand and promoting dissociation by addition of 25 μM GTPγS and the orthosteric antagonist, 100 μM 8-CPT. The resulting K-score ('kinetic score') reported in Table 1 denotes the percentage of the ternary complex remaining after 10 min of radioligand dissociation, and is a measure of the strength of the allosteric interaction between a given concentration of test ligand and the pre-equilibrated ^{125}I-ABA-A_1 receptor complex; the higher this K-score percentage, the greater the ability of the test ligand to allosterically stabilize the binding of the orthosteric radioligand.[11] Because the dissociation kinetic assays were performed on a receptor that had been pre-equilibrated with orthosteric radioligand, any effect of the test ligand on the dissoci-



Scheme 2. Reagents: (i) EtOH, H_2O, NaOH; (ii) Boc_2O, DMAP, dioxane; (iii) BOP, DMF, DIPEA amine; (iv) 6 M HCl, EtOH.

Table 1
AE activity of 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines



No.	R	R^1	K-score[a] (%)	EC_{50}[b] (μM)
PD 81,723	—	—	28.0 ± 1.1	13.6 ± 2.1
PD117,915	Bn	C(O)4-ClPh	32.6 ± 3.8	>20
2a	Bn	C(O)OEt	44.9 ± 3.4	10.3 ± 0.10
2b	Me	C(O)OEt	14.9 ± 7.1	>20
2c	Boc	C(O)OEt	32.8 ± 3.5	13.2 ± 4.4
2d	Boc-L-Phe	C(O)OEt	82.5 ± 12.4	7.2 ± 3.9
2e	Boc-*N*Me-D-Phe(4-Cl)	C(O)OEt	92.9 ± 2.0	9.1 ± 0.6
2f	Boc-L-Cys(Trt)	C(O)OEt	93.2 ± 0.4	7.9 ± 5.5
2g	H	C(O)OEt	58.5 ± 1.8	11.7 ± 1.1
3a	Bn	C(O)OH	68.3 ± 4.7	8.0 ± 2.9
3b	Me	C(O)OH	77.1 ± 9.9	11.6 ± 6.1
3c	Boc	C(O)OH	66.1 ± 1.8	12.1 ± 6.6
6a	Bn	C(O)NHBn	37.6 ± 7.3	13.1 ± 5.8
6b	Bn	C(O)NH(3-ClBn)	36.0 ± 4.1	>20
6c	Bn	C(O)NH(3-CF_3Bn)	46.9 ± 3.3	15.5 ± 1.1
6d	Bn	$C(O)NHNH_2$	82.9 ± 13.0	8.9 ± 3.9
6e	Bn	C(O)NHNHPh	55.4 ± 11.1	7.8 ± 3.4
6f	Me	C(O)NHBn	87.2 ± 7.7	12.0 ± 6.3
6g	Me	C(O)NH(3-ClBn)	88.0 ± 6.9	13.2 ± 4.5
6h	Me	$C(O)NHNH_2$	85.7 ± 9.2	4.4 ± 3.4
6i	Me	C(O)NHNHPh	83.1 ± 11.9	11.5 ± 2.8
7a	Bn	$CONH_2$	19.1 ± 11.3	>20
7b	Me	$CONH_2$	19.8 ± 6.6	>20
8a	Bn	CN	52.0 ± 0.15	15.2 ± 6.0
8b	Me	CN	14.8 ± 0.70	>20
8c	Boc	CN	22.9 ± 7.3	>20

[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 pre-treatment with the candidate AE (50 μM).
[b] Concentration of modulator producing half-maximal allosteric effect on [^{125}I]-ABA dissociation.

ation of the radioligand must arise from a purely allosteric mechanism because the orthosteric site is already occupied by the ^{125}I-ABA. Thus, this kinetic assay is biased towards detecting purely allosteric effects; it should be noted, however, that this biased assay design cannot differentiate modulators that solely recognize the allosteric site from those that can bind to both the orthosteric and allosteric sites. The determination of a K-score for multiple concentrations of test ligand, up to a concentration of 50 μM, also allowed for the determination of the potency (EC_{50}) range for many of the compounds to mediate their half-maximal allosteric effects on radioligand dissociation; these latter values provide a semi-quantitative estimate of the apparent affinity of the modulators for the allosteric site on the agonist-occupied receptor. Where incomplete curves were attained due to low potency, the EC_{50} value is reported as >20 μM and the K-score reported as the percent change in radioligand dissociation at 50 μM of modulator (Table 1).

From the results obtained in the dissociation kinetic assays and the resultant K-scores shown in Table 1, it was evident that the greatest degree of allosteric stabilization of agonist–receptor complexes was achieved by compounds that contained an ethyl ester in the 3-position together with *N*-Boc amino acid substitution in the 6-position (e.g., compounds **2d**–**2f**), or a methyl substitution of the 6-position together with an amide or hydrazide substitution in the 3-position (e.g., compounds **6f**–**6i**). The potency of these compounds to retard radioligand dissociation was similar to, or slightly better than, that displayed by PD 81,723.

Subsequently, we determined the functional effects of the novel compounds using an AlphaScreen plate-based assay of A_1AR-mediated phosphorylation of ERK1/2 (pERK1/2) in intact CHO cells.[12] For each compound, two concentrations (3 μM and 10 μM) were tested alone and against an EC_{50} concentration of the orthosteric agonist, R-PIA. Under these conditions an increase in functional response represents either an allosteric enhancement of R-PIA function or intrinsic agonism of the test compound (or both). In contrast, a reduction in R-PIA response indicates an antagonistic effect of the compounds.

