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Structure–activity relationships of 2-amino-3-aroyl-4-[(4-arylpiperazin-1-yl)methyl]thiophenes. Part 2: Probing the influence of diverse substituents at the phenyl of the arylpiperazine moiety on allosteric enhancer activity at the A_1 adenosine receptor

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ABSTRACT

In a preliminary article, we reported the potent allosteric enhancer activity at the A_1 adenosine receptor of a small series of 2-amino-3-(4-chlorobenzoyl)-4-[4-(aryl)piperazin-1-yl)methyl]thiophene derivatives bearing electron-withdrawing or electron-releasing groups at the *para*-position of the phenylpiperazine moiety. In the present study, we report the development of the compounds previously studied by modifying both the number and position of substituents on the phenylpiperazine moiety, aimed at establishing a structure–activity relationship identifying additional compounds with improved activity.

The nature and the position of substituents on the phenyl ring tethered to the piperazine seemed to exert a fundamental influence on the allosteric enhancer activity, with the 3,4-difluoro **4i**, 3-chloro-4-fluoro **4o**, and 4-trifluoromethoxy **4ak** derivatives being the most active compounds in binding (saturation and competition experiments) and functional cAMP studies. This study shows that it is also possible to obtain a good separation between allosteric enhancement and antagonistic activity at the A_1 adenosine receptor.

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1. Introduction

Adenosine is an endogenous nucleoside modulator able to mediate several effects through activation of four G-protein coupled adenosine receptors (ARs) named as A_1, A_{2A}, A_{2B} and A_3.[1] In particular A_1 and A_3ARs are coupled to Gi proteins mediating a decrease of cAMP production whilst A_{2A} and A_{2B}ARs are linked to Gs proteins and increase cAMP accumulation. The adenosine A_1AR is highly and widely expressed in the Central Nervous System (CNS) with high levels in brain, cortex, cerebellum and hippocampus, but also in other tissues such as fat cells, bladder and heart. The A_1ARs modulate the activity of the CNS mediating anticonvulsant and anxiolytic effects and playing a role in adenosine-mediated analgesia.[2] Adenosine accumulates rapidly at sites of tissue hypoxia or injury exerting protective effects through mechanisms such as ischemic preconditioning (IPC) or modulating the actions of inflammatory cells, as in reperfusion injury.

Due to their ubiquitous presence, the ability to selectively modulate A_1ARs within specific target tissues may be of great therapeutic importance. The side effects induced by the A_1 activation limit the clinical application of A_1 agonists. A more controlled and selective 'tuning' action is feasible through the allosteric modulation of G protein-coupled receptors (GPCRs).[3] 'Allosteric' refers to binding sites that are different from the 'orthosteric' primary substrate or ligand binding site, to which the binding of modulators results in conformation changes that might profoundly influence GPCR function.[4]

The binding of an allosteric enhancer (AE) to its site causes conformational changes in the A_1AR, slowing the dissociation of endogenous agonist adenosine from the orthosteric site.[4] By the screening of chemical libraries, Bruns and colleagues identified various 2-amino-3-benzoylthiophene derivatives capable of slowing the dissociation of the agonist N^6-cyclopentyladenosine (CPA) from the A_1AR. They also reported that these compounds were

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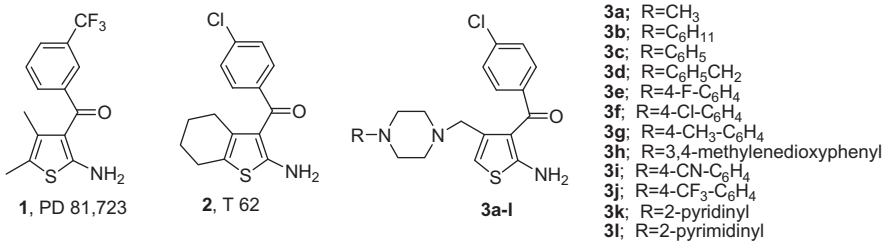
capable of acting as competitive antagonists at the same receptor subtype at higher concentrations.[6,7] Further investigations have shown that this class of molecules is able to act as AEs at the A_1AR, both in binding and in vitro functional assays.[7] Among the tested compounds, PD 81,723 (2-amino-4,5-dimethylthien-3-yl)-[3-(trifluoromethyl)phenyl]-methanone (compound **1**) was pharmacologically studied and recognized as the first specific and selective AE of the A_1AR.[7–9]

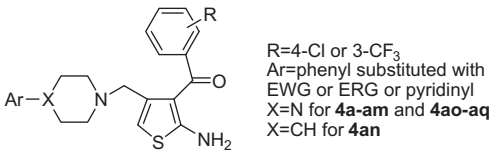
Since the structure of the allosteric site of the A_1ARs has not been resolved at the atomic level and intrigued by the peculiarity of the PD 81,723 structure, several groups started a molecular modulation of this ligand to elucidate the binding site of the 2-amino-3-aroyl-thiophene moiety.[10–19] These studies highlighted that the 2-amino group is essential for activity. In addition, electron-withdrawing substituents, such as chloro and trifluoromethyl, at either the *meta* or *para*-position on the benzoyl moiety at the 3-position of the thiophene ring greatly increased enhancement activity. A range of alkyl and aryl groups in the 4-and 5-positions of the thiophene ring also favored allosteric enhancing activity.

Among the synthesized compounds, derivative **2** {T-62, (2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)(4-chlorophenyl)methanone, was taken into Phase II clinical trials by King Pharmaceutical for the treatment of neuropathic pain.[20]

In a preliminary article, we have reported the AE activity at the A_1ARs of a series of 2-amino-3-(4-chlorobenzoyl)thiophene derivatives **3a–l**, with various alkyl- (**3a–b**) or aryl- (**3c–l**) piperazine moieties attached to the 4-position of the thiophene ring by a methylene unit (Chart 1).[21] In detail, we have evaluated the effect on AE activity by electron-withdrawing substituents such as F, Cl, CN and CF_3, (compounds **3e**, **3f, 3i** and **3j**, respectively) and electron-donating groups (CH_3 and 3,4-methylenedioxy for **3g** and **3h**, respectively) introduced as substituents on the *N*-phenylpiperazine moiety. The results obtained with compounds **3a–l** make it possible to isolate in the phenylpiperazine moiety a region that is critical for interaction with the allosteric site of the A_1ARs, and whose manipulation can greatly improve enhancer activity. It may be possible that the phenyl ring of the phenylpiperazine moiety contributes to activity with a π–π interaction (such as charge-transfer), interacting with a lipophilic pocket. This was confirmed by replacement of the phenyl moiety with electron-deficient heteroaromatic pyridin-2-yl (**3k**) or pyrimidin-2-yl (**3l**) rings, which incorporate one or two basic nitrogens in the critical region of binding, causing a reduction of the AE activity.

For these reasons, derivatives **3a–l** were the starting point for achieving potent and efficacious compounds with reduced competitive antagonistic activity that is not required for the enhancement at A_1ARs. The nature of group at the *para*-position of the phenylpiperazine moiety seemed to exert an important influence on AE activity, although only a limited series of substituents were considered to investigate their effect. Therefore, in the current study we have investigated the structural modification of the phenyl piperazine moiety at the 4-position of the 2-amino-3-(4-chlorobenzoyl)-





Ar=$(CH_3)_2$CH, R=4-Cl, **4a**
Ar=cyclopentyl, R=4-Cl, **4b**
Ar=cycloheptyl, R=4-Cl, **4c**
Ar=1-naphthyl, R=4-Cl, **4d**
Ar=4-F-$C_6H_4CH_2$, R=4-Cl, **4e**
Ar=3-F-C_6H_4, R=4-Cl, **4f**
Ar=2-F-C_6H_4, R=4-Cl, **4g**
Ar=2,4-diF-C_6H_3, R=4-Cl, **4h**
Ar=3,4-diF-C_6H_3, R=4-Cl, **4i**
Ar=3,5-diF-C_6H_3, R=4-Cl, **4j**
Ar=2,6-diF-C_6H_3, R=4-Cl, **4k**
Ar=2,3-diF-C_6H_3, R=4-Cl, **4l**
Ar=2,5-diF-C_6H_3, R=4-Cl, **4m**
Ar=2,4,6-triF-C_6H_2, R=4-Cl, **4n**
Ar=3-Cl,4-F-C_6H_3, R=4-Cl, **4o**
Ar=4-Cl-C_6H_4CO, R=4-Cl, **4p**
Ar=4-Cl-$C_6H_4CH_2$, R=4-Cl, **4q**
Ar=4-Cl-$C_6H_4(CH_2)_2$, R=4-Cl, **4r**
Ar=4-Cl-$C_6H_4(CH_2)_3$, R=4-Cl, **4s**
Ar=4-Cl-$C_6H_4CH_2CO$, R=4-Cl, **4t**
Ar=4-Cl-$C_6H_4COCH_2$, R=4-Cl, **4u**
Ar=3-Cl-C_6H_4, R=4-Cl, **4v**
Ar=2-Cl-C_6H_4, R=4-Cl, **4w**
Ar=2,6-diCl-C_6H_3, R=4-Cl, **4x**
Ar=2,5-diCl-C_6H_3, R=4-Cl, **4y**
Ar=3,4-diCl-C_6H_3, R=4-Cl, **4z**
Ar=2,4-diCl-C_6H_3, R=4-Cl, **4aa**
Ar=2,3-diCl-C_6H_3, R=4-Cl, **4ab**
Ar=3,5-diCl-C_6H_3, R=4-Cl, **4ac**
Ar=2-Cl,4-F-C_6H_3, R=4-Cl, **4ad**
Ar=2-F,4-Cl-C_6H_3, R=4-Cl, **4ae**
Ar=4-CH_3O-C_6H_4, R=4-Cl, **4af**
Ar=4-NO_2-C_6H_4, R=4-Cl, **4ag**
Ar=3-CF_3-C_6H_4, R=4-Cl, **4ah**
Ar=2-CF_3-C_6H_4, R=4-Cl, **4ai**
Ar=4-Cl,3-CF_3-C_6H_3, R=4-Cl, **4aj**
Ar=4-CF_3O-C_6H_4, R=4-Cl, **4ak**
Ar=3-pyridinyl, R=4-Cl, **4al**
Ar=4-pyridinyl, R=4-Cl, **4am**
Ar=4-Cl-C_6H_4, R=4-Cl, **4an**
Ar=4-CF_3-C_6H_4, R=3-CF_3, **4ao**
Ar=3-F-C_6H_4, R=3-CF_3, **4ap**
Ar=2,6-diF-C_6H_4, R=3-CF_3, **4aq**

Chart 1. 2-Amino-3-aroyl thiophene derivatives, allosteric modulators for A_1 adenosine receptor.

thiophene scaffold, by the synthesis of a wide series of compounds with one or more electron withdrawing substituents (mainly, but not exclusively, F, Cl and CF_3) in all five positions of the phenyl ring linked to the piperazine moiety. By the synthesis of compounds **4e** and **4p–u**, we have also investigated the effect on the AE activity due to the presence of a linker (one, two or three methylene units for compounds **4e–q**, **4r** and **4s**, respectively), carbonyl for **4p**, acetyl and 'reversed acetyl' for **4t** and **4u**, between the aromatic ring and the *N*1-nitrogen of the piperazine ring.

