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The synthesis and biological evaluation of 2-amino-4,5,6,7,8,9-hexahydrocycloocta[*b*]thiophenes as allosteric modulators of the A_1 adenosine receptor

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ABSTRACT

A series of 2-amino-4,5,6,7,8,9-hexahydrocycloocta[*b*]thiophenes were prepared and evaluated as potential allosteric modulators of the A_1 adenosine receptor (AR). The structure–activity relationships of the 3-position were explored along with varying the size of the cycloalkyl ring. 2-Aminothiophenes with amide and hydrazide groups in the 3-position were completely inactive in an A_1-AR-mediated ERK1/2 phosphorylation assay, yet most of the 3-benzoyl substituted compounds exhibited allosteric effects on responses mediated by the orthosteric agonist, *R*-PIA. Despite finding an increase in both agonistic and allosteric activities by going from a cyclopentyl ring to a cyclohexyl ring in the 3-benzoyl series, decreases were observed when further increasing the ring size. Varying the substituents on the phenyl ring of the 3-benzoyl group also affected the activity of these compounds.

During hypoxic, ischemic and inflammatory events, adenosine is endogenously released to act as a cardio- and neuro-protective agent via interaction with multiple subtypes of GPCRs. Efforts to selectively target these GPCR subtypes using modified adenosine analogs have been of limited therapeutic value due to poor subtype selectivity. One way to increase the selectivity of an endogenous ligand for a receptor is by targeting an allosteric site on that receptor with an allosteric enhancer (AE). The positive allosteric modulation of the A_1-AR by 2-aminothiophenes is well documented and has been explored by a number of research groups.[1–3] The pioneering work of Bruns et al.[4,5] investigated compound motifs that were crucial for activity, and ultimately yielded four potent and selective allosteric enhancers (the 'PD series') that also displayed weak antagonism of the A_1-AR at high concentrations (Fig. 1), with PD 81,723[6] showing the most favourable ratio of allosteric enhancement to antagonism.[4] Subsequently, the discovery of two allosteric modulators LUF 5484 and T-62 that are structurally related to PD 71,605, were shown to have improved potency compared to PD 81,723.[2,7,8]

In a previous study,[9] it was shown that increasing the ring size of 2-aminocycloalkyl[*b*]thiophenes from five to seven membered rings improved the ability of these 2-aminothiophenes to act as AEs to stabilize the agonist-receptor-G protein ternary complex in an in vitro dissociation kinetic binding assay of the A_1-AR.[9] By extrapolation, further increases in ring size may improve allosteric ligand activity, and thus the aim of the current study was to investigate this hypothesis by exploring the effect of introducing eight-membered rings into 2-aminocycloalkyl[*b*]thiophenes.

To the best of our knowledge, the allosteric effects of 2-aminocycloocta[*b*]thiophenes at the A_1-AR have only been investigated once previously,[10] and this study only included one example. We

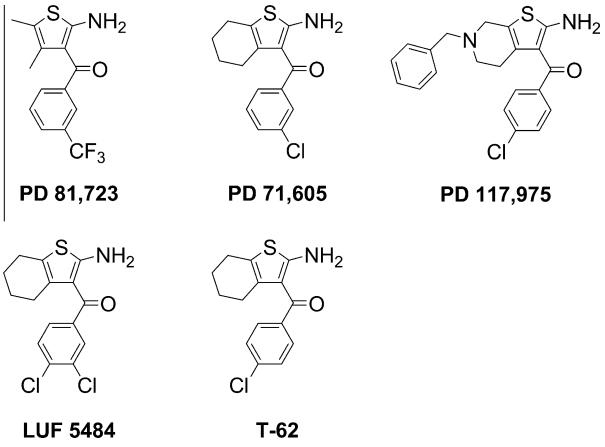

Figure 1. The PD series of allosteric enhancers discovered by Bruns and co-workers, and the second generation allosteric enhancers LUF 5484 and T-62.

