Synthesis and Biological Evaluation of a New Series of 2-Amino-3-Aroyl Thiophene Derivatives as Agonist Allosteric Modulators of the A_1 Adenosine Receptor. A Position-Dependent Effect Study.

Romeo Romagnoli[a]*, Pier Giovanni Baraldi[a], Carlota Lopez Cara[a], Olga Cruz-Lopez[a], Allan R. Moorman[b,e], Arnault Massink[c], Adriaan P. IJzerman[c], Fabrizio Vincenzi[d], Pier Andrea Borea[d], Katia Varani[d]

[a]Dipartimento di Scienze Chimiche e Farmaceutiche, Via Fossato di Mortara 17-19, Università di Ferrara, 44121, Ferrara, Italy;

[b]King Pharmaceuticals Inc., Research and Development, 4000 CentreGreen Way, Suite 300, Cary, North Carolina 27513;

[c]Leiden Academic Centre for Drug Research, Division of Medicinal Chemistry, P. O. Box 9502, 2300 RA Leiden, The Netherlands;

[d]Dipartimento di Scienze Mediche, Sezione di Farmacologia, Università di Ferrara, Ferrara, Italy

* To whom correspondence should be addressed. Phone: 39-(0)532455303. Fax: 39-(0)532455953. E-mail:rmr@unife.it (R.R.).

Present Address:

[e]Alta Vetta Pharmaceutical Consulting, L.L.C., 3 Skipwith Court, Durham, North Carolina 27707

Abstract: The 2-amino-3-(*p*-chlorobenzoyl)thiophene scaffold has been widely employed as a pharmacophore for the identification of small molecules acting as allosteric modulators at the adenosine A_1 receptor. A new series of 2-amino-3-(*p*-chlorobenzoyl)-4-benzyl-5-arylthiophene derivatives, characterized by the absence as well as the presence of electron-releasing or electron-withdrawing groups on the phenyl ring at the 4- and 5-positions of the thiophene ring, were identified as positive allosteric enhancers at the adenosine A_1 receptor in binding (saturation, competition and dissociation kinetics) and functional assays. To better understand the positional requirements of substituents on the 2-amino-3-(*p*-chlorobenzoyl)thiophene core, the corresponding regioisomeric 4-aryl-5-benzylthiophene analogues were synthesized and found to possess reduced allosteric enhancer activity.

Keywords: A_1 adenosine receptor; allosteric modulator, G protein-coupled receptors; anti-nociceptive effect.

1. Introduction

Adenosine, an important tissue-protective endogenous hormone released from almost all cells, mediates its effect through binding interactions with four G protein-coupled receptors (GPCRs), named A_1, A_{2A}, A_{2B}, and A_3 [1]. Activation of the A_1 adenosine receptor (A_1AR) causes inhibition of the activity of the enzyme adenylate cyclase coupled by an inhibitory G-protein (Gi), reducing intracellular levels of cyclic adenosine monophosphate (cAMP) [2]. A_1ARs are highly and extensively expressed in brain, cortex, cerebellum and ippocampus and also present in other tissues such as kidney, fat cells, bladder, and heart [3]. As a consequence, the potential therapeutic applications of the A_1AR modulation have been investigated for the treatment of several diseases such as cardiovascular and central nervous system disorders and cancer [4]. In particular, selective A_1AR antagonists could be used as potential target for the treatment of Alzheimer's and Parkinson's diseases [5]. It is well known that the antiamnesic activities is exhibited by adenosine A_1AR antagonists which are effective in altering memory, promoting a facilitation and a recognition

memory impairment [6]. Moreover, A_1AR agonists are able to mediate sedative, anticonvulsant, anxiolytic, analgesic and locomotor depressant effects induced by adenosine [3, 7]. Thus, A_1AR agonist administration decreased nociceptive responses in different animal models of pain, suggesting their involvement in pain modulation. Although potent and selective A_1AR agonists have been developed, none are available for clinical use due to their cardiac and renal side-effects and poor accessibility to the central nervous system [7]. To address the problem of side effects, the approach of allosteric modulators for A_1ARs as potential therapeutic agents has been developed [8]. A positive allosteric enhancer (AE) is a molecule that binds to a receptor at a site topographically distinct from the orthosteric site that binds the natural ligand, amplifying the activity or increasing the affinity of an endogenous ligand or a synthetic agonist at its receptor [9]. The probable location of the allosteric site on the A_1AR, as well as the amino acids involved in the interaction of the allosteric binding site, are unknown [10]. Narlawar et al. speculated that the allosteric site of the A_1AR is located in close proximity to the orthosteric site [11].

AEs of the A_1AR amplify the action of adenosine locally released in high concentrations during ischemic, hypoxic or inflammatory events, presumably avoiding the side effects associated with the indiscriminate activation of the A_1AR throughout the body [12]. Hence, an AE might be useful therapeutically in hypoxic or ischemic tissues where it was essential to enhance locally the action of released adenosine [13]. AEs at the A_1AR may have great potential as analgesics and neuroprotective agents considering that anti-nociception represents one of the most important roles of A_1AR stimulation [14].

The 2-amino-3-aroylthiophene scaffold has been widely employed as a pharmacophore for the identification of molecules acting as allosteric modulators at the adenosine A_1 receptor [15]. It has already been observed that the presence of the 2-amino group and 3-benzoyl moiety seem to be essential parts of the molecule for allosteric enhancer activity at the A_1AR. In addition, the presence of a lipophilic substitution on the phenyl of the 3-benzoyl moiety, such as a 3-trifluoromethyl present in PD 81,723 (**1a**) [16a, 16b], the 3,4-dichloro in LUF5484 (**1b**) [16c], the 4-chloro in T62

(**1c**) [16a, 16b], the 3-chloro in PD 71,605 (**1d**) [16a, 16b] and the 4-methyl in **1e** [16c] favours allosteric enhancer activity at the A_1AR (Chart 1). Several research groups have highlighted the crucial importance of 4- and 5-thiophene substitution, modification of which allows the AE activity to be greatly modulated [17]. The 4-position is known to tolerate the presence of a wide variety of substituents, which include alkyl chains, phenyl rings with electron-releasing or electron-withdrawing groups (ERG's or EWG's, respectively) [17c] as well as a methylene unit linked to arylpiperazine [19] or *tert*-butyl [19] moieties (compounds with general structure **2** and **3**, respectively). Aryl/heteroaryl rings linked directly [18c, 19] or by an acetylene spacer [20] at the C-5 position of the thiophene nucleus, concomitant with substitution at the C-4 position, were tolerated and contributed additively to the AE activity, decreasing in the order aryl>H>arylethynyl and arylethynyl>aryl>H for compounds with general structures **2** and **3**, respectively. It has been previously shown that aryl rings at the 5-position, along with hydrogen or methyl substitution at the 4-position favoured antagonistic properties [21, 17f]. Many authors have postulated the existence of two different regions in the allosteric binding site of the A_1AR [16b, 22]. One region is able to accommodate the 2-amino and 3-benzoyl groups, which were found to be crucial for the AE activity. A second wide, hydrophobic domain is able to interact with alkyl and aryl substituents at the C-4 and C-5 positions of thiophene ring.

Thus, once the 2-amino-3-(*p*-chlorobenzoyl) thiophene scaffold was identified as the minimum structural requirement for the AE activity, and the presence of hydrophobic substituents, such as aryl moieties at C-5, was deemed critical for optimal interaction with the allosteric site of the A_1AR, our strategy for further improvement of the activity was to perform modification at the 4-position of the thiophene ring. From the previously described series of 2-amino-3-(*p*-chlorobenzoyl)-4-neopentyl-5-arylthiophene structure (**3**) [19], the first round of optimization included replacement of the neopentyl moiety with a benzyl group, to furnish a new series of 2-amino-3-(*p*-chlorobenzoyl)-4-(substituted)benzyl-5-arylthiophene analogues, exemplified by structure **4a** (Chart 2).

Replacing the phenyl group of the 4-benzyl moiety with the more lipophilic and sterically more bulky naphth-1-yl group led to compound **4b**. For compounds **4c-m**, we fixed the aryl group at the C-5 position of the thiophene ring as an unsubstituted phenyl, and focused modifications on the phenyl of the benzyl moiety at the C-4 position by adding electron-withdrawing (F, Cl, CF_3 and OCF_3) and electron-releasing (CH_3, alkoxy and SCH_3) groups. To analyze the effects of additional methyl and methoxy groups on the 4-benzyl moiety, we synthesized analogues **4n-o** and **4p-q**, respectively. In addition to an unsubstituted phenyl moiety at C-5 of the thiophene nucleus (compounds **4a**-**q**), we have also introduced substituents on the C-5 phenyl with opposite electronic effects, including halogens (fluorine and chlorine for compounds **4r-w** and **4x-ab**, respectively) and methoxy (derivatives **4ac-af**).

We continued to explore the potential of molecules of this type by the synthesis of a second series of analogues with general structure **5**, containing a phenyl moiety at the 4-positions of the 2-amino-3-(*p*-chlorobenzoyl)thiophene core and various substituted benzyl groups at the 5-position (Chart 2). These latter molecules can be viewed as positional isomers of the compounds with general formulae **4**, which are characterized by the presence of a benzyl moiety at the 4-position and a phenyl group at the 5-position of the thiophene nucleus, respectively.

2. Chemistry

The 2-amino-3-(*p*-chlorobenzoyl)-4-benzyl-5-arylthiophene derivatives **4a-af** were prepared following the procedure reported in the Scheme 1. The 4-bromomethyl thiophenes **6a-d** [23] were subjected to palladium-catalyzed cross-coupling conditions in the presence of the appropriate heterogeneous conditions [$Pd(Ph_3P)_4$, K_2CO_3] in toluene at 100 °C to yield the 4-benzylthiophene analogues **7a-af**, which were transformed to the final compounds **4a-af** by treatment with hydrazine in ethanol at reflux.

The isomeric 2-amino-3-(*p*-chlorobenzoyl)-4-phenyl-5-benzylthiophene derivatives **5a-f** were synthesized by the approach shown in Scheme 2. Phthaloyl protection of the 2-amino group of **8** [19] with phthalic anhydride in acetic acetic at reflux furnished almost quantitatively the

phthalimido derivative **9**. Subsequent bromination with NBS and a catalytic amount of benzoyl peroxide in refluxing CCl_4 afforded the 5-bromomethyl analogue **10**, used as a common building block for the synthesis of the final compounds **5a-f** by the same two-step procedure employed for the synthesis of compounds **4a-af**. The intermediate **10** was coupled with the appropriate arylboronic acid by a standard Suzuki cross-coupling reaction to furnish the 5-benzylthiophene derivatives **11a-i**, followed by the removal of the phthaloyl protecting group with hydrazine in refluxing ethanol to afford final compounds **5a-i**.

3. Biological Results and Discussion

3.1. Functional assays.

The allosteric enhancement by the 4-benzyl-5-arylthiophenes **4a-af** and the corresponding regioisomeric analogues **5a-i** was initially measured using a functional assay, evaluating their ability to inhibit forskolin-stimulated cAMP accumulation in CHO cells expressing the cloned hA_1AR. Activation of this receptor causes a measurable inhibition of adenylate cyclase activity and a reduction of cAMP content in the CHO cells. AEs are thought to act by stabilizing the active conformation of the A_1AR, leading to a reduction in the cAMP content of the cells [24].

The reference compounds **1a** (PD 81,723), **2a**, and **3a**, along with the new synthesized derivatives **4a-af** and **5a-i** were tested at a concentration of 10 μM alone and at a 100-fold lower concentration (100 nM) in the presence of the orthosteric agonist CCPA (1 pM) to assess enhancement of the A_1AR agonist activity. Figures 1A and 1B show histograms of the reduction in cAMP levels mediated by PD 81,723, as well as representative compounds **4g**, **4j**, and **4z**, at the two aforementioned concentrations (10 μM alone and 100 nM in the presence of CCPA, respectively). A reduction in cAMP content is indicated in Table 1 as a percentage inhibition of cAMP production relative to control (absence of the test compound), in the absence or presence of the orthosteric agonist. CCPA alone caused a slight reduction of cAMP content in CHO cells by activation of A_1ARs (data not shown). The degree of inhibition of cAMP production by each of the

novel AEs was similar under the two conditions tested. The novel allosteric enhancers are thought to shift the A_1 adenosine receptor from the inactive to the active conformational state, presumably increasing the constitutive activity of the receptors and inhibiting the forskolin-induced cAMP production, even in the absence of the orthosteric agonist.

In the series of 5-phenylthiophene derivatives **4a-q**, the data shown in Table 1 indicate that the activity of the AEs can be influenced by steric, electronic, and lipophilic effects of substituents on the 4-benzyl moiety. An increase of lipophilicity, by replacement of the phenyl of the benzyl moiety in **4a** with a naphth-1-yl (**4b**), was tolerated and maintained the percent reduction of cAMP production (51% and 58% of control, respectively). Introduction of either electron-releasing or electron-withdrawing groups at the *para*-position on the phenyl of the 4-benzyl moiety (**4c-k**) was found to have beneficial effects on the activity, producing an enhanced reduction of cAMP content compared with the unsubstituted benzyl analogue **4a**, with the 4-(*p*-methoxybenzyl)thiophene **4g** as one of the most active compounds.

Starting from the 4-benzyl-5-phenylthiophene derivative **4a**, introduction of the electron-withdrawing fluorine atom (**4c**) increased the inhibition of cAMP production, which was further improved increasing the size of the halogen from fluorine to chlorine (**4d**). The presence of electron-releasing groups, such as methyl (**4e**) or methoxy (**4g**), also enhanced potency relative to the unsubstituted 4-benzyl derivative **4a**, and this effect was more evident for *p*-methoxy derivative **4g**. The position and the number of methoxy groups on the phenyl of the benzyl moiety had influence on the AE activity, which was reduced by moving the methoxy moiety from the *para*- to *ortho*-position (compound **4m**). Because the electronic properties of **4g** and **4m** are similar, the reduced activity seen with **4m** is presumably due to steric factors, caused by the methoxy group at the *ortho*-position of the phenyl ring. Since the *p*-methoxy substitution of **4g** was favorable for potency, we examined the effect on activity caused by the introduction of additional electron-releasing methyl or methoxy groups. The insertion of a second and especially, a third strongly electron-releasing methoxy group, to furnish the corresponding *o*, *p*-dimethoxy (**4p**) and *m*, *m'*, *p*-

trimethoxy (**4q**) derivatives, caused a reduction in the percent inhibition of cAMP production relative to **4g**. The reduction of potency was less pronounced by the introduction of one or two additional methyl moieties on the *meta*-positions, to furnish compounds **4n** and **4o**, respectively, suggesting that the reduced activity observed with compounds **4o-q** may be accounted for largely by an increase in the steric hindrance at the *ortho*- and *meta*-positions on the phenyl of the 4-benzyl moiety.

Replacement of the weakly electron-releasing methyl group (**4e**) by the electron-withdrawing trifluoromethyl moiety (**4f**), with opposite electronic effects and a larger steric effect, caused a substantial increase in the inhibition of cAMP production. By substitution of the trifluoromethyl moiety in **4f** with the bulkier trifluoromethoxy group (**4h**) the AE activity was maintained.

In an effort to further understand the steric effects of the alkoxy substituent at the *para*-position of the 4-benzyl moiety, replacing the methoxy with the ethoxy homologue (**4j**) or a bulkier isopropoxy moiety (**4k**) largely maintained the potency. Replacement of the methoxy group with the electronically neutral, but more lipophilic and larger methylthio moiety resulted in derivative (**4i**) with somewhat reduced activity relative to **4g**.

Compounds **4r-af** examined the effect of adding an electron-withdrawing (F and Cl) or electron-releasing (methoxy) group in the *para*-position of the phenyl moiety at the C-5 position of the thiophene ring. In general, compounds that possessed a *p*-chlorophenyl group in the 5-position (**4x-ab**) showed greater activity than the corresponding compounds with a *p*-fluorophenyl or *p*-methoxyphenyl moiety at the C-5 position when the C-4 benzyl substituent was held constant (i.e. **4x** *vs*. **4r** and **4ac**, **4y** *vs*. **4s**, **4z** *vs*. **4t** and **4ad**, **4ab** *vs*. **4v** and **4ae**). Interestingly, this analysis becomes more complicated when the corresponding compounds with an unsubstituted phenyl group at C-5 are included. If the C-4 benzyl moiety contains an electron-withdrawing substituent in the *para*-position, then the 5-phenyl compounds (**4c** and **4d**) were found to have activities intermediate between the C-5 *p*-chlorophenyl (**4y** and **4z**) and *p*-fluorophenyl (**4s** and **4t**) derivatives. Comparing the C-5 *p*-fluorophenylthiophene derivatives **4r-v** with the *p*-chlorophenyl

counterparts **4x**-**ab**, it can be suggested that increasing the size of the halide leads to a large increase in the percent inhibition of cAMP production, from 45-59% to 69-81%, with the greatest difference observed between **4t** and **4z**. However, by including the derivatives bearing an unsubstituted phenyl at the C-5 position, it can be seen that factors beyond the steric bulk of the *para*-substituents are influencing AE activity. The results also showed that, with the notable exception of 4-(*p*-methylthiobenzyl) derivatives **4i**, **4w** and **4af**, the derivatives bearing a *p*-methoxyphenyl moiety at C-5 of the thiophene ring were generally less active than the corresponding phenyl and *p*-fluorophenyl counterparts (**4ac** *vs.* **4a** and **4r**, **4ad** *vs.* **4d** and **4t**, **4ae** *vs.* **4g** and **4v**).

Among the 5-(*p*-fluorophenyl)thiophene derivatives **4r-w**, replacement of the *p*-methylbenzyl moiety at the 4-position of the thiophene ring with a *p*-methoxybenzyl (compounds **4u** and **4v**, respectively) had little effect on AE activity. However, replacing the electron-releasing *p*-methoxy or *p*-methyl groups on the C-4 benzyl with hydrogen (**4r**), the sterically more demanding, but electronically neutral methylthio group (**4w**), or an electron-withdrawing halogen (F and Cl for **4s** and **4t**, respectively) led to a reduction in activity, which was more evident for compound **4s**. In contrast, the potency of the 5-(*p*-methoxyphenyl)thiophene derivative with a 4-(*p*-methylthiobenzyl) moiety (**4af**) was substantially greater than seen with the derivatives containing benzyl (**4ac**), *p*-chlorobenzyl (**4ad**) or *p*-methoxybenzyl (**4ae**) groups. For the C-5 *p*-(chlorophenyl)thiophene analogues **4x**-**ab**, the insertion of methyl or methoxy moieties on the *para*-position of the phenyl of the 4-benzyl moiety was tolerated and maintained the AE activity (compounds **4aa** and **4ab**, respectively) relative to the unsubstituted benzyl derivative **4x**, while the presence of *p*-fluoro and *p*-chloro for the corresponding derivatives **4y** and **4z**, was associated with increased AE activity.

The contribution of the phenyl and benzyl groups at the 4- and 5-positions of the 2-amino-3-(*p*-chlorobenzoyl)thiophene scaffold was clearly position dependent and their relative position was critical for the AE activity. Placing the benzyl group at the C-5 position of the thiophene ring

resulted in a series of compounds (**5a-i**) which proved to be from 1.5 to 2.5-fold less efficacious in inhibiting cAMP production than the corresponding isomeric 4-benzyl counterparts (i.e. **5a** *vs.* **4a**, **5b** *vs.* **4b**, **5c** *vs.* **4f**, **5d** *vs.* **4g**, **5e** *vs.* **4l**, **5f** *vs.* **4h**, **5g** *vs.* **4j**, **5h** *vs.* **4k**, **5i** *vs.* **4n**). It is clear that the AE activity is not regulated by the simple presence of hydrophobic groups, regardless of location, but by the presence of specific groups at specific positions. In summation, the SAR studies showed that the most active derivatives had a substituted benzyl along with a phenyl or a *p*-chlorophenyl in the 4- and 5-positions, respectively, on the 2-amino-3-(*p*-chlorobenzoylthiophene scaffold.