2. Chemistry

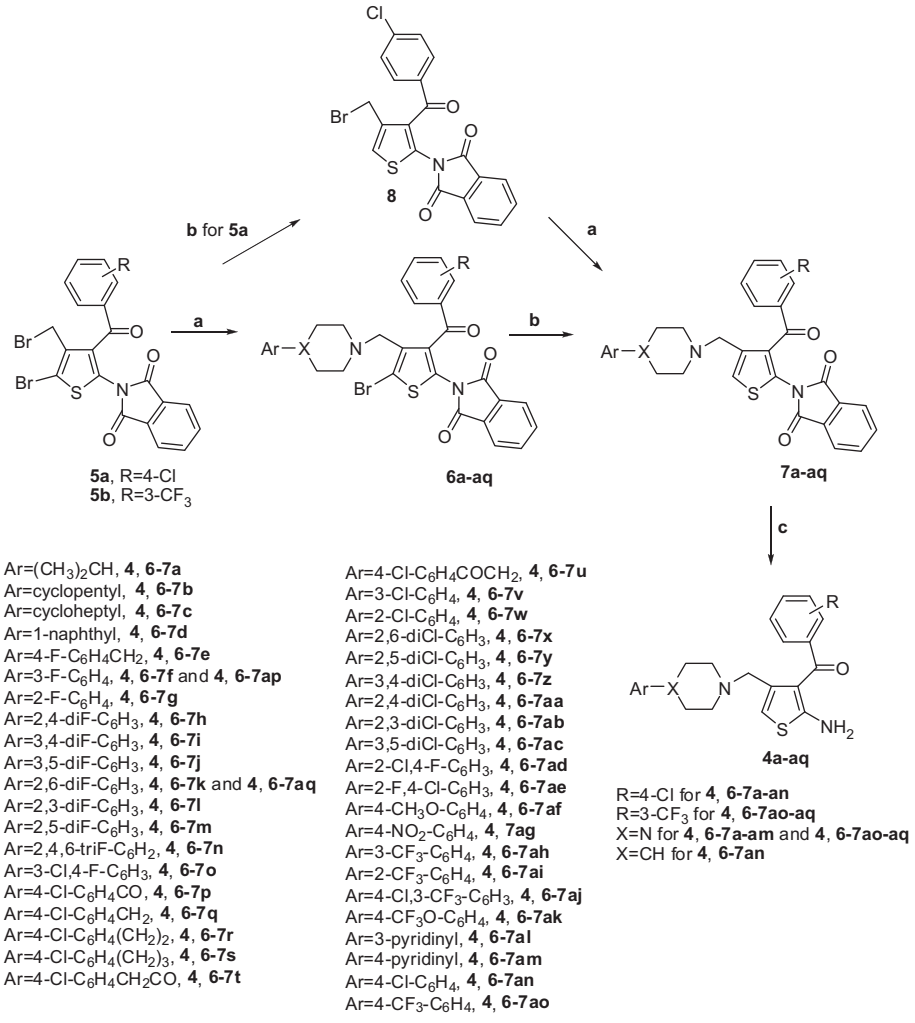
The approach taken for the preparation of compounds **4a–aq** is shown in Scheme 1. Preparation of the studied compounds was done according to literature methods for the derivatives **3a–l**.[21] The 4-bromomethyl-5-bromo thiophene analogs **5a** and **5b** were used as common intermediates for the synthesis of final compounds **4a–an** and **4ao–aq**, respectively. Derivatives **5a** and **5b** were coupled with the appropriate *N*-alkyl/arylpiperazines or p-chlorophenylpiperidine (2 equiv) in dichloromethane to afford the derivatives **6a–aq** in good yields. Dehalogenation by catalytic hydrogenolysis using 10% Pd/C furnished the 5-unsubstituted thiophenes **7a–aq**, with the exception of **7ag**. This latter compound was obtained by the condensation of **8** (prepared in poor yield by the chemoselective dehalogenation of **5a**) with 1-(4-nitrophenyl)piperazine. Treatment of **7a–aq** with ethanolic hydrazine afforded the final compounds **4a–aq**.

3. Results and discussion

3.1. Functional assays

Functional assays were performed using CHO cells stably transfected with the recombinant human A_1ARs In these experiments the ability to reduce the cAMP levels in hA_1CHO cells by the novel compounds **4a–aq** and by the reference compound (PD 81,723) at four different concentrations (0.01, 0.1, 1 and 10 μM) was measured (Table 1). The effect of each tested compound on cAMP production was reported as a percentage in comparison with the controls (100%). Novel compounds with the potential role of AEs were able to activate human A_1ARs showing a decrease of the cAMP production in hA_1CHO cells.

Among the forty-three synthesized compounds, thirteen compounds (**4a–d**, **4g**, **4t**, **4w–x**, **4ag**, **4al–am, 4ao** and **4aq**) were less active than PD 81,723. The remaining derivatives decreased the percentage of cAMP production more than (from 70% to 82% for **4e–f, 4h–o, 4q–s, 4v, 4aa–ae, 4ah** and **4ak**) or comparable (**4p, 4u, 4y–z, 4af, 4ai–aj, 4an** and **4ap**) to PD 81,723 at the highest concentration tested (10 μM). At 10-fold lower concentrations (1 μM), derivatives **4f, 4h–o, 4q, 4y–ac, 4ae, 4ah, 4aj** and **4ak** cause a



Scheme 1. Reagents and conditions: (a) *N*-Alkyl/aryl piperazine or *p*-chlorophenylpiperidine, K_2CO_3, CH_2Cl_2, +4 °C for 30 min then rt for 2 h; (b) H_2, 10% Pd/C, DMF, rt; (c) NH_2NH_2, EtOH, rx.

Table 1
Effect of compounds **3b–f** and **3j–k**, **4a–aq** and PD 81,723 in cAMP assays on hA_1CHO cells

Compound	% Change in cAMP production (mean ± SEM)[a] concentration of compounds			
	0.01 μM	0.1 μM	1.0 μM	10 μM
PD 81,723	−8.0 ± 0.5	−3.4 ± 0.6	−31 ± 6.2	−57 ± 2.3
3b[21]	+5.3 ± 0.5	+3.8 ± 0.7	−13 ± 1.4	−52 ± 3.9
3c[21]	−4.7 ± 0.1	−26 ± 1.5	−62 ± 11	−78 ± 4.8
3d[21]	+3.2 ± 0.4	+4.6 ± 0.2	−30 ± 11	−78 ± 4.9
3e[21]	+3.5 ± 0.7	−19 ± 3.4	−63 ± 4.9	−77 ± 2.8
3f[21]	+10 ± 4.2	−48 ± 14	−65 ± 4.9	−78 ± 2.8
3j[21]	−10 ± 1.7	−53 ± 5.8	−84 ± 7.9	−87 ± 5.9
3k[21]	+9.4 ± 1.4	−5.6 ± 1.6	−46 ± 14	−69 ± 1.8
4a	−1.0 ± 0.1	+3.6 ± 0.2	+7.3 ± 0.7	−7.4 ± 1.2
4b	+9.1 ± 1.2	+2.1 ± 0.4	+10 ± 2.3	−30 ± 7.2
4c	+2.9 ± 0.5	+13 ± 4.2	+3.7 ± 0.5	−25 ± 9.1
4d	+8.7 ± 1.1	0.0 ± 0.0	+2.9 ± 0.1	−21 ± 8.1
4e	+2.2 ± 0.3	−3.9 ± 0.7	−34 ± 11	−82 ± 3.9
4f	−18 ± 2.0	−37 ± 5.9	−86 ± 5.8	−82 ± 2,7
4g	+2.1 ± 0.7	−1.0 ± 0.1	−9.2 ± 0.8	−15 ± 2.2
4h	0.0 ± 0.0	−17 ± 2.8	−66 ± 3.7	−82 ± 2.9
4i	−3.2 ± 0.6	−34 ± 4.3	−78 ± 2.8	−87 ± 2.8
4j	−13 ± 2.6	−5.0 ± 1.3	−66 ± 10	−79 ± 4.8
4k	−12 ± 1.7	−23 ± 4.2	−65 ± 3.6	−80 ± 1.9
4l	−13 ± 1.6	−5.2 ± 1.3	−66 ± 10	−79 ± 4.3
4m	+3.1 ± 0.2	−13 ± 2.2	−59 ± 4.1	−80 ± 3.9
4n	+4.2 ± 0.3	−3.9 ± 0.1	−56 ± 3.2	−78 ± 3.9
4o	+4.2 ± 0.5	−21 ± 2.3	−60 ± 8.3	−78 ± 6.7
4p	−6.2 ± 0.7	−10 ± 1.4	−34 ± 2.8	−60 ± 4.7
4q	−2.2 ± 0.4	+4.2 ± 0.3	−43 ± 1.7	−82 ± 2.9
4r	+1.2 ± 0.4	−3.1 ± 0.7	−32 ± 5.2	−76 ± 3.8
4s	0.0 ± 0.0	−3.0 ± 0.3	−30 ± 5.2	−76 ± 5.2
4t	+2.1 ± 0.2	0.0 ± 0.0	−8.1 ± 0.4	−42 ± 3.2
4u	−5.0 ± 0.6	+1.0 ± 0.0	−25 ± 5.2	−62 ± 6.2
4v	−3.1 ± 0.3	−20 ± 1.6	−59 ± 4.1	−77 ± 3.2
4w	−2.1 ± 0.0	+2.0 ± 0.7	−5.2 ± 0.1	−18 ± 2.6
4x	+15 ± 1.5	0.0 ± 0.0	−2.2 ± 0.4	−47 ± 1.2
4y	+13 ± 1.2	−6.2 ± 0.5	−42 ± 2.9	−62 ± 5.2
4z	−1.2 ± 0.0	−13 ± 1.4	−60 ± 8.2	−62 ± 1.8
4aa	+2.0 ± 0.0	−11 ± 1.4	−54 ± 5.2	−74 ± 1.2
4ab	+1.0 ± 0.0	+3.2 ± 0.1	−57 ± 6.4	−77 ± 3.5
4ac	−1.0 ± 0.0	−22 ± 7.2	−69 ± 4.2	−74 ± 3.5
4ad	+6.1 ± 0.0	−6.2 ± 0.2	−39 ± 3.2	−70 ± 2.3
4ae	−9.2 ± 0.6	−9.4 ± 0.4	−57 ± 1.2	−81 ± 3.2
4af	−1.2 ± 0.0	0.0 ± 0.0	−15 ± 1.1	−52 ± 4.1
4ag	−12 ± 1.3	−13 ± 1.5	−8.3 ± 0.7	−17 ± 1.0
4ah	−8.2 ± 0.6	−30 ± 4.2	−72 ± 0.2	−80 ± 3.3
4ai	0.0 ± 0.0	−7.5 ± 0.4	−39 ± 7.2	−60 ± 4.2
4aj	−2.5 ± 0.4	−12 ± 1.4	−47 ± 5.2	−60 ± 3.3
4ak	0.0 ± 0.0	−13 ± 1.7	−51 ± 3.2	−74 ± 10
4al	+3.2 ± 0.9	+3.4 ± 0.8	−2.7 ± 0.7	−23 ± 2.0
4am	−4.0 ± 0.3	+1.4 ± 0.0	−8.3 ± 1.5	−15 ± 3.1
4an	0.0 ± 0.0	−2.3 ± 0.5	−24 ± 3.2	−61 ± 4.3
4ao	−12 ± 3.2	−11 ± 4.0	−27 ± 5.0	−47 ± 4.1
4ap	−11 ± 2.5	−12 ± 1.5	−35 ± 3.3	−62 ± 4.2
4aq	+2.1 ± 0.7	+1.3 ± 0	+1.3 ± 0.0	+4.0 ± 0.5

[a] The results are the average of four experiments at each of four concentrations of tested compounds.

greater than 42% decrease of cAMP accumulation (31% for PD, 81,723), appearing to be considerably more active than PD 81,723; while compounds**4e, 4p, 4r–s, 4ad, 4ai** and **4ap** were comparable to PD 81,723. In addition, the compounds **4f, 4j–k, 4o, 4v, 4ac** and **4ah** caused significantly greater inhibition of cAMP production than PD 81,723 at 0.1 μM concentration.

The *N*-isopropyl, *N*-cyclopropyl and *N*-cyclohepthyl derivatives (compounds **4a–c**) appeared less active as an allosteric enhancer than the reference compound PD 81,723 at all concentrations tested, and were less active than the *N*-cyclohexyl analog (**3b**) or the *N*-phenyl analog (**3c**). A further increase in lipophilicity and size of the ring linked to the *N*-4 position of the piperazine, by the replacement of phenyl ring in **3c** with a naphthyl (compound **4d**) caused a decrease of enhancement. Because other substitution patterns that increased lipophilicity (i.e., compounds **4i**, **4j**, and **4ac**) without significantly increasing the steric size of the aryl moiety did not show this decrease in enhancement, we suggest that the detrimental effect of the naphthyl moiety is primarily associated with the increase in ring size. Among the isomeric pyridine derivatives **3k**, **4al** and **4am**, the pyridin-2-yl derivative **3k** was more active than the pyridin-3-yl and pyridin-4-yl analogs **4al** and **4am**, respectively, at concentrations of 10 nM–1 μM.