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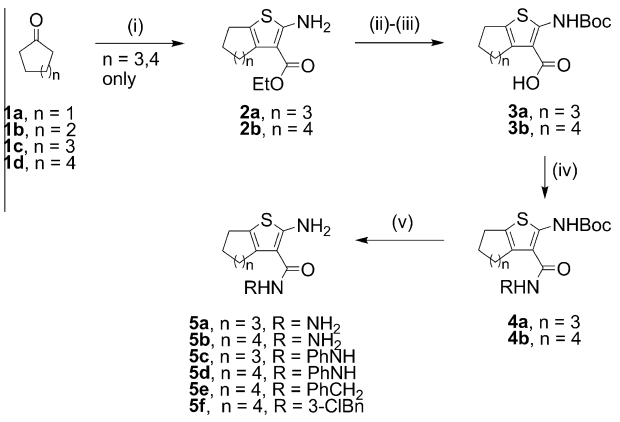
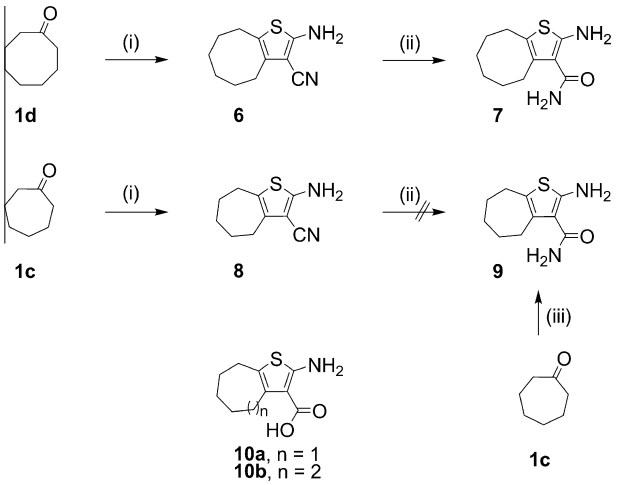
have also revisited some compounds with smaller ring sizes (i.e., **12a**–**f**) to enable a direct comparison of the effects of ring size to be made using data obtained from the same functional assay. We have previously observed that certain compounds which have produced allosteric effects in kinetic binding assays at higher concentrations, act as orthosteric antagonists in functional assays which measure A_1-AR-mediated phosphorylation of ERK1/2 (pERK1/2) in intact CHO cells.[11,12]

The synthesis of the 2-aminocycloalkyl[*b*]thiophenes **2a**,**b** began with the condensation of cycloalkanones **1c**,**d** with activated nitriles. When utilising ethyl cyanoacetate the classical Gewald[13] conditions with ethanol solvent, nitrile, elemental sulphur and base did not provide the compounds **2a**,**b** and therefore alternate conditions were employed. In the case of cycloheptanone **1c**, a 1:1 mixture of DMF:Et_3N provided the 2-aminothiophene **2a** in adequate yield, but in the case of cyclooctanone **1d** no reaction occurred under these conditions. The two step procedure by initially forming the Knöevenagel condensation product under microwave irradiation and then cyclising with sulphur provided the crude thiophene **2b** (Scheme 1) as a viscous resin, that is, contaminated with the starting ketone **1d**. Purification via column chromatography on silica gel failed to separate the thiophene **2b** from the ketone **1d** as they co-elute. In previous work,[14] thiophene **2a** was hydrolysed to the acid **10a** under basic conditions and then used for subsequent amidation reactions. In the case of **2b**, hydrolysis under the same conditions resulted in decomposition and therefore the alternate route depicted in Scheme 1 was employed. The thiophenes **2a**,**b** were readily carbamoylated with Boc anhydride and then hydrolysed to the acids **3a**,**b** under basic conditions, which were amidated with BOP reagent to the carboxamides **4** in adequate yield and purity. The crude carbamates **4** were treated with TFA to remove the Boc group and the crude 2-aminothiophenes were chromatographed and then recrystallized to purity.