In comparing the effects of ERG's and EWG's on the phenyl of the benzyl moiety at the C-4 position of the thiophene ring, no consistent difference in influence on allosteric enhancement activity occurred. In fact, several compounds, characterized by the presence of substituents with opposite electronic effects, showed the same potency. For example, compound **4f** containing the strongly electron-withdrawing trifluoromethyl group showed an activity little different from that of compound **4g** containing the electron-releasing methoxy group.

Among the newly synthesized compounds **4a-af** and **5a-i**, the most efficacious and potent derivative was **4z**, which possessed *p*-chlorobenzyl and *p*-chlorophenyl substituents in the 4- and 5-positions of 2-amino-3-(*p*-chlorobenzoyl)thiophene core, respectively. Comparing **4z** to reference compounds **2a** and **3a,** each characterized by the presence of a common 2-amino-3-(*p*-chlorobenzoyl)-5-(*p*-chlorophenyl)thiophene skeleton and differing only in their 4-substituent, it appeared that replacing the 4-neopentyl moiety of **3a** with a *p*-chlorobenzyl (**4z**) led to a 1.5-fold increase in the percent inhibition of cAMP, while **4z** showed activity comparable to that of the 4-(*p*-chlorophenyl)piperazin-1-ylmethylene derivative **2a**. All of the 4-benzyl-5-arylthiophene derivatives **4a** and **4c-af** were from 3- to 4-fold more potent than PD 81,723 at the concentration tested and reduced the amount of cAMP production from 43% to 84%. The isomeric 4-phenyl-5-benzyl derivatives showed AE activity as high as that of PD 81,723, with several compounds (**5d**, **5e** and **5i**) having substantially higher activity.

3.2. Antagonistic activity.

The therapeutic potential of many of the currently available allosteric enhancers of agonist binding to the hA_1AR can be hampered by several non-specific actions, which include the propensity to cause antagonism at the A_1 adenosine receptor, especially at higher concentrations. The ability of compounds **4a-af** and **5a-i** to displace the binding of [^{3}H]DPCPX, [^{3}H]ZM241385 and [^{3}H]MRE-3008-F20 at human A_1, A_{2A} and A_3 ARs was evaluated in CHO cells at a concentration of 10 μM. The prototype enhancer PD 81,723 did not inhibit the binding of the radiolabeled antagonists to A_1 and A_{2A} ARs, but at 10 μM, it reduced by 21% the binding of [^{3}H]MRE-3008-F20 to hA_3ARs [25]. None of the examined derivatives significantly inhibited the specific binding of the radioligands to A_1, A_{2A} and A_3ARs, causing inhibition of radioligand binding of 10% or less (data not shown). For the most active compounds in functional assays, such as **4f-h**, **4j-n**, and **4x-ab**, it was possible to achieve a good separation between high efficacy in the inhibition of cAMP production and binding at the orthosteric site.

3.3. Effect of enhancers on A_1 AR binding parameters.

Saturation and competition experiments of the selective adenosine A_1 agonist [^{3}H]CCPA to A_1 receptors were performed to determine if the novel compounds modified the agonist binding parameters. From these experiments, A_1 receptor affinity (K_D) and density (Bmax) were evaluated in the presence and in the absence of the examined compounds [PD 81,723 (**1a**), **2a**, **3a**, **4a-af**, and **5a-i** at a concentration of 10 μM] and were used to calculate the increase of A_1 density (Bmax shift) (Table 2). The reference compound PD 81,723 induced a Bmax shift to human A_1 adenosine receptors of 1.3-fold. Under the same experimental conditions, all of the newly synthesized compounds were more potent than PD 81,723. From the receptor density calculated in the presence and in the absence of the novel enhancers, the derivatives **4b-p, 4t-ab**, **4ae**, and **4af** were the most active compounds, each causing a Bmax shift of more than five-fold, with **4z** causing an eleven-fold shift. Figure 2 shows the effect of the allosteric modulators PD 81,723, **4g**, **4j** and **4z** at 10 μM

concentration in [^{3}H]CCPA saturation binding experiments on A_1AR binding parameters such as affinity and density. Interestingly, no differences were found in affinity values, suggesting that the enhancers were not able to modify the K_D values of the high affinity binding sites labeled by [^{3}H]CCPA (K_D ranged from 1.0 ± 0.1 to 1.2± 0.1).

Table 2 also reports the derived apparent affinity (Ki) values for CCPA, based on a one-state model of analysis, in the absence and in the presence of the tested enhancers. This table also shows the CCPA shift, representing the ratio of apparent Ki values in the absence and in the presence of the tested compounds at 10 μM concentration. In the hA_1CHO membranes, by using [^{3}H]DPCPX as radioligand, the Ki value of CCPA was 15.1 ± 1.4 nM. Interestingly, a significant decrease in the apparent Ki value was due to the presence of the putative allosteric enhancers, suggesting an increase in the high-affinity binding sites. In the presence of PD 81,723, the affinity of CCPA increased by 1.4-fold. With the exception of compounds **5b-c** and **5g**, the CCPA affinity data in the presence of the newly synthesized derivatives **4a-af** and **5a-i** reveals that the displacement curves are shifted left, suggesting even lower Ki values for CCPA. In particular, the largest affinity shift was observed for compounds **4f-g, 4j** and **4x-z.** These molecules enhanced the apparent affinity of CCPA approximately from 8- to 11-fold, with the most active compound of the series being **4z**, which appeared three-fold more potent than the 4-neopentyl derivative **3a** in this assay (Table 2). Comparing the CCPA affinity data of compounds **3a** and **4z**, we hypothesize that the phenyl ring of the benzyl moiety may establish a π–π interaction with a specific amino acid residue located in the hydrophobic domain at the allosteric site surrounding the C-4 position of the thiophene ring. Compound **4z** also induced a higher apparent CCPA affinity than the corresponding arylpiperazine derivative **2a**, with Ki values of 1.3±0.1 and 1.8±0.1 nM, respectively. Thus, the enhancers were able to mediate a shift of the A_1 receptors toward the high affinity state, as suggested by the increase of the CCPA affinity expressed as Ki values (Table 2). In Figure 3, representative binding curves for the displacement of [^{3}H]DPCPX by different concentrations of CCPA alone and in the presence of PD 81,723, **4g**, **4j** and **4z** at 10 μM concentration are shown, demonstrating an

apparent increase of the CCPA binding affinity in the presence of these novel enhancers. Starting from the unsubstituted 4-benzyl derivative **4a**, the addition of substituents with opposite electronic effects at the phenyl of the benzyl moiety, such as electron-withdrawing trifluoromethyl and electron-releasing methoxy moieties for compounds **4f** and **4g**, respectively, resulted in a 2-fold increase of apparent CCPA affinity. The results obtained from the competition and saturation experiments confirmed that most of the newly synthesized compounds had good enhancer activity at the A_1AR, in agreement with the data from the cAMP functional assay.

3.4. Effect of 4z on kinetic parameters of agonist binding to A_1AR.

Association and dissociation experiments were performed on hA_1AR with the agonist radioligand [^{3}H]NECA. From these experiments, association and dissociation rates (k_{on} and k_{off}, respectively) were calculated in the presence and absence of PD81,723 and **4z** at a concentration of 1 μM (Fig. 4 and Table 3). Alteration of the dissociation kinetics of ligand binding from the orthosteric site indicates an allosteric effect by the added compound. PD81,723 was used as a reference allosteric modulator, which is known to enhance agonist binding at a concentration of 10 μM [16a]. The association rate constant of [^{3}H]NECA was not influenced by either 1 μM PD81,723 or **4z**. The dissociation rate constant of [^{3}H]NECA was not changed significantly by 1 μM PD81,723 either. However, in the presence of 1 μM of compound **4z**, the dissociation rate constant of [^{3}H]NECA decreased by 2.5-fold. Additionally, the affinity of [^{3}H]NECA was derived from the association and dissociation rate constants, yielding the kinetic K_D. In the presence of 1 μM PD81,723 the affinity of the radioligand was not changed, whereas in the presence of 1 μM **4z,** the affinity of [^{3}H]NECA increased by 2.3-fold, These results confirm the allosteric enhancing effect of **4z** on agonist binding at the hA_1AR.

3.5. Formalin assay.

To investigate the potential therapeutic properties of the novel A_1AR allosteric enhancers, the most active compound of the series (**4z**) was also tested in the formalin test, a widely used model for the

study of nociception. Administration of **4z** resulted in a significant suppression of pain behaviours relative to vehicle-treated mice at doses of 0.3 and 3 mg/kg, with a peak inhibition of nociceptive responses of 49% and 82%, respectively (Fig. 5). The data obtained in the formalin assay confirmed the potential anti-nociceptive effect of the novel A_1AR allosteric enhancers.

4. Conclusions

In summary, two different regioisomeric series of 4,5-disubstituted 2-amino-3-(*p*-chlorobenzoyl) thiophene derivatives, with general structures **4** and **5**, were prepared and evaluated as AEs at the A_1AR. These two series of analogues were designed to evaluate the effect of interchanging the benzyl and phenyl substituents at the C-4 and C-5 positions of the thiophene ring. Starting with an unsubstituted benzyl at C-4 and an unsubstituted phenyl at C-5 (**4a**), structural optimization was conducted by introducing a number of ERG's or EWG's on the phenyl group of the benzyl moiety at the C-4 position. A range of substituted phenyl groups in the C-5 position was also evaluated, with R_5 as an unsubstituted, *p*-fluoro, *p*-chloro, or *p*-methoxy phenyl. The data show no clear steric or electronic effects for substituents on the benzyl moiety at C-4, except that the introduction of substituents in the *ortho*- or *meta*-position or the presence of multiple substituents on the phenyl ring of the benzyl group led to the least active compounds **4l**, **4m**, and **4o-q**.

We have also shown that the appropriate orientation of substituents at the 4- and 5-positions of the thiophene nucleus was fundamental to the AE activity. The relative positions of the benzyl and phenyl moieties on the 2-amino-3-(*p*-chlorobenzoyl)thiophene scaffold were critical for optimal AE activity, with the 4-benzyl-5-arylthiophene derivatives more potent than 4-aryl-5-benzylthiophene counterparts. This may suggest that the introduction of a methylene spacer between the phenyl ring and the 5-position of the thiophene nucleus was detrimental for the AE activity.

A characteristic feature of many AEs at the A_1AR is a propensity to cause antagonism at higher concentrations. None of the new synthesized molecules **4a-af** and **5a-i** significantly inhibited antagonist binding at the hA_1AR, hA_2AR, or hA_3AR. Among these, derivatives **4f-g**, **4j** and **4x-aa**

were the most active compounds in binding (saturation and displacement) experiments and functional cAMP assays. Compound **4z** slowed agonist dissociation from the hA_1AR, without affecting agonist association, confirming its allosteric enhancing effect on this receptor. When tested in the formalin assay, compound **4z** showed a good anti-nociceptive effect, confirming that AEs at the A_1AR could be useful in pain modulation.

5. Experimental Section

5.1. Chemistry.

5.1.1. Materials and Methods.

^{1}H-NMR and ^{13}C-NMR spectra were determined in $CDCl_3$ or d_6-DMSO solutions and recorded with a Varian VXR-200 spectrometer or a Varian Mercury Plus 400 spectrometer. Chemical shifts (δ) are given in parts per million (ppm) downfield and *J* values are given in hertz. Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing ESI Micromass ZMD 2000 mass spectrometer. Melting points (mp) were determined on a Buchi-Tottoli apparatus and are uncorrected. Elemental analyses were conducted by the Microanalytical Laboratory of the Chemistry Department of the University of Ferrara and were performed on a Yanagimoto MT-5 CHN recorder analyzer. All tested compounds yielded data consistent with a purity of at least 95% as compared with the theoretical values. All reactions were performed under an inert atmosphere of dry nitrogen, unless otherwise described. Reaction courses and product mixtures were routinely monitored by TLC on silica gel (precoated F_{254} Merck plates) and visualized with aqueous $KMnO_4$. Flash chromatography was performed using 230-400 mesh silica gel and the solvent system indicated in the procedure. All commercially available compounds were used without further purification. Organic solutions were dried over anhydrous Na_2SO_4. Petroleum ether refers to the fraction boiling at 40-60 °C.

5.1.2. General procedure (A) for the synthesis of compounds 7a-af and 11a-i.

A mixture of thiophene derivatives **6a-d** or **10** (1 mmol), K_2CO_3 (207 mg, 1.5 mmol, 1.5 equiv.), the appropriate aryl boronic acid (2 mmol, 2 equiv.) and tetrakis(triphenylphosphine)palladium (28 mg, 0.024 mmol) in dry toluene (10 mL) was stirred at 100 °C under nitrogen for 18 h. After cooling to room temperature, the reaction mixture was diluted with methylene chloride (10 mL), filtered through a pad of Celite, and the combined filtrates concentrated. The residue was dissolved with methylene chloride (20 mL), and the resulting solution was washed sequentially with 5% $NaHCO_3$ (5 mL), water (5 mL), and brine (5 mL). The organic layer was dried, filtered, and evaporated, and the residue was purified by flash chromatography on silica gel.

5.1.2.1. 2-[4-Benzyl-3-(4-chlorobenzoyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7a). Following general procedure A, after workup as described previously, the crude residue was purified by column chromatography on silica gel (eluent EtOAc-petroleum ether 1.5-8.5), to furnish the desired product **7a** as a yellow solid. Yield: 71%, mp 195-196 °C. ^{1}H NMR ($CDCl_3$) δ: 4.18 (s, 2H), 6.91 (d, J=8.6 Hz, 2H), 7.05 (m, 2H), 7.08 (d, J=8.6 Hz, 2H), 7.43 (m, 4H), 7.53 (m, 4H), 7.72 (m, 4H). MS (ESI): $[M+1]^+$=535.2.

5.1.2.2. 2-[3-(4-Chlorobenzoyl)-4-(1-naphthylmethyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7b). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent ethyl ether), furnished the desired product **7b** as a colour cream solid. Yield: 79%, mp 253-255 °C. ^{1}H NMR ($CDCl_3$) δ: 4.61 (s, 2H), 6.96 (d, J=8.4 Hz, 2H), 7.10 (d, J=7.2 Hz, 1H), 7.22 (d, J=7.2 Hz, 1H), 7.28 (d, J=8.8 Hz, 2H), 7.35 (d, J=8.8 Hz, 2H), 7.42 (m, 8H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=585.1.

5.1.2.3. 2-[3-(4-Chlorobenzoyl)-4-(4-fluorobenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7c). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 3-7), furnished the desired product **7c** as a white solid. Yield: 67%, mp 74-76 °C. ^{1}H NMR ($CDCl_3$) δ: 4.14 (s, 2H), 6.74 (m,

4H), 7.10 (d, J=8.8 Hz, 2H), 7.43 (d, J=8.8 Hz, 2H), 7.51 (m, 5H), 7.76 (m, 4H). MS (ESI): $[M+1]^+$=553.1

5.1.2.4. 2-[3-(4-Chlorobenzoyl)-4-(4-chlorobenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7d). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 2-8), furnished the desired product **7d** as a yellow oil. Yield: 57%. ^{1}H NMR ($CDCl_3$) δ: 4.13 (s, 2H), 6.82 (d, J=8.6 Hz, 2H), 7.02 (d, J=8.6 Hz, 2H), 7.10 (d, J=8.6 Hz, 2H), 7.43 (m, 7H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=569.5.

5.1.2.5. 2-[3-(4-Chlorobenzoyl)-4-(4-methylbenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7e). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7e** as a yellow solid. Yield: 63%, mp 118-120 °C. ^{1}H NMR ($CDCl_3$) δ: 2.15 (s, 3H), 4.12 (s, 2H), 6.77 (d, J=8.8 Hz, 2H), 6.83 (d, J=8.8 Hz, 2H), 7.10 (d, J=8.8 Hz, 2H), 7.41 (d, J=8.8 Hz, 2H), 7.44 (m, 3H), 7.52 (m, 2H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=549.2.

5.1.2.6. 2-{3-(4-Chlorobenzoyl)-5-phenyl-4-[4-(trifluoromethyl)benzyl]-2-thienyl}-1H-isoindole-1,3(2H)-dione (7f). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 2-8), furnished the desired product **7f** as a yellow oil. Yield: 95%. ^{1}H NMR ($CDCl_3$) δ: 4.22 (s, 2H), 6.92 (d, J=8.4 Hz, 2H), 7.11 (d, J=8.4 Hz, 2H), 7.26 (d, J=8.8, Hz, 2H), 7.38 (d, J=8.8 Hz, 2H), 7.48 (m, 5H), 7.73 (m, 4H). MS (ESI): $[M+1]^+$=603.1.

5.1.2.7. 2-[3-(4-Chlorobenzoyl)-4-(4-methoxybenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7g). Following general procedure A, after workup as described previously, the crude residue purified purified by column chromatography (eluent EtOAc-petroleum ether 2-8), furnished the desired product **7g** as a white solid. Yield 73%, mp 83-85 °C. ^{1}H NMR ($CDCl_3$) δ: 3.65 (s, 3H),

4.09 (s, 2H), 6.60 (d, J=8.0 Hz, 2H), 6.76 (d, J=8.0 Hz, 2H), 7.10 (d, J=8.8 Hz, 2H), 7.43 (d, J=7.6 Hz, 2H), 7.44 (m, 5H), 7.76 (m, 4H). MS (ESI): $[M+1]^+$=565.2.

5.1.2.8. 2-{3-(4-Chlorobenzoyl)-5-phenyl-4-[4-(trifluoromethoxy)benzyl]-2-thienyl}-1H-isoindole-1,3(2H)-dione (7h). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7h** as a white solid. Yield 83%, mp 154-156 °C. ^{1}H NMR ($CDCl_3$) δ: 4.17 (s, 2H), 6.89 (d, J=8.0 Hz, 2H), 7.06 (d, J=8.0 Hz, 2H), 7.36 (d, J=8.8 Hz, 2H), 7.44 (m, 7H), 7.76 (m, 4H). MS (ESI): $[M+1]^+$=619.1.

5.1.2.9. 2-[3-(4-Chlorobenzoyl)-4-(4-methylthiobenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7i). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7i** as a yellow solid. Yield 84%, mp 187-189 °C. ^{1}H NMR ($CDCl_3$) δ: 2.33 (s, 3H), 4.11 (s, 2H), 6.82 (d, J=8.4 Hz, 2H), 6.93 (d, J=8.4 Hz, 2H), 7.09 (d, J=8.6 Hz, 2H), 7.44 (d, J=8.6 Hz, 2H), 7.44 (m, 5H), 7.73 (m, 4H). MS (ESI): $[M+1]^+$=581.2.

5.1.2.10. 2-[3-(4-Chlorobenzoyl)-4-(4-ethoxybenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7j). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 2-8), furnished the desired product **7j** as a white solid. Yield: 68%, mp 89-90 °C. ^{1}H NMR ($CDCl_3$) δ: 1.29 (t, J=7.2 Hz, 3H), 3.85 (q, J=7.2 Hz, 2H), 4.08 (s, 2H), 6.55 (d, J=8.8 Hz, 2H), 6.75 (d, J=8.8 Hz, 2H), 7.08 (d, J=8.8 Hz, 2H), 7.42 (d, J=8.8 Hz, 2H), 7.44 (m, 5H), 7.72 (m, 4H). MS (ESI): $[M+1]^+$=579.1.