With the exception of PD81,723, which displayed some allosteric agonism in the absence of R-PIA (yielding approx. 40% of the maximum R-PIA response at 10 μM), none of the test compounds (including PD117,915) displayed any significant effects on basal ERK1/2 signaling in the absence of agonist (not shown). Interestingly, when tested against a concentration of R-PIA that yields approximately 50% of the maximal response, it was also noted that none of the compounds, other than PD81,723 and PD 117,975, caused any appreciable potentiation of agonist responsiveness (Fig. 2). Instead, we observed either no appreciable effect on agonist function for the two concentrations of test compound utilized or an antagonistic effect on R-PIA-mediated ERK1/2 phosphorylation, which was particularly striking for compounds **2c**, **2d** and **2e** (Fig. 3). To confirm this finding, therefore, we constructed complete R-PIA concentration–response curves in the absence or presence of increasing concentrations of each of these three test compounds to ascertain and quantify the nature of their interaction with the agonist at the A_1AR (Fig. 2). Analysis of these data according to a simple model of antagonism[13] yielded the following pA_2 values: **2c**: 6.08 ± 05; **2d**: 6.43 ± 0.09; **2e**: 5.74 ± 0.07; ($n = 3$), which are empirical measures of antagonist potency.[14] These pA_2 values indicate that the compounds are actually more potent as orthosteric antagonists of R-PIA function than allosteric modulators of agonist dissociation, that is, their antagonistic effects are expressed at lower concentrations than any potential allosteric effects.

3. Conclusions

Despite finding that 3- and 6-substituted 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines possess the ability to recognize an allosteric site on the agonist-occupied A_1AR at relatively high concentrations, we conclude that the structural modifications we have performed on the 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine scaffold actually favor the expression of orthosteric antagonist properties over allosteric properties. This finding is similar to that made in a recent study by our group on 2-aminothienopyridazines.[12] Thus, in addition to identifying 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines as novel class of orthosteric antagonist of the A_1AR, our results indicate that the close relationship between structural elements governing allosteric modulation and orthosteric antagonism of agonist function at the A_1AR may imply that A_1AR allosteric site on this GPCR is in very close proximity to the orthosteric site.

4. Experimental

Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. All ^{1}H NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer at 300.13 MHz. All ^{13}C NMR spectra were recorded on a Varian Unity Inova 600 spectrometer at 150.8 MHz, unless stated otherwise or on a Bruker Avance DPX 300 spectrometer at 75.4 MHz. Unless stated otherwise, samples were dissolved in $CDCl_3$. High resolution mass spectra were obtained on a Waters LCT Premier XE (TOF) mass spectrometer fitted with an ESI ion source. Thin-layer

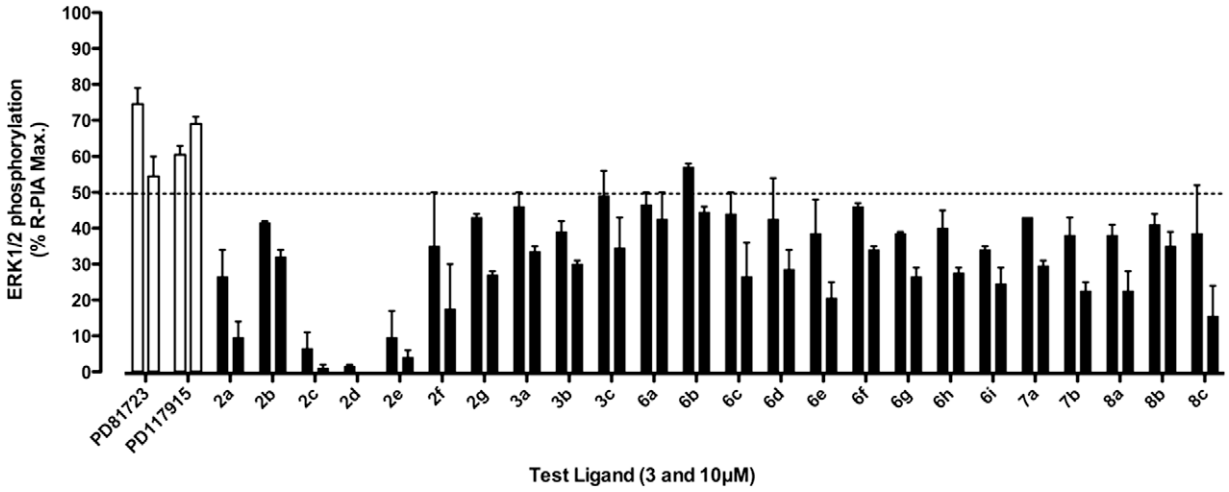


Figure 2. Effect of two different concentrations (3 μM, left bar; 10 μM, right bar) of test ligands on A_1AR-mediated stimulation of ERK1/2 phosphorylation in intact CHO FlpIn cells, in the presence of an EC_{50} concentration of R-PIA (determined on the same day as each assay); dashed line denotes 50% response level. The effects of PD81,723 and PD117,915 are also shown for comparison. Data represent the mean ± standard deviation of two experiments conducted in triplicate.

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).

4.1. Ethyl 2-amino-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (2a)

N-Benzyl-4-piperidone **1a** (5 g, 26.42 mmol), ethyl cyanoacetate (3.09 mL, 29.06 mmol) and sulfur (1.02 g, 31.70 mmol) were suspended in EtOH (55 mL). Morpholine (4.62 mL, 52.84 mmol) was added and the mixture was refluxed gently with stirring for 2 h. The cooled solution was diluted with water and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were washed with water, then brine, dried ($MgSO_4$), filtered and concentrated. The resultant residue was triturated with hot MeOH (20 mL) and washed with ice cold MeOH to afford the desired product as an off white powder (7.1 g, 85% yield).

Mp 110–111 °C. ^{1}H NMR δ 7.41–7.26 (m, 5H, aromatic), 6.09 (br s, 2H, NH_2), 4.26 (q, J = 7.2 Hz, 2H, CH_2CH_3), 3.69 (s, 2H, NCH_2Ph), 3.42 (s, 2H, 7-CH_2), 2.89–2.71 (m, 4H, 5-CH_2 and 4-CH_2), 1.33 (t, J = 7.2 Hz, 3H, CH_2CH_3). ^{13}C NMR (75.4 MHz) δ 166.0, 162.3, 138.2, 131.0, 129.2, 128.35, 127.2, 114.7, 105.2, 62.0, 59.5, 51.3, 50.3, 27.3, 14.5. HR-ESMS calcd for $C_{17}H_{21}N_2O_2S^+$ (M+1) 317.1318, found 317.1292.