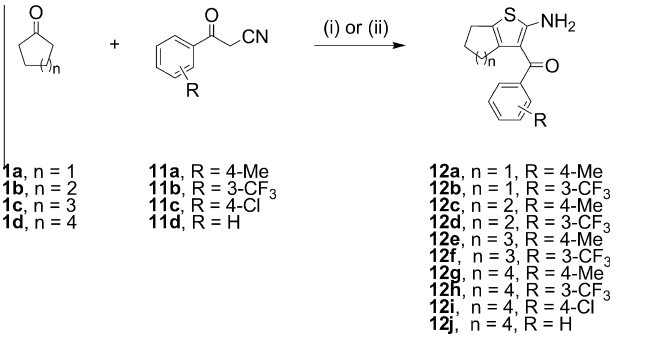
The carboxamides **7** and **9** (Scheme 2) required a different route in their synthesis and it was envisaged that simple hydrolysis from the nitriles **6** and **8** under acidic conditions would provide the amides. This was the case with **6**, which was dissolved in concentrated sulphuric acid giving clean conversion to the amide **7**, yet nitrile **8** was insoluble and even in 85% phosphoric acid only returned **8**. The carboxamide **9** was ultimately synthesised under the same conditions as for **2a** except ethyl cyanoacetate was replaced with cyanoacetamide.



Scheme 1. Reagents: (i) **1c**, DMF, Et_3N, $EtOCOCH_2CN$, S_8 and **1d**, PhMe, $EtOCOCH_2CN$, $AcONH_4$, MW then S_8, EtOH, morpholine; (ii) dioxane, DMAP, Boc_2O; (iii) EtOH, H_2O, NaOH; (iv) BOP, collidine, amine, CH_2Cl_2; (v) TFA, CH_2Cl_2.

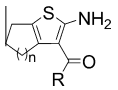


Scheme 2. Reagents: (i) DMF, Et_3N, $NCCH_2CN$, S_8; (ii) concd H_2SO_4; (iii) DMF, Et_3N, H_2NCOCH_2CN, S_8.



Scheme 3. Reagents: (i) $TiCl_4$, pyridine, $EtOCOCH_2CN$ then S_8, Et_2NH, THF; (ii) EtOH, morpholine, AcOH, S_8.

Table 1
Effect of test compounds on A_1-AR-mediated stimulation of ERK1/2 phosphorylation in intact CHO FlpIn cells in the presence of an EC_{50} concentration of *R*-PIA



Compound	R	*n*	Activity[a]	
			3 μM	10 μM
5a	NH_2NH	3	52 ± 3	51 ± 1
5b	NH_2NH	4	48 ± 1	48 ± 1
5c	PhNHNH	3	44 ± 3	42 ± 4
5d	PhNHNH	4	47 ± 1	43 ± 4
5e	$PHCH_2NH$	4	48 ± 2	50 ± 1
5f	3-ClPhCH_2NH	4	49 ± 1	47 ± 2
7	NH_2	4	48 ± 1	48 ± 1
9	NH_2	3	47 ± 1	50 ± 3
12a	4-CH_3Ph	1	50 ± 3	56 ± 2
12b	3-CF_3Ph	1	75 ± 6	98 ± 3
12c	4-CH_3Ph	2	70 ± 8	91 ± 5
12d	3-CF_3Ph	2	73 ± 3	88 ± 3
12e	4-CH_3Ph	3	55 ± 2	68 ± 3
12f	3-CF_3Ph	3	53 ± 4	74 ± 7
12g	4-CH_3Ph	4	56 ± 12	75 ± 1
12h	3-CF_3Ph	4	57 ± 2	65 ± 11
12i	4-ClPh	4	56 ± 8	63 ± 10
12j	Ph	4	49 ± 3	36 ± 9

[a] Effect of two concentrations (3 and 10 μM) of the test compound on A_1-AR-mediated stimulation of ERK1/2 phosphorylation in intact CHO FlpIn cells, in presence of an EC_{50} concentration of *R*-PIA (determined on the same day as each assay). Data represent the mean ± standard deviation of two experiments conducted in triplicate. 0% is defined as basal ERK1/2 phosphorylation in the absence of *R*-PIA, whereas 100% is defined as the maximal response to a saturating concentration (100 nM) of *R*-PIA.