5.1.2.11. 2-[3-(4-Chlorobenzoyl)-4-(4-isopropoxybenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7k). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7k** as a white solid. Yield 81%, mp 99-101 °C. ^{1}H NMR ($CDCl_3$) δ: 1.17 (d, J=6.2

Hz, 6H), 4.08 (s, 2H), 4.34 (m, 1H), 6.52 (d, J=8.6 Hz, 2H), 6.73 (d, J=8.6 Hz, 2H), 7.05 (d, J=8.6 Hz, 2H), 7.44 (d, J=8.6 Hz, 2H), 7.53 (m, 5H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=593.3.

5.1.2.12. 2-[3-(4-Chlorobenzoyl)-4-(3-methoxybenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7l). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7l** as a white solid. Yield 72%, mp 180-182 °C. ^{1}H NMR ($CDCl_3$) δ: 3.59 (s, 3H), 4.14 (s, 2H), 6.48 (m, 2H), 6.96 (t, J=8.4 Hz, 1H), 7.10 (d, J=8.2 Hz, 2H), 7.42 (d, J=8.2 Hz, 2H), 7.45 (m, 4H), 7.51 (m, 2H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=565.3.

5.1.2.13. 2-[3-(4-Chlorobenzoyl)-4-(2-methoxybenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7m). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7m** as a yellow solid. Yield 67%, mp 223-225 °C. ^{1}H NMR ($CDCl_3$) δ: 3.54 (s, 3H), 4.02 (s, 2H), 6.63 (t, J=8.4 Hz, 1H), 6.78 (d, J=8.4 Hz, 2H), 7.00 (m, 1H), 7.32 (d, J=8.4 Hz, 2H), 7.39 (d, J=8.4 Hz, 2H), 7.52 (m, 5H), 7.83 (m, 4H). MS (ESI): $[M+1]^+$=565.2.

5.1.2.14. 2-[3-(4-Chlorobenzoyl)-4-(4-methoxy-3-methylbenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7n). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7n** as a white solid. Yield 68%, mp 100-102 °C. ^{1}H NMR ($CDCl_3$) δ: 2.01 (s, 3H), 3.73 (s, 3H), 4.07 (s, 2H), 6.62 (d, J=7.2 Hz, 1H), 6.74 (m, 1H), 6.81 (s, 1H), 7.10 (d, J=8.6 Hz, 2H), 7.44 (m, 7H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=579.2.

5.1.2.15. 2-[3-(4-Chlorobenzoyl)-4-(3,5-dimethyl-4-methoxybenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7o). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 2-8), furnished the desired product **7o** as a yellow oil. Yield 63%. ^{1}H NMR ($CDCl_3$) δ: 2.00 (s, 6H),

4.03 (s, 3H), 4.74 (s, 2H), 6.45 (s, 2H), 7.12 (d, J=8.8 Hz, 2H), 7.22 (d, J=8.8 Hz, 2H), 7.49 (m, 5H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=593.1.

5.1.2.16. 2-[3-(4-Chlorobenzoyl)-4-(2,4-dimethoxybenzyl)-5-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (7p). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7p** as a yellow solid. Yield 58%, mp 92-94 °C. ^{1}H NMR ($CDCl_3$) δ: 3.48 (s, 3H), 3.67 (s, 3H), 3.96 (s, 2H), 6.18 (m, 3H), 6.64 (d, J=8.6 Hz, 2H), 7.12 (d, J=8.8 Hz, 2H), 7.38 (m, 4H), 7.52 (d, J=8.8 Hz, 1H), 7.78 (m, 4H). MS (ESI): $[M+1]^+$=595.2.

5.1.2.17. 2-[3-(4-Chlorobenzoyl)-5-phenyl-4-(3,4,5-trimethoxybenzyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7q). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 2-8), furnished the desired product **7q** as a white solid. Yield 52%, mp 80-82 °C. ^{1}H NMR ($CDCl_3$) δ: 3.57 (s, 6H), 3.66 (s, 3H), 4.08 (s, 2H), 5.99 (s, 2H), 7.14 (d, J=8.6 Hz, 2H), 7.46 (d, J=8.6 Hz, 2H), 7.53 (m, 5H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=625.2.

5.1.2.18. 2-[4-Benzyl-3-(4-chlorobenzoyl)-5-(4-fluorophenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7r). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 2-8), furnished the desired product **7r** as a yellow solid. Yield 62%, mp 196-198 °C. ^{1}H NMR ($CDCl_3$) δ: 4.12 (s, 2H), 6.86 (d, J=6.4 Hz, 2H), 7.00 (d, J=6.4 Hz, 2H), 7.06 (d, J=6.4 Hz, 2H), 7.08 (d, J=6.4 Hz, 2H), 7.44 (m, 5H), 7.72 (m, 4H). MS (ESI): $[M+1]^+$=553.1.

5.1.2.19. 2-[3-(4-Chlorobenzoyl)-4-(4-fluorobenzyl)-5-(4-fluorophenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7s). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7s** as a yellow solid. Yield 52%, mp 87-89 °C. ^{1}H NMR ($CDCl_3$) δ: 4.08 (s, 2H),

6.75 (d, J=8.6 Hz, 2H), 6.78 (d, J=7.6 Hz, 2H), 7.08 (d, J=8.4 Hz, 2H), 7.15 (d, J=8.6 Hz, 2H), 7.40 (d, J=8.8 Hz, 2H), 7.43 (m, 2H), 7.73 (m, 4H). MS (ESI): $[M+1]^+$=571.3.

5.1.2.20. 2-[3-(4-Chlorobenzoyl)-4-(4-chlorobenzyl)-5-(4-fluorophenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7t). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7t** as a yellow solid. Yield 73%, mp 224-226 °C. ^{1}H NMR ($CDCl_3$) δ: 4.08 (s, 2H), 6.77 (d, J=8.2 Hz, 2H), 7.00 (d, J=8.2 Hz, 2H), 7.08 (m, 4H), 7.41 (m, 4H), 7.74 (m, 4H). MS (ESI): $[M+1]^+$=587.5.

5.1.2.21. 2-[3-(4-Chlorobenzoyl)-5-(4-fluorophenyl)-4-(4-methylbenzyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7u). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7u** as a brown solid. Yield 65%, mp 218-220 °C. ^{1}H NMR ($CDCl_3$) δ: 2.14 (s, 3H), 4.06 (s, 2H), 6.75 (d, J=8.8 Hz, 2H), 6.82 (d, J=8.8 Hz, 2H), 7.03 (d, J=8.8 Hz, 2H), 7.12 (d, J=8.8 Hz, 2H), 7.39 (d, J=8.8 Hz, 2H), 7.45 (m, 2H), 7.7 (m, 4H). MS (ESI): $[M+1]^+$=567.1.

5.1.2.22. 2-[3-(4-Chlorobenzoyl)-4-(4-methoxybenzyl)-5-(4-fluorophenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7v). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7v** as a white solid. Yield 62%, mp 175-177 °C. ^{1}H NMR ($CDCl_3$) δ: 3.63 (s, 3H), 4.03 (s, 2H), 5.59 (d, J=8.4 Hz, 2H), 6.73 (d, J=8.4 Hz, 2H), 7.06 (d, J=8.4 Hz, 2H), 7.11 (m, 2H), 7.38 (d, J=8.4 Hz, 2H), 7.42 (m, 2H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=583.1.

5.1.2.23. 2-{3-(4-Chlorobenzoyl)-5-(4-fluorophenyl)-4-[4-(methylthio)benzyl]-2-thienyl}-1H-isoindole-1,3(2H)-dione (7w). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7w** as a colour cream solid. Yield 78%, mp 81-83 °C. ^{1}H

NMR ($CDCl_3$) δ: 2.32 (s, 3H), 4.06 (s, 2H), 6.79 (d, J=8.8 Hz, 2H), 6.93 (d, J=8.8 Hz, 2H), 7.08 (d, J=8.6 Hz, 2H), 7.13 (d, J=8.6 Hz, 2H), 7.41 (d, J=8.2 Hz, 2H), 7.45 (m, 2H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=599.2.

5.1.2.24. 2-[4-Benzyl-3-(4-chlorobenzoyl)-5-(4-chlorophenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7x). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7x** as a yellow solid. Yield 67%, mp 80-82 °C. ^1H NMR ($CDCl_3$) δ: 4.13 (s, 2H), 6.84 (d, J=8.2 Hz, 2H), 7.02 (d, J=8.4 Hz, 2H), 7.08 (d, J=8.4 Hz, 2H), 7.43 (m, 7H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=569.5.

5.1.2.25. 2-[3-(4-Chlorobenzoyl)-4-(4-fluorobenzyl)-5-(4-chlorophenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7y). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7y** as a yellow solid. Yield 61%, mp 197-199 °C. ^1H NMR ($CDCl_3$) δ: 4.09 (s, 2H), 6.74 (d, J=8.6 Hz, 2H), 6.78 (d, J=8.6 Hz, 2H), 7.07 (d, J=8.8 Hz, 2H), 7.38 (d, J=8.8 Hz, 2H), 7.43 (m, 4H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=587.6.

5.1.2.26. 2-[3-(4-Chlorobenzoyl)-4-(4-chlorobenzyl)-5-(4-chlorophenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7z). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7z** as a yellow solid. Yield 73%, mp 235-237 °C. ^1H NMR ($CDCl_3$) δ: 4.09 (s, 2H), 6.78 (d, J=8.4 Hz, 2H), 7.01 (d, J=8.4 Hz, 2H), 7.08 (d, J=8.4 Hz, 2H), 7.43 (m, 6H), 7.75 (m, 6H). MS (ESI): $[M+1]^+$=603.9.

5.1.2.27. 2-[3-(4-Chlorobenzoyl)-4-(4-methylbenzyl)-5-(4-chlorophenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7aa). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 2-8) furnished

the desired product **7aa** as a yellow solid. Yield 84%, mp 224-226 °C. ^{1}H NMR ($CDCl_3$) δ: 2.15 (s, 3H), 4.08 (s, 2H), 6.71 (d, J=8.0 Hz, 2H), 6.83 (t, J=8.0 Hz, 2H), 7.07 (d, J=8.8 Hz, 2H), 7.41 (d, J=8.8 Hz, 2H), 7.44 (m, 4H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=583.5.

5.1.2.28. 2-[3-(4-Chlorobenzoyl)-4-(4-methoxylbenzyl)-5-(4-chlorophenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7ab). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7ab** as a colour cream solid. Yield 53%, mp 91-93 °C. ^{1}H NMR ($CDCl_3$) δ: 3.64 (s, 3H), 4.04 (s, 2H), 6.55 (d, J=8.8 Hz, 2H), 6.73 (d, J=8.8 Hz, 2H), 7.08 (d, J=8.8 Hz, 2H), 7.43 (m, 6H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=599.5.

5.1.2.29. 2-[4-Benzyl-3-(4-chlorobenzoyl)-5-(4-methoxyphenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7ac). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7ac** as a pink solid. Yield 80%, mp 94-96 °C. ^{1}H NMR ($CDCl_3$) δ: 3.84 (s, 3H), 4.14 (s, 2H), 6.82 (m, 5H), 6.94 (d, J=8.8 Hz, 2H), 7.03 (d, J=8.8 Hz, 2H), 7.37 (m, J=8.8 Hz, 2H), 7.42 (d, J=8.8 Hz, 2H), 7.75 (m, 4H). MS (ESI): $[M+1]^+$=565.2.

5.1.2.30. 2-[3-(4-Chlorobenzoyl)-4-(4-chlorobenzyl)-5-(4-methoxyphenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7ad). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **7ad** as a yellow solid. Yield 90%, mp 82-84 °C. ^{1}H NMR ($CDCl_3$) δ: 3.84 (s, 3H), 4.09 (s, 2H), 6.84 (d, J=8.8 Hz, 2H), 6.94 (d, J=8.8 Hz, 2H), 7.00 (d, J=8.8 Hz, 2H), 7.12 (d, J=8.8 Hz, 2H), 7.41 (m, 4H), 7.73 (m, 4H). MS (ESI): $[M+1]^+$=599.7.

5.1.2.31. 2-[3-(4-Chlorobenzoyl)-4-(4-methoxybenzyl)-5-(4-methoxyphenyl)-2-thienyl]-1H-isoindole-1,3(2H)-dione (7ae). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 2-

8), furnished the desired product **7ae** as a pink solid. Yield 73%, mp 95-97 °C. ^{1}H NMR ($CDCl_3$) δ: 3.63 (s, 3H), 3.84 (s, 3H), 4.05 (s, 2H), 6.55 (d, J=8.8 Hz, 2H), 6.75 (d, J=8.8 Hz, 2H), 6.98 (d, J=8.8 Hz, 2H), 7.09 (d, J=8.8 Hz, 2H), 7.38 (d, J=8.8 Hz, 2H), 7.46 (d, J=8.8 Hz, 2H), 7.74 (m, 4H). MS (ESI): $[M+1]^+$=595.2.

5.1.2.32. 2-{3-(4-chlorobenzoyl)-5-(4-methoxyphenyl)-4-[4-(methylthio)benzyl]-2-thienyl}-1H-isoindole-1,3(2H)-dione (7af). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 2-8), furnished the desired product **7af** as a pink solid. Yield 49%, mp 88-90 °C. ^{1}H NMR ($CDCl_3$) δ: 2.32 (s, 3H), 3.85 (s, 3H), 4.08 (s, 2H), 6.78 (d, J=8.4 Hz, 2H), 6.92 (d, J=8.4 Hz, 2H)), 6.94 (d, J=8.4 Hz, 2H)), 7.09 (d, J=8.6 Hz, 2H), 7.39 (d, J=8.4 Hz, 2H), 7.44 (d, J=8.4 Hz, 2H), 7.74 (m, 4H). MS (ESI): $[M+1]^+$=611.2.

5.1.2.33. 2-[5-Benzyl-3-(4-chlorobenzoyl)-4-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (11a). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **11a** as an orange oil. Yield 52%. ^{1}H NMR ($CDCl_3$) δ: 4.11 (s, 2H), 7.09 (d, J=8.6 Hz, 2H), 7.12 (m, 2H), 7.18 (d, J=8.6 Hz, 2H), 7.23 (m, 6H), 7.53 (d, J=88 Hz, 2H), 7.74 (m, 4H). MS (ESI): $[M+1]^+$=535.1.

5.1.2.34. 2-[3-(4-Chlorobenzoyl)-5-(1-naphthylmethyl)-4-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (11b). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **11b** as a yellow oil. Yield 38%. ^{1}H NMR ($CDCl_3$) δ: 4.59 (s, 2H), 6.98 (d, J=8.4 Hz, 2H), 7.24 (d, J=7.2 Hz, 1H), 7.32 (d, J=7.2 Hz, 1H), 7.42 (d, J=8.8 Hz, 2H), 7.44 (d, J=8.8 Hz, 2H), 7.48 (m, 8H), 7.80 (m, 4H). MS (ESI): $[M+1]^+$=585.2.

5.1.2.35. 2-{3-(4-Chlorobenzoyl)-4-phenyl-5-[4-(trifluoromethyl)benzyl]-2-thienyl}-1H-isoindole-1,3(2H)-dione (11c). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **11c** as a yellow oil. Yield 35%. ^{1}H NMR ($CDCl_3$) δ: 4.19 (s, 2H), 6.94 (d, J=8.4 Hz, 2H), 7.17 (d, J=8.4 Hz, 2H), 7.34 (d, J=8.8, Hz, 2H), 7.44 (d, J=8.8 Hz, 2H), 7.52 (m, 5H), 7.72 (m, 4H). $[M+1]^+$=603.2.

5.1.2.36. 2-[3-(4-Chlorobenzoyl)-5-(4-methoxybenzyl)-4-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (11d). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **11d** as a yellow oil. Yield 43%. ^{1}H NMR ($CDCl_3$) δ: 3.86 (s, 3H), 4.04 (s, 2H), 6.87 (d, J=8.0 Hz, 2H), 7.11 (d, J=8.0 Hz, 2H), 7.17 (d, J=8.8 Hz, 2H), 7.24 (m, 5H), 7.53 (d, J=7.6 Hz, 2H), 7.76 (m, 4H). MS (ESI): $[M+1]^+$=565.1.

5.1.2.37. 2-[3-(4-Chlorobenzoyl)-5-(3-methoxybenzyl)-4-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (11e). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **11e** as a yellow oil. Yield 48%. ^{1}H NMR ($CDCl_3$) δ: 3.77 (s, 3H), 4.07 (s, 2H), 6.73 (m, 2H), 6.81 (t, J=8.4 Hz, 1H), 7.08 (d, J=8.2 Hz, 2H), 7.19 (m, 4H), 7.23 (m, 2H), 7.53 (d, J=8.6 Hz, 2H), 7.79 (m, 4H). MS (ESI): $[M+1]^+$=565.3.

5.1.2.38. 2-{3-(4-Chlorobenzoyl)-4-phenyl-5-[4-(trifluoromethyl)benzyl]-2-thienyl}-1H-isoindole-1,3(2H)-dione (11f). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **11f** as a yellow oil. Yield 34%. ^{1}H NMR ($CDCl_3$) δ: 4.12 (s, 2H), 7.17 (m, 7H), 7.26 (d, J=8.0 Hz, 2H), 7.39 (d, J=8.8 Hz, 2H), 7.67 (d, J=8.4 Hz, 2H), 7.72 (m, 4H). MS (ESI): $[M+1]^+$=619.1.

5.1.2.39. 2-[3-(4-Chlorobenzoyl)-5-(4-ethoxybenzyl)-4-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (11g). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **11g** as a yellow oil. Yield 42%. ^{1}H NMR ($CDCl_3$) δ: 1.26 (t, J=7.2 Hz, 3H), 4.04 (q, J=7.2 Hz, 2H), 4.08 (s, 2H), 6825 (d, J=8.8 Hz, 2H), 6.88 (d, J=8.8 Hz, 2H), 7.11 (d, J=8.8 Hz, 2H), 7.22 (d, J=8.8 Hz, 2H), 7.36 (m, 5H), 7.67 (m, 4H). MS (ESI): $[M+1]^+$=579.1.

5.1.2.40. 2-[3-(4-Chlorobenzoyl)-5-(4-isopropoxybenzyl)-4-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (11h). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **11h** as a yellow oil. Yield 43%. ^{1}H NMR ($CDCl_3$) δ: 0.88 (d, J=6.2 Hz, 6H), 4.03 (s, 2H), 6.74 (m, 3H), 6.85 (d, J=8.6 Hz, 2H), 7.09 (d, J=8.6 Hz, 2H), 7.17 (m, 3H), 7.55 (d, J=8.8 Hz, 2H), 7.74 (m, 4H). MS (ESI): $[M+1]^+$=593.2.

5.1.2.41. 2-[3-(4-Chlorobenzoyl)-5-(4-methoxy-3-methylbenzyl)-4-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (11i). Following general procedure A, after workup as described previously, the crude residue purified by column chromatography (eluent EtOAc-petroleum ether 1.5-8.5), furnished the desired product **11i** as a yellow oil. Yield 38%. ^{1}H NMR ($CDCl_3$) δ: 2.19 (s, 3H), 3.82 (s, 3H), 4.01 (s, 2H), 6.78 (d, J=7.2 Hz, 1H), 6.98 (m, 2H), 7.09 (d, J=8.4 Hz, 2H), 7.20 (m, 5H), 7.54 (d, J=8.6 Hz, 2H), 7.82 (m, 4H). MS (ESI): $[M+1]^+$=579.1.