Several electron-withdrawing substituents (Cl, F and CF_3) at the *ortho*, *meta* and *para* positions of the phenyl moiety linked to the piperazine were investigated. As reported previously, replacement of the phenyl (**3c**) with a benzyl group (**3d**) was detrimental to the allosteric enhancement activity at 0.01, 0.1 and 1 μM concentrations, though the two compounds appeared equivalent at the highest concentration. Starting from the 4-fluorophenyl derivative **3e**, the 4-fluorobenzyl derivative (**4e**) displayed a similar pattern, with **3e** and **4e** appearing to have similar efficacies at a concentration of 10 μM. Shifting the fluorine atom from the *para* position of **3e** to the *meta* (derivative **4f**), increased the reduction of the cAMP level at the concentrations of 1, 0.1 and 0.01 μM, while **3e** and **4f** showed comparable activity at the higher concentration (10 μM).

The *meta*-fluorine derivative **4f** was found one of the most potent derivatives at any concentration, showing the same potency both at 1 and 10 μM of concentration, being able to reduce the cAMP level of 82%. Moreover this compound did not increase the content of cAMP of hA_1CHO cells at any concentration. In contrast, a single *ortho*-fluoro substitution (**4g**) is resolutely unfavorable. In fact, compound **4g** is almost inactive as an allosteric enhancer. Derivative **4f** showed a profile comparable to that of the *para*-trifluoromethyl derivative **3j**, the most active compound of the previously published series. As with the three fluorophenyl isomers, the *para*- and *meta*-chloro derivatives **3f** and **4v**, respectively, showed comparable activities at the higher concentrations (1 and 10 μM), which was reduced at lower concentrations (10 and 100 nM). Similarly, the 2-chloro derivative (**4w**) was inactive, indicating that the mono-substitution at the *ortho* position is adverse for activity.

Starting from the *para*-chlorophenyl piperazine derivative **3f**, by the addition of one-, two- or three-methylene units between the *p*-chlorophenyl and the N_4-nitrogen of the piperazine (**4q**, 4r and **4s**, respectively), a similar efficacy was observed at the 10 μM concentration. At 1 μM, the activity decreased to increase the length of the alkyl chain, suggesting that the introduction of a spacer between the phenyl and piperazine was detrimental for the activity. Moreover, the replacement of the alkyl spacer with a different linker, such as carbonyl, acetyl and 'reversed' acetyl (compounds **4p**, 4t and **4u**, respectively) led to a substantial decline in activity at any concentration relative to **3f**. The presence of a basic nitrogen in the piperazine ring was important for the activity. In fact, its replacement with the isosteric carbon, to furnish the piperdine analog **4an**, has a detrimental effect on AE activity at each concentration tested.

With the aim of verifying if the presence of a second fluorine atom on the phenyl ring would lead to an increase of activity, the difluorophenyl derivatives **4h–m** were synthesized. All of these latter compounds maintain an AE activity comparable to those of the mono-fluoro substituted derivatives **3e** and **4f** at 1 and 10 μM. Among the compounds with two fluorine atoms, derivative **4i** in which the fluorines are adjacent to each other at the 3- and 4-positions on the phenyl ring exhibited the highest activity at any concentration. Starting from the 2,6-difluoroderivative **4k**, the incorporation of an additional fluorine at the 4-position, furnishing the 2,4,6-trifluoro derivative **4n**, was tolerated at 1 and 10 μM. Replacement of the *meta*-fluoro substituent in compound **4i** (3,4-diF) with a more bulky Cl (3-Cl, 4-F, derivative **4o**) increased the

inhibition of cAMP production at all concentrations, resulting in one of the most active compounds of the whole series, along with **4i**.

Based upon these results, the addition of a second chlorine atom to the 4-chloro derivative (**3f**) at different positions of the phenyl ring was investigated, affording the di-chlorinated derivatives **4x–ac**. The di-chloro derivatives **4y–ac** maintained the same activity of *para*- and *meta*-chloro derivatives **3f** and **4v** at 1 and 10 μM concentrations, while the 2,6-dichloro derivative (**4x**) was found to be the least active of the series. Staring from the 2,4-dichlorophenyl derivative **4aa**, replacement of *ortho*-chloro moiety with a fluorine to furnish the 2-F, 4-Cl analog **4ae**, retained the activity. From this latter compound, exchange each other the substituents on the phenylpiperazine moiety, to yield the 2-Cl, 4-F analog **4ad**, reduced slightly the enhancement at A_1ARs. Replacement of chlorine in **3f** with a more electron-withdrawing and less lipophilic nitro group (derivative **4ag**) was detrimental to activity at all concentrations.

Among the previously published *para*-substituted phenyl piperazine derivatives **3a–l**, the strong electron-withdrawing trifluoromethyl group led to the most potent compound of the whole series (analog **3j**). Shifting the trifluoromethyl moiety from the *para*- to *meta*-position (**4ah**) maintained the activity at 10 μM, with slightly reduced activity at lower concentrations (1 and 0.1 μM). It was confirmed that mono-substitution of the phenyl ring at the 2-position is unfavorable. In fact, the *ortho*-CF_3 compound **4ai** showed diminished activity with respect to the *para*- and *meta*-isomers (**3j** and **4ah**, respectively), though not as pronounced as with the corresponding 2-fluoro- and 2-chloro derivatives. Staring from the *meta*-trifluoromethyl derivative **4ah**, the introduction of a less hydrophobic electron-withdrawing chlorine group to furnish the 4-Cl, 3-CF_3 derivative **4aj**, caused a reduction in the enhancement activity.

The replacement of the *para*-trifluoromethyl group of **3j** with a less lipophilic and electron-releasing trifluoromethoxy moiety (compound **4ak**), maintained the enhancement at the higher concentration of 10 μM, while the activity decreased at the lower concentrations with respect to **3j**.

The beneficial role of electron-withdrawing substituents was confirmed by the synthesis of the derivative **4af**, characterized by the presence of electron-releasing methoxy moiety on the *para*-position of phenyl ring. This compound was less active as an AE than the unsubstituted phenylpiperazine derivative (**3c**) at all concentrations tested.

Comparing compounds characterized by the presence of the same phenylpiperazine moiety at the 4-position of the thiophene ring (**4f** vs **4ap**, **4k** vs **4aq** and **3j** vs **4ao**), replacement of 4-chlorobenzoyl group at the 3-position of the thiophene with a 3-(trifluoromethyl)benzoyl moiety reduced allosteric enhancer activity.

3.2. Antagonistic activity

The ability of compounds **4a–aq** to displace the binding of [^{3}H]DPCPX, [^{3}H]ZM241385 and [^{3}H]MRE3008F20 to human A_1, A_{2A} and A_3ARs were evaluated in CHO cells. 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 3008F20 to A_3ARs. None of the examined compounds (10 μM) significantly inhibited the specific binding of the radioligands to A_1, A_{2A} and A_3ARs, reaching a very low percentage of inhibition (see Table 1 Supplementary data). Binding and functional experiments demonstrated that it was possible to achieve a good separation between enhancing activity and the binding to the orthosteric site. Twenty compounds (**4e–f, 4h–j, 4l, 4m–o, 4q–s, 4u–v, 4y, 4aa, 4ac–ae** and **4ak**) were more active than PD 81,723 in the enhancing activity, and at the same time were unable to displace the radioligands from the orthosteric site as suggested from competition binding experiments.[22]

3.3. Effect on enhancers on A_1 AR binding parameters

Saturation binding experiments of the selective adenosine A_1 agonist [^{3}H]CCPA and A_1 antagonist [^{3}H]DPCPX were performed to verify if the novel compounds modified the A_1ARs binding parameters. From these experiments, A_1AR affinity (K_D) and density (B_{max}) were evaluated in the presence and absence of the examined compounds (PD 81,723, **3a–l** and **4a–aq** at a concentration of 10 μM). No differences were found in affinity values (K_D) derived from saturation binding experiments as reported in Table 2. From the receptor density calculated in the presence and in the absence of enhancers, B_{max} shift was evaluated (Table 2, column A). [^{3}H]CCPA competition binding experiments were also performed, with the aim of verifying the specific in the absence and in the presence of examined enhancers (Bound shift). In [^{3}H]CCPA saturation binding experiments, the reference compound PD 81,723 induced a B_{max} shift to human A_1ARs of 1.3-fold. Under the same experimental conditions, compounds **4e–f, 4h–t, 4v, 4x–af, 4ah–ak** and **4an–ap** were significantly more potent than PD 81,723. The derivatives **4h, 4i–k, 4o, 4ak** and **4ap** were the most active compounds, causing a B_{max} shift of 6.1-, 7.2-, 6.2-, 6.3-, 7.0-, 6.8- and 6.0-fold, respectively. In [^{3}H]DPCPX saturation binding experiments, the tested compounds did not modify either affinity or receptor density in comparison to control conditions (Table 2, column B). Interestingly, the presence of enhancers mediated an increase in B_{max} shift derived from [^{3}H]CCPA saturation binding experiments, suggesting the ability of these novel compounds to mediate a shift from the ground state (R) to the activated state (R*) of the A_1ARs. B_{max} values from [^{3}H]DPCPX saturation binding experiments were generally greater than the B_{max} values obtained from [^{3}H]CCPA saturation binding experiments, suggesting that DPCPX as a typical adenosine antagonist, was able to label both the R and R* states of the A_1ARs.

Table 2 also reports the derived apparent affinity (K_i) values for CCPA (column C), based on a one-state model of analysis, in the absence and in the presence of tested enhancers. This table shows that the CCPA shift, representing the ratio of apparent K_i 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 K_i value of CCPA was 15.1 ± 1.6 nM. Interestingly, a significant decrease in the apparent K_i value was observed in the presence of the putative allosteric enhancers suggesting an increase in the number of the high affinity binding sites. In the presence of PD 81,723, the apparent affinity of CCPA increased 1.7-fold. The CCPA affinity data in the presence of the derivatives**4e–f, 4h–o, 4q–r, 4v, 4x–af, 4ah–ak** and **4an–ap** reveal that the displacement curves are shifted left, suggesting lower K_i values for CCPA. In particular, the largest affinity shift was observed for compounds **4i**, **4o** and **4ak**. These molecules decreased the apparent K_i values of CCPA approximately of 5.5-, 5.4- and 5.2-fold, respectively (Table 2). Compounds **4h**, **4j–k**, **4ac–ae**, **4ah**, **4aj** and **4ap** caused a similar shift of the apparent CCPA affinity, ranging from four to fivefold, while derivatives **4e–f**, **4m–n**, **4q**, **4r**, **4v–z**, **4aa–ab**,**4ae**, **4ai** and **4an–ao** afforded an apparent increase ranging from three to fourfold. Thus, the enhancers were able to mediate a shift of the A_1ARs towards the high affinity state, as suggested from the increase of the CCPA affinity expressed as K_i values (Table 2).