The aroylthiophenes **12a–f** were synthesised via the method of Tormyshev et al.[15] with slight modification (Scheme 3). In this method, the Knöevenagel intermediate (not shown) is preformed in EtOH, morpholine and acetic acid without removing water in the process and finally adding elemental sulphur in the one pot. In this fashion low to moderate yields of products were obtained with the best yields derived from cyclohexanone **1b** and cycloheptanone **1c** (**12c–e**). However, this method was unfruitful when cyclooctanone **1d** was used. Therefore the Lewis acid based condensation of ketones and malonates with titanium tetrachloride devised by Lehnert[16] was employed in the synthesis of **12g–j**. Although poor yields were obtained sufficient material was isolated for further work.

In order to assess the biological activity of the novel series of compounds, we initially screened all compounds using a plate-based assay of A_1-AR-mediated ERK1/2 phosphorylation (pERK1/2) in intact CHO cells.[17] For each compound, two concentrations (3 and 10 μM) were tested alone to assess intrinsic agonism, and against an EC_{50} concentration of the orthosteric agonist *R*-PIA, to assess enhancement or inhibition of the A_1-AR agonist activity (Table 1). With the exception of **12b–d** (each yielding approximately 50% of the maximum *R*-PIA response at 10 μM; Fig. 2A), none of the compounds displayed any substantial ability to activate the receptor on their own. In the presence of an EC_{50} concentration of *R*-PIA, a large number of compounds were found to be inactive in modulating the response of the orthosteric agonist at 3 and 10 μM (Fig. 2B). In fact, all 2-aminothiophenes with amide or hydrazide functionality in the 3-position (compounds **5a–f**, **7** and **9**) were completely inactive, irrespective of the size of the cycloalkyl ring. Regarding the 2-amino-3-benzoyl compounds, it is interesting to observe that most of them exhibited a positive allosteric effect on *R*-PIA-mediated response. In this series of compounds **12a–j**, 4-CH_3 and 3-CF_3 substituents were the main groups investigated. Within the 4-CH_3 substituted family, increasing the size of the cycloalkyl ring induced bi-phasic behaviour, whereby an increase in both the agonistic and allosteric activities of the compounds was noted when going from cyclopentyl **12a** to cyclohexyl **12c**, whereas a decrease of activity was observed when extending further to the cycloheptyl **12e** and cyclooctyl **12g**. In contrast, within the 3-CF_3 substituted family, increasing the size of the cycloalkyl ring seemed to have no particular effect on the allosteric activity of the compounds, as evidenced by the fact that no substantial variation of activity was observed between **12b**, **12d**, **12f** and **12h**. As a control, the compound presented in Baraldi's study, **12j**, was also investigated and appeared to show modest allosteric enhancing activity in ERK1/2 phosphorylation. Finally, comparison of the unsubstituted benzoyl group in **12j**, revealed that substitution with either a methyl (**12g**), trifluoromethyl (**12h**) or chloro (**12i**) group was beneficial for the allosteric activity, while not influencing the agonist properties of the compounds.

It is interesting to observe that the best allosteric enhancers are also the compounds that exhibit agonist properties in their own right, such that **12b–d** are strong allosteric agonists, while **12i**