5.1.3. General procedure (B) for the synthesis of compounds 4a-af and 5a-i.

A stirred suspension of thiophene derivatives **7a-af** or **11a-i** (0.5 mmol) and hydrazine monohydrate (29 μL, 0.6 mmol, 1.2 equiv.) in absolute EtOH (10 mL) was heated at reflux for 1 h. The solvent was evaporated, and the residue partitioned between CH_2Cl_2 (10 mL) and water (5 mL). The organic phase was washed with brine (2 mL), dried (Na_2SO_4), and concentrated *in vacuo* to obtain a residue that was purified by column chromatography on silica gel.

5.1.3.1. [2-Amino-4-benzyl-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4a). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to yield the desired product **4a** as a yellow solid. Yield 68%, mp 134-135 °C. ^{1}H NMR (d_6-DMSO) δ: 3.67 (s, 2H), 6.49 (d, J=8.0 Hz, 2H), 7.04 (m, 3H), 7.28 (m, 3H), 7.39 (m, 6H), 7.66 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 33.46, 114.19, 120.81, 125.58 (2C), 127.31 (2C), 127.84 (2C), 127.90 (2C), 128.70 (2C), 129.05 (2C), 129.73 (2C), 131.35, 133.53, 135.39, 139.07, 139.59, 164.18, 190.11. MS (ESI): $[M+1]^+$=404.9. Anal. calcd for $C_{24}H_{18}ClNOS$: C, 71.36; H, 4.49; N, 3.47; found: C, 71.23; H, 4.25; N, 3.32.

5.1.3.2. (2-Amino-4-(1-naphthylmethyl)-5-phenyl-3-thienyl)(4-chlorophenyl)methanone (4b). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 1.5-8.5, to furnish the desired product **4b** as a yellow solid. Yield 91%, mp 178-180 °C. ^{1}H NMR ($CDCl_3$) δ: 4.07 (s, 2H), 6.67 (bs, 2H), 6.74 (d, J=8.8 Hz, 2H), 6.83 (d, J=8.8 Hz, 2H), 6.84 (d, J=7.2 Hz, 1H), 6.94 (d, J=7.2 Hz, 1H), 7.33 (m, 8H), 7.63 (d, J=7.2 Hz, 1H), 7.74 (d, J=7.2 Hz, 1H). MS (ESI): $[M+1]^+$=455.0. Anal. calcd for $C_{28}H_{20}ClNOS$: C, 74.08; H, 4.44; N, 3.09; found: C, 73.83; H, 4.26; N, 2.94.

5.1.3.3. [2-Amino-4-(4-fluorobenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4c). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4c** as a yellow oil. Yield 72%. ^{1}H NMR ($CDCl_3$) δ: 3.67 (s, 2H), 6.36 (bs, 2H), 6.48 (dd, J=8.8 and 1.2 Hz, 2H), 6.74 (t, J=8.8 Hz, 2H), 7.28 (d, J=8.6 Hz, 2H), 7.36 (m, 7H). MS (ESI): $[M+1]^+$=422.9. Anal. calcd for $C_{24}H_{17}ClFNOS$: C, 68.32; H, 4.06; N, 3.32; found: C, 68.21; H, 3.94; N, 3.23.

5.1.3.4. [2-Amino-4-(4-chlorobenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4d). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4d** as a yellow solid. Yield 72%, mp 83-85 °C. ^{1}H NMR (d_6-DMSO) δ: 3.65 (s, 2H), 6.51 (d, J=8.4 Hz, 2H), 7.11 (d, J=8.4 Hz, 2H),

7.30 (dd, J=8.0 and 1.2 Hz, 2H), 7.35 (m, 2H), 7.40 (m, 5H), 7.67 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 32.93, 114.03, 121.09, 127.36, 127.78 (2C), 127.97 (2C), 128.74 (2C), 129.01 (2C), 129.12 (2C), 129.74 (2C), 130.15, 130.83, 133.37, 135.50, 138.66, 138.96, 164.27, 190.00. MS (ESI): $[M+1]^+$=439.4. Anal. calcd for $C_{24}H_{17}Cl_2NOS$: C, 65.76; H, 3.91; N, 3.20; found: C, 65.58; H, 3.77; N, 3.05.

5.1.3.5. [2-Amino-4-(4-methylbenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4e). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4e** as a yellow solid. Yield 57%, mp 60-62 °C. ^{1}H NMR (d_6-DMSO) δ: 2.14 (s, 3H), 3.59 (s, 2H), 6.36 (d, J=8.0 Hz, 2H), 6.84 (d, J=8.0 Hz, 2H), 7.28 (d, J=8.4 Hz, 2H), 7.35 (m, 5H), 7.38 (d, J=8.4 Hz, 2H), 7.60 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 20.98, 33.56, 114.82, 121.22, 127.76 (2C), 127.92, 129.47 (2C), 128.99 (2C), 129.25 (2C), 129.54 (2C), 130.37 (2C), 132.18, 134.12, 135.04, 135.99, 137.08, 139.63, 164.59, 190.68. MS (ESI): $[M+1]^+$=418.9. Anal. calcd for $C_{25}H_{20}ClNOS$: C, 71.84; H, 4.82; N, 3.35; found: C, 71.68; H, 4.59; N, 3.21.

5.1.3.6. {2-Amino-5-phenyl-4-[4-(trifluoromethyl)benzyl]-3-thienyl}(4-chlorophenyl)methanone (4f). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to yield the desired product **4f** as a yellow solid. Yield 66%, mp 72-74 °C. ^{1}H NMR (d_6-DMSO) δ: 3.75 (s, 3H), 6.72 (d, J=8.0 Hz, 2H), 7.26 (m, 2H), 7.34 (m, 6H), 7.39 (d, J=8.0 Hz, 2H), 7.67 (bs, 2H). ^{13}C NMR (100 MHz, d_6-DMSO) δ: 33.78, 114.28, 121.61, 123.09, 124.92, 127.66 (2C), 128.19 (2C), 128.32 (2C), 129.01 (2C), 129.25 (2C), 129.91 (2C), 130.58, 133.56, 135.75, 139.12, 144.86, 164.55, 177.67, 190.23. MS (ESI): $[M+1]^+$=472.8. Anal. calcd for $C_{25}H_{17}ClF_3NOS$: C, 63.63; H, 3.63; N, 2.97; found: C, 63.48; H, 3.51; N, 2.78.

5.1.3.7. [2-Amino-4-(4-methoxybenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4g). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 1.5-8.5, to furnish the desired product **4g** as a yellow solid. Yield 78%,

mp 130-131 °C. ^{1}H NMR ($CDCl_3$) δ: 3.56 (s, 2H), 3.65 (s, 3H), 6.26 (bs, 2H), 6.32 (d, J=8.4 Hz, 2H), 6.52 (d, J=8.4 Hz, 2H), 7.29 (m, 9H). MS (ESI): $[M+1]^+$=434.9. Anal. calcd for $C_{25}H_{20}ClNO_2S$: C, 69.19; H, 4.64; N, 3.23; found: C, 69.02; H, 4.48; N, 3.12.

5.1.3.8. {2-Amino-5-phenyl-4-[4-(trifluoromethoxy)benzyl]-3-thienyl}(4-chlorophenyl)methanone (4h). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 1.5-8.5, to furnish the desired product **4h** as a yellow oil. Yield 84%. ^{1}H NMR (d_6-DMSO) δ: 3.68 (s, 2H), 6.58 (d, J=8.8 Hz, 2H), 7.01 (d, J=8.8 Hz, 2H), 7.26 (m, 2H), 7.34 (m, 4H), 7.69 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 33.55, 114.52, 121.04 (2C), 121.70, 127.95, 128.44 (2C), 129.30 (2C), 129.56 (2C), 129.63 (2C), 130.13 (2C), 131.36, 133.92, 135.99, 139.51, 139.66, 146.78, 164.90, 190.60. MS (ESI): $[M+1]^+$=488.8. Anal. calcd for $C_{25}H_{17}ClF_3NO_2S$: C, 61.54; H, 3.51; N, 2.87; found: C, 61.38; H, 3.36; N, 2.72.

5.1.3.9. [2-Amino-4-(4-methylthiobenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4i). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4i** as a yellow solid. Yield 63%, mp 58-60 °C. ^{1}H NMR (d_6-DMSO) δ: 2.37 (s, 3H), 3.62 (s, 2H), 6.43 (d, J=8.0 Hz, 2H), 6.96 (d, J=8.0 Hz, 2H), 7.31 (d, J=8.8 Hz, 2H), 7.39 (m, 7H), 7.65 (bs, 2H). ^{13}C NMR (100 MHz, d_6-DMSO) δ: 14.83, 32.91, 114.16, 120.82, 125.78 (2C), 127.29 (2C), 127.94 (2C), 128.72 (2C), 129.00 (2C), 129.78 (2C), 131.32, 133.49 (2C), 134.91, 135.47, 136.39, 139.02, 164.11, 190.08. MS (ESI): $[M+1]^+$=451.1. Anal. calcd for $C_{25}H_{20}ClNOS_2$: C, 66.72; H, 4.48; N, 3.11; found: C, 66.58; H, 4.32; N, 2.96.

5.1.3.10. [2-Amino-4-(4-ethoxybenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4j). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 1.5-8.5, to furnish the desired product **4j** as a yellow solid. Yield 95%, mp 150-152 °C. ^{1}H NMR (d_6-DMSO) δ: 1.24 (t, J=6.8 Hz, 3H), 3.58 (s, 2H), 3.87 (q, J=6.8 Hz, 2H), 6.35 (d, J=8.8 Hz, 2H), 6.59 (d, J=8.8 Hz, 2H), 7.29 (d, J=8.4 Hz, 2H), 7.44 (m, 7H), 7.64 (bs,

2H). ^{13}C NMR (100 MHz, d_6-DMSO) δ: 14.52, 32.57, 62.66, 113.78 (2C), 114.21, 120.55, 127.23, 127.88 (2C), 128.28 (2C), 128.69 (2C), 129.03 (2C), 129.74 (2C), 131.23, 131.92, 133.58, 135.39, 139.10, 156.42, 164.05, 190.16. MS (ESI): $[M+1]^+$=449.0. Anal. calcd for $C_{26}H_{22}ClNO_2S$: C, 69.71; H, 4.95; N, 3.13; found: C, 69.56; H, 4.69; N, 2.92.

5.1.3.11. [2-Amino-4-(4-isopropoxybenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4k). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to yield the desired product **4k** as a yellow solid. Yield 70%, mp 70-72 °C. ^{1}H NMR ($CDCl_3$) δ: 1.27 (d, J=6.2 Hz, 6H), 3.63 (s, 2H), 4.23 (m, 1H), 6.36 (bs, 2H), 6.41 (d, J=8.2 Hz, 2H), 6.58 (d, J=8.2 Hz, 2H), 7.34 (m, 7H), 7.42 (m, 2H). MS (ESI): $[M+1]^+$=462.5. Anal. calcd for $C_{27}H_{24}ClNO_2S$: C, 70.19; H, 5.24; N, 3.03; found: C, 69.93; H, 5.06; N, 2.89.

5.1.3.12. [2-Amino-4-(3-methoxybenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4l). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4l** as a yellow solid. Yield 81%, mp 58-60 °C. ^{1}H NMR (d_6-DMSO) δ: 3.57 (s, 3H), 3.64 (s, 2H), 5.97 (d, J=2.0 Hz, 1H), 6.11 (d, J=7.6 Hz, 1H), 6.59 (d, J=8.0 and 2.0 Hz, 1H), 6.98 (t, J=8.0 Hz, 1H), 7.28 (m, 3H), 7.37 (m, 6H), 7.71 (bs, 2H). ^{13}C NMR (100 MHz, d_6-DMSO) δ: 33.48, 54.52, 111.15, 112.88, 114.13, 119.53, 120.75, 127.29, 127.88 (2C), 128.72 (2C), 128.84, 129.05 (2C), 129.68 (2C), 131.23, 133.55, 135.31, 139.14, 141.21, 158.79, 164.30, 190.15. MS (ESI): $[M+1]^+$=434.7. Anal. calcd for $C_{25}H_{20}ClNO_2S$: C, 69.19; H, 4.64; N, 3.23; found: C, 68.98; H, 4.43; N, 3.09.

5.1.3.13. [2-Amino-4-(2-methoxybenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4m). Following the general procedure B, the residue was purified by column chromatography, eluting with EtOAc-petroleum ether 1-9, to furnish the desired product **4m** as a yellow solid. Yield 85%, mp 76-78 °C. ^{1}H NMR (d_6-DMSO) δ: 3.47 (s, 3H), 3.49 (s, 2H), 6.55 (d, J=7.6 and 1.6 Hz, 1H), 6.65 (d, J=8.4 Hz, 1H), 6.73 (t, J=8.4 Hz, 1H), 7.06 (m, 3H), 7.25 (m, 5H), 7.31 (m, 2H), 7.96 (bs,

2H). ^{13}C NMR (100 MHz, d_6-DMSO) δ: 27.39, 54.46, 109.26, 113.95, 119.65, 120.53, 126.86, 127.21, 127.50 (2C), 127.76 (2C), 128.64 (2C), 128.72, 128.80 (2C), 130.68 (2C), 133.59, 134.47, 139.45, 156.13, 165.18, 190.64. MS (ESI): $[M+1]^+$=434.8. Anal. calcd for $C_{25}H_{20}ClNO_2S$: C, 69.19; H, 4.64; N, 3.23; found: C, 68.93; H, 4.50; N, 3.02.

5.1.3.14. [2-Amino-4-(4-methoxy-3-methylbenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4n). Following the general procedure B, the residue was purified by column chromatography. eluting with EtOAc-petroleum ether 1.5-8.5, to furnish the desired product **4n** as a yellow solid. Yield 90%, mp 78-80 °C. ^{1}H NMR ($CDCl_3$)) δ: 2.01 (s, 3H), 3.62 (s, 2H), 3.81 (s, 3H), 6.38 (m, 4H), 6.61 (d, J=8.6 Hz, 2H), 7.31 (m, 8H). MS (ESI): $[M+1]^+$=449.1. Anal. calcd for $C_{26}H_{22}ClNO_2S$: C, 69.71; H, 4.95; N, 3.13; found: C, 69.56; H, 4.78; N, 3.02.

5.1.3.15. [2-Amino-4-(3,5-dimethyl-4-methoxybenzyl)-5-phenyl-3-thienyl](4-chlorophenyl) methanone (4o). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether, to furnish the desired product **4o** as a yellow solid. Yield 95%, mp 69-71 °C. ^{1}H NMR ($CDCl_3$) δ: 2.09 (s, 6H), 3.62 (s, 3H), 3.64 (s, 2H), 6.08 (bs, 2H), 6.44 (s, 2H), 7.24 (d, J=8.8 Hz, 2H), 7.32 (d, J=8.8 Hz, 2H), 7.37 (m, 5H). MS (ESI): $[M+1]^+$=463.1. Anal. calcd for $C_{27}H_{24}ClNO_2S$: C, 70.19; H, 5.24; N, 3.03; found: C, 70.03; H, 5.08; N, 2.89.

*5.1.3.16. [2-Amino-4-(2,4-dimethoxybenzyl)-5-phenyl-3-thienyl](4-chlorophenyl)methanone (4p). F*ollowing the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4p** as a yellow solid. Yield 89%, mp 169-171 °C. ^{1}H NMR ($CDCl_3$) δ: 3.45 (s, 3H), 3.50 (s, 2H), 3.75 (s, 3H), 6.14 (s, 1H), 6.34 (dd, J=7.4 and 1.2 Hz, 1H), 6.63 (m, 3H), 7.11 (d, J=8.8 Hz, 2H), 7.17 (d, J=8.8 Hz, 2H), 7.33 (m, 5H). MS (ESI): $[M+1]^+$=465.1. Anal. calcd for $C_{26}H_{22}ClNO_3S$: C, 67.30; H, 4.78; N, 3.02; found: C, 67.11; H, 4.59; N, 2.89.

5.1.3.17. [2-Amino-5-phenyl-4-(3,4,5-trimethoxybenzyl)-3-thienyl](4-chlorophenyl)methanone (4q). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 3-7, to yield the desired product **4q** as a yellow solid. Yield 89%, mp 181-183 °C. ^{1}H NMR ($CDCl_3$) δ: 3.64 (s, 6H), 3.67 (s, 2H), 3.76 (s, 3H), 5.70 (s, 2H), 6.41 (bs, 2H), 7.28 (m, 4H), 7.37 (m, 5H). MS (ESI): $[M+1]^+$=495.2. Anal. calcd for $C_{27}H_{24}ClNO_4S$: C, 65.65; H, 4.90; N, 2.84; found: C, 65.49; H, 4.78; N, 2.68.

5.1.3.18. [2-Amino-4-benzyl-5-(4-fluorophenyl)-3-thienyl](4-chlorophenyl)methanone (4r). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4r** as a yellow solid. Yield 93%, mp 58-60 °C. ^{1}H NMR ($CDCl_3$) δ: 3.62 (s, 2H), 6.35 (bs, 2H), 6.48 (d, J=6.4 Hz, 2H), 7.00 (d, J=6.4 Hz, 2H), 7.04 (m, 2H), 7.29 (m, 7H). MS (ESI): $[M+1]^+$=422.9. Anal. calcd for $C_{24}H_{17}ClFNOS$: C, 68.32; H, 4.06; N, 3.32; found: C, 68.12; H, 3.89; N, 3.12.

5.1.3.19. [2-Amino-4-(4-fluorobenzyl)-5-(4-fluorophenyl)-3-thienyl](4-chlorophenyl)methanone (4s). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4s** as a yellow solid, yield 95%, mp 141-143 °C. ^{1}H NMR ($CDCl_3$) δ: 3.61 (s, 2H), 6.38 (bs, 2H), 6.42 (d, J=8.8 Hz, 1H), 6.46 (d, J=8.8 Hz, 1H), 7.46 (d, J=8.8 Hz, 2H), 6.75 (d, J=8.8 Hz, 2H), 6.99 (d, J=8.6 Hz, 1H), 7.06 (d, J=8.8 Hz, 1H), 7.31 (m, 4H). MS (ESI): $[M+1]^+$=440.9. Anal. calcd for $C_{24}H_{16}ClF_2NOS$: C, 65.53; H, 3.67; N, 3.18; found: C, 65.38; H, 3.48; N, 3.03.

5.1.3.20. [2-Amino-4-(4-chlorobenzyl)-5-(4-fluorophenyl)-3-thienyl](4-chlorophenyl)methanone (4t). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4t** as a yellow solid. Yield 88%, mp 72-74 °C. ^{1}H NMR (d_6-DMSO) δ: 3.61 (s, 2H), 6.51 (d, J=8.4 Hz, 2H), 7.11 (d, J=8.4 Hz, 2H), 7.23 (t, J=8.8 Hz, 2H), 7.30 (d, J=8.4 Hz, 2H), 7.38 (m, 4H), 7.68 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 32.87, 113.90, 115.56, 115.78, 119.88, 127.78 (2C), 127.97, 128.21 (2C), 129.12 (2C),

129.74 (2C), 130.18, 131.09, 131.17, 135.53, 138.55, 138.93, 160.14, 162.57, 164.18, 189.63. MS (ESI): $[M+1]^+$=457.4. Anal. calcd for $C_{24}H_{16}Cl_2FNOS$: C, 63.16; H, 3.53; N, 3.07; found: C, 63.01; H, 3.39; N, 2.88.