4.2. Ethyl 2-amino-6-methyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (2b)

N-Methyl-4-piperidone (1b) (10 mL, 86.60 mmol), ethyl cyanoacetate (9.22 mL, 86.60 mmol) and sulfur (3.05 g, 95.26 mmol) were suspended in EtOH (150 mL). Morpholine (15.15 mL, 173.21 mmol) was added and the mixture was refluxed gently with stirring for 2 h. The cooled solution was diluted with water (250 mL). The resultant yellow precipitate was collected by suction filtration and washed with water until the filtrate was colorless (13.4 g of yellow powder, 64% yield). A small portion was recrystallised from EtOH providing an off white powder.

Mp 101–103 °C. ^{1}H NMR δ 6.03 (br s, 2H, NH_2), 4.27 (q, J = 7.2 Hz, 2H, CH_2CH_3), 3.39 (s, 2H, 7-CH_2), 2.85 (t, 2H, J = 5.7 Hz, 2H, 5-CH_2), 2.68 (t, 2H, J = 5.8 Hz, 2H, 4-CH_2), 2.45 (s, 3H, NCH_3), 1.34 (t, J = 7.2 Hz, 3H, CH_2CH_3). ^{13}C NMR (75.4 MHz) δ 166.0, 162.5, 130.5, 114.1, 104.9, 59.4, 53.2, 52.3, 45.3, 27.2, 14.4. HR-ESMS calcd for $C_{11}H_{17}N_2O_2S^+$ (M+1) 241.1005, found 241.1001.

4.3. 6-*tert*-Butyl 3-ethyl 2-amino-4,5-dihydrothieno[2,3-*c*]pyridine-3,6(7*H*)-dicarboxylate (2c)

N-Boc-4-piperidone **1c** (8.93 g, 44.89 mmol), ethyl cyanoacetate (5.25 mL, 49.38 mmol) and sulfur (1.73 g, 53.87 mmol) were suspended in EtOH (90 mL). Morpholine (7.85 mL, 89.79 mmol) was added and the mixture was refluxed gently with stirring for 3 h then allowed to stir at room temperature overnight. The resultant precipitate was collected by suction filtration and washed with ice cold EtOH until the filtrate was colorless (9.71 g of white powder, 66% yield).

Mp 140–144 °C. ^{1}H NMR δ 6.23 (br s, 2H, NH_2), 4.37 (s, 2H, 7-CH_2), 4.29 (q, J = 7.2 Hz, 2H, CH_2CH_3), 3.64 (t, 2H, J = 5.8 Hz, 2H, 5-CH_2), 2.83 (t, 2H, J = 5.7 Hz, 2H, 4-CH_2), 1.50 (s, 9H, $C(CH_3)_3$), 1.36 (t, J = 7.2 Hz, 3H, CH_2CH_3). ^{13}C NMR (75.4 MHz) δ 165.8, 162.7, 154.7, 131.2, 113.7, 104.8, 79.9, 59.5, 42.8–40.5, 28.5, 27.1, 14.5. HR-ESMS calcd for $C_{15}H_{23}N_2O_4S^+$ (M+1) 327.1373, found 327.1363.

4.4. (*S*)-Ethyl 2-amino-6-(2-(*tert*-butoxycarbonylamino)-3-phenylpropanoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (2d)

N-Boc-L-Phe-OH (0.5 g, 1.89 mmol) was dissolved in DMF (6 mL) and 4-piperidone hydrochloride monohydrate **9** (347 mg, 2.26 mmol) was added, followed by BOP reagent (1.0 g, 2.26 mmol) and DIPEA (1.3 mL, 7.54 mmol). After 3 h stirring at room temperature, the solution was diluted with EtOAc and washed with water followed by 10% citric acid, water, saturated bicarbonate solution, water and finally brine. The organic layer was dried ($MgSO_4$), filtered and concentrated to yield a clear colorless resin (645 mg, 99% yield). This compound (240 mg, 0.693 mmol) was dissolved in EtOH (2 mL) and ethyl cyanoacetate (81 μL, 0.762 mmol) was added followed by sulfur (23 mg, 0.693 mmol). Morpholine (200 μL, 2.29 mmol) was added and the mixture was stirred at ~40 °C for 2 h. After cooling on an ice bath, the resultant precipitate was collected by suction filtration and washed with ice cold EtOH to afford the desired product as a white solid (240 mg, 73% yield).

Mp 143–145 °C. ^{1}H NMR (complex rotamers) δ 7.25–7.13 (m, 5H, aromatic), 5.50–4.20 (m, 6H, NH, NH_2, CH_2CH_3 and NCHCO), 3.84–2.25 (m, 8H, 7-CH_2, 5-CH_2, 4-CH_2 and $PhCH_2$), 1.41 (s, 9H, $C(CH_3)_3$), 1.37–1.29 (m, 3H, CH_2CH_3). HR-ESMS calcd for $C_{24}H_{32}N_3O_5S^+$ (M+1) 474.2057, found 474.2052.