In Figure 1, we have shown the effects of the allosteric modulators PD 81,723, **3j**, **4i** and **4o** at 10 μM concentration in [^{3}H]CCPA saturation binding experiments on A_1AR binding parameters such as affinity and density. In Figure 2A, representative binding curves showing the displacement of [^{3}H]DPCPX by different concentrations of CCPA alone and in the presence of PD81,723, **3j**, **4i** and

Table 2
Saturation binding assays in hA_1 CHO membranes obtained by using [^{3}H]CCPA (A) and [^{3}H]DPCPX (B) as radioligands. Modulation of enhancers (10 μM) on CCPA affinity (K_i) and CCPA (K_i) shift obtained from [^{3}H]DPCPX competition binding experiments (C)

Compound	(A) [^{3}H]CCPA saturation binding experiments			(B) [^{3}H]DPCPX saturation binding experiments			(C) [^{3}H]DPCPX competition binding experiments	
	K_D (nM)	B_{max} (fmol/mg protein)	B_{max} shift (fold of increase)	K_D (nM)	B_{max} (fmol/mg protein)	B_{max} shift (fold of increase)	CCPA K_i (nM)	CCPA K_i shift (fold of increase)
PD 81,723	1.1 ± 0.1	670 ± 52	1.3 ± 0.1	1.8 ± 0.2	3340 ± 372	1.0 ± 0.1	9.2 ± 0.8	1.7 ± 0.2
4a	1.0 ± 0.1	567 ± 53	1.1 ± 0.1	1.8 ± 0.1	3089 ± 286	0.9 ± 0.1	12.6 ± 1.3	1.2 ± 0.1
4b	1.1 ± 0.1	567 ± 59	1.1 ± 0.1	1.6 ± 0.2	3106 ± 346	0.9 ± 0.1	11.7 ± 1.2	1.3 ± 0.1
4c	1.0 ± 0.1	567 ± 47	1.1 ± 0.1	1.6 ± 0.1	3009 ± 281	0.9 ± 0.1	13.1 ± 0.1	1.2 ± 0.1
4d	1.1 ± 0.1	567 ± 42	1.1 ± 0.1	1.9 ± 0.1	3024 ± 369	0.9 ± 0.1	11.6 ± 1.1	1.3 ± 0.1
4e	1.1 ± 0.1	1906 ± 184	3.7 ± 0.3	1.6 ± 0.1	3145 ± 275	0.9 ± 0.1	4.9 ± 0.4	3.1 ± 0.3
4f	1.0 ± 0.1	2472 ± 264	4.8 ± 0.4	1.6 ± 0.2	3364 ± 333	1.0 ± 0.1	4.0 ± 0.3	3.9 ± 0.4
4g	1.1 ± 0.1	585 ± 52	1.1 ± 0.1	1.7 ± 0.2	3341 ± 328	1.0 ± 0.1	11.3 ± 1.1	1.4 ± 0.1
4h	1.1 ± 0.1	3142 ± 312	6.1 ± 0.6	1.7 ± 0.1	3465 ± 322	1.0 ± 0.1	3.2 ± 0.3	4.8 ± 0.5
4i	1.2 ± 0.1	3708 ± 384	7.2 ± 0.6	1.7 ± 0.2	3652 ± 412	1.1 ± 0.1	2.8 ± 0.3	5.5 ± 0.4
4j	1.1 ± 0.1	3193 ± 329	6.2 ± 0.6	1.8 ± 0.1	3524 ± 367	1.1 ± 0.1	3.2 ± 0.3	4.8 ± 0.5
4k	1.2 ± 0.1	3245 ± 324	6.3 ± 0.6	1.7 ± 0.1	3588 ± 394	1.1 ± 0.1	3.1 ± 0.2	5.0 ± 0.4
4l	1.1 ± 0.1	2472 ± 238	4.8 ± 0.4	1.9 ± 0.1	3263 ± 323	1.0 ± 0.1	3.9 ± 0.4	4.0 ± 0.3
4m	1.2 ± 0.1	2421 ± 226	4.7 ± 0.4	1.6 ± 0.2	3498 ± 363	1.0 ± 0.1	4.1 ± 0.4	3.8 ± 0.3
4n	1.1 ± 0.1	2318 ± 227	4.5 ± 0.5	1.7 ± 0.1	3361 ± 277	1.0 ± 0.1	4.3 ± 0.5	3.6 ± 0.3
4o	1.1 ± 0.1	3605 ± 376	7.0 ± 0.6	1.8 ± 0.1	3678 ± 358	1.1 ± 0.1	2.8 ± 0.3	5.5 ± 0.5
4p	1.0 ± 0.1	824 ± 85	1.6 ± 0.1	1.8 ± 0.2	3076 ± 366	0.9 ± 0.1	9.4 ± 0.5	1.6 ± 0.2
4q	1.0 ± 0.1	2318 ± 216	4.5 ± 0.4	1.9 ± 0.2	3355 ± 393	1.0 ± 0.1	4.2 ± 0.4	3.7 ± 0.3
4r	1.1 ± 0.1	1582 ± 147	3.1 ± 0.3	1.7 ± 0.2	3263 ± 372	1.0 ± 0.1	4.5 ± 0.4	3.4 ± 0.3
4s	1.1 ± 0.1	824 ± 71	1.6 ± 0.2	1.9 ± 0.1	3054 ± 295	0.9 ± 0.1	9.4 ± 0.9	1.6 ± 0.2
4t	1.0 ± 0.1	773 ± 68	1.5 ± 0.1	1.9 ± 0.1	3052 ± 378	0.9 ± 0.1	10.1 ± 1.2	1.5 ± 0.1
4u	1.1 ± 0.1	721 ± 70	1.4 ± 0.1	2.0 ± 0.2	2996 ± 257	0.9 ± 0.1	11.0 ± 1.1	1.4 ± 0.1
4v	1.1 ± 0.1	2266 ± 231	4.4 ± 0.4	1.8 ± 0.1	3226 ± 304	1.0 ± 0.1	4.2 ± 0.4	3.7 ± 0.3
4w	1.0 ± 0.1	618 ± 57	1.2 ± 0.1	1.6 ± 0.1	2987 ± 246	0.9 ± 0.1	11.1 ± 1.1	1.4 ± 0.1
4x	1.0 ± 0.1	1700 ± 167	3.3 ± 0.3	1.7 ± 0.1	3111 ± 258	0.9 ± 0.1	5.4 ± 0.5	2.9 ± 0.3
4y	1.1 ± 0.1	1648 ± 172	3.2 ± 0.3	1.6 ± 0.2	3154 ± 352	0.9 ± 0.1	5.6 ± 0.6	2.8 ± 0.3
4z	1.1 ± 0.1	2369 ± 242	4.6 ± 0.5	2.0 ± 0.2	3264 ± 296	1.0 ± 0.1	4.1 ± 0.3	3.8 ± 0.4
4aa	1.0 ± 0.1	2266 ± 214	4.4 ± 0.4	1.9 ± 0.1	3441 ± 425	1.0 ± 0.1	4.2 ± 0.4	3.7 ± 0.4
4ab	1.1 ± 0.1	2163 ± 189	4.2 ± 0.3	1.9 ± 0.2	3274 ± 294	1.0 ± 0.1	4.4 ± 0.4	3.5 ± 0.4
4ac	1.1 ± 0.1	2987 ± 285	5.8 ± 0.6	1.8 ± 0.1	3266 ± 316	1.0 ± 0.1	3.4 ± 0.3	4.5 ± 0.4
4ad	1.0 ± 0.1	2678 ± 273	5.2 ± 0.3	1.6 ± 0.2	3630 ± 304	1.1 ± 0.1	3.7 ± 0.3	4.2 ± 0.4
4ae	1.0 ± 0.1	2730 ± 286	5.3 ± 0.5	1.9 ± 0.1	3426 ± 342	1.0 ± 0.1	3.6 ± 0.4	4.3 ± 0.4
4af	1.0 ± 0.1	1670 ± 181	3.3 ± 0.3	2.0 ± 0.1	3153 ± 311	0.9 ± 0.1	5.5 ± 0.6	2.8 ± 0.2
4ag	1.1 ± 0.1	567 ± 49	1.1 ± 0.1	1.6 ± 0.2	3102 ± 266	0.9 ± 0.1	3.7 ± 0.4	4.2 ± 0.4
4ah	1.2 ± 0.1	2730 ± 273	5.3 ± 0.5	1.7 ± 0.1	3408 ± 346	1.0 ± 0.1	3.6 ± 0.3	4.3 ± 0.4
4ai	1.2 ± 0.1	2266 ± 263	4.4 ± 0.3	1.8 ± 0.2	3479 ± 319	1.0 ± 0.1	4.3 ± 0.3	3.6 ± 0.3
4aj	1.2 ± 0.1	2936 ± 318	5.7 ± 0.6	1.8 ± 0.2	3394 ± 314	1.0 ± 0.1	3.4 ± 0.3	4.5 ± 0.5
4ak	1.1 ± 0.1	3502 ± 341	6.8 ± 0.6	1.9 ± 0.1	3588 ± 264	1.1 ± 0.1	2.9 ± 0.3	5.3 ± 0.5
4al	1.1 ± 0.1	670 ± 63	1.3 ± 0.1	1.8 ± 0.2	3021 ± 293	0.9 ± 0.1	10.8 ± 1.0	1.4 ± 0.1
4am	1.0 ± 0.1	567 ± 52	1.1 ± 0.1	1.8 ± 0.1	3112 ± 278	0.9 ± 0.1	12.6 ± 1.2	1.2 ± 0.1
4an	1.2 ± 0.1	2009 ± 193	3.9 ± 0.3	1.7 ± 0.1	3321 ± 259	1.0 ± 0.1	4.7 ± 0.5	3.3 ± 0.3
4ao	1.1 ± 0.1	2266 ± 237	4.4 ± 0.4	1.9 ± 0.2	3332 ± 286	1.0 ± 0.1	4.6 ± 0.5	3.4 ± 0.3
4ap	1.1 ± 0.1	3090 ± 314	6.0 ± 0.6	2.0 ± 0.2	3589 ± 411	1.1 ± 0.1	3.3 ± 0.3	4.7 ± 0.5
4aq	1.2 ± 0.1	567 ± 59	1.1 ± 0.1	1.6 ± 0.1	3022 ± 297	0.9 ± 0.1	12.6 ± 1.3	1.2 ± 0.1

The results are mean values (±SEM) of three independent experiments.
(A) = K_D (nM), B_{max} (fmol/mg protein) and B_{max} shift obtained in [^{3}H]CCPA saturation binding experiments performed in the absence (K_D = 1.1 ± 0.1 nM, B_{max} = 512 ± 43 fmol/mg protein) or in the presence of 10 μM enhancers.
(B) = K_D (nM), B_{max} (fmol/mg protein) and B_{max} shift obtained in [^{3}H]DPCPX saturation binding experiments performed in the absence (K_D = 1.7 ± 0.2 nM, B_{max} = 3350 ± 320 fmol/mg protein) and in the presence of 10 μM enhancers.
(C) = K_i values of CCPA in the presence of 10 μM tested compounds and CCPA shift = K_i(CCPA)/K_i(CCPA + 10 μM enhancers) where the K_i of CCPA was 15.4 ± 1.7 nM.

4o at 10 μM concentration. Figure 2B shows a very high correlation of the binding data for the examined compounds, expressed as B_{max} shift from saturation binding experiments and CCPA shift from competition binding experiments.

4. Conclusions

In conclusion, as an extension of the series of arylpiperazine derivatives **3a–l** previously synthesized, we have now reported the results of a systematic modification of the lead molecules **3e**, **3f** and **3j** aimed at defining the interplay between structure and activity in the new (**4a–aq**) and old (**3a–l**) synthesized compounds. Of the 43 newly synthesized molecules, only nine compounds showed AE activity lower than that PD 81,723: seven derivatives showed activity at least as high as that of PD 81,723, with 27 analogs having substantially higher activity, among these the 3,4-difluoro-**4i**, 3-chloro,4-fluoro-**4o**, and 4-(trifluoromethoxy)-**4ak** derivatives being the most active compounds in binding (saturation and displacement experiments) and functional cAMP studies. In competition binding experiments, the K_i values of CCPA in the presence of compounds **4i**, **4o** and **4ak** was decreased approximately 5.5, 5.4 and 5.2-fold, respectively. The results clearly show that moving a single fluorine and chlorine atom from the *para* to the *ortho*-position of the phenyl ring was detrimental for activity (i.e., **3e** and **3f** vs **4g** and **4w**, respectively), while the 2,6-difluoro derivative **4k** was equipotent to **3e**. This presumably reflects the importance of the relative position in space between the piperazine and phenyl rings, and suggests that a detrimental *ortho*-effect, as indicated by compound **4g** and **4w**, can be overcome by the number and position of other substituents on the aryl moiety, as shown by compound **4k**. Moreover, the relative size of the detrimental substituent at the *ortho*-position and the positive

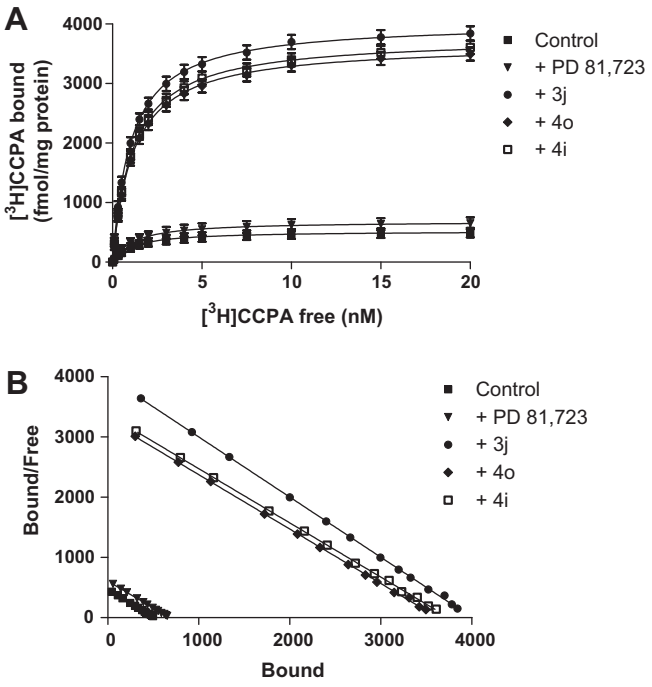


Figure 1. [^{3}H]-CCPA saturation binding curves to human A_1ARs (A). In control conditions, K_D value was 1.1 ± 0.1 nM and the B_{max} was 515 ± 47 fmol/mg protein. In the presence of novel enhancers (10 μM), K_D values were similar to those obtained in controls and B_{max} values were reported in Table 2. Scatchard plots of the same experimental data (B). Values are the means and vertical lines are the SEM of three separate experiments as described in experimental procedures.