5.1.3.21. 2-Amino-5-(4-fluorophenyl)-4-(4-methylbenzyl)-3-thienyl](4-chlorophenyl)methanone (4u). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4u** as a yellow solid. Yield 89%, mp 71-73 °C. ^{1}H NMR (d_6-DMSO) δ: 2.16 (s, 3H), 3.57 (s, 2H), 6.37 (d, J=8.0 Hz, 2H), 6.85 (d, J=8.0 Hz, 2H), 7.22 (t, J=8.8 Hz, 2H), 7.30 (d, J=8.4 Hz, 2H), 7.38 (m, 4H), 7.63 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 20.41, 32.94, 114.13, 115.51, 115.73, 119.43, 127.20 (2C), 127.91 (2C), 128.45 (2C), 129.81, 129.91, 131.04, 131.12, 131.89, 134.51, 135.46, 136.41, 139.02, 160.07, 162.50, 163.96, 190.09. MS (ESI): $[M+1]^+$=436.9. Anal. calcd for $C_{25}H_{19}ClFNOS$: C, 68.88; H, 4.39; N, 3.21; found: C, 68.69; H, 4.27; N, 3.04.

5.1.3.22. [2-Amino-5-(4-fluorophenyl)-4-(4-methoxybenzyl)-3-thienyl](4-chlorophenyl)methanone (4v). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4v** as a yellow solid. Yield 91%, mp 77-79 °C. ^{1}H NMR (d_6-DMSO) δ: 3.52 (s, 2H), 3.61 (s, 3H), 6.35 (d, J=8.8 Hz, 2H), 6.59 (d, J=8.8 Hz, 2H), 7.19 (t, J=8.8 Hz, 2H), 7.28 (d, J=8.6 Hz, 2H), 7.30 (m, 4H), 7.62 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 32.59, 54.88, 113.40 (2C), 114.14, 115.60, 115.82, 119.43, 127.99 (2C), 128.36 (2C), 129.85, 130.04, 131.17, 131.25, 131.35, 132.25, 135.51, 139.16, 157.28, 160.17, 162.60, 164.12, 190.19. MS (ESI): $[M+1]^+$=452.9. Anal. calcd for $C_{25}H_{19}ClFNO_2S$: C, 66.44; H, 4.24; N, 3.10; found: C, 66.26; H, 4.09; N, 2.95.

5.1.3.23. [2-Amino-5-(4-fluorophenyl)-4-(4-methylthiolbenzyl)-3-thienyl](4-chlorophenyl) methanone (4w). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4w** as a yellow solid. Yield 88%, mp 60-62 °C. ^{1}H NMR ($CDCl_3$) δ: 2.37 (s, 3H), 3.58 (s, 2H), 6.44 (d,

J=8.8 Hz, 2H), 6.96 (d, J=8.8 Hz, 2H), 7.23 (m, 2H), 7.31 (d, J=8.4 Hz, 2H), 7.40 (m, 4H), 7.64 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 14.84, 32.86, 114.07, 115.54, 115.75, 119.62, 125.81 (2C), 127.94 (2C), 129.78 (2C), 131.07 (2C), 131.15 (2C), 131.60, 134.95, 135.50, 136.28, 138.98, 160.10, 162.54, 164.01, 190.05. MS (ESI): $[M+1]^+$=469.1. Anal. calcd for $C_{25}H_{19}ClFNOS_2$: C, 64.16; H, 4.09; N, 2.99; found: C, 64.02; H, 3.96; N, 2.79.

5.1.3.24. [2-Amino-4-benzyl-5-(4-chlorophenyl)-3-thienyl](4-chlorophenyl)methanone (4x). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4x** as a yellow solid. Yield 87%, mp 153-155 °C. ^{1}H NMR (d_6-DMSO) δ: 3.66 (s, 2H), 6.49 (dd, J=7.6 and 2.0 Hz, 2H), 7.04 (m, 3H), 7.29 (d, J=8.4 Hz, 2H), 7.38 (m, 4H), 7.43 (d, J=8.8 Hz, 2H), 7.66 (bs, 2H). ^{13}C NMR (d_6-DMSO) δ: 33.44, 114.29, 119.35, 125.63, 127.33 (2C), 127.87 (2C), 127.93 (2C), 128.71 (2C), 129.78 (2C), 130.70 (2C), 131.90, 132.16, 132.45, 135.50, 138.96, 139.40, 164.14, 190.09. MS (ESI): $[M+1]^+$=439.4. Anal. calcd for $C_{24}H_{17}Cl_2NOS$: C, 65.76; H, 3.91; N, 3.20; found: C, 65.58; H, 3.74; N, 3.03.

5.1.3.25. [2-Amino-5-(4-chlorophenyl)-4-(4-fluorobenzyl)-3-thienyl](4-chlorophenyl)methanone (4y). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4y** as a yellow solid. Yield 76%, mp 193-195 °C. ^{1}H NMR (d_6-DMSO) δ: 3.82 (s, 2H), 6.47 (d, J=5.6 Hz, 1H), 6.49 (d, J=5.6 Hz, 1H), 6.84 (t, J=8.8 Hz, 2H), 7.28 (dd, J=6.4 and 2.0 Hz, 2H), 7.34 (dd, J=6.8 and 2.0 Hz, 2H), 7.40 (dd, J=6.8 and 2.0 Hz, 2H), 7.44 (dd, J=6.4 and 2.0 Hz, 2H), 7.67 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 32.80, 114.18, 114.57, 114.79, 119.55, 128.06 (2C), 128.82 (2C), 129.07, 129.14, 129.84, 130.81 (2C), 132.06, 132.11, 132.44, 135.54, 135.64, 139.01, 159.20, 161.60, 164.36, 190.13. MS (ESI): $[M+1]^+$=457.5. Anal. calcd for $C_{24}H_{16}Cl_2FNOS$: C, 63.16; H, 3.53; N, 3.07; found: C, 62.97; H, 3.39; N, 2.88.

5.1.3.26. [2-Amino-4-(4-chlorobenzyl)-5-(4-chlorophenyl)-3-thienyl](4-chlorophenyl)methanone (4z). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4z** as a yellow solid. Yield 61%, mp 72-74 °C. ^{1}H NMR (d_6-DMSO) δ: 3.62 (s, 2H), 6.49 (d, J=8.4 Hz, 2H), 7.09 (d, J=8. Hz, 2H), 7.31 (d, J=6.0 Hz, 2H), 7.35 (d, J=8.8 Hz, 2H), 7.40 (d, J=6.0 Hz, 2H), 7.44 (d, J=8.8 Hz, 2H), 7.66 (bs, 2H). ^{13}C NMR (100 MHz, d_6-DMSO) δ: 33.00, 114.21, 119.72, 127.92 (2C), 128.11 (2C), 128.86 (2C), 129.24 (2C), 129.88 (2C), 130.32, 130.78 (2C), 131.72, 132.08, 132.39, 135.70, 138.58, 138.94, 164.33, 190.09. MS (ESI): $[M+1]^+$=473.8. Anal. calcd for $C_{24}H_{16}Cl_3NOS$: C, 60.97; H, 3.41; N, 2.96; found: C, 60.77; H, 3.29; N, 2.78.

5.1.3.27. [2-amino-5-(4-chlorophenyl)-4-(4-methylbenzyl)-3-thienyl](4-chlorophenyl)methanone (4aa). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4aa** as a yellow solid. Yield 84%, mp 181-183 °C. ^{1}H NMR (d_6-DMSO) δ: 2.14 (s, 3H), 3.58 (s, 2H), 6.35 (d, J=8.0 Hz, 2H), 6.84 (d, J=8.0 Hz, 2H), 7.29 (dd, J=6.8 and 2.4 Hz, 2H), 7.34 (dd, J=6.8 and 2.4 Hz, 2H), 7.41 (m, 4H), 7.62 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 20.51, 33.06, 114.43, 119.27, 127.30 (2C), 128.04 (2C), 128.57 (2C), 128.80 (2C), 129.94 (2C), 130.72 (2C), 131.94, 132.50, 132.58, 134.65, 135.64, 136.41, 139.04, 164.11, 190.21. MS (ESI): $[M+1]^+$=453.4. Anal. calcd for $C_{25}H_{19}Cl_2NOS$: C, 66.37; H, 4.23; N, 3.10; found: C, 66.19; H, 4.04; N, 2.97.

5.1.3.28. [2-amino-5-(4-chlorophenyl)-4-(4-methoxybenzyl)-3-thienyl](4-chlorophenyl)methanone (4ab). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to yield the desired product **4ab** as a yellow oil. Yield 95%. ^{1}H-NMR (d_6-DMSO) δ: 3.57 (s, 2H), 3.64 (s, 3H), 6.37 (d, J=8.8 Hz, 2H), 6.61 (d, J=8.8 Hz, 2H), 7.31 (d, J=8.4 Hz, 2H), 7.36 (d, J=8.8 Hz, 2H), 7.41 (d, J=8.4 Hz, 2H), 7.44 (d, J=8.8 Hz, 2H), 7.64 (bs, 2H). ^{13}C-NMR (d_6-DMSO) δ: 32.52, 54.80, 113.34 (2C), 114.29, 119.09, 127.93 (2C), 128.28 (2C), 128.70 (2C), 129.78 (2C), 130.67 (2C), 131.18, 131.85, 132.50,

132.68, 135.50, 138.98, 157.22, 164.04, 190.14. MS (ESI): $[M+1]^+$=469.4. Anal. calcd for $C_{25}H_{19}Cl_2NO_2S$: C, 64.11; H, 4.09; N, 2.99; found: C, 63.97; H, 3.89; N, 2.79.

5.1.3.29. [2-Amino-4-benzyl-5-(4-methoxyphenyl)-3-thienyl](4-chlorophenyl)methanone (4ac). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4ac** as a yellow solid. Yield 91%, mp 76-78 °C. ^{1}H NMR ($CDCl_3$) δ: 3.65 (s, 2H), 3.76 (s, 2H), 6.35 (bs, 2H), 6.40 (d, J=8.8 Hz, 2H), 6.84 (d, J=8.8 Hz, 2H), 7.03 (d, J=8.8 Hz, 2H), 7.06 (d, J=8.8 Hz, 2H), 7.35 (m, 6H). MS (ESI): $[M+1]^+$=435.0. Anal. calcd for $C_{25}H_{20}ClNO_2S$: C, 69.19; H, 4.65; N, 3.23; found: C, 69.04; H, 4.49; N, 3.09.

5.1.3.30. [2-Amino-4-(4-chlorobenzyl)-5-(4-methoxyphenyl)-3-thienyl](4-chlorophenyl)methanone (4ad). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **4ad** as a yellow solid. Yield 62%, mp 80-82 °C. ^{1}H NMR ($CDCl_3$) δ: 3.61 (s, 2H), 3.79 (s, 3H), 6.36 (bs, 2H), 6.42 (d, J=8.6 Hz, 2H), 6.86 (d, J=8.8 Hz, 2H), 7.00 (d, J=8.8 Hz, 2H), 7.28 (m, 6H). MS (ESI): $[M+1]^+$=469.3. Anal. calcd for $C_{25}H_{19}Cl_2NO_2S$: C, 64.11; H, 4.09; N, 2.99; found: C, 63.89; H, 3.92; N, 2.79.

5.1.3.31. [2-Amino-4-(4-methoxybenzyl)-5-(4-methoxyphenyl)-3-thienyl](4-chlorophenyl) methanone (4ae). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-ptroleum ether 2-8, to yield the desired product **4ae** as a yellow solid. Yield 92%, mp 77-79 °C. ^{1}H NMR ($CDCl_3$) δ: 3.57 (s, 3H), 3.76 (s, 3H), 3.82 (s, 2H), 6.31 (bs, 2H), 6.38 (d, J=8.4 Hz, 2H), 6.57 (d, J=8.4 Hz, 2H), 6.84 (d, J=8.4 Hz, 2H), 7.29 (m, 6H). MS (ESI): $[M+1]^+$=465.0. Anal. calcd for $C_{26}H_{22}ClNO_3S$: C, 67.30; H, 4.78; N, 3.02; found: C, 67.16; H, 4.59; N, 2.88.

5.1.3.32. {2-Amino-5-(4-methoxyphenyl)-4-[4-(methylthio)benzyl]-3-thienyl}(4-chlorophenyl) methanone (4af). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-ptroleum ether 2-8, to yield the desired product **4af** as a yellow solid. Yield 95%, mp 71-73 °C. ^{1}H NMR (d_6-DMSO) δ: 2.37 (s, 3H), 3.56 (s, 2H), 3.74 (s, 3H), 6.41 (d, J=8.4 Hz, 2H), 6.94 (d, J=5.4 Hz, 2H), 6.96 (d, J=5.5 Hz, 2H), 7.27 (t, J=8.8 Hz, 4H), 7.39 (d, J=8.8 Hz, 2H), 7.65 (bs, 2H). ^{13}C NMR (d_6-DMSO) δ: 14.84, 32.91, 55.04, 113.96, 114.15 (2C), 120.78, 125.56, 125.77 (2C), 127.91 (2C), 127.98, 129.72 (2C), 130.36 (2C), 130.51 (2C), 134.85, 135.34, 136.49, 139.15, 158.54, 163.98, 189.98. MS (ESI): $[M+1]^+$=481.1. Anal. calcd for $C_{26}H_{22}ClNO_2S_2$: C, 65.05; H, 4.62; N, 2.92; found: C, 64.88; H, 4.48; N, 2.78.

5.1.3.33. (2-Amino-5-benzyl-4-phenyl-3-thienyl)(4-chlorophenyl)methanone (5a). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 1.5-8.5, to furnish the desired product **5a** as a yellow oil. Yield 63%. ^{1}H NMR ($CDCl_3$) δ: 3.88 (s, 2H), 6.64 (bs, 2H), 6.88 (d, J=8.4 Hz, 2H), 6.97 (d, J=8.4 Hz, 2H), 7.11 (m, 4H), 7.28 (m, 6H). MS (ESI): $[M+1]^+$=404.9. Anal. calcd for $C_{24}H_{18}ClNOS$: C, 71.36; H, 4.49; N, 3.47; found: C, 71.18; H, 4.33; N, 3.26.

5.1.3.34. [2-Amino-5-(1-naphthylmethyl)-4-phenyl-3-thienyl](4-chlorophenyl)methanone (5b). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 1.5-8.5, to furnish the desired product **5b** as a yellow oil. Yield 59%. ^{1}H NMR ($CDCl_3$) δ: 4.36 (s, 2H), 6.62 (bs, 2H), 6.94 (d, J=8.8 Hz, 2H), 7.06 (d, J=8.8 Hz, 2H), 7.12 (d, J=7.2 Hz, 1H), 7.36 (d, J=7.2 Hz, 1H), 7.42 (m, 8H), 7.48 (d, J=7.2 Hz, 1H), 7.86 (d, J=7.2 Hz, 1H). MS (ESI): $[M+1]^+$=455.0. Anal. calcd for $C_{28}H_{20}ClNOS$: C, 74.08; H, 4.44; N, 3.09; found: C, 73.88; H, 4.31; N, 2.90.

5.1.3.35. {2-Amino-4-phenyl-5-[4-(trifluoromethyl)benzyl]-3-thienyl}(4-chlorophenyl)methanone (5c). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 1.5-8.5, to furnish the desired product **5c** as a

yellow oil. Yield 52%. ^{1}H NMR ($CDCl_3$) δ: 3.94 (s, 2H), 6.79 (bs, 2H), 7.12 (d, J=7.8 Hz, 2H), 7.22 (d, J=7.8 Hz, 2H), 7.26 (m, 5H), 7.45 (t, J=7.8 Hz, 2H), 7.69 (d, J=8.2 Hz, 2H). MS (ESI): $[M+1]^+$=472.9. Anal. calcd for $C_{25}H_{17}ClF_3NOS$: C, 63.63; H, 3.63; N, 2.97; found: C, 63.42; H, 3.54; N, 2.69.

5.1.3.36. [2-Amino-5-(4-methoxybenzyl)-4-phenyl-3-thienyl](4-chlorophenyl)methanone (5d). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **5d** as a yellow oil. Yield 53%. ^{1}H NMR ($CDCl_3$) δ: 3.78 (s, 2H), 3.81 (s, 3H), 6.84 (bs, 2H), 6.79 (d, J=8.4 Hz, 2H), 6.92 (d, J=8.4 Hz, 2H), 7.02 (m, 9H). MS (ESI): $[M+1]^+$=435.0. Anal. calcd for $C_{25}H_{20}ClNO_2S$: C, 69.19; H, 4.64; N, 3.23; found: C, 68.99; H, 4.47; N, 3.06.

5.1.3.37. [2-Amino-5-(3-methoxybenzyl)-4-phenyl-3-thienyl](4-chlorophenyl)methanone (5e). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 1.5-8.5, to furnish the desired product **5e** as a yellow oil. Yield 54%. ^{1}H NMR ($CDCl_3$) δ: 3.76 (s, 2H), 3.85 (s, 3H), 6.61 (s, 1H), 6.74 (d, J=7.4 Hz, 2H), 6.83 (bs, 2H), 6.92 (d, J=8.4 Hz, 2H), 7.11 (d, J=8.0 Hz, 1H), 7.38 (m, 7H). MS (ESI): $[M+1]^+$=435.1. Anal. calcd for $C_{25}H_{20}ClNO_2S$: C, 69.19; H, 4.64; N, 3.23; found: C, 68.92; H, 4.50; N, 3.03.

5.1.3.38. {2-Amino-4-phenyl-5-[4-(trifluoromethoxy)benzyl]-3-thienyl}(4-chlorophenyl)methanone (5f). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **5f** as a yellow oil. Yield 61%. ^{1}H NMR ($CDCl_3$) δ: 3.89 (s, 3H), 6.74 (bs, 2H), 6.94 (m, 4H), 7.11 (d, J=8.8 Hz, 2H), 7.29 (m, 4H), 7.44 (d, J=8.8 Hz, 2H). MS (ESI): $[M+1]^+$=488.9. Anal. calcd for $C_{25}H_{17}ClF_3NO_2S$: C, 61.54; H, 3.51; N, 2.87; found: C, 61.41; H, 3.38; N, 2.59.

5.1.3.39. [2-Amino-5-(4-ethoxybenzyl)-4-phenyl-3-thienyl](4-chlorophenyl)methanone (5g). Following the general procedure B, the crude residue was purified by column chromatography,

eluting with EtOAc-petroleum ether 3-7, to furnish the desired product **5g** as a yellow oil. Yield 95%. ^{1}H NMR ($CDCl_3$) δ: 1.43 (t, J=7.2 Hz, 3H), 3.83 (s, 2H), 4.04 (q, J=7.2 Hz, 2H), 6.59 (bs, 2H), 6.79 (d, J=8.6 Hz, 2H), 6.92 (m, 3H), 7.08 (d, J=8.6 Hz, 2H), 7.12 (d, J=8.2 Hz, 2H), 7.23 (d, J=8.2 Hz, 2H), 7.37 (d, J=8.6 Hz, 2H). MS (ESI): $[M+1]^+$=449.0. Anal. calcd for $C_{26}H_{22}ClNO_2S$: C, 69.71; H, 4.95; N, 3.13; found: C, 69.49; H, 4.78; N, 2.98.