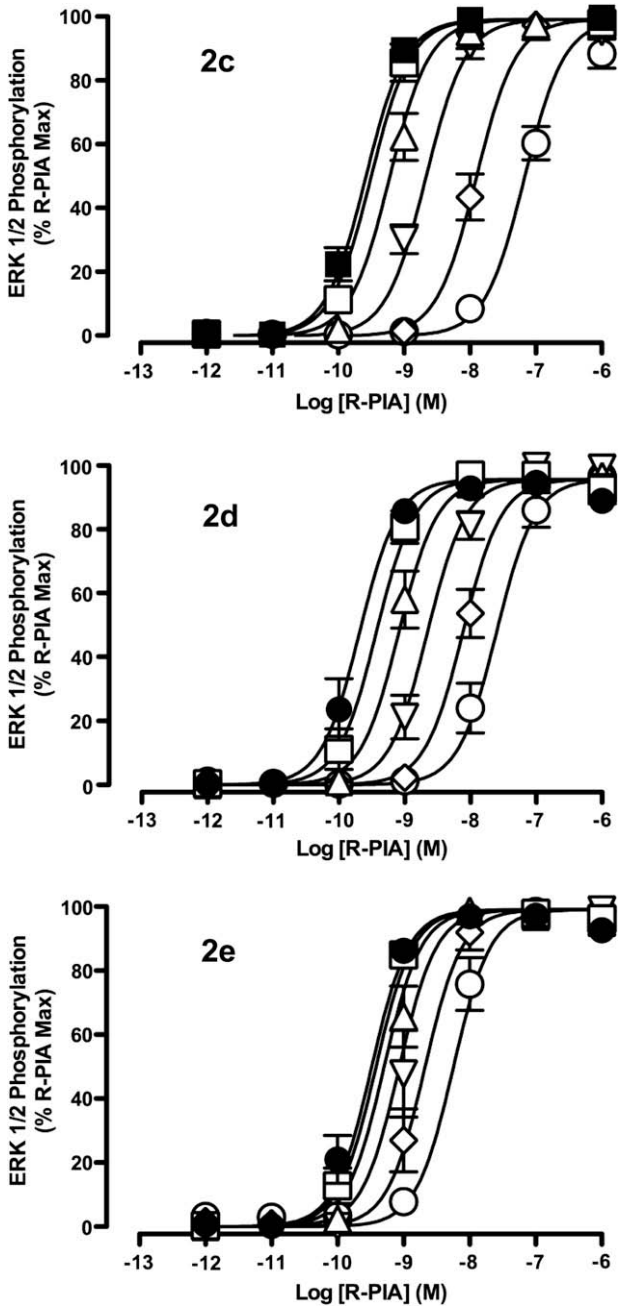


Figure 3. R-PIA mediated stimulation of ERK1/2 phosphorylation in the absence (●) or presence of 0.3 μM (□), 1 μM (△), 3 μM (▽), 10 μM (◇) or 30 μM (○) of the indicated test compound in intact CHO FlpIn A_1AR cells. Data points represent the mean ± s.e.m. of three experiments conducted in triplicate. Curves drawn through the points represent the best global fit of a competitive model of agonist–antagonist interaction.

4.5. (*R*)-Ethyl 2-amino-6-(2-(*tert*-butoxycarbonyl(methyl)-amino)-3-(4-chlorophenyl)propanoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (2e)

N-Boc-*N*-Me-D-Phe(4-Cl)-OH (0.5 g, 1.59 mmol) was dissolved in DMF (5 mL) and 4-piperidone hydrochloride monohydrate (**9**) (293 mg, 1.91 mmol) was added, followed by BOP (846 mg, 1.91 mmol) and DIPEA (1.11 mL, 6.37 mmol). The mixture was stirred at room temperature overnight and the solution was diluted with EtOAc and washed with water followed by 10% citric acid, water, saturated bicarbonate solution, water and finally brine. The organic layer was dried ($MgSO_4$), filtered and concentrated to provide a solid that was recrystallised from ether (455 mg, 72% yield). This amide (400 mg, 1.01 mmol) was dissolved in EtOH (1 mL) and ethyl cyanoacetate (118 μL, 1.11 mmol) was added followed by sulfur (34 mg, 1.06 mmol). Morpholine (211 μL, 2.41 mmol) was added and the mixture was stirred at ~60 °C for 3 h and then at room temperature overnight. The resultant precipitate was collected by suction filtration and washed with ice cold EtOH to afforded the desired product as a white solid (228 mg, 43% yield).

Mp 148–150 °C. ^{1}H NMR (complex rotamers) δ 7.26–7.09 (m, 4H, aromatic), 5.50–2.60 (m, 16H, NH_2, NCH_3, CH_2CH_3, NCHCO, 7-CH_2, 5-CH_2, 4-CH_2 and $PhCH_2$,), 1.42–1.22 (m, 12H, $C(CH_3)_3$ and CH_2CH_3). HR-ESMS calcd for $C_{25}H_{33}ClN_3O_5S^+$ (M+1) 522.1824, found 522.1822.

4.6. (*R*)-Ethyl 2-amino-6-(2-(*tert*-butoxycarbonylamino)-3-(tritylthio)propanoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate (2f)

L-Cys(Trt)-OH (0.5 g, 1.38 mmol) was suspended in DMF (6 mL) and triethylamine (400 μL, 2.87 mmol) was added followed by Boc_2O (315 mg, 1.44 mmol). After stirring at room temperature for 10 min all of the solids had dissolved. After stirring a further hour the mixture was diluted with water, acidified with 10% citric acid and extracted with $CHCl_3$ (×3). The combined organics were washed with water, dried ($MgSO_4$), filtered and concentrated to provide clear colorless oil. The oil was dissolved in DMF (6 mL) and 4-piperidone hydrochloride monohydrate (**9**) (254 mg, 1.65 mmol) was added followed by $HOBt.H_2O$ (253 mg, 1.65 mmol) and TBTU (663 mg, 2.06 mmol). Finally DIPEA (1.2 mL, 6.88 mmol) was added and the mixture was stirred at room temperature overnight. A precipitate formed upon dilution with water. This precipitate was collected by suction filtration and washed with copious amounts of water and air dried to yield a white/cream solid (741 mg, 99% yield). This amide (300 mg, 0.551 mmol) was dissolved in EtOH (1.6 mL) and ethyl cyanoacetate (65 μL, 0.606 mmol) was added, followed by sulfur (18 mg, 0.551 mmol) and morpholine (160 μL, 1.83 mmol). The mixture was stirred at ~60 °C for 3 h then at room temperature overnight. The mixture was concentrated to a resin and taken up in 2-propanol, filtered and then diluted with water while stirring. The resultant precipitate was collected by suction filtration and washed with copious amounts of water and air dried to afford a pale yellow solid (328 mg, 88% yield).

Mp 92–95 °C. ^{1}H NMR (complex rotamers) δ 7.41–7.17 (m, 15H, aromatic), 5.30–2.35 (m, 14H, NH_2, NH, CH_2CH_3, NCHCO, 7-CH_2, 5-CH_2, 4-CH_2 and $TrtSCH_2$,), 1.48–1.30 (m, 12H, $C(CH_3)_3$ and CH_2CH_3). HR-ESMS calcd for $C_{37}H_{42}N_3O_5S_2^+$ (M+1) 672.2560, found 672.2549.

4.7. Ethyl 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate hydrochloride (2g)

Compound **2c** (1.0 g, 3.06 mmol) was dissolved in dioxane (10 mL) and 6 M HCl (1 mL) was added dropwise with stirring. The mixture was heated to 80 °C for 5 min when a precipitate developed. After stirring a further 5 min at this temperature, the mixture was allowed to cool to room temperature and the precipitate was collected by suction filtration. The filter cake was washed with dioxane and finally EtOAc to afford the desired product as a hydrochloride salt (717 mg, 89% yield).