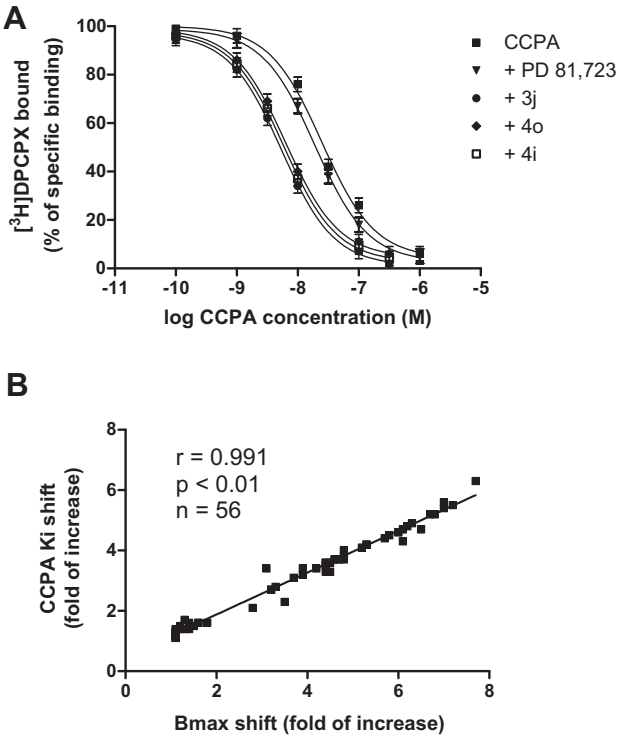


Figure 2. Inhibition curves of specific [^{3}H]-DPCPX binding to hA_1ARs of CCPA in the absence and in the presence of novel enhancers (10 μM) values are the means and vertical lines are the SEM of three separate experiments as described in experimental procedures (A). Correlation between CCPA K_i shift calculated by using the affinity of the agonist in the absence and in the presence of novel enhancers (10 μM) and B_{max} shift obtained from [^{3}H]-CCPA saturation binding experiments in the absence and in the presence of novel enhancers (10 μM) (B).

substituent at an alternative position may influence the magnitude of the overall effect, as seen with compounds **4ad** and **4ae** (2-chloro-4-fluoro vs 2-fluoro-4-chloro, respectively). In contrast, substitution in the *meta*-position (F for **4f** and Cl for **4v**) gave results comparable to that of the *para*-position. The effects of compounds **4a–aq** on functional assays (cAMP content) were consistent with their behavior in binding studies.

5. Experimental section

5.1. Chemistry

5.1.1. Materials and methods

4-Chlorobenzoylacetonitrile and 3-trifluorobenzoylacetonitrile are commercially available. All aryl piperazines were commercially available or were synthesized as described in the literature (see Supplementary data).

^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer. Chemical shifts (δ) are given in ppm relative to tetramethylsilane as internal standard, and the spectra were recorded in appropriate deuterated solvents indicated in the procedure. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing Finnigan MAT 95 instrument with BE geometry. 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. Results were within ±0.4% of the theoretical values. All reactions were performed under an inert atmosphere of dry nitrogen, unless otherwise described. Standard syringe techniques were applied for transferring dry solvents. 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. Dichloromethane (DCM) and DMF were distilled from calcium chloride and stored over molecular sieves (3 Å). In high-pressure hydrogenation experiments, a Parr shaker on a high-pressure autoclave was used.

5.2. General procedure (A) for the synthesis of compounds 6a–aq

To a stirred solution of compound **5a–b** (2 mmol) in dry DCM (10 mL) was added K_2CO_3 (1.1 equiv, 2.2 mmol, 304 mg). The mixture was cooled with a bath of ice/water, and then the appropriate N-substituted piperazine (2 equiv, 4 mmol), dissolved in DCM (2 mL), was added slowly over 30 min. The mixture was then stirred at room temperature for two hours, diluted with DCM (10 mL), washed with water (10 mL) and then with brine (10 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo to give a brown residue that was purified by column chromatography to furnish the derivatives **6a–aq**.

5.3. General procedure (B) for the synthesis of compounds (7a–aq)

A solution of piperazine derivative **6a–aq** (2 mmol) in DMF (20 mL), containing Et_3N (0.3 mL, 2 mmol, 1 equiv) was hydrogenated over 120 mg of 10% Pd/C at 60 psi for 3 h. The catalyst was removed by filtration, the filtrate concentrated, and the residue dissolved in DCM (20 mL), washed with water (5 mL), brine

(5 mL), dried (Na_2SO_4). After filtering, the solvent was removed in vacuo to obtain a residue that was purified by column chromatography.

5.4. General procedure (C) for the synthesis of compounds (4a–aq)

A stirred suspension of thiophene derivatives **7a–aq** (0.5 mmol) and 100% hydrazine monohydrate (1.2 equiv, 0.6 mmol, 29 μl) in abs ethanol (10 mL) was refluxed for 3 h, then the resulting solution was kept at room temperature for 1 h. The solvent was evaporated and the residue was partitioned between EtOAc (10 mL) and water (5 mL). The separated organic phase was washed with brine (2 mL), dried, then concentrated in vacuo to obtain a residue that was purified by column chromatography to give the desired products **4a–aq**.

5.4.1. [2-Amino-4-((4-(isopropyl)piperazin-1-yl)methyl) thiophen-3-yl](4-chlorophenyl) methanone (4a)

Following the general procedure (C), derivative **4a** was purified by column chromatography (EtOAc/MeOH 3:7). Yield: 39%. Yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.02 (m, 6H), 1.99 (t, J = 5.2 Hz, 4H), 2.35 (t, J = 5.2 Hz, 4H), 2.57 (m, 1H), 2.93 (s, 2H), 6.05 (br s, 2H), 6.10 (s, 1H), 7.36 (d, J = 8.6 Hz, 2H), 7.52 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 378.1. Anal. ($C_{19}H_{24}ClN_3OS$): C, H, N.

5.4.2. [2-Amino-4-((4-(cyclopentyl)piperazin-1-yl)methyl) thiophen-3-yl](4-chlorophenyl)-methanone (4b)

Following the general procedure (C), derivative **4b** was purified by column chromatography (EtOAc/MeOH 6:4 as eluent). Yield: 36%. Yellow solid, mp 95–97 °C. ^{1}H NMR ($CDCl_3$) δ: 1.16 (m, 4H), 1.48 (m, 5H), 1.82 (t, J = 5.2 Hz, 4H), 2.79 (s, 2H), 2.85 (t, J = 5.2 Hz, 4H), 5.72 (br s, 2H), 6.90 (s, 1H), 7.32 (d, J = 7.0 Hz, 2H), 8.00 (d, J = 7.0 Hz, 2H). MS (ESI): $[M+1]^+$ = 404.2. Anal. ($C_{21}H_{26}ClN_3OS$): C, H, N

5.4.3. [2-Amino-4-((4-(cycloheptyl)piperazin-1-yl)methyl) thiophen-3-yl](4-chlorophenyl) methanone (4c)

Following the general procedure (C), derivative **4c** was purified by column chromatography (EtOAc/MeOH 7:3 as eluent). Yield: 38%. Yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.26 (m, 12H), 1.79 (t, J = 5.2 Hz, 4H), 2.09 (t, J = 5.2 Hz, 4H), 2.81 (m, 1H), 3.03 (s, 2H), 6.03 (br s, 2H), 6.09 (s, 1H), 7.31 (d, J = 8.8 Hz, 2H), 7.45 (d, J = 8.8 Hz, 2H). MS (ESI): $[M+1]^+$ = 432.3. Anal. ($C_{23}H_{30}ClN_3OS$): C, H, N.

5.4.4. [2-Amino-4-((4-(naphthalen-1-yl)piperazin-1-yl)methyl) thiophen-3-yl](4-chlorophenyl) methanone (4d)

Following the general procedure (C), derivative **4d** was purified by column chromatography (EtOAc/DCM 2:8 as eluent). Yield: 46%. Brown solid, mp 178 °C. ^{1}H NMR ($CDCl_3$) δ: 2.42 (t, J = 5.4 Hz, 4H), 2.54 (t, J = 5.4 Hz, 4H), 3.03 (s, 2H), 6.04 (s, 2H), 6.11 (s, 1H), 6.93 (d, J = 7.2 Hz, 2H), 7.06 (d, J = 7.2 Hz, 2H), 7.33 (m, 3H), 7.56 (d, J = 7.8 Hz, 2H), 7.87 (d, J = 7.8 Hz, 2H). MS (ESI): $[M+1]^+$ = 462.2. Anal. ($C_{26}H_{24}ClN_3OS$): C, H, N.

5.4.5. {2-Amino-4-[(4-(4-fluorobenzyl)piperazin-1-yl)methyl] thiophen-3-yl}(4-chlorophenyl) methanone (4e)

Following the general procedure (C), derivative **4e** was purified by column chromatography (MeOH/DCM 0.5:9.5 as eluent). Yield: 53%. Yellow solid, mp 230–231 °C. ^{1}H NMR ($CDCl_3$) δ: 1.55 (t, J = 4.6 Hz, 4H), 1.88 (t, J = 4.6 Hz, 4H), 2.92 (s, 2H), 2.92 (s, 2H), 6.02 (br s, 2H), 6.09 (s, 1H), 6.96 (t, J = 8.8 Hz, 2H), 7.21 (dd, J = 8.8 and 5.6 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H). MS (ESI): $[M+1]^+$ = 444.1. Anal. ($C_{23}H_{23}ClFN_3OS$): C, H, N.

5.4.6. {2-Amino-4-[(4-(3-fluorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4f)

Following the general procedure (C), derivative **4f** was purified by column chromatography (EtOAc/DCM 2:8 as eluent). Yield: 48%. Yellow solid, mp 150–152 °C. ^{1}H NMR ($CDCl_3$): δ: 2.02 (t, J = 4.8 Hz, 4H), 2.93 (t, J = 4.8 Hz, 4H), 3.00 (s, 2H), 6.08 (s, 2H), 6.13 (s, 1H), 6.45 (t, J = 7.0 Hz, 1H), 6.53 (m, 2H), 7.17 (q, J = 7.6 Hz, 1H), 7.40 (d, J = 8.2 Hz, 2H), 7.56 (d, J = 8.2 Hz, 2H). MS (ESI): $[M+1]^+$ = 430.1. Anal. ($C_{22}H_{21}ClFN_3OS$): C, H, N.

5.4.7. {2-Amino-4-[(4-(2-fluorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4g)

Following the general procedure (C), derivative **4g** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 48%. Yellow solid, mp 102–104 °C. ^{1}H NMR ($CDCl_3$) δ: 2.04 (t, J = 5.2 Hz, 4H), 2.86 (t, J = 5.2 Hz, 4H), 3.02 (s, 2H), 6.04 (s, 2H), 6.12 (s, 1H), 6.74 (d, J = 7.8 Hz, 1H), 6.82 (d, J = 7.8 Hz, 1H), 6.86 (t, J = 7.6 Hz, 1H), 6.92 (t, J = 7.6 Hz, 1H), 7.38 (d, J = 8.2 Hz, 2H), 7.54 (d, J = 8.2 Hz, 2H). MS (ESI): $[M+1]^+$ = 430.2. Anal. ($C_{22}H_{21}ClF_2N_3OS$): C, H, N.