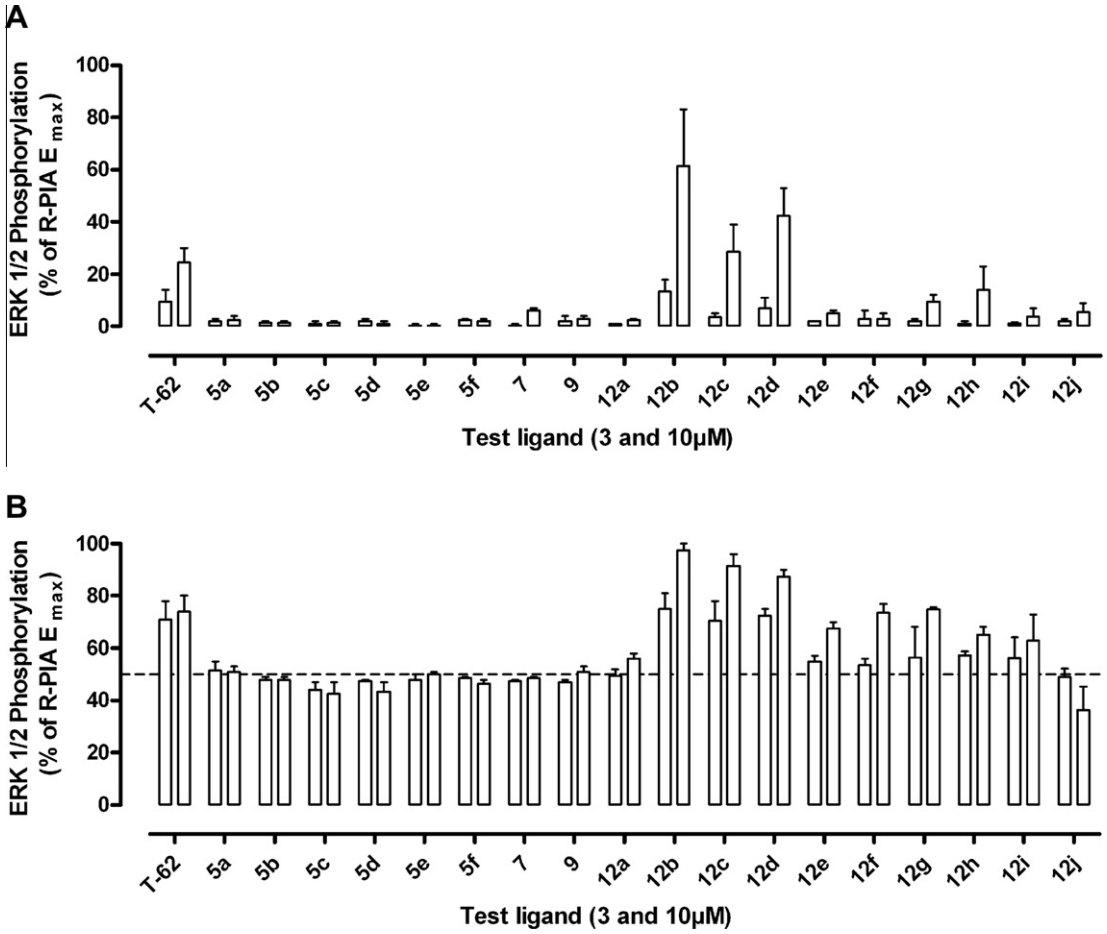


Figure 2. Effect of two different concentrations (3 μM, left bar; 10 μM right bar) of test ligands on A_1-AR-mediated stimulation of ERK1/2 phosphorylation in intact CHO FlpIn cells, in absence (A) or in presence (B) of an EC_{50} concentration (0.3 nM) of *R*-PIA (determined on the same day as each assay); dashed line denotes 50% response level. 0% is defined as basal ERK1/2 phosphorylation in the absence of *R*-PIA, whereas 100% is defined as the maximal response to a saturating concentration (100 nM) of *R*-PIA. Data represent the mean ± standard deviation of two experiments conducted in triplicate.