Mp 255 °C dec. ^{1}H NMR (D_2O) δ 4.27 (q, J = 7.2 Hz, 2H, CH_2CH_3), 4.17 (s, 2H, 7-CH_2), 3.47 (t, J = 6.2 Hz, 2H, 5-CH_2), 3.05 (t, J = 6.2 Hz, 2H, 4-CH_2), 1.33 (t, J = 7.2 Hz, 2H, CH_2CH_3). ^{13}C NMR (D_2O) δ 166.4, 164.1, 129.8, 107.9, 103.5, 60.7, 41.4, 23.2, 13.4. HR-ESMS calcd for $C_{10}H_{15}N_2O_2S^+$ (M+1) 227.0849, found 227.0850.

4.8. 2-Amino-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylic acid (3a)

Compound **2a** (4.0 g, 12.64 mmol) was suspended in EtOH (40 mL) and water (20 mL), dioxane (15 mL) and NaOH (2.02 g, 50.56 mmol) were added. The mixture was gently refluxed for 4 h. The solution was concentrated to a residue and partitioned between water (30 mL) and ether. The ether layer was discarded and the aqueous phase chilled in an ice bath and slowly acidified with 6 M HCl with stirring to pH ≈ 7. The resultant precipitate was collected by suction filtration and washed with ice cold water (10 mL). This solid was further triturated in ice cold EtOH (15 mL) to afford the desired product as a gray solid (2.16 g, 59% yield).

Mp 198 °C dec. ^{1}H NMR (DMSO-d_6) δ 12.0 (br s, 1H, CO_2H), 7.62–7.29 (m, 7H, NH_2 and aromatic), 4.40–3.72 (m, 4H, 7-CH_2 and NCH_2Ph), 3.25 (m, 2H, 5-CH_2), 2.95 (m, 2H, 4-CH_2). ^{13}C NMR (DMSO-d_6) δ 166.4, 164.3, 131.4, 130.3, 130.0, 129.4, 128.8, 106.5, 102.2, 57.8, 48.6, 48.0, 23.9. HR-ESMS calcd for $C_{15}H_{17}N_2O_2S^+$ (M+1) 289.1005, found 289.0997.

4.9. 2-Amino-6-methyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylic acid (3b)

Compound **2b** (6.4 g, 26.63 mmol) was suspended in EtOH (40 mL) and water (30 mL) and NaOH (4.26 g, 106.52 mmol) were added. The mixture stirred at ~80 °C for 5.5 h. The solution was concentrated to a residue and partitioned between water (30 mL) and ether. The ether layer was discarded and the aqueous phase chilled in an ice bath and slowly acidified with 6 M HCl with stirring to pH ≈ 7. The resultant precipitate was collected by suction filtration and washed with ice cold water (20 mL) and 1:1 ether/ethanol (10 mL) to afford the product as a light brown solid (4.58 g, 81% yield).

Mp 185 °C dec. ^{1}H NMR (DMSO-d_6) δ 10.9 (br s, 1H, CO_2H), 7.43 (br s, 2H, NH_2), 4.08 (s, 2H, 7-CH_2), 3.39–3.29 (m, 2H, 5-CH_2), 3.01–2.91 (m, 2H, 4-CH_2), 2.81 (s, 3H, NCH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 166.4, 164.2, 129.5, 106.7, 102.3, 50.2, 41.8, 24.0. HR-ESMS calcd for $C_9H_{13}N_2O_2S^+$ (M+1) 213.0692, found 213.0685.

4.10. 2-Amino-6-(*tert*-butoxycarbonyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylic acid (3c)

Compound **2c** (1.0 g, 3.06 mmol) was suspended in EtOH (5 mL) and water (4 mL), dioxane (2 mL) and KOH (687 mg, 12.25 mmol) were added. The mixture was stirred with gentle reflux for 2.5 h. The cooled solution was diluted with water (100 mL) and washed with ether. The aqueous phase was cooled in an ice bath and carefully acidified to pH ≈ 5 with 10% citric acid and extracted with $CHCl_3$ (×2) and EtOAc (×1). The combined organic portions were washed with brine dried ($MgSO_4$), filtered and concentrated to a solid (0.6 g). Trituration with MeOH afforded the desired product as a white powder (200 mg, 22% yield).

Mp 167–170 °C. ^{1}H NMR δ 11.9 (br s, 1H, CO_2H), 7.29 (br s, 2H, NH_2), 4.25 (s, 2H, 7-CH_2), 3.51 (t, J = 5.8 Hz, 2H, 5-CH_2), 2.66 (t, 2H, J = 5.7 Hz, 2H, 4-CH_2), 1.42 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 166.5, 163.4, 153.9, 130.8, 111.8, 102.7, 79.0, 41.9, 41.4, 28.0, 26.6. HR-ESMS calcd for $C_{13}H_{17}N_2O_4S^-$ (M-1) 297.0915, found 297.0915.

4.11. 6-Benzyl-2-(*tert*-butoxycarbonylamino)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylic acid (4a)

Compound **2a** (5.15 g, 16.28 mmol) was dissolved in dioxane (30 mL) and Boc_2O (7.82 g, 35.81 mmol) was added followed by DMAP (200 mg, 1.64 mmol) and the mixture was heated with stirring at ~70–80 °C for 3 h then at room temperature overnight. The solution was concentrated and then partitioned between water and $CHCl_3$. The organic layer was set aside and the aqueous layer was extracted with $CHCl_3$ (×2). The combined organic portions were washed with water then brine, dried ($MgSO_4$), filtered and concentrated to a residue. This residue was suspended in EtOH (50 mL) and water (50 mL) and KOH (3.65 g, 65.10 mmol) were added. The mixture was at ~70 °C for 3 h and then concentrated to remove EtOH. The aqueous residue was diluted with water until all solids had dissolved and then washed with ether (×3). The aqueous layer was chilled on ice and acidified with 10% citric acid to pH ≈ 7. After extraction with EtOAc (4 × 250 mL), the combined organics were dried ($MgSO_4$), filtered and concentrated to yield a yellow powder. This powder was triturated with ether and filtered on a Buchner funnel and washed with ether to afford a yellow solid (4.62 g, 73%).