5.4.8. {2-Amino-4-[(4-(2,4-difluorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4h)

Following the general procedure (C), derivative **4h** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 51%. Yellow solid, mp 170–172 °C. ^{1}H NMR ($CDCl_3$) δ: 2.04 (t, J = 4.8 Hz, 4H), 2.77 (t, J = 4.8 Hz, 4H), 3.00 (s, 2H), 6.06 (br s, 2H), 6.13 (s, 1H), 6.80 (m, 3H), 7.35 (d, J = 8.6 Hz, 2H), 7.56 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 448.1. Anal. ($C_{22}H_{20}ClF_2N_3OS$): C, H, N.

5.4.9. {2-Amino-4-[(4-(3,4-difluorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4i)

Following the general procedure (C), derivative **4i** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 48%. Yellow solid, mp 143–145 °C. ^{1}H NMR ($CDCl_3$) δ: 2.01 (t, J = 4.8 Hz, 4H), 2.84 (t, J = 4.8 Hz, 4H), 3.00 (s, 2H), 6.07 (br s, 2H), 6.13 (s, 1H), 6.52 (m, 2H), 7.02 (m, 1H), 7.36 (d, J = 8.2 Hz, 2H), 7.53 (d, J = 8.2 Hz, 2H). MS (ESI): $[M+1]^+$ = 448.2. Anal. ($C_{22}H_{20}ClF_2N_3OS$): C, H, N.

5.4.10. {2-Amino-4-[(4-(3,5-difluorophenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4j)

Following the general procedure (C), derivative **4j** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 48%. Yellow solid, mp 162–163 °C. ^{1}H NMR ($CDCl_3$) δ: 2.00 (t, J = 4.8 Hz, 4H), 2.93 (t, J = 4.8 Hz, 4H), 3.00 (s, 2H), 6.10 (br s, 2H), 6.13 (s, 1H), 6.26 (m, 3H), 7.38 (d, J = 8.8 Hz, 2H), 7.55 (d, J = 8.8 Hz, 2H). MS (ESI): $[M+1]^+$ = 448.1. Anal. ($C_{22}H_{20}ClF_2N_3OS$): C, H, N.

5.4.11. {2-Amino-4-[(4-(2,6-difluorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4k)

Following the general procedure (C), derivative **4k** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 47%, Yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.99 (t, J = 4.8 Hz, 4H), 2.95 (t, J = 4.8 Hz, 4H), 2.98 (s, 2H), 6.08 (br s, 2H), 6.13 (s, 1H), 6.82 (m, 3H), 7.36 (d, J = 6.6 Hz, 2H), 7.58 (d, J = 6.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 448.1. Anal. ($C_{22}H_{20}ClF_2N_3OS$): C, H, N.

5.4.12. {2-Amino-4-[(4-(2,3-difluorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4l)

Following the general procedure (C), derivative **4l** was purified by column chromatography (EtOAc/DCM 0.5:9.5 as eluent). Yield: 52%. Yellow solid, mp 138 °C. ^{1}H NMR ($CDCl_3$) δ: 1.99 (t, J = 4.8 Hz, 4H), 2.78 (t, J = 4.8 Hz, 4H), 2.95 (s, 2H), 6.00 (br s, 2H), 6.07 (s, 1H), 6.54 (t, J = 7.6 Hz, 1H), 6.67 (d, J = 7.8 Hz, 1H), 6.85

(t, J = 8.2 Hz, 1H), 7.31 (d, J = 8.4 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H). MS (ESI): $[M+1]^+$ = 448.2. Anal. ($C_{22}H_{20}ClF_2N_3OS$): C, H, N.

5.4.13. {2-Amino-4-[(4-(2,5-difluorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4m)

Following the general procedure (C), derivative **4m** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 49%. Yellow solid, mp 62–63 °C. ^{1}H NMR ($CDCl_3$) δ: 1.99 (t, J = 4.8 Hz, 4H), 2.95 (t, J = 4.8 Hz, 4H), 2.98 (s, 2H), 6.08 (br s, 2H), 6.13 (s, 1H), 6.82 (m, 3H), 7.36 (d, J = 8.6 Hz, 2H), 7.58 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 448.3. Anal. ($C_{22}H_{20}ClF_2N_3OS$): C, H, N.

5.4.14. {2-Amino-4-[(4-(2,4,6-trifluorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4n)

Following the general procedure (C), derivative **4n** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 48%. Yellow solid, mp 153–155 °C. ^{1}H NMR ($CDCl_3$) δ: 1.98 (t, J = 4.6 Hz, 4H), 2.87 (t, J = 4.8 Hz, 4H), 2.98 (s, 2H), 6.08 (br s, 2H), 6.13 (s, 1H), 6.58 (t, J = 9.0 Hz, 2H), 7.37 (d, J = 8.6 Hz, 2H), 7.56 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 466.2. Anal. ($C_{22}H_{19}ClF_3N_3OS$): C, H, N.

5.4.15. {2-Amino-4-[(4-(3-chloro-4-fluorophenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4o)

Following the general procedure (C), derivative **4o** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 56%. Yellow solid, mp 161–163 °C. ^{1}H NMR ($CDCl_3$) δ: 2.01 (t, J = 4.6 Hz, 4H), 2.85 (t, J = 4.6 Hz, 4H), 3.00 (s, 2H), 6.07 (br s, 2H), 6.13 (s, 1H), 6.68 (m, 1H), 6.81 (dd, J = 6.4 and 3.0 Hz, 1H), 6.93 (t, J = 8.8 Hz, 1H), 7.38 (d, J = 8.6 Hz, 2H), 7.56 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 464.2. Anal. ($C_{22}H_{20}Cl_2FN_3OS$): C, H, N.

5.4.16. {2-Amino-4-[(4-(4-chlorobenzoyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4p)

Following the general procedure (C), derivative **4p** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 54%. Yellow solid, mp 185–186 °C. ^{1}H NMR ($CDCl_3$) δ: 1.83 (t, J = 4.8 Hz, 2H), 1.92 (t, J = 4.8 Hz, 2H), 2.99 (s, 2H), 3.15 (t, J = 5.0 Hz, 2H), 3.49 (t, J = 5.0 Hz, 2H), 6.08 (s, 1H), 6.10 (s, 2H), 7.24 (d, J = 8.2 Hz, 2H), 7.28 (d, J = 8.2 Hz, 2H), 7.36 (d, J = 8.2 Hz, 2H), 7.54 (d, J = 8.2 Hz, 2H). MS (ESI): $[M]^+$ = 473.1. Anal. ($C_{23}H_{21}Cl_2N_3OS$): C, H, N.

5.4.17. {2-Amino-4-[(4-(4-chlorobenzyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4q)

Following the general procedure (C), derivative **4q** was purified by column chromatography (EtOAc/DCM 1:1 as eluent). Yield: 53%. Yellow solid, mp 65–67 °C. ^{1}H NMR ($CDCl_3$) δ: 1.88 (t, J = 4.6 Hz, 4H), 2.21 (m, 4H), 2.92 (s, 2H), 3.36 (s, 2H), 6.02 (br s, 2H), 6.09 (s, 1H), 7.24 (m, 4H), 7.34 (d, J = 8.6 Hz, 2H), 7.52 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 460.2. Anal. ($C_{23}H_{23}Cl_2N_3OS$): C, H, N.

5.4.18. (2-Amino-4-{(4-[2-(4-chlorophenyl)ethyl]piperazin-1-yl)methyl}thiophen-3-yl)(4-chlorophenyl) methanone (4r)

Following the general procedure (C), derivative **4r** was purified by column chromatography (DCM/MeOH 9.5:0.5 as eluent). Yield: 48%. Yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.92 (t, J = 4.6 Hz, 4H), 2.27 (m, 4H), 2.47 (t, J = 7.2 Hz, 2H), 2.67 (t, J = 7.2 Hz, 2H), 2.95 (s, 2H), 6.09 (br s, 3H), 7.08 (d, J = 8.6 Hz, 2H), 7.23 (d, J = 8.6 Hz, 2H), 7.36 (d, J = 8.6 Hz, 2H), 7.52 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 474.1. Anal. ($C_{24}H_{25}Cl_2N_3OS$): C, H, N.

5.4.19. (2-Amino-4-{(4-[2-(4-chlorophenyl)propyl]piperazin-1-yl)methyl}thiophen-3-yl)(4-chlorophenyl)methanone (4s)

Following the general procedure (C), derivative **4s** was purified by column chromatography (DCM/MeOH 9.5:0.5 as eluent). Yield: 48%. Yellow solid, mp 60–61 °C. ^{1}H NMR ($CDCl_3$) δ: 1.62 (m, 2H), 1.90 (t, J = 4.8 Hz, 4H), 2.24 (m, 4H), 2.58 (t, J = 4.8 Hz, 4H), 2.95 (s, 2H), 6.02 (br s, 2H), 6.09 (s, 1H), 7.10 (d, J = 8.6 Hz, 2H), 7.20 (d, J = 8.6 Hz, 2H), 7.34 (d, J = 8.6 Hz, 2H), 7.52 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 488.2. Anal. ($C_{25}H_{27}Cl_2N_3OS$): C, H, N.

5.4.20. 1-{4-[5-Amino-4-(4-chlorobenzoyl)-thiophen-3-ylmethyl]-piperazin-1-yl}-2-(4-chlorophenyl)-ethanone (4t)

Following the general procedure (C), derivative **4t** was purified by column chromatography (EtOAc-DCM 2–8 as eluent). Yield: 46%. Yellow solid, mp 60–61 °C. ^{1}H NMR ($CDCl_3$) δ: 1.73 (t, J = 4.8 Hz, 2H), 1.78 (t, J = 4.8 Hz, 2H), 2.93 (s, 2H), 3.19 (t, J = 5.2 Hz, 2H), 3.64 (t, J = 5.2 Hz, 2H), 3.59 (s, 2H), 6.06 (s, 1H), 6.09 (s, 2H), 7.10 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 7.39 (d, J = 8.6 Hz, 2H), 7.53 (d, J = 8.6 Hz, 2H). MS (ESI): $[M]^+$ = 487.1. Anal. ($C_{24}H_{23}Cl_2N_3OS$): C, H, N.

5.4.21. 2-{4-[5-Amino-4-(4-chlorobenzoyl)-thiophen-3-ylmethyl]-piperazin-1-yl}-1-(4-chlorophenyl)-ethanone (4u)

Following the general procedure (C), derivative **4u** was purified by column chromatography (EtOAc-MeOH 9.5–0.5 as eluent). Yield: 46%. Yellow solid, mp 77–78 °C. ^{1}H NMR ($CDCl_3$) δ: 2.73 (t, J = 4.8 Hz, 4H), 3.42 (t, J = 4.8 Hz, 4H), 3.56 (s, 2H), 3.72 (s, 2H), 6.06 (br s, 2H), 6.08 (s, 1H), 7.22 (d, J = 8.4 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 7.44 (d, J = 8.4 Hz, 2H), 7.62 (d, J = 8.4 Hz, 2H). MS (ESI): $[M+1]^+$ = 488.1. Anal. ($C_{24}H_{23}Cl_2N_3OS$): C, H, N.

5.4.22. {2-Amino-4-[(4-(3-chlorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4v)

Following the general procedure (C), derivative **4v** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 51%. Yellow solid, mp 197–199 °C. ^{1}H NMR ($CDCl_3$) δ: 2.01 (t, J = 5.2 Hz, 4H), 2.93 (t, J = 5.2 Hz, 4H), 3.00 (s, 2H), 6.08 (br s, 2H), 6.13 (s, 1H), 6.68 (d, J = 7.6 Hz, 1H), 6.78 (d, J = 7.6 Hz, 1H), 6.81 (s, 1H), 7.12 (t, J = 8.0 Hz, 1H), 7.37 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H). MS (ESI): $[M+1]^+$ = 446.3. Anal. ($C_{22}H_{21}Cl_2N_3OS$): C, H, N.