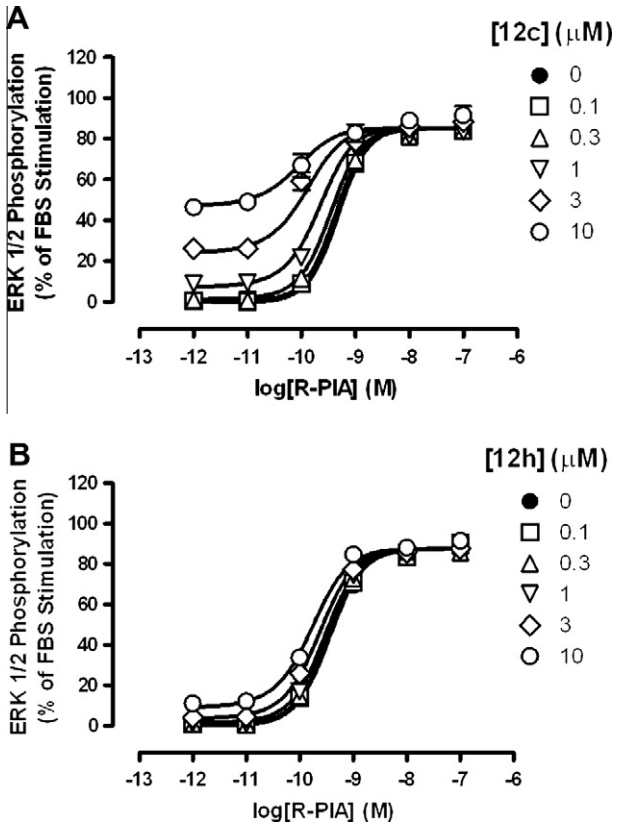


Figure 3. *R*-PIA mediated stimulation of ERK1/2 phosphorylation in the absence or presence of the indicated concentrations of test compounds in intact CHO FlpIn A_1-AR cells. Data represent the mean ± standard deviation of three experiments conducted in duplicate. Curves drawn through the points represent the best global fit of an allosteric model of ligand–receptor interaction.

appears as a weak allosteric agonist. To investigate this observation, we constructed complete *R*-PIA concentration–response curves in the absence or presence of increasing concentrations of two of the compounds. We chose **12c** as an allosteric enhancer with strong agonism and **12h** as an allosteric enhancer with weak agonsim. Increasing concentrations of **12c** promoted a leftward shift of the orthosteric agonist concentration–response curve, with concomitant strong (allosteric) agonist activity (Fig. 3A). In contrast, increasing concentrations of **12h** more modestly potentiated the *R*-PIA concentration–response curve, while exhibiting minimal allosteric agonism (Fig. 3B). Application of an operational model of allosterism[17,18] to the data yielded pK_B estimates of the affinity of the compounds **12c** and **12h** for the allosteric site of 5.10 ± 0.14 and 5.07 ± 0.17, respectively, and estimates of the positive cooperativity factors of the interactions with $\log \alpha\beta = 0.98 \pm 0.13$ (i.e., $\alpha\beta = 10$) and 0.46 ± 0.07 (i.e., $\alpha\beta = 3$), respectively. It appears, therefore, that, neither the type of substituent on the 3-benzoyl group nor the size of the cycloalkyl ring are the key points for engendering affinity to the allosteric site, but rather influence the cooperativity of these compounds exerted on *R*-PIA.

In conclusion, a novel series of 2-amino-4,5,6,7,8,9-hexahydrocycloocta[*b*]thiophenes were synthesised and evaluated as allosteric enhancers at the A_1-AR. As a consequence the assumption that increasing the cycloalkyl ring size to improve the allosteric effects did not hold. Also, as evidenced by the AlphaScreen plate-based assay, that increased allosterism is accompanied by antagonism. It is also evident that 3-benzoyl substitution is highly desirably and that methyl or trifluoromethyl groups on the 3-benzoyl are beneficial for the allosteric activity. All of the 2-aminothiophenes with amide and hydrazide functionality in the 3-position proved to be inactive in this functional assay.

Acknowledgments

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Supplementary data

Supplementary data (full experimental procedures including ^1H and ^{13}C NMR data of compounds **5a**–**f**, **7**, **9** and **12a**–**j**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.04.080.

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