Mp 187 °C dec. ^{1}H NMR (DMSO-d_6) δ 12.5 (br s, 1H, CO_2H), 10.5 (br s, 1H, NH), 7.36–7.28 (m, 5H, aromatic), 3.69 (s, 2H, 7-CH_2), 3.50 (s, 2H, NCH_2Ph), 2.66–2.83 (m, 4H, 5-CH_2 and 4-CH_2), 1.49 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 167.2, 151.2, 148.2, 137.5, 130.0, 128.9, 128.3, 127.2, 121.5, 110.9, 81.6, 60.6, 50.3, 49.3, 27.7, 26.1.

4.12. 2-(*tert*-Butoxycarbonylamino)-6-methyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylic acid (4b)

Compound **2b** (5.0 g, 20.81 mmol) was dissolved in dioxane (38 mL) and Boc_2O (9.31 g, 42.65 mmol) was added followed by DMAP (254 mg, 2.08 mmol). The mixture was stirred at ~70–80 °C for 3 h then at room temperature overnight. The solution was concentrated and then partitioned between water and $CHCl_3$. The organic layer was set aside and the aqueous layer extracted with $CHCl_3$ (×2). The combined organic layers were then washed with water then brine, dried ($MgSO_4$), filtered and concentrated to a residue. This residue was suspended in EtOH (30 mL) and water (15 mL) and NaOH (3.33 g, 83.25 mmol) was added. The mixture was stirred at ~70 °C for 3 h and then concentrated to remove EtOH. The aqueous residue was diluted with water until all solids dissolved and then washed with ether (×3). The aqueous layer was chilled on ice and acidified with 10% citric acid to pH ≈ 7. The resultant precipitate was collected by suction filtration and washed with water. Drying under high vacuum afforded the product as a yellow powder (4.09 g, 63% yield).

Mp 171 °C dec. ^{1}H NMR (DMSO-d_6) δ 11.21 (br s, 1H, NH), 4.45 (br s, 1H, CO_2H), 3.77 (br s, 2H, 7-CH_2), 2.91 (br s, 2H, 5-CH_2), 2.56 (br s, 2H, 4-CH_2), 1.48 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 168.2, 151.4, 146.6, 130.0, 117.8, 113.7, 81.1, 51.1, 50.7, 42.9, 27.8, 24.8.

4.13. General method for the synthesis of compounds 6a–i

Compound **4a**/**4b** (200 mg) and the appropriate amine (1.2 equiv) were dissolved in DMF (3 mL). BOP (1.2 equiv) and DIPEA (3 equiv) were added and the reaction was stirred at room temperature overnight. Following dilution EtOAc, the solution was washed with water (×4), brine, dried ($MgSO_4$), filtered and concentrated to a foam (60–80% yield). The crude product was subsequently chromatographed on silica gel (30–50% EtOAc/petroleum ether). The resultant amide was dissolved in EtOH (1 mL) and stirred with 6 M HCl (300 μL) at room temperature overnight. Evaporation of the solvents afforded a residue which was triturated with dioxane. The solid was collected by suction filtration and washed with dioxane then ether and the crude products were recrystallised from EtOH/ether to afford compounds **6a–i** as the hydrochloride salts.

4.14. 2-Amino-*N*,6-dibenzyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide hydrochloride (6a)

The title compound **6a** was synthesized as per the general method from **4a** to afford **6a** as a yellow solid in 31% yield. Mp 135–140 °C. ^{1}H NMR (DMSO-d_6) δ 11.15 (br s, 1H, $(CH_2)_3N^+H$), 7.65–7.31 (m, 13H, aromatic, NH and NH_2), 4.49–4.36 (m, 4H, 2 × NCH_2Ph), 4.09 (s, 2H, 7-CH_2), 3.60–3.56 (m, 2H, 5-CH_2), 3.09–3.06 (m, 2H, 4-CH_2). HR-ESMS calcd for $C_{22}H_{24}N_3OS^+$ (M+1) 378.1635, found 378.1646.

4.15. 2-Amino-6-benzyl-*N*-(3-chlorobenzyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide hydrochloride (6b)

The title compound **6b** was synthesized as per the general method from **4a** to afford **6b** as a yellow solid in 35% yield. Mp 210 °C dec. ^{1}H NMR (DMSO-d_6) δ 10.93 (br s, 1H, $(CH_2)_3N^+H$), 7.65–7.31 (m, 10H, aromatic and NH), 7.00 (br s, 2H, NH_2), 4.47–4.40 (m, 4H, 2 × NCH_2Ph), 4.09 (s, 2H, 7-CH_2), 3.65–3.55 (m, 2H, 5-CH_2), 3.05–3.10 (m, 2H, 4-CH_2). HR-ESMS calcd for $C_{22}H_{23}ClN_3OS^+$ (M+1) 412.1245, found 412.1245.

4.16. 2-Amino-6-benzyl-*N*-(3-(trifluoromethyl)benzyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide hydrochloride (6c)

The title compound **6c** was synthesized as per the general method from **4a** to afford **6c** as a yellow solid in 36% yield. Mp 155–160 °C. ^{1}H NMR (DMSO-d_6) δ 11.19 (br s, 1H, $(CH_2)_3N^+H$), 7.66–7.43 (m, 12H, aromatic, NH and NH_2), 4.50–4.42 (m, 4H, 2 × NCH_2Ph), 4.08 (s, 2H, 7-CH_2), 3.66–3.56 (m, 2H, 5-CH_2), 3.07–3.13 (m, 2H, 4-CH_2). HR-ESMS calcd for $C_{23}H_{23}F_3N_3OS^+$ (M+1) 446.1508, found 446.1468.

4.17. 2-Amino-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carbohydrazide hydrochloride (6d)

The title compound **6d** was synthesized as per the general method from **4a** to afford **6d** as a yellow solid in 65% yield. Mp 191 °C dec. ^{1}H NMR (DMSO-d_6) δ 11.60 (br s, 1H, $(CH_2)_3N^+H$), 10.20 (br s, 1H, $NHNH_2$), 7.73–7.68 and 7.49–7.46 (2 m, 7H, aromatic and NH_2), 4.43 (br s, 2H, NCH_2Ph), 4.07 (s, 2H, 7-CH_2), 3.66–3.30 (m, 4H, 5-CH_2 and $NHNH_2$), 3.07–3.17 (m, 2H, 4-CH_2). ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 165.7, 163.4, 131.5, 129.9, 129.5, 128.8, 127.8, 107.3, 103.1, 57.1, 48.2, 47.2, 22.6. HR-ESMS calcd for $C_{15}H_{19}N_4OS^+$ (M+1) 303.1274, found 303.1279.