5.1.3.40. [2-Amino-5-(4-isopropoxybenzyl)-4-phenyl-3-thienyl](4-chlorophenyl)methanone (5h). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product **5h** as a colourless oil. Yield 70%. ^{1}H NMR ($CDCl_3$) δ: 1.28 (d, J=6.2 Hz, 6H), 3.80 (s, 2H), 4.52 (m, 1H), 6.56 (bs, 2H), 6.77 (d, J=8.2 Hz, 2H), 6.82 (d, J=8.2 Hz, 2H), 6.92 (m, 2H), 6.98 (m, 5H), 7.15 (d, J=8.2 Hz, 2H). MS (ESI): $[M+1]^+$=463.4. Anal. calcd for $C_{27}H_{24}ClNO_2S$: C, 70.19; H, 5.24; N, 3.03; found: C, 69.98; H, 5.10; N, 2.92.

5.1.3.41. [2-Amino-5-(4-methoxy-3-methylbenzyl)-4-phenyl-3-thienyl](4-chlorophenyl)methanone (5i). Following the general procedure B, the crude residue was purified by column chromatography, eluting with EtOAc-petroleum ether 2-8, to furnish the desired product as a yellow oil. Yield 61%. ^{1}H NMR ($CDCl_3$) δ: 2.17 (s, 3H), 3.78 (s, 2H), 3.81 (s, 3H), 6.66 (bs, 2H), 6.74 (d, J=8.6 Hz, 2H), 6.88 (m, 3H), 6.94 (m, 5H), 7.02 (d, J=8.6 Hz, 2H). MS (ESI): $[M+1]^+$=449.5. Anal. calcd for $C_{26}H_{22}ClNO_2S$: C, 69.71; H, 4.95; N, 3.13; found: C, 69.58; H, 4.81; N, 2.98.

5.1.4. Preparation of 2-[3-(4-chlorobenzoyl)-5-methyl-4-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (9). To a solution of 2-methyl-4-phenyl-thiophene **8** (1.3 g., 4 mmol) in acetic acid (25 mL) was added phthalic anhydride (0.74 g., 5 mmol.) and the resulting mixture heated under reflux for 4 h. The solvent was evaporated *in vacuo* and the residue dissolved in methylene chloride (20 mL). The organic solution was washed with a saturated aqueous solution of $NaHCO_3$ (5 mL), water (5 mL), brine (5 mL), dried (Na_2SO_4), and concentrated *in vacuo*. The crude residue was stirred for 1 h with petroleum ether (20 mL), and after filtration furnished the product **9** as a yellow powder.

Yield: 83%, mp 184-186 °C. ^{1}H NMR ($CDCl_3$) δ: 2.45 (s, 3H), 7.09 (d, J=7.2 Hz, 2H), 7.15 (m, 5H), 7.54 (d, J=7.2 Hz, 2H), 7.74 (m, 2H), .8.50 (m, 2H). MS (ESI): $[M]^+$=458.0.

5.1.5. Preparation of 2-[5-(bromomethyl)-3-(4-chlorobenzoyl)-4-phenyl-2-thienyl]-1H-isoindole-1,3(2H)-dione (10). To a refluxing suspension of **9** (2.3 g., 5 mmol) in CCl_4 (50 mL) was added a mixture of NBS (1.9 g., 10 mmol.) and 75% benzoyl peroxide (140 mg, 0.6 mmol). After one hour at reflux, the yellow solution was cooled to room temperature, the succinimide that separated upon cooling was filtered, and the filter cake was rinsed with CCl_4 (10 mL). The filtrate was washed with a saturated aqueous solution of $NaHCO_3$ (10 mL), water (10 mL), brine (10 mL), dried (Na_2SO_4), and concentrated under reduced pressure to give a yellow oil. The crude residue, purified by column chromatography on silica gel eluting with a mixture of EtOAc-petroleum ether 2-8, furnished compound **10** as a yellow solid. Yield: 52%, mp 223-225 °C. ^{1}H NMR ($CDCl_3$) δ: 4.63 (s, 2H), 7.11 (d, J=8.4 Hz, 2H), 7.23 (m, 5H), 7.56 (d, J=8.4 Hz, 2H), 7.78 (m, 2H), 7.87 (m, 2H). MS (ESI): $[M]^+$=536.8, 538.9.

5.2. Biology Experiments

5.2.1. Materials.

[^{3}H]DPCPX ([^{3}H]1,3-dipropyl-8-cyclopentyl-xanthine; specific activity, 120 Ci/mmol), [^{3}H]CCPA ([^{3}H]2-chloro-N^6-cyclopentyladenosine; specific activity, 55 Ci/mmol), and [^{3}H]NECA ([^{3}H]5'-*N*-ethylcarboxamidoadenosine; specific activity, 29.4 Ci/mmol) were obtained from Perkin Elmer Research Products (Boston, MA); [^{3}H]ZM 241385 ([^{3}H](4-(2-[7-amino-2-(2-furil)[1,2.4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol); specific activity, 17 Ci/mmol) was obtained from Biotrend (Cologne, Germany); [^{3}H]MRE-3008-F20 ([^{3}H]5-N-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo [1,5-c]pyrimidine; specific activity, 67 Ci/mmol) was obtained from GE Healthcare (Buckinghamshire, UK). DPCPX (1,3-dipropyl-8-cyclopentyl-xanthine), R-PIA ((R)- N^6-(L-2-Phenylisopropyl)adenosine) and CCPA (2-chloro-N^6-cyclopentyladenosine) were obtained from

Sigma (St. Louis, MO, USA). hA_1, hA_{2A} and hA_3CHO cells were obtained from Perkin Elmer Research Products (Boston, MA). All other reagents were of analytical grade and obtained from commercial sources.

5.2.2. Cell membrane preparation.

For the [^{3}H]CCPA saturation, and the [^{3}H]DPCPX, [^{3}H]ZM 241385, and [^{3}H]MRE-3008-F20 competition binding experiments the hA_1CHO, hA_{2A}CHO and hA_3CHO cells were grown adherently and maintained in Dulbecco's modified Eagle's medium with nutrient mixture F12, containing 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), geneticine (G418) 0.2 mg/mL at 37 °C in 5% CO_2/95% air. For membrane preparation the culture medium was removed and the cells were washed with phosphate-buffered saline and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl, 1 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron, the homogenate was spun for 10 min at 1000 x g and the supernatant was then centrifuged for 30 min at 100,000 x g. The membrane pellet was suspended in 50 mM Tris HCl buffer (pH 7.4) for A_1ARs, in 50 mM Tris HCl, 10 mM $MgCl_2$ (pH 7.4) for A_{2A}ARs, in 50 mM Tris HCl, 10 mM $MgCl_2$, 1 mM EDTA (pH 7.4) for A_3ARs. The membranes were incubated with 2-3 IU/mL of adenosine deaminase to reduce the endogenous adenosine. The protein concentration was determined according to a Bio-Rad method with bovine albumin as a standard reference [26]. For the [^{3}H]NECA kinetic binding experiments, hA_1CHO cells were grown in Ham's F12 medium, containing 10% (v/v) normal adult bovine serum, streptomycin (100 µg/mL), penicillin (100 IU/mL), and G418 (0.4 mg/mL), at 37 °C in 5% CO_2. Cells were detached from 10-cm ø culture plates by scraping them into 5 mL phosphate-buffered saline (PBS), collected, and centrifuged at 700 g (3000 rpm) for 5 min. Cell pellets were pooled and resuspended in 50 mM Tris-HCl buffer (pH 7.4), supplemented with 5 mM $MgCl_2$. An UltraThurrax (Heidolph Instruments, Schwabach, Germany) was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 g (31,000 rpm) in a Beckman Optima LE-80K ultracentrifuge (Beckman Coulter, Fullerton, CA), at 4

°C for 20 min. The pellet was resuspended in 50 mM Tris-HCl, 5 mM $MgCl_2$ buffer, and the homogenization and centrifugation step was repeated. The same Tris-HCl buffer was used to resuspend the pellet, and ADA was added (0.8 IU/mL). Membranes were stored in 250 µL aliquots at -80 °C. Concentrations of the membrane protein were measured using a Pierce BCA protein assay kit [26].

5.2.3. Binding Experiments in hA_1CHO membranes

5.2.3.1. [^{3}H]CCPA Binding Experiments.

Saturation binding experiments of [^{3}H]CCPA (0.05 to 20 nM) to hA_1CHO membranes were performed in triplicate at 25 °C for 90 min in 50 mM Tris-HCl, pH 7.4, in the absence and presence of the tested compounds at the final concentration of 10 µM [27]. Non-specific binding was defined as binding in the presence of 1 µM R-PIA.

5.2.3.2. [^{3}H]DPCPX Competition Binding Experiments.

Competition binding experiments of 1 nM [^{3}H]DPCPX were performed in triplicate in 50 mM Tris-HCl, pH 7.4, for 90 min at 25 °C. The effect of the different tested compounds at a concentration of 10 µM on the CCPA curve (0.01 nM -1 µM) was investigated [28]. Non-specific binding was defined as binding in the presence of 1 µM DPCPX.

5.2.3.3. Assay of the Antagonist Activity versus A_1, A_{2A} and A_3 ARs.

A_1, A_{2A}, and A_3 AR competition binding experiments were performed using 1 nM [^{3}H]DPCPX, 1 nM [^{3}H]ZM 241385, and 2 nM [^{3}H]MRE-3008-F20 as radioligands, respectively [28-30]. Membrane suspensions were incubated in 50 mM Tris HCl, pH 7.4, at 25 °C for 120 min, in 50 mM Tris HCl, 10 mM $MgCl_2$, pH 7.4, at 4 °C for 60 min, or in 50 mM Tris HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4 at 4 °C for 120 min to study A_1, A_{2A}, and A_3 ARs, respectively. Non-specific binding was defined as the binding in the presence of 1 µM DPCPX or ZM 241385 or MRE-3008-F20 for A_1, A_{2A}, and A_3ARs, respectively. Inhibition was expressed as percentage of control specific

binding (100%). Test agents were dissolved in DMSO and added to the assay from a 100-fold concentrated solution in DMSO. Control incubations also contained 1% DMSO.

Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fiber filters using a Brandel cell harvester (Brandel Instruments, Unterföhring, Germany). The filter bound radioactivity was counted by Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

5.2.4. Effect of the novel compounds in cyclic AMP assays.

Human A_1 CHO cells (10^6 cells/mL) were prepared as described above and were suspended in 0.5 mL incubation mixture phosphate buffer, containing 1.0 IU adenosine deaminase/mL and 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as a phosphodiesterase inhibitor and preincubated for 10 min in a shaking bath at 37 °C. The allosteric enhancers were studied at 10 μM concentration that was added to the mixture for a further 10 min. The effect of the allosteric enhancers (100 nM) was also studied in the presence of a low concentration of CCPA (1 pM). Forskolin 1 μM was added for 5 min and was used to stimulate the activity of adenylate cyclase. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged at 2,000 *g* for 10 min at 4 °C and the supernatant was extracted four times with water saturated diethyl ether. The final aqueous solution was tested for cAMP levels by a competition protein binding assay. Samples of cAMP standards (0-10 pmol) were added to each test tube containing trizma base 0.1 M, aminophylline 8.0 mM, mercaptoethanol 6.0 mM, pH 7.4, and [^{3}H]-cAMP (at the final concentration of 1 nM). The binding protein, previously prepared from beef adrenals, was added to the samples and incubated at 4 °C for 150 min. At the end of the incubation time and after the addition of charcoal, the samples were centrifuged at 2,000 *g* for 10 min. The clear supernatant was mixed with 4 mL of Ultima Gold (Perkin Elmer) and counted in a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

5.2.5. Kinetic radioligand binding experiments.

Association and dissociation experiments were performed at 37 °C in a total volume of 100 µL assay buffer (50 mM Tris-HCl (pH 7.4) supplemented with 5 mM $MgCl_2$). Membrane aliquots containing 13 µg of protein were incubated with 7 nM [^{3}H]NECA, in the presence or absence of 1 µM PD81,723 or **4z**. Total binding was less than 10% of the total radioactivity added, to prevent radioligand depletion. Non-specific binding was determined in the presence of 10 µM DPCPX. In association experiments, the amount of radioligand bound to the receptor was measured at different time intervals during a total incubation time of 45 min. For dissociation experiments, the reaction mix was preincubated for 45 min, to allow full association of the radioligand to the receptor. After the preincubation, radioligand dissociation was initiated by the addition of 10 µM unlabeled DPCPX. The amount of radioligand still bound to the receptor was measured at various time intervals for a total duration of 2 h.

Incubations were terminated by rapid vacuum filtration to separate bound from free radioligand through 96-well GF/B filter plates, using a Filtermate harvester (Perkin Elmer, Groningen, The Netherlands). Filters were subsequently washed ten times with ice-cold wash buffer (50 mM Tris-HCl [pH 7.4] supplemented with 5 mM $MgCl_2$). The filter-bound radioactivity was determined by scintillation spectrometry using a 1450 Microbeta Wallac Trilux scintillation counter (Perkin Elmer).

5.2.6. Formalin Assay.

Male CD1 mice (22-24 g) were obtained from Charles River (Milan, Italy). The animals were kept under standard environmental temperature and humidity-controlled conditions (22±2°C) with 12 h light/dark cycle with food and water *ad libitum*. The animals were acclimated to the laboratory settings for at least 1 h before testing and were used only once throughout the experiments. All the procedures used in the present study were carried out in accordance with European Communities Council directives (86/609/EEC) and National Laws and Policies (D.L.116/92) with authorization from the Italian Ministry for Health (122/2011-B).

The formalin test measures nociception responses to an injection in the plantar surface of the hind paw of a formalin dilution (1.5% in saline, 20 µl). After the injection each mouse was immediately returned to a plexiglass observation chamber. The degree of pain intensity was assessed as the total time spent by the animal licking, biting, shaking, favouring, and flinching of the injected paw, measured by visual observation and a digital time-out stopwatch for 45 min [31]. Each mouse was randomly assigned to one of the experimental groups (n=6) and received intraperitoneal administration of vehicle or compound **4z** (0.03, 0.3 or 3 mg/kg) 15 min before formalin injection.

5.2.7. Data Analysis.

Saturation and competition binding experiments were analysed with the program LIGAND, which performed a weighted, non-linear, least squares curve fitting program [32]. Inhibitory binding constants, Ki, were also calculated from the IC_{50} values according to the Cheng and Prusoff equation $Ki=IC_{50}/(1+[C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^* its dissociation constant [33]. Data from the kinetic radioligand binding experiments were analyzed using the non-linear regression curve fitting program Prism 5.1 (GraphPad, San Diego, CA, U.S.A.). Kinetic k_{on} and k_{off} values were obtained by analysis of the association and dissociation data. The association data was preferentially fit to a two-phase association curve for the control situation and in the presence of PD81,723, but fit better to a one-phase association curve in the presence of **4z**. The dissociation data fit best to a two-phase dissociation equation in all cases. However, for the purpose of clarity and unambiguous comparability between the situations, we chose to present all values from association and dissociation data fit to one-phase kinetics. The kinetic K_D was calculated from the one-phase association and dissociation rates, by dividing the k_{off} by the k_{on} values. All experimental data are expressed as mean ± standard error of the mean (S.E.M.) of three or four independent experiments performed in duplicate. For the kinetic radioligand binding and formalin test, statistical analysis of the data was performed using an unpaired 2-sided Student's t-test.

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Supporting information available. Synthetic procedure for the preparation of compound **6d**.

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25. In our experiments, the reference compound PD 81,723 (at a concentration of 10 μM) did not inhibit [^{3}H]DPCPX binding to human A_1 receptors transfected in CHO cells. For the same reference compound, Bruns (Reference 16a) showed a K_i value of 11 μM obtained in competition binding experiments by using [^{3}H]DPCPX as radioligand on rat membranes. Furthermore, data performed on CHO-K1cells stably expressing the human A_1 receptors (reference 17f) reported an inhibition of [^{3}H]DPCPX binding to human A_1 receptors by PD 81,723 only of 42±7%, when tested at 100 μM. We speculate that species differences in affinity binding of PD 81,723 may explain the discrepancy between the data.

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Figure, Chart and Scheme Captions

Figure 1. Histograms showing the cAMP inhibition, expressed in pmol/10^6 cells, mediated by novel allosteric enhancers **4g**, **4j** and **4z** at 10 μM concentration (A). The effect of the examined compounds PD 81,723, **4g**, **4j** and **4z** (100 nM) was also studied in the presence of 1 pM CCPA (B). Values are expressed as mean ± SEM of three separate experiments, as described in Experimental Section.

Figure 2. [^{3}H]-CCPA saturation binding curves at human A_1 adenosine receptors (A). Under control conditions, K_D value was 1.1±0.1 nM and the Bmax was 521±47 fmol/mg protein. In the presence of novel enhancers **4g**, **4j** and **4z** (10 μM), K_D values were similar to those obtained in controls and Bmax values were as reported in Table 2. Values are the means and vertical lines are the SEM of three separate experiments, as described in Experimental Section. Scatchard plots of the same experimental data (B).

Figure 3. Inhibition curves of specific [^{3}H]-DPCPX binding to human A_1ARs by CCPA in the absence and in the presence of novel enhancers **4g**, **4j** and **4z** (10 μM). Affinity values were calculated by using a one-state model of analysis. Values are the means and vertical lines are the SEM of three separate experiments as described in Experimental Section.

Figure 4. Association (A) and dissociation (B) of [^{3}H]NECA (7 nM), in the presence or absence of 1 μM PD 81,423 or **4z**, at 37 °C to hA_1AR stably expressed on CHO membranes. In the association experiments, the amount of radioligand bound to the receptor was measured at each time point. Before dissociation, [^{3}H]NECA was allowed to fully associate to hA_1AR CHO membranes. After addition of 10 μM DPCPX (final concentration), the amount of radioligand bound to the receptor was measured at each time point. The figure shows graphs from a representative experiment performed in duplicate.

Figure 5. Time response curve of nociceptive responses in the formalin test after intraperitoneal administration of vehicle or **4z** at three different doses (0.03, 0.3 and 3.0 mg/kg). Data are presented as mean ± SEM relative to n=6 mice/group.

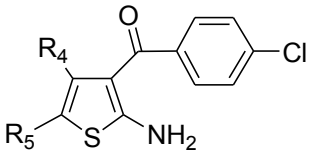
Chart 1. Structures of previously described 2-amino-3-aroyl thiophene derivatives **1a-e**, **2a** and **3a-b** as allosteric modulators for the A_1 adenosine receptor

Chart 2. Structures of 2-amino-3-(p-chlorobenzoyl)-4.5-disubstituted thiophene derivatives **4a-af**, and **5a-i** evaluated in this study as allosteric ligands for the A_1 adenosine receptor

Scheme 1. Reagents. a: $ArB(OH)_2$, $Pd(Ph_3P)_4$, K_2CO_3, $PhCH_3$, 100 °C; **b:** NH_2NH_2, EtOH, reflux.