5.4.23. {2-Amino-4-[(4-(2-chlorophenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4w)

Following the general procedure (C), derivative **4w** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 44%. Yellow solid, mp 115–117 °C. ^{1}H NMR ($CDCl_3$) δ: 2.04 (t, J = 5.2 Hz, 4H), 2.78 (t, J = 5.2 Hz, 4H), 3.10 (s, 2H), 6.06 (br s, 2H), 6.11 (s, 1H), 6.60 (d, J = 7.4 Hz, 1H), 6.72 (d, J = 7.4 Hz, 1H), 6.80 (t, J = 7.4 Hz,, 1H), 7.02 (t, J = 7.4 Hz, 1H), 7.42 (d, J = 8.2 Hz, 2H), 7.52 (d, J = 8.2 Hz, 2H). MS (ESI): $[M+1]^+$ = 446.0. Anal. ($C_{22}H_{21}Cl_2N_3OS$): C, H, N.

5.4.24. {2-Amino-4-[(4-(2,6-dichlorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4x)

Following the general procedure (C), derivative **4x** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 42%. Yellow solid, mp 128–130 °C. ^{1}H NMR ($CDCl_3$) δ: 2.02 (t, J = 4.8 Hz, 4H), 2.82 (t, J = 4.6 Hz, 4H), 3.02 (s, 2H), 6.05 (br s, 2H), 6.09 (s, 1H), 7.01 (d, J = 8.6 Hz, 1H), 7.17 (t, J = 8.6 Hz, 1H), 7.29 (d, J = 2.6 Hz, 1H), 7.34 (d, J = 8.8 Hz, 2H), 7.52 (d, J = 8.8 Hz, 2H). MS (ESI): $[M+1]^+$ = 480.2. Anal. ($C_{22}H_{20}Cl_3N_3OS$): C, H, N.

5.4.25. {2-Amino-4-[(4-(2,5-dichlorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4y)

Following the general procedure (C), derivative **4y** was purified by column chromatography (EtOAc/DCM 0.5:9.5 as eluent). Yield: 49%. Yellow solid, mp 78–80 °C. ^{1}H NMR ($CDCl_3$) δ: 2.07 (t, J = 4.6 Hz, 4H), 2.81 (t, J = 4.6 Hz, 4H), 3.03 (s, 2H), 6.08 (br s, 2H), 6.15 (s, 1H), 6.93 (s, 1H), 7.24 (d, J = 9.2 Hz, 2H), 7.39 (d, J = 8.6 Hz, 2H), 7.57 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 480.4. Anal. ($C_{22}H_{20}Cl_3N_3OS$): C, H, N.

5.4.26. {2-Amino-4-[(4-(3,4-dichlorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4z)

Following the general procedure (C), derivative **4z** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 41%. Yellow solid, mp 102–104 °C. ^{1}H NMR ($CDCl_3$) δ: 2.04 (t, J = 4.6 Hz, 4H), 2.90 (t, J = 4.6 Hz, 4H), 3.00 (s, 2H), 6.08 (br s, 2H), 6.13 (s, 1H), 6.66 (dd, J = 9.0 and 2.8 Hz, 1H), 6.87 (d, J = 2.8 Hz, 1H), 7.21 (s, 1H), 7.40 (d, J = 8.6 Hz, 2H), 7.56 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 480.0. Anal. ($C_{22}H_{20}Cl_3N_3OS$): C, H, N.

5.4.27. {2-Amino-4-[(4-(2,4-dichlorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4aa)

Following the general procedure (C), derivative **4aa** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 44%. Yellow solid, mp 142–143 °C. ^{1}H NMR ($CDCl_3$) δ: 2.04 (t, J = 4.6 Hz, 4H), 2.76 (t, J = 4.6 Hz, 4H), 3.01 (s, 2H), 6.05 (br s, 2H), 6.14 (s, 1H), 6.88 (d, J = 8.6 Hz, 1H), 7.14 (dd, J = 11 and 2.6 Hz, 1H), 7.31 (d, J = 2.6 Hz, 1H), 7.38 (d, J = 8.8 Hz, 2H), 7.56 (d, J = 8.8 Hz, 2H). MS (ESI): $[M+1]^+$ = 480.1. Anal. ($C_{22}H_{20}Cl_3N_3OS$): C, H, N.

5.4.28. {2-Amino-4-[(4-(2,3-dichlorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4ab)

Following the general procedure (C), derivative **4ab** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 47%. Yellow oil. ^{1}H NMR ($CDCl_3$) δ: 2.05 (t, J = 4.4 Hz, 4H), 2.78 (t, J = 4.4 Hz, 4H), 3.01 (s, 2H), 6.07 (br s, 2H), 6.14 (s, 1H), 6.89 (d, J = 4.8 Hz, 1H), 7.11 (d, J = 5.2 Hz, 2H), 7.43 (t, J = 7.0 Hz, 2H), 7.58 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 480.2. Anal. ($C_{22}H_{20}Cl_3N_3OS$): C, H, N.

5.4.29. {2-Amino-4-[(4-(3,5-dichlorophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4ac)

Following the general procedure (C), derivative **4ac** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 44%. Yellow solid, mp 185–187 °C. ^{1}H NMR ($CDCl_3$) δ: 1.99 (t, J = 4.8 Hz, 4H), 2.92 (t, J = 4.8 Hz, 4H), 2.99 (s, 2H), 6.09 (br s, 2H), 6.13 (s, 1H), 6.64 (s, 2H), 6.76 (s, 1H), 7.38 (d, J = 8.6 Hz, 2H), 7.53 (d, J = 8.6 Hz, 2H). MS MS (ESI): $[M+1]^+$ = 480.1. Anal. ($C_{22}H_{20}Cl_3N_3OS$): C, H, N.

5.4.30. {2-Amino-4-[(4-(2-chloro-4-fluorophenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4ad)

Following the general procedure (C), derivative **4ad** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 51%. Yellow solid, mp 59–61 °C. ^{1}H NMR ($CDCl_3$) δ: 2.06 (t, J = 4.6 Hz, 4H), 2.75 (t, J = 4.6 Hz, 4H), 3.02 (s, 2H), 6.07 (br s, 2H), 6.15 (s, 1H), 6.92 (d, J = 8.4 Hz, 2H), 7.08 (d, J = 8.4 Hz, 1H), 6.93 (t, J = 8.8 Hz, 1H), 7.38 (d, J = 8.6 Hz, 2H), 7.58 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 464.1. Anal. ($C_{22}H_{20}Cl_2FN_3OS$): C, H, N.

5.4.31. {2-Amino-4-[(4-(2-fluoro-4-chlorophenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4ae)

Following the general procedure (C), derivative **4ae** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 54%. Yellow solid, mp 161–163 °C. ^{1}H NMR ($CDCl_3$) δ: 2.04 (t, J = 4.8 Hz, 4H), 2.80 (t, J = 4.8 Hz, 4H), 3.01 (s, 2H), 6.07 (br s, 2H), 6.14 (s, 1H), 6.78 (t, J = 7.6 Hz, 1H), 7.01 (m, 2H), 7.37 (d, J = 8.6 Hz, 2H), 7.55 (d, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 464.4. Anal. ($C_{22}H_{20}Cl_2FN_3OS$): C, H, N.

5.4.32. {2-Amino-4-[(4-(4-(methoxy)phenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4af)

Following the general procedure (C), derivative **4af** was purified by column chromatography (EtOAc/DCM 2:8 as eluent). Yield: 38%. Yellow oil. ^{1}H NMR ($CDCl_3$) δ: 2.04 (t, J = 5.4 Hz, 4H), 2.83 (t, J = 5.4 Hz, 4H), 3.10 (s, 2H), 3.75 (s, 3H), 6.08 (br s, 2H), 6.14 (s, 1H), 6.82 (d, J = 10.2 Hz, 2H), 6.86 (d, J = 10.2 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H). MS (ESI): $[M+1]^+$ = 442.2. Anal. ($C_{23}H_{24}ClN_3OS$): C, H, N.

5.4.33. {2-Amino-4-[(4-(4-nitrophenyl)piperazin-1-yl) methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4ag)

Following the general procedure (C), derivative **4ag** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 43%. Yellow solid, mp 110–112 °C. ^{1}H NMR ($CDCl_3$) δ: 2.46 (t, J = 5.4 Hz, 4H), 2.84 (t, J = 5.4 Hz, 4H), 3.03 (s, 2H), 6.21 (br s, 2H), 6.24 (s, 1H), 6.81 (d, J = 9.6 Hz, 2H), 7.46 (d, J = 9.2 Hz, 2H), 7.65 (d, J = 9.6 Hz, 2H), 8.13 (d, J = 9.2 Hz, 2H). MS (ESI): $[M+1]^+$ = 457.5. Anal. ($C_{22}H_{21}ClN_4O_3S$): C, H, N.

5.4.34. {2-Amino-4-[(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4ah)

Following the general procedure (C), derivative **4ah** was purified by column chromatography (EtOAc/DCM 0.5:9.5 as eluent). Yield: 48%. Yellow solid, mp 167–169 °C. ^{1}H NMR ($CDCl_3$) δ: 2.04 (t, J = 5.2 Hz, 4H), 2.97 (t, J = 5.2 Hz, 4H), 3.00 (s, 2H), 6.07 (br s, 2H), 6.14 (s, 1H), 7.01 (m, 3H), 7.26 (t, J = 9.6 Hz, 1H), 7.38 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.4 Hz, 2H). MS (ESI): $[M+1]^+$ = 480.1. Anal. ($C_{23}H_{21}ClN_4O_3S$): C, H, N.

5.4.35. {2-Amino-4-[(4-(2-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4ai)

Following the general procedure (C), derivative **4ai** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 52%. Yellow oil. ^{1}H NMR ($CDCl_3$) δ: 2.01 (t, J = 4.8 Hz, 4H), 2.67 (t, J = 5.2 Hz, 4H), 3.00 (s, 2H), 6.05 (br s, 2H), 6.13 (s, 1H), 7.12 (t, J = 8.4 Hz, 1H), 7.18 (m, 2H), 7.36 (d, J = 8.8 Hz, 2H), 7.44 (m, 1H), 7.57 (d, J = 8.8 Hz, 2H). MS (ESI): $[M+1]^+$ = 480.2. Anal. ($C_{23}H_{21}ClF_3N_3OS$): C, H, N.

5.4.36. {2-Amino-4-[(4-(4-chloro-3-(trifluoromethyl) phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4aj)

Following the general procedure (C), derivative **4aj** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 44%. Yellow solid, mp 141–143 °C. ^{1}H NMR ($CDCl_3$) δ: 1.96 (t, J = 4.4 Hz, 4H), 2.87 (t, J = 4.4 Hz, 4H), 2.94 (s, 2H), 6.01 (br s, 2H), 6.07 (s, 1H), 6.78 (dd, J = 8.2 and 2.8 Hz, 1H), 7.00 (s, 1H), 7.19 (d, J = 8.2 Hz, 1H), 7.33 (d, J = 8.2 Hz, 2H), 7.48 (d, J = 8.2 Hz, 2H). MS (ESI): $[M+1]^+$ = 514.1. Anal. ($C_{23}H_{20}Cl_2F_3N_3OS$): C, H, N.

5.4.37. {2-Amino-4-[(4-(4-(trifluoromethoxy)phenylpiperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (4ak)

Following the general procedure (C), derivative **4ak** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 40%. Yellow solid, mp 151–153 °C. ^{1}H NMR ($CDCl_3$) δ: 2.04 (t, J = 4.8 Hz, 4H), 2.92 (t, J = 4.8 Hz, 4H), 3.01 (s, 2H), 6.09 (br s, 2H), 6.14 (s, 1H), 6.80 (d, J = 8.6 Hz, 1H), 7.07 (d, J = 8.6 Hz, 2H), 7.38 (d, J = 7.8 Hz, 2H), 7.56 (d, J = 7.8 Hz, 2H). MS (ESI): $[M+1]^+$ = 496.4. Anal. ($C_{23}H_{21}ClF_3N_3O_2S$): C, H, N.