4.18. 2-Amino-6-benzyl-*N*'-phenyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carbohydrazide hydrochloride (6e)

The title compound **6a** was synthesized as per the general method from **4a** to afford **6e** as a yellow solid in 33% yield. Mp 180 °C dec. ^{1}H NMR (DMSO-d_6) δ 11.15 (br s, 1H, $(CH_2)_3N^+H$), 8.99 (br s, 1H, $NHNHPh$), 7.73–7.68 and 7.49–7.46 (2 m, 12H, aromatic and NH_2), 4.43 (br s, 2H, NCH_2Ph), 4.07 (s, 2H, 7-CH_2), 3.66–3.30 (m, 4H, 5-CH_2 and $NHNH_2$), 3.17–3.07 (m, 2H, 4-CH_2). HR-ESMS calcd for $C_{21}H_{23}N_4OS^+$ (M+1) 379.1587, found 379.1606.

4.19. 2-Amino-*N*-benzyl-6-methyl-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridine-3-carboxamide hydrochloride (6f)

The title compound **6f** was synthesized as per the general method from **4b** to afford **6f** as a yellow solid in 39% yield. Mp 200 °C dec. ^{1}H NMR (DMSO-d_6) δ 10.98 (br s, 1H, $(CH_2)_2MeN^+H$), 7.57–7.23 (m, 8H, aromatic, NH and NH_2), 4.41 (m, 2H, NCH_2Ph), 4.06 (m, 2H, 7-CH_2), 3.56 (m, 2H, 5-CH_2), 3.10–3.01 (m, 2H, 4-CH_2), 2.85 (s, 3H, NCH_3).). ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 164.7, 156.9, 140.0, 128.2, 128.0, 127.2, 126.6, 109.6, 108.7, 50.0, 49.6, 42.3, 41.3, 22.9. HR-ESMS calcd for $C_{16}H_{20}N_3OS^+$ (M+1) 302.1322, found 302.1318.

4.20. 2-Amino-*N*-(3-chlorobenzyl)-6-methyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide hydrochloride (6g)

The title compound **6f** was synthesized as per the general method from **4b** to afford **6g** as a yellow solid in 25% yield. Mp 190–195 °C. ^{1}H NMR (DMSO-d_6) δ 10.66 (br s, 1H, $(CH_2)_2MeN^+H$), 7.64–7.56 (m, 1H, NH), 7.41–7.23 (m, 4H, aromatic), 7.02 (br s, 2H, NH_2), 4.40–4.38 (m, 2H, NCH_2Ph), 4.32–4.27 and 4.11–3.98 (m, 2H, 7-CH_2), 3.62–3.50 (m, 2H, 5-CH_2), 3.10–3.05 (m, 2H, 4-CH_2), 2.87 (s, 3H, NCH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 165.1, 159.8, 142.8, 132.8, 130.0, 127.4, 127.0, 126.4, 125.9, 107.1, 50.1, 49.7, 41.8, 41.3, 22.9. HR-ESMS calcd for $C_{16}H_{19}ClN_3OS^+$ (M+1) 336.0932, found 336.0931.

4.21. 2-Amino-6-methyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carbohydrazide hydrochloride (6h)

The title compound **6h** was synthesized as per the general method from **4b** to afford **6h** as a yellow solid in 46% yield. Mp 195 °C dec. ^{1}H NMR (D_2O) δ 4.39–4.34 and 4.14–4.09 (m, 2H, 7-CH_2), 3.72–3.66 and 3.42–3.33 (m, 2H, 5-CH_2), 3.07–2.97 (m, 5H, 4-CH_2 and NCH_3). ^{13}C NMR (D_2O) δ 166.1, 162.4, 127.5, 109.4, 105.1, 51.2, 51.1, 41.9, 22.8. HR-ESMS calcd for $C_9H_{15}N_4OS^+$ (M+1) 227.0961, found 227.0948.

4.22. 2-Amino-6-methyl-*N*'-phenyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carbohydrazide hydrochloride (6i)

The title compound **6i** was synthesized as per the general method from **4b** to afford **6i** as a yellow solid in 22% yield. Mp 190 °C dec. ^{1}H NMR (DMSO-d_6) δ 10.56 (br s, 1H, $(CH_2)_3N^+H$), 8.99 (br s, 1H, $NHNHPh$), 7.17–7.12 and 6.81–6.68 (m, 5H, aromatic), 4.33–4.28 (m, 1H, $NHNHPh$), 4.08–4.04 (m, 2H, 7-CH_2), 3.62 (m, 2H, 5-CH_2), 3.17–3.10 (m, 2H, 4-CH_2), 2.89 (s, 3H, NCH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 165.8, 160.1, 149.7, 128.7, 127.8, 118.5, 114.5, 112.5, 107.4, 50.1, 49.7, 41.2, 22.8. HR-ESMS calcd for $C_{15}H_{19}N_4OS^+$ (M+1) 303.1274, found 303.1279.

4.23. 2-Amino-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide hydrochloride (7a)

N-Benzyl-4-piperidone **1a** (1.67 g, 8.84 mmol), 2-cyanoacetamide (743 mg, 8.84 mmol) were suspended in EtOH (2.6 mL). Sulfur (309 mg, 9.64 mmol) and morpholine (885 μL, 10.12 mmol) were added and the mixture was heated on an oil bath at 60 °C for 3 h during which time a solid precipitated. After cooling, the precipitate was collected by suction filtration and washed with ice cold EtOH (2.21 g, 87% yield) and recrystallised from MeOH. A sample of recrystallised material (0.5 g) was dissolved in dioxane (10 mL) and 6 M HCl was added dropwise until no further precipitation occurred. The hydrochloride salt was collected by suction filtration and washed with dioxane and finally EtOAc.