Scheme 2. Reagents. a: phthalic anhydride, AcOH, reflux; **b:** NBS, benzoyl peroxide (cat.), CCl_4, reflux; **c:** $ArB(OH)_2$, Pd $(Ph_3P)_4$, K_2CO_3, toluene, reflux; **d:** NH_2NH_2, EtOH, reflux**.**

Table 1. Effect of the novel allosteric enhancers **4a-af, 5a-i** and reference compounds PD 81,723 (**1a**), **2a** and **3a** in cAMP assay in hA_1 CHO cells



	R_4	R_5	% inhibition of cAMP production[a]	% inhibition of cAMP production + CCPA[b]
4a	$C_6H_4CH_2$	C_6H_5	51±5	50±5
4b	Naphth-1-ylmethyl	C_6H_5	57±6	58±6
4c	*p*-F-$C_6H_4CH_2$	C_6H_5	58±6	57±6
4d	*p*-Cl-$C_6H_4CH_2$	C_6H_5	61±6	64±5
4e	*p*-CH_3-$C_6H_4CH_2$	C_6H_5	56±6	58±6
4f	*p*-CF_3-$C_6H_4CH_2$	C_6H_5	69±7	66±6
4g	*p*-OCH_3-$C_6H_4CH_2$	C_6H_5	71±7	74±7
4h	*p*-OCF_3-$C_6H_4CH_2$	C_6H_5	68±7	69±7
4i	*p*-SCH_3-$C_6H_4CH_2$	C_6H_5	63±6	65±7
4j	*p*-OC_2H_5-$C_6H_4CH_2$	C_6H_5	68±6	69±7
4k	*p*-$OCH(CH_3)_2$-$C_6H_4CH_2$	C_6H_5	65±6	67±6
4l	*m*-OCH_3-$C_6H_4CH_2$	C_6H_5	66±6	63±6
4m	*o*-OCH_3-$C_6H_4CH_2$	C_6H_5	55±5	51±5
4n	*m*-CH_3-*p*-OCH_3-$C_6H_3CH_2$	C_6H_5	66±6	66±6
4o	*m,m'*-$(CH_3)_2$-*p*-OCH_3-$C_6H_2CH_2$	C_6H_5	59±6	61±6
4p	*o,p*-$(OCH_3)_2$-$C_6H_3CH_2$	C_6H_5	52±5	53±5
4q	*m,m',p*-$(OCH_3)_3$-$C_6H_2CH_2$	C_6H_5	47±5	43±4
4r	$C_6H_4CH_2$	*p*-F-C_6H_4	50±5	49±5
4s	*p*-F-$C_6H_4CH_2$	*p*-F-C_6H_4	45±4	41±4
4t	*p*-Cl-$C_6H_4CH_2$	*p*-F-C_6H_4	53±4	55±5
4u	*p*-CH_3-$C_6H_4CH_2$	*p*-F-C_6H_4	59±6	62±6
4v	*p*-OCH_3-$C_6H_4CH_2$	*p*-F-C_6H_4	58±6	59±6
4w	*p*-SCH_3-$C_6H_4CH_2$	*p*-F-C_6H_4	52±5	55±4
4x	$C_6H_4CH_2$	*p*-Cl-C_6H_4	69±7	67±7
4y	*p*-F-$C_6H_4CH_2$	*p*-Cl-C_6H_4	75±7	73±7
4z	*p*-Cl-$C_6H_4CH_2$	*p*-Cl-C_6H_4	81±7	84±8

4aa	*p*-CH_3-$C_6H_4CH_2$	*p*-Cl-C_6H_4	71±6	69±7
4ab	*p*-OCH_3-$C_6H_4CH_2$	*p*-Cl-C_6H_4	70±7	67±6
4ac	$C_6H_4CH_2$	*p*-OCH_3-C_6H_4	46±4	47±4
4ad	*p*-Cl-$C_6H_4CH_2$	*p*-OCH_3-C_6H_4	49±4	48±4
4ae	*p*-OCH_3-$C_6H_4CH_2$	*p*-OCH_3-C_6H_4	52±5	50±5
4af	*p*-SCH_3-$C_6H_4CH_2$	*p*-OCH_3-C_6H_4	62±6	62±6
5a	C_6H_5	$C_6H_5CH_2$	34±3	35±3
5b	C_6H_5	napht-1-yl	21±2	22±2
5c	C_6H_5	*p*-CF_3-$C_6H_4CH_2$	25±2	24±2
5d	C_6H_5	*p*-OCH_3-$C_6H_4CH_2$	35±4	36±4
5e	C_6H_5	*m*-OCH_3-$C_6H_4CH_2$	32±3	31±3
5f	C_6H_5	*p*-OCF_3-$C_6H_4CH_2$	24±2	25±2
5g	C_6H_5	*p*-OC_2H_5-$C_6H_4CH_2$	22±2	21±2
5h	C_6H_5	*p*-$OCH(CH_3)_2$-$C_6H_4CH_2$	26±3	26±2
5i	C_6H_5	*m*-CH_3-*p*-OCH_3-$C_6H_3CH_2$	31±3	30±3
2a	*p*-Cl-$C_6H_4N(CH_2CH_2)_2NCH_2$	*p*-Cl-C_6H_4	85±9	71±7
3a	$(CH_3)_3CCH_2$	*p*-Cl-C_6H_4	57±6	58±6
1a (PD 81,723)			19±2	21±2

(a) Inhibition of the forskolin-stimulated cAMP production (in percentage) by the novel allosteric enhancers (10 μM); (b) Inhibition of the cAMP production (in percentage) by the novel allosteric enhancers (100 nM) in the presence of CCPA (1 pM). The values are expressed as the mean ± SEM, n=3 independent experiments.

Table 2. A_1AR density expressed as Bmax values (A) obtained by [^{3}H]CCPA binding assays in hA_1CHO membranes in the presence of reference compounds PD 81,723 (**1a**), **2a** and **3a** along with new molecules **4a-af** and **5a-ai** (10 μM). Modulation by the novel allosteric enhancers (10 μM) on the CCPA affinity (CCPA Ki shift) in [^{3}H]DPCPX competition binding experiments (B).[a]

Compound	(A)		(B)	
	Bmax (fmol/ mg protein)	Bmax shift (fold of increase)	CCPA Ki (nM)	CCPA Ki shift (fold of increase)
4a	2538±234	4.9±0.5	3.6±0.4	4.2±0.4
4b	3168±267	6.1±0.6	3.5±0.4	4.3±0.4
4c	3289±286	6.3±0.6	2.8±0.3	5.4±0.5
4d	3334±298	6.4±0.6	2.7±0.3	5.6±0.6
4e	2918±249	5.6±0.5	3.0±0.3	5.1±0.5
4f	4752±462	9.1±0.9	1.7±0.2	8.9±0.9
4g	4889±469	9.4±0.9	1.7±0.2	8.9±0.9
4h	4272±397	8.2±0.8	2.3±0.2	6.6±0.7
4i	3442±317	6.6±0.6	2.4±0.2	6.3±0.6
4j	4749±436	9.1±0.9	1.9±0.2	8.0±0.8
4k	3950±325	7.6±0.7	2.6±0.2	5.8±0.6
4l	3803±336	7.3±0.7	2.2±0.2	6.9±0.7
4m	2918±228	5.6±0.5	3.1±1.3	4.9±0.5
4n	4217±401	8.1±0.8	2.5±0.2	6.1±0.6
4o	3451±328	6.6±0.6	3.3±0.3	4.6±0.5
4p	2918±288	5.6±0.5	3.6±0.3	4.2±0.4
4q	2229±179	4.3±0.4	4.0±0.4	3.8±0.4
4r	2564±219	4.9±0.5	3.9±0.4	3.9±0.4
4s	2489±209	4.8±0.4	4.1±0.4	3.7±0.4
4t	2759±238	5.3±0.4	3.7±0.3	4.1±0.4
4u	3385±308	6.5±0.7	2.7±0.3	5.6±0.6
4v	3289±286	6.3±0.6	2.8±0.3	5.4±0.5
4w	2772±251	5.3±0.5	2.9±0.2	5.2±0.4
4x	3737±335	7.2±0.7	1.8±0.2	8.4±0.8
4y	4753±465	9.1±0.9	1.6±0.1	9.5±0.9

4z	5743±508	11.0±1.2	1.3±0.1	11.7±1.1
4aa	4467±427	8.6±0.9	2.1±0.2	7.2±0.7
4ab	4418±411	8.5±0.8	2.3±0.2	6.6±0.6
4ac	2498±215	4.8±0.4	4.3±0.4	3.5±0.3
4ad	2598±222	5.0±0.4	3.9±0.4	3.9±0.4
4ae	2652±187	5.1±0.5	3.3±0.3	4.6±0.5
4af	3394±318	6.5±0.5	2.5±0.2	6.1±0.6
5a	1355±127	2.6±0.3	7.9±0.8	1.9±0.2
5b	771±68	1.5±0.1	10.6±1.2	1.4±0.1
5c	944±83	1.8±0.2	9.6±0.8	1.6±0.2
5d	1450±144	2.8±0.3	7.5±0.8	2.0±0.2
5e	1351±131	2.6±0.3	7.8±0.8	1.9±0.2
5f	949±87	1.8±0.2	8.3±0.8	1.8±0.2
5g	789±65	1.5±0.1	9.9±0.9	1.5±0.1
5h	947±81	1.8±0.2	7.6±0.7	2.0±0.2
5i	1407±133	2.7±0.3	6.4±0.6	2.4±0.2
2a	6008±512	11.4±1.1	1.8±0.1	8.7±0.7
3a	3022±309	5.8±0.6	3.8±0.4	4.0±0.4
1a (PD 81,723)	692±63	1.3±0.1	10.9±1.1	1.4±0.1

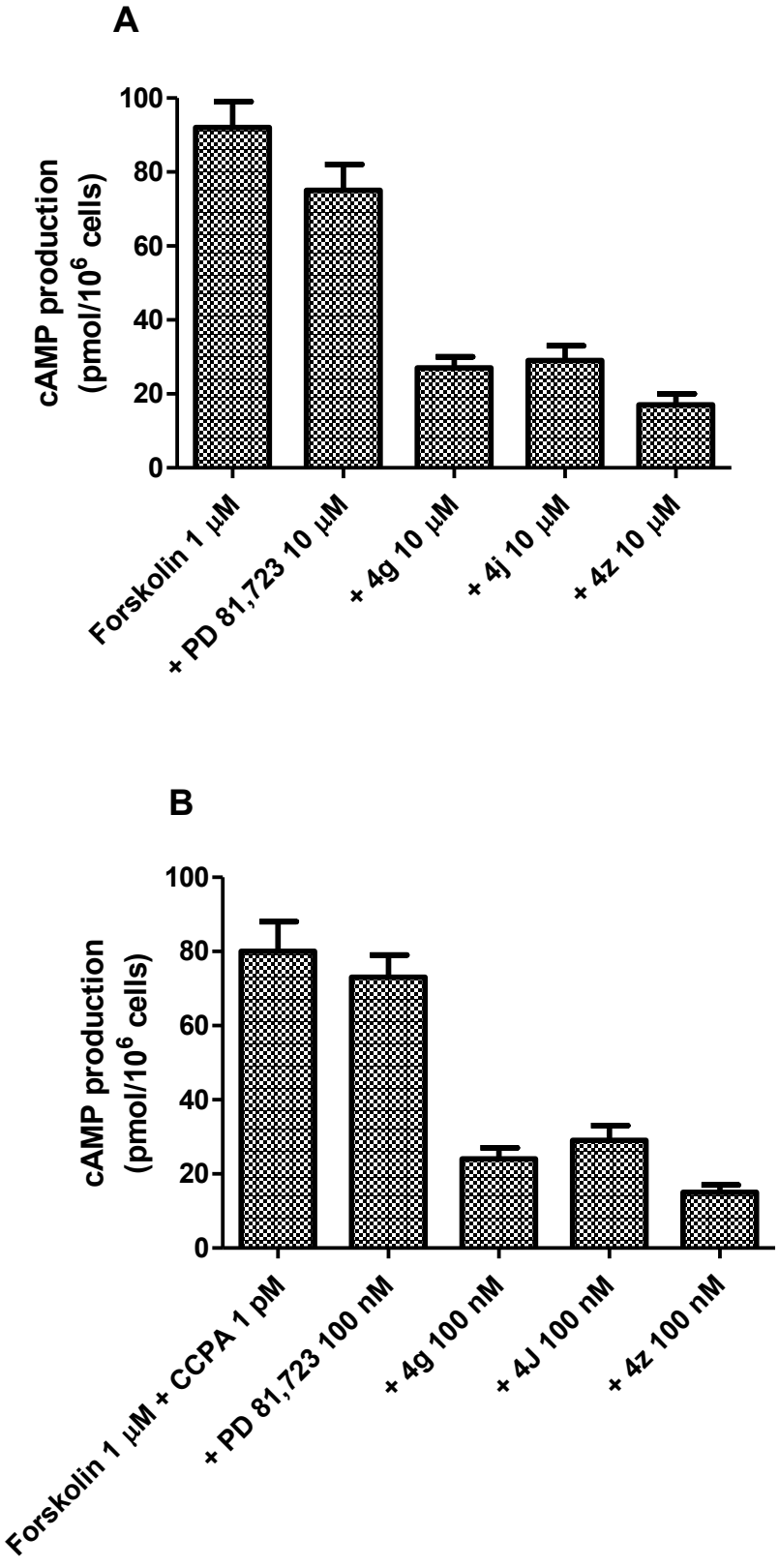
[a]The values are expressed as the mean ± SEM, n=3 independent experiments.
(A)=Bmax (fmol/mg protein) and Bmax shift obtained in [^{3}H]CCPA saturation binding experiments performed in the absence (Bmax=521±47 fmol/mg protein) or in the presence of 10 μM enhancers.
(B)=Ki values of CCPA in the presence of 10 μM test compounds and CCPA shift = Ki(CCPA)/Ki(CCPA+10 μM enhancers) where the Ki of CCPA was 15.1±1.4 nM.

Table 3. Association rate constants (k_{on}), dissociation rate constants (k_{off}), and affinity (K_D) values of [^{3}H]NECA (7 nM), in the presence or absence of 1 μM PD81,423 or **4z**, derived from association and dissociation experiments at 37 °C on hA_1AR stably expressed on CHO membranes.

	k_{on} *($nM^{-1} \cdot min^{-1}$)*	**k_{off}** *(min^{-1})*	**K_D** *(nM)*
Control	0.0222 ± 0.0023	0.0194 ± 0.0009	0.87 ± 0.10
+ 1 μM PD81,723	0.0251 ± 0.0020	0.0157 ± 0.0012	0.63 ± 0.07
+ 1 μM **4z**	0.0204 ± 0.0007	0.0079 ± 0.0005***	0.39 ± 0.03**

K_D was calculated by $K_D = k_{off} / k_{on}$.
Significantly different from control with ** $p < 0.01$ or *** $p < 0.001$ (unpaired Student's t-test).
Values are means ± S.E.M. of three separate experiments, each performed in duplicate.

Figure 1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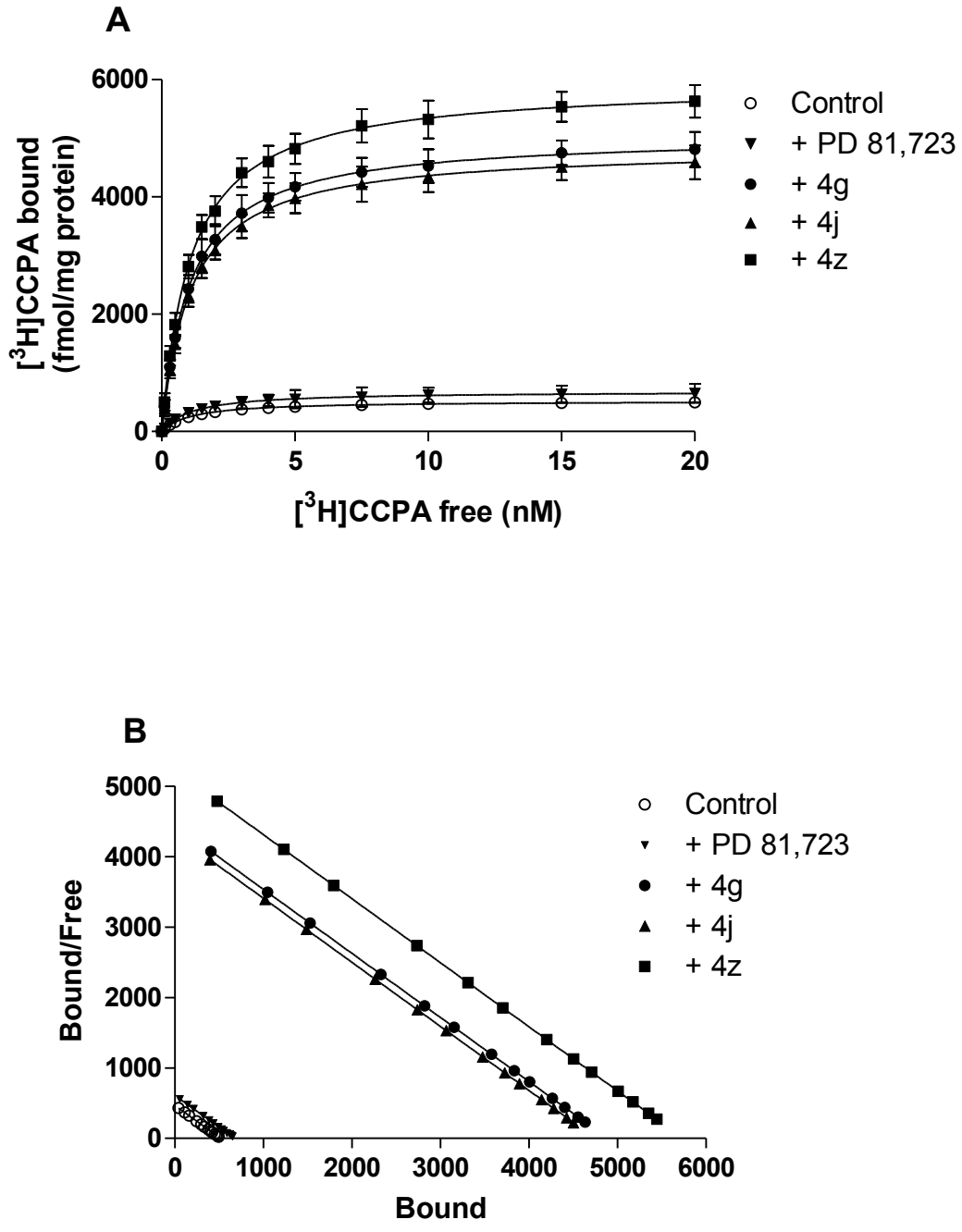
cAMP production (pmol/10^6 cells)
100
80
60
40
20
0
Forskolin 1 μM
+ PD 81,723 10 μM
+ 4g 10 μM
+ 4j 10 μM
+ 4z 10 μM
B
cAMP production (pmol/10^6 cells)
100
80
60
40
20
0
Forskolin 1 μM + CCPA 1 pM
+ PD 81,723 100 nM
+ 4g 100 nM
+ 4J 100 nM
+ 4z 100 nM

Figure 2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4000
2000
0
[3H]CCPA bound
(fmol/mg protein)
0
5
10
15
20
[3H]CCPA free (nM)
Control
+ PD 81,723
+ 4g
+ 4j
+ 4z
B
5000
4000
3000
2000
1000
0
Bound/Free
0
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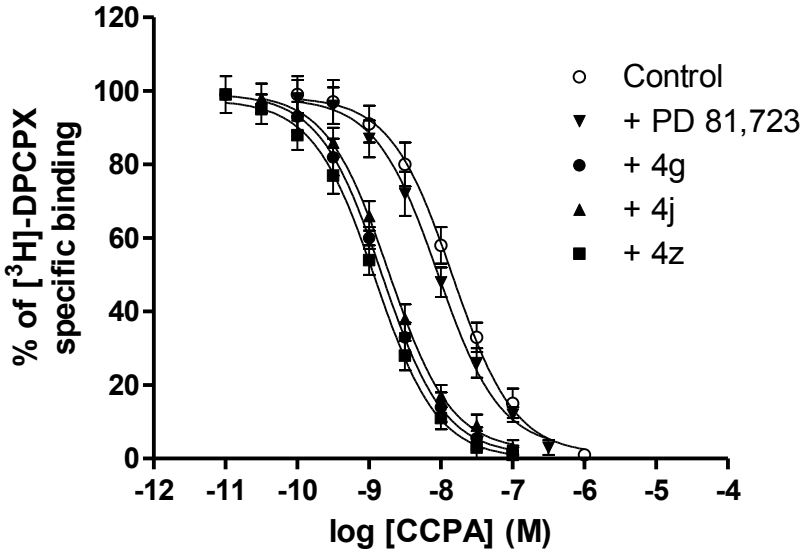
2000
3000
4000
5000
6000
Bound
Control
+ PD 81,723
+ 4g
+ 4j
+ 4z