5.4.38. [2-Amino-4-(4-(pyridin-3-yl)piperazin-1-yl)methyl) thiophen-3-yl](4-chlorophenyl) methanone (4al)

Following the general procedure (C), derivative **4al** was purified by column chromatography (EtOAc/MeOH 6:4 as eluent). Yield: 34%. Yellow solid, mp 101–103 °C. ^{1}H NMR ($CDCl_3$) δ: 2.01 (t, J = 4.8 Hz, 4H), 2.84 (t, J = 4.8 Hz, 4H), 3.00 (s, 2H), 6.09 (br s, 2H), 6.13 (s, 1H), 6.52 (d, J = 7.8 Hz, 1H), 6.78 (m, 1H), 7.42 (d, J = 8.6 Hz, 2H), 7.57 (d, J = 8.6 Hz, 2H), 8.07 (s, 1H), 8.24 (d, J = 9.0 Hz, 1H). MS (ESI): $[M+1]^+$ = 413.1. Anal. ($C_{21}H_{21}ClN_4OS$): C, H, N.

5.4.39. [2-Amino-4-((4-(pyridin-4-yl)piperazin-1-yl)methyl)thiophen-3-yl](4-chlorophenyl) methanone (4am)

Following the general procedure (C), derivative **4am** was purified by column chromatography (EtOAc/MeOH 7:3 as eluent). Yield: 38%. Yellow solid, mp 86–88 °C. ^{1}H NMR ($CDCl_3$) δ: 2.01 (t, J = 5.2 Hz, 4H), 3.00 (s, 2H), 3.24 (t, J = 5.2 Hz, 4H), 6.09 (br s, 2H), 6.12 (s, 1H), 6.72 (d, J = 5.6 Hz, 2H), 6.87 (d, J = 5.6 Hz, 2H), 8.06 (d, J = 7.4 Hz, 2H), 8.19 (d, J = 7.4 Hz, 2H). MS (ESI): $[M+1]^+$ = 413.2. Anal. ($C_{21}H_{21}ClN_4OS$): C, H, N.

5.4.40. {2-Amino-4-[(4-(4-chlorophenyl)piperidin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl) methanone (4an)

Following the general procedure (C), derivative **4an** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 45%. Yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.67 (m, 4H), 2.27 (m, 1H), 2.54 (t, J = 5.4 Hz, 4H), 3.04 (s, 2H), 6.09 (s, 2H), 6.16 (s, 1H), 7.33 (s, 4H), 7.39 (d, J = 8.6 Hz, 2H), 7.51 (t, J = 8.6 Hz, 2H). MS (ESI): $[M+1]^+$ = 445.2. Anal. ($C_{23}H_{22}Cl_2N_2OS$): C, H, N.

5.4.41. {2-Amino-4-[(4-(4-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(3-(trifluoromethyl)phenyl)methanone (4ao)

Following the general procedure (C), derivative **4ao** was purified by column chromatography (EtOAc/DCM 0.25:9.75 as eluent). Yield: 52%. Yellow solid, mp: 143–145 °C. ^{1}H NMR ($CDCl_3$) δ: 1.95 (t, J = 4.8 Hz, 4H), 2.94 (s, 2H), 2.98 (t, J = 4.8 Hz, 4H), 6.14 (s, 1H), 6.28 (br s, 2H), 6.82 (t, J = 8.8 Hz, 2H), 7.42 (d, J = 8.8 Hz, 2H), 7.54 (t, J = 8.0 Hz, 1H), 7.74 (m, 2H), 7.85 (s, 1H). MS (ESI): $[M+1]^+$ = 514.5. Anal. ($C_{25}H_{22}F_6N_2OS$): C, H, N.

5.4.42. {2-Amino-4-[(4-(3-fluorophenyl)piperazin-1-yl)methyl]thiophen-3-yl}(3-(trifluoromethyl)phenyl)methanone (4ap)

Following the general procedure (C), derivative **4ap** was purified by column chromatography (EtOAc/DCM 1:9 as eluent). Yield: 55%. Yellow solid, mp: 100–102 °C. ^{1}H NMR ($CDCl_3$) δ: 1.94 (t, J = 4.8 Hz, 4H), 2.90 (t, J = 4.8 Hz, 4H), 2.93 (s, 2H), 6.14 (s, 1H), 6.29 (br s, 2H), 6.49 (m, 2H), 6.57 (d, J = 9.6 Hz, 1H), 7.15 (q, J = 7.2 Hz, 1H), 7.54 (t, J = 8.0 Hz, 1H), 7.74 (m, 2H), 7.84 (s, 1H). MS (ESI): $[M+1]^+$ = 464.4. Anal. ($C_{24}H_{22}F_4N_2OS$): C, H, N.

5.4.43. {2-Amino-4-[(4-(2,6-difluorophenyl)piperazin-1-yl)methyl]thiophen-3-yl}(3-(trifluoromethyl)phenyl)methanone (4aq)

Following the general procedure (C), derivative **4aq** was purified by column chromatography (EtOAc/DCM 0.5:9.5 as eluent). Yield: 62%. Yellow solid, mp: 163–165 °C. ^{1}H NMR ($CDCl_3$) δ: 1.92 (t, J = 4.8 Hz, 4H), 2.89 (t, J = 4.8 Hz, 4H), 3.02 (s, 2H), 6.73 (s, 1H), 6.84 (m, 5H), 7.34 (t, J = 8.0 Hz, 1H), 7.46 (d, J = 8.0 Hz, 1H), 7.84 (d, J = 7.8 Hz, 1H), 8.01 (s, 1H). MS (ESI): $[M+1]^+$ = 482.1. Anal. ($C_{24}H_{21}F_5N_2OS$): C, H, N.

5.5. Biology experiments

5.5.1. Cyclic AMP accumulation in CHO cells

Chinese hamster ovary cells expressing human recombinant A_1 adenosine receptors (hA_1CHO cells) at a density of approximately 8000 fmol/mg protein were prepared as previously described[5] and aliquots of these cells at low passage numbers were frozen and stored in liquid nitrogen. Upon the arrival of a group of compounds for testing in the laboratory, an aliquot of cells was removed from liquid nitrogen storage and grown in Ham's F-12 culture medium with 10% fetal bovine serum and 0.5 mg/mL of antibiotic G-418.[23] Cells were passaged thrice weekly and aliquots of cells were placed into 12-well culture plates with culture medium, serum, and antibiotic for 48 h, by which time the cells had grown to a confluent monolayer. To begin an experiment, growth medium was removed from the culture plates and cells were washed once with Hanks' buffered saline solution. The wash solution was then removed and replaced with fresh Hanks' solution containing forskolin (1 μM), rolipram (20 μM), CPA (0.01 nM), adenosine deaminase (2 U/mL), and the allosteric enhancer to be tested. Forskolin was used to stimulate the activity of adenylyl cyclase, rolipram to inhibit cAMP phosphodiesterase, adenosine deaminase to degrade endogenous adenosine, and CPA to cause a small increase of the number of activated adenosine receptors. After 6 min of incubation at 36 °C in the presence of drugs, the incubation solution was removed and hydrochloric acid (final concentration, 50 mM) was added to cells to terminate drug action. The content of cAMP in acidified extracts of cells was determined by radioimmunoassay as previously described.[5] Because the magnitude of the effects of allosteric enhancers on hA_1CHO cells changed subtly with passage number and differed slightly among different aliquots of cells, the action of tested compounds and the action of the reference compound PD 81,723 were assayed in each experiment. Allosteric enhancement was measured as the action of a test compound at different concentrations (0.01, 0.1, 1 and 10 μM) to reduce the cAMP content of hA_1CHO cells in the presence of CPA (0.05–0.1 nM) which causes a slight reduction of cAMP content by activation of A_1ARs. Allosteric enhancement of the action of CPA causes a further reduction of the cAMP content of hA_1-CHO cells. Because the spontaneous activity of adenosine receptors in hA_1CHO cells causes an inhibition of adenylyl cyclase activity even in the absence of an agonist,[23] antagonists of adenosine receptors increase cAMP content of cells. Therefore, compounds that increased cAMP content of cells in this study were provisionally identified as A_1 adenosine receptor antagonists.

5.5.2. Saturation and competition binding experiments

5.5.2.1. Materials. [^{3}H]DPCPX ([^{3}H]1,3-dipropyl-8-cyclopentyl-xanthine; specific activity, 120 Ci/mmol) and [^{3}H]CCPA ([^{3}H]2-chloro-N^6-cyclopentyladenosine; specific activity, 55 Ci/mmol) were obtained from Perkin Elmer (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 3008F20 ([^{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 Amersham International (Buckinghamshire, UK). DPCPX (1,3-dipropyl-8-cyclopentyl-xanthine), R-PIA ((*R*)-N^6-(L-2-Phenylisopropyl)adenosine) and CPA (N^6-cyclopentyladenosine) were obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade and obtained from commercial sources.

5.5.2.2. Membrane preparation from CHO cells transfected with hA_1, hA_{2A} and hA_3ARs. The hA_1CHO, hA_{2A}CHO and hA_3-CHO 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.[24] Cells were splitted two or three times weekly at a ratio of 1:5. For membrane preparation the culture medium was removed, the cells were washed with PBS and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris–HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with Polytron and the homogenate was spun for 10 min at 1,000×*g*. The supernatant was then centrifuged for 30 min at 100,000×*g*. The membrane pellet was resuspended in 50 mM Tris–HCl buffer pH 7.4 for A_1ARs, 50 mM Tris–HCl buffer pH 7.4, 10 mM $MgCl_2$ for A_{2A}ARs, 50 mM Tris–HCl buffer pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA for A_3ARs and incubated with 3 UI/mL of adenosine deaminase for 30 min at

37 °C. The protein concentration was determined according to a Bio-Rad method (Bradford, 1976) with bovine albumin as a standard reference.

5.5.2.3. [^{3}H]CCPA Binding Experiments in hA$_1$CHO membranes. Saturation binding experiments of [^{3}H]CCPA (0.05–20 nM) to hA$_1$CHO 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.[14] Non specific binding was defined as binding in the presence of 1 μM R-PIA.

Competition binding experiments were carried out in triplicate in a final volume of 250 μl containing 1 nM [^{3}H]CCPA, 50 mM Tris–HCl, pH 7.4 and 100 μl of diluted membranes and at least six to eight different concentrations of the tested compounds in the range from 1 nM to 10 μM for 90 min at 25 °C.[15] Non specific binding was defined as binding in the presence of 1 μM R-PIA.

5.5.2.4. [^{3}H]DPCPX Binding experiments in hA$_1$CHO membranes. Saturation binding experiments of [^{3}H]DPCPX (0.05–20 nM) to hA$_1$CHO 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.[14] Non specific binding was defined as binding in the presence of 1 μM DPCPX.

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 different tested compounds at the concentration 10 μM on CCPA curve (0.01 nM–1 μM) was investigated. Non specific binding was defined as binding in the presence of 1 μM DPCPX.

5.5.2.5. Assay of adenosine antagonist activity. A$_1$, A$_{2A}$ and A$_3$ AR competition binding experiments were performed using 1 nM [^{3}H]DPCPX,[25] 1 nM [^{3}H]ZM 241385[26] and 2 nM [^{3}H]MRE 3008F20[27] as radioligands, respectively. 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, and 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 3008F20 for A$_1$, A$_{2A}$ and A$_3$ARs, 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 Scintillation Counter Packard Tri Carb 2810 TR (Perkin Elmer).

5.6. Data analysis

Saturation and competition binding experiments were analyzed with the program LIGAND[28] which performed weighted, non-linear, least squares curve fitting program. Inhibitory binding constants, K_i, were also calculated from the IC_{50} values according to the Cheng and Prusoff equation $K_i = IC_{50}/(1 + [C*]/K_D*)$, where [C*] is the concentration of the radioligand and K_D* its dissociation constant.[29] All experimental data are expressed as mean ± standard error of the mean (S.E.M.) of three or four independent experiments performed in duplicate.

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Supplementary data

Supplementary data (data on antagonist activity of compounds **PD** 81,723, **3a–l** and **4a–aq**, synthetic procedure for the preparation of compound **5b**, detailed characterization of compounds **6a–aq**, **7a–aq** and **8**. Elemental analyses of compounds **4a–aq**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.11.044.

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