Mp 200–205 °C. ^{1}H NMR (D_2O) δ 4.56-4.39 4.56–4.39 (m, 2H, 7-CH_2), 4.18–4.17 (m, 2H, NCH_2Ph), 3.85–3.78 and 3.46–3.36 (2 m, 2H, 5-CH_2), 3.06–3.02 (m, 2H, 4-CH_2). ^{13}C NMR (D_2O) δ 168.9, 158.2, 131.0, 130.3, 129.3, 128.4, 128.1, 110.3, 109.6, 59.2, 49.3, 48.9, 22.8. HR-ESMS calcd for $C_{15}H_{18}N_3OS^+$ (M+1) 288.1165, found 288.1144.

4.24. 2-Amino-6-methyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide hydrochloride (7b)

N-Methyl-4-piperidone **1b** (1.0 g, 8.84 mmol) and 2-cyanoacetamide (743 mg, 8.84 mmol) were suspended in EtOH (2.6 mL). Sulfur (309 mg, 9.64 mmol) and morpholine (885 μL, 10.12 mmol) were added and the mixture was heated on an oil bath at 60 °C for 3 h during which time a solid precipitated. After cooling, the precipitate was collected by suction filtration and washed with ice cold EtOH (1.69 g, 90% yield) and recrystallised from MeOH. A sample of the recrystallised material (0.5 g) was dissolved in dioxane (10 mL) and 6 M HCl was added dropwise until no further precipitation occurred. The hydrochloride salt was filtered by suction filtration and washed with dioxane and finally EtOAc.

Mp 207 °C dec. ^{1}H NMR (D_2O) δ 4.20-4.15 4.20–4.15 and 3.95–3.90 (2 m, 2H, 7-CH_2), 3.53–3.45 and 3.21–3.12 (2 m, 2H, 5-CH_2), 2.91–2.79 (m, 5H, 4-CH_2 and NCH_3). ^{13}C NMR (75.4 MHz, D_2O) δ 168.4, 154.8, 127.9, 112.4, 112.0, 51.3, 51.0, 42.0, 22.8. HR-ESMS calcd for $C_9H_{14}N_3OS^+$ (M+1) 212.0852, found 212.0850.

4.25. 2-Amino-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carbonitrile (8a)

N-Benzyl-4-piperidone **1a** (5.0 g, 26.42 mmol) and malononitrile (1.75 g, 26.42 mmol) were dissolved in EtOH (50 mL). Sulfur (932 mg, 29.06 mmol) and morpholine (4.62 mL, 52.84 mmol) were added and the mixture was refluxed for 2 h. After cooling, the solution was diluted with water (100 mL). A precipitate formed which was collected by suction filtration and washed with water. This afforded the product as a dark brown solid (6.4 g, 90% yield).

Mp 138–141 °C. ^{1}H NMR δ 7.40–7.30 (m, 5H, aromatic), 4.68 (br s, 2H, NH_2), 3.76 (s, 2H, NCH_2Ph), 3.45 (s, 2H, 7-CH_2), 2.85 (t, J = 5.7 Hz, 2H, 5-CH_2), 2.66 (t, J = 5.7 Hz, 2H, 4-CH_2). ^{13}C NMR (75.4 MHz) δ 161.0, 137.9, 131.0, 129.2, 128.7, 128.5, 127.5, 117.8, 115.4, 61.8, 50.8, 49.61, 24.7.

4.26. 2-Amino-6-methyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carbonitrile (8b)

N-Methyl-4-piperidone **1b** (2.0 g, 17.67 mmol) and malononitrile (1.17 g, 17.67 mmol) were dissolved in EtOH (10 mL). Sulfur (577 mg, 18.0 mmol) and diethylamine (2 mL) were added and the mixture was heated to 60 °C for 1 h then left to stir at room temperature overnight. The resultant precipitate was collected by suction filtration and washed to EtOH to form the product as a yellow solid (1.75 g, 51% yield).

Mp 183–185 °C. ^{1}H NMR (DMSO-d_6) δ 7.00 (br s, 2H, NH_2), 3.24 (s, 2H, 7-CH_2), 2.62–2.58 (m, 2H, 5-CH_2), 2.48–2.40 (m, 2H, 4-CH_2), 2.32 (s, 3H, NCH_3). ^{13}C NMR (DMSO-d_6) δ 163.9, 130.0, 116.5, 114.9, 83.2, 52.7, 51.6, 45.3, 24.8. HR-ESMS calcd for $C_9H_{12}N_3S^+$ (M+1) 194.0746, found 194.0732.

4.27. *tert*-Butyl 2-amino-3-cyano-4,5-dihydrothieno[2,3-*c*]pyridine-6(7H)-carboxylate (8c)

N-Boc-4-piperidone **1c** (0.5 g, 2.51 mmol) and malononitrile (182 mg, 2.76 mmol) were dissolved in EtOH (5 mL). Sulfur (85 mg, 2.64 mmol) and morpholine (200 μL, 2.29 mmol) were added and the mixture was heated to 70–80 °C for 2 h. The mixture was cooled on an ice bath and the resultant precipitate was collected by suction filtration and washed with ice cold EtOH. This afforded the product as a pale peach solid (440 mg, 63% yield).

Mp 195–197 °C. ^{1}H NMR δ 4.36 (s, 2H, 7-CH_2), 3.66 (t, J = 5.7 Hz, 2H, 5-CH_2), 2.59 (t, J = 5.7 Hz, 2H, 4-CH_2), 1.48 (s, 9H, C(CH_3)$_3$). ^{13}C NMR (rotamers) δ 161.3, 154.6, 131.0, 116.7, 116.1, 115.1, 87.6, 80.4, 42.6, 42.0, 41.4, 40.0, 28.4, 24.7. HR-ESMS calcd for $C_{13}H_{18}N_3O_2S^+$ (M+1) 280.1114, found 280.1109.

4.28. Dissociation kinetic assays

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 doses 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 were 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 an AE, B_o = residual binding (cpm) at the end of 10 min of dissociation in the absence of an AE 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.

4.29. 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 (3 or 10 μM for initial screens; more detailed concentration–response analyses for compounds **2c**, **2d** and **2e**) at 37 °C. R-PIA was then added and stimulation allowed to proceed for 5 min before the reaction was terminated by the re-

moval 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.

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