Figure 3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100
80
60
40
20
0
% of [3H]-DPCPX specific binding
-12
-11
-10
-9
-8
-7
-6
-5
-4
log [CCPA] (M)
Control
+ PD 81,723
+ 4g
+ 4j
+ 4z

Figure 4

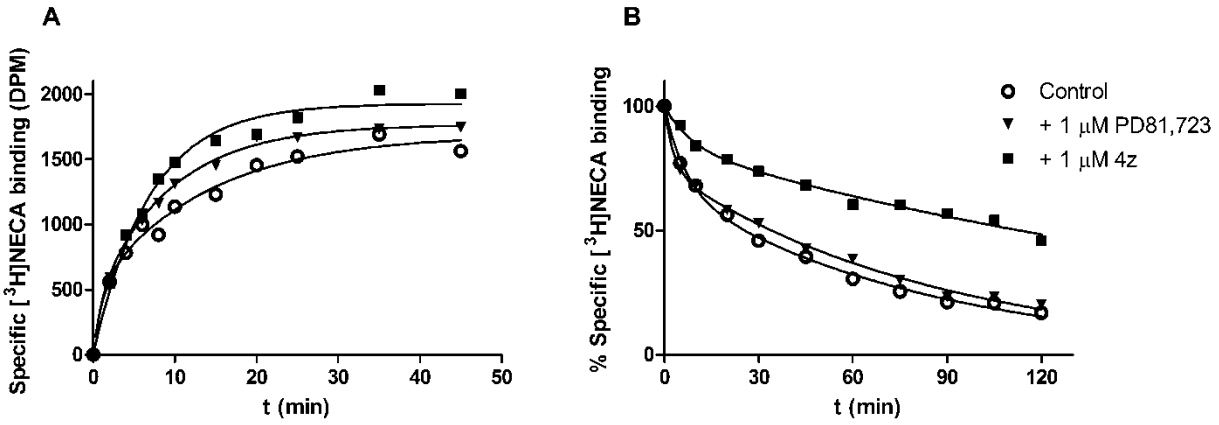

A
Specific [3H]NECA binding (DPM)
2000
1500
1000
500
0
0
10
20
30
40
50
t (min)
B
% Specific [3H]NECA binding
100
50
0
0
30
60
90
120
t (min)
Control
+ 1 μM PD81,723
+ 1 μM 4z

Figure 5

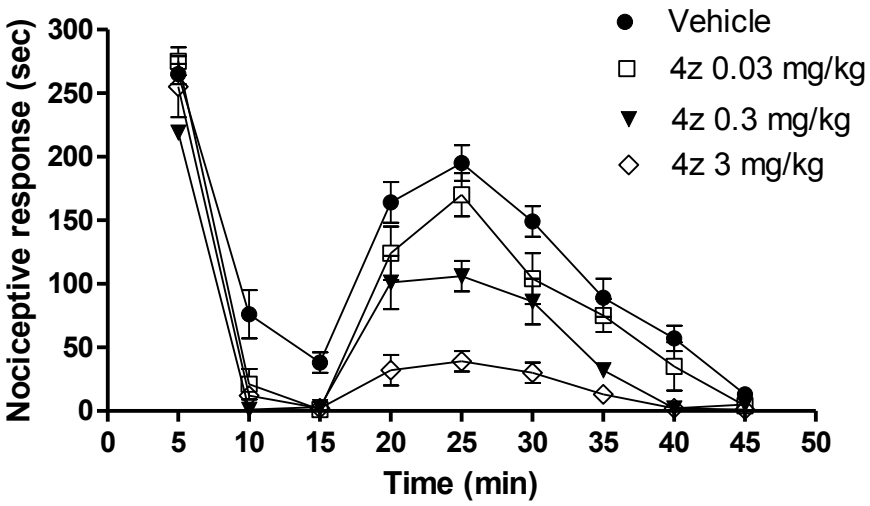
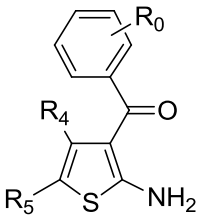
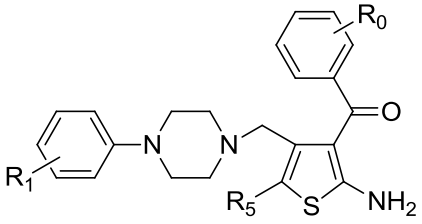

Vehicle
4z 0.03 mg/kg
4z 0.3 mg/kg
4z 3 mg/kg
Nociceptive response (sec)
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
45
50
Time (min)

Chart 1. Structures of previously described 2-amino-3-aroyl thiophene derivatives **1a-e**, **2a** and **3a-b** as allosteric modulators for the A_1 adenosine receptor

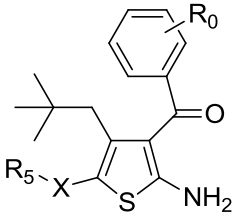


1a (PD 81,723); R_0=3-CF_3, R_4=R_5=CH_3
1b (LUF5484); R_0=3,4-Cl_2, R_4,R_5=$(CH_2)_4$
1c (T62); R_0=4-Cl, R_4,R_3=$(CH_2)_4$
1d (PD 71,605); R_0=3-Cl, R_4,R_5=$(CH_2)_4$
1e; R_0=4-CH_3, R_4,R_5=$(CH_2)_4$



2

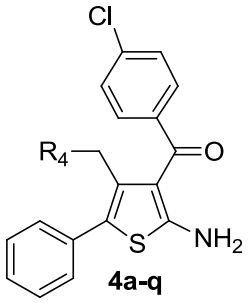
R_0=4-Cl or 3-CF_3
R_1= EWG (F, Cl, CF_3, NO_2) or ERG (Me or OMe)
R_5=H, Me, Et, Br or aryl
2a, R_0=4'-Cl, R_5=4'-Cl-C_6H_4



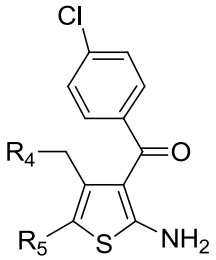
3

R_0=2'- 3'-or 4'-Cl, 3',4'-Cl_2, 3'-CF_3 or 4'-CH_3.
R_5=H, Br, heteroaryl (thiophene, furan, pyridine, pyrazole, isoxazole), phenyl with EWG (Cl or F) or ERG (alkyl, alkoxy or OCF_3).
X=nothing or -C≡C-.
3a, R_0=4'-Cl, X=nothing, R_5=4'-Cl-C_6H_4
3b, R_0=4'-Cl, X= -C≡C-, R_5=C_6H_5

Chart 2. Structures of 2-amino-3-(p-chlorobenzoyl)-4.5-disubstituted thiophene derivatives **4a-af**, and **5a-i** evaluated in this study as allosteric ligands for the A_1 adenosine receptor



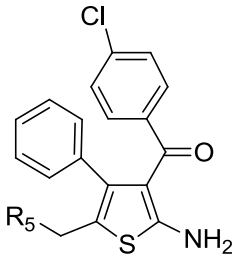
4a-q



4r-af

4a; R_4=C_6H_5
4b; R_4=1-naphthyl
4c; R_4=*p*-F-C_6H_4
4d; R_4=*p*-Cl-C_6H_4
4e; R_4=*p*-CH_3-C_6H_4
4f; R_4=*p*-CF_3-C_6H_4
4g; R_4=*p*-OCH_3-C_6H_4
4h; R_4=*p*-OCF_3-C_6H_4
4i; R_4=*p*-SCH_3-C_6H_4
4j; R_4=*p*-OCH_2CH_3-C_6H_4
4k; R_4=*p*-$OCH(CH_3)_2$-C_6H_4
4l; R_4=*m*-OCH_3-C_6H_4
4m; R_4=*o*-OCH_3-C_6H_4
4n; R_4=*m*-CH_3-*p*-OCH_3-C_6H_3
4o; R_4=*m,m'*-$(CH_3)_2$-*p*-OCH_3-C_6H_2
4p; R_4=*o,p*-$(OCH_3)_2$-C_6H_3
4q; R_4=*m,m',p*-$(OCH_3)_3$-C_6H_2

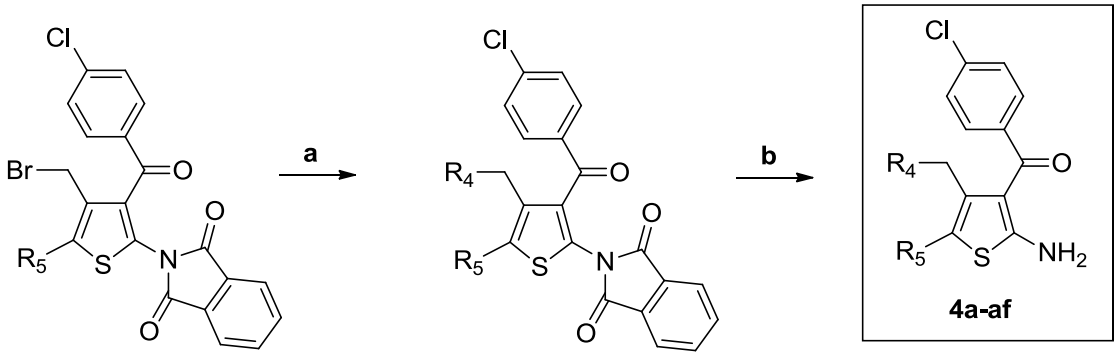
4r; R_4=C_6H_5, R_5=*p*-F-C_6H_4
4s; R_4=R_5=*p*-F-C_6H_4
4t; R_4=*p*-Cl-C_6H_4, R_5=*p*-F-C_6H_4
4u; R_4=*p*-CH_3-C_6H_4, R_5=*p*-F-C_6H_4
4v; R_4=*p*-OCH_3-C_6H_4, R_5=*p*-F-C_6H_4
4w; R_4=*p*-SCH_3-C_6H_4, R_5=*p*-F-C_6H_4
4x; R_4=C_6H_5, R_5=*p*-Cl-C_6H_4
4y; R_4=*p*-F-C_6H_4, R_5=*p*-Cl-C_6H_4
4z; R_4=R_5=*p*-Cl-C_6H_4
4aa; R_4=*p*-CH_3-C_6H_4, R_5=*p*-Cl-C_6H_4
4ab; R_4=*p*-OCH_3-C_6H_4, R_5=*p*-Cl-C_6H_4
4ac; R_4=C_6H_5, R_5=*p*-OMe-C_6H_4
4ad; R_4=*p*-Cl-C_6H_4, R_5=*p*-OMe-C_6H_4
4ae; R_4=R_5=*p*-OMe-C_6H_4
4af; R_4=*p*-SCH_3-C_6H_4, R_5=*p*-OMe-C_6H_4



5a-h

5a; R_5=C_6H_5
5b; R_5=1-naphthyl
5c; R_5=*p*-CF_3-C_6H_4
5d; R_5=*p*-OCH_3-C_6H_4
5e; R_5=*m*-OCH_3-C_6H_4
5f; R_5=*p*-OCF_3-C_6H_4
5g; R_5=*p*-OCH_2CH_3-C_6H_4
5h; R_5=*p*-$OCH(CH_3)_2$-C_6H_4
5i; R_5=*m*-CH_3-*p*-OCH_3-C_6H_3

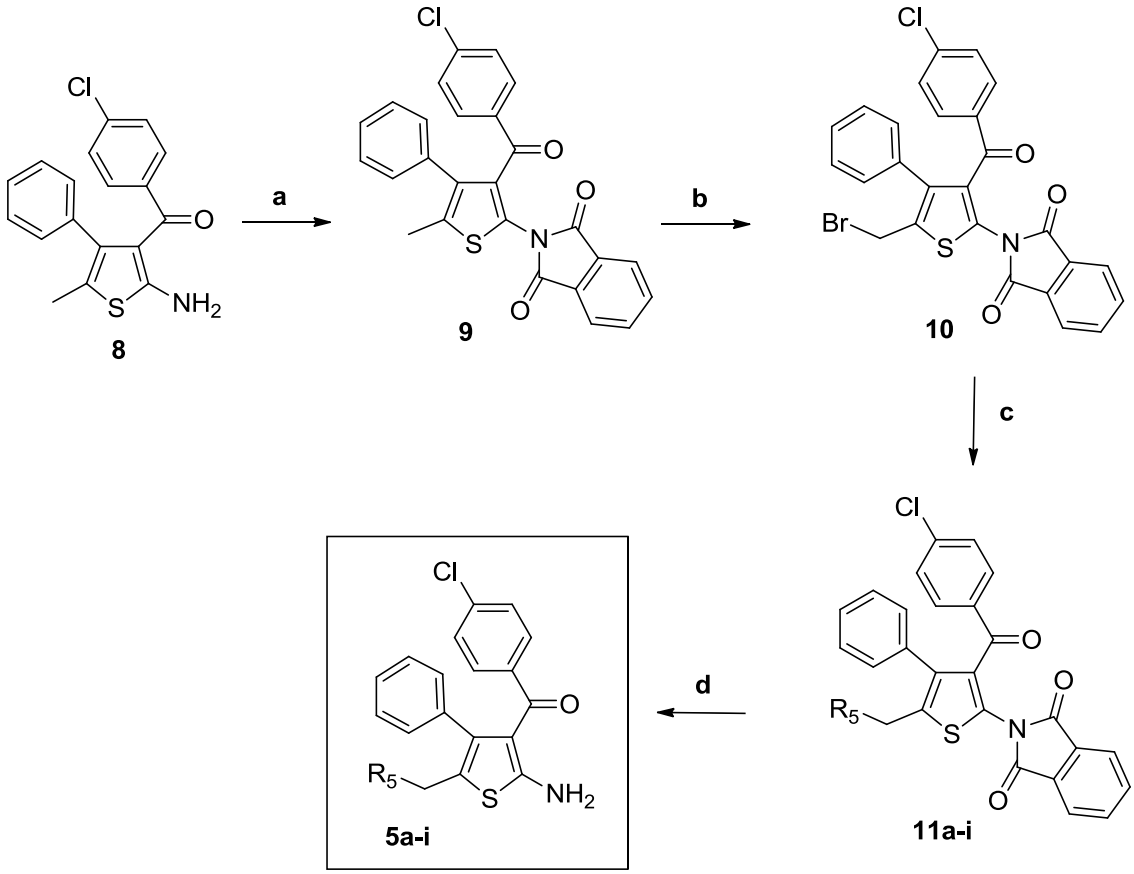
Scheme 1



6a; $R_5=C_6H_5$
6b;$R_5=p$-F-C_6H_4
6c; $R_5=p$-Cl-C_6H_4
6d; $R_5=p$-OMe-C_6H_4

7a, 4a; $R_4=R_5=C_6H_5$
7b, 4b; R_4=1-naphthyl, $R_5=C_6H_5$
7c, 4c; $R_4=p$-F-C_6H_4, $R_5=C_6H_5$
7d, 4d; $R_4=p$-Cl-C_6H_4, $R_5=C_6H_5$
7e, 4e; $R_4=p$-CH_3-C_6H_4, $R_5=C_6H_5$
7f, 4f; $R_4=p$-CF_3-C_6H_4, $R_5=C_6H_5$
7g, 4g; $R_4=p$-OCH_3-C_6H_4, $R_5=C_6H_5$
7h, 4h; $R_4=p$-OCF_3-C_6H_4, $R_5=C_6H_5$
7i, 4i; $R_4=p$-SCH_3-C_6H_4, $R_5=C_6H_5$
7j, 4j; $R_4=p$-OCH_2CH_3-C_6H_4, $R_5=C_6H_5$
7k, 4k; $R_4=p$-$OCH(CH_3)_2$-C_6H_4, $R_5=C_6H_5$
7l, 4l; $R_4=m$-OCH_3-C_6H_4, $R_5=C_6H_5$
7m, 4m; $R_4=o$-OCH_3-C_6H_4, $R_5=C_6H_5$
7n, 4n; $R_4=m$-CH_3-p-OCH_3-C_6H_3, $R_5=C_6H_5$
7o, 4o; $R_4=m,m'$-$(CH_3)_2$-p-OCH_3-C_6H_2, $R_5=C_6H_5$
7p, 4p; $R_4=o,p$-$(OCH_3)_2$-C_6H_3, $R_5=C_6H_5$
7q, 4q; $R_4=m,m',p$-$(OCH_3)_3$-C_6H_2, $R_5=C_6H_5$
7r, 4r; $R_4=C_6H_5$, $R_5=p$-F-C_6H_4
7s, 4s; $R_4=R_5=p$-F-C_6H_4
7t, 4t; $R_4=p$-Cl-C_6H_4, $R_5=p$-F-C_6H_4
7u, 4u; $R_4=p$-CH_3-C_6H_4, $R_5=p$-F-C_6H_4
7v, 4v; $R_4=p$-OCH_3-C_6H_4, $R_5=p$-F-C_6H_4
7w, 4w; $R_4=p$-SCH_3-C_6H_4, $R_5=p$-F-C_6H_4
7x, 4x; $R_4=C_6H_5$, $R_5=p$-Cl-C_6H_4
7y, 4y; $R_4=p$-F-C_6H_4, $R_5=p$-Cl-C_6H_4
7z, 4z; $R_4=R_5=p$-Cl-C_6H_4
7aa, 4aa; $R_4=p$-CH_3-C_6H_4, $R_5=p$-Cl-C_6H_4
7ab, 4ab; $R_4=p$-OCH_3-C_6H_4, $R_5=p$-Cl-C_6H_4
7ac, 4ac; $R_4=C_6H_5$, $R_5=p$-OMe-C_6H_4
7ad, 4ad; $R_4=p$-Cl-C_6H_4, $R_5=p$-OMe-C_6H_4
7ae, 4ae; $R_4=R_5=p$-OMe-C_6H_4
7af, 4af; $R_4=p$-SCH_3-C_6H_4, $R_5=p$-OMe-C_6H_4

Scheme 2



5a, $R_5=C_6H_5$
5b, R_5=1-naphthyl
5c, $R_5=p$-CF_3-C_6H_4
5d, $R_5=p$-OCH_3-C_6H_4
5e, $R_5=m$-OCH_3-C_6H_4
5f, $R_5=p$-OCF_3-C_6H_4
5g, $R_5=p$-OCH_2CH_3-C_6H_4
5h, $R_5=p$-$OCH(CH_3)_2$-C_6H_4
5i, $R_5=m$-CH_3-p-OCH_3-C_6H_3

11a, $R_5=C_6H_5$
11b, R_5=1-naphthyl
11c, $R_5=p$-CF_3-C_6H_4
11d, $R_5=p$-OCH_3-C_6H_4
11e, $R_5=m$-OCH_3-C_6H_4
11f, $R_5=p$-OCF_3-C_6H_4
11g, $R_5=p$-OCH_2CH_3-C_6H_4
11h, $R_5=p$-$OCH(CH_3)_2$-C_6H_4
11i, $R_5=m$-CH_3-p-OCH_3-C_6H_3