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Synthesis and Biological Evaluation of 2-Amino-3-(4-chlorobenzoyl)-4-[(4-arylpiperazin-1-yl)methyl]-5-substituted-thiophenes. Effect of the 5-Modification on Allosteric Enhancer Activity at the A_1 Adenosine Receptor

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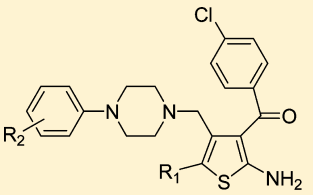
ABSTRACT: We have recently reported a detailed structure−activity relationship study around a wide series of 2-amino-3-(4-chlorobenzoyl)-4-[(4-arylpiperazin-1-yl)methyl]thiophene derivatives as potent allosteric enhancers of the A_1 adenosine receptor. In the current study, we have continued to explore the potential of these molecules by synthesizing of a novel series of analogues that share a common 2-amino-3-(4-chlorobenzoyl)thiophene nucleus. Modifications were focused on varying the nature and the position of electron-withdrawing or electron-releasing groups on the phenyl of an arylpiperazine moiety attached at the 4-position of the thiophene ring by a methylene chain, combined with the presence of small alkyl groups (methyl or ethyl), bromine, or aryl moieties at the thiophene C-5 position. In this series of compounds, substitution at the 5-position had a fundamental effect on activity, with the 5-aryl group contributing additively to the allosteric enhancer activity. The thiophene C-5 aryl derivatives **4ad**, **4ak**, and **4al** were the most active compounds in binding and functional experiments.



R_1=Me, Et, Br or aryl
R_2=H, halogen, CH_3, OCH_3, CN, NO_2, CF_3, OCF_3

INTRODUCTION

Adenosine is an endogenous nucleoside modulator released from almost all cells and is generated in the extracellular space by breakdown of ATP.[1] This nucleoside mediates its effects through activation of a family of four G protein-coupled adenosine receptors (ARs), named A_1, A_{2A}, A_{2B}, and A_3.[2] These receptors differ in their affinity for adenosine, in the type of G proteins that they recruit, and finally in the downstream signaling pathways that are activated in the target cells. The A_1AR is coupled to members of the Gi/Go family of G proteins, inducing inhibition of adenylate cyclase activity.[3] The A_1AR is widely distributed in the central nervous system (CNS), with high levels in brain, cortex, cerebellum, hippocampus, and the dorsal horn of the spinal cord. It modulates the activity of the nervous system at the cellular level and is responsible for sedative, anticonvulsant, anxiolytic, and locomotor depressant effects induced by adenosine. Importantly, the A_1AR has been reported to play a role in adenosine-mediated analgesia.[4] Stimulation of A_1ARs in the heart exerts a cardioprotective effect by inhibiting norepinephrine release from sympathetic nerve endings.[5] Adenosine also protects tissues through its effect in ischemic preconditioning (IPC), a brief period of ischemia and reperfusion, which can protect myocardium from a subsequent prolonged ischemic insult.[6]

Efforts to selectively target the A_1AR with modified adenosine analogues have resulted in therapeutics that are limited by side effects due to their ubiquitous nature and poor receptor subtype selectivity. In contrast to ligands that target the orthosteric site, allosteric ligands interact with binding domains that are topographically distinct from the orthosteric site.[7] The binding of an allosteric ligand to its site causes a conformational change in the receptor protein that is transmitted to the orthosteric site (and vice versa), enhancing or inhibiting activation of the receptor by its natural ligand.[7a] Allosteric enhancers (AEs) at the A_1AR have potential as therapeutic agents because they increase the efficacy of potent and highly selective exogenous agonists, thereby allowing lower doses, or they may amplify the action of endogenous adenosine by stabilizing the agonist−receptor−G protein ternary complex that forms when local adenosine concentrations increase under conditions of metabolic stress, such as hypoxia.[8]

One of the first compounds recognized as an AE at the A_1AR was the 2-amino-3-aroylthiophene derivative named PD 81,723 (**1**, Chart 1)[9] that selectively enhanced agonist radioligand binding at the A_1AR with no major effect on agonist binding at the other adenosine receptor subtypes.[10]

Several studies emphasized the presence of two regions in the allosteric binding site in the hA_1AR: one region able to interact with the 2-amino-3-aroyl thiophene moiety, while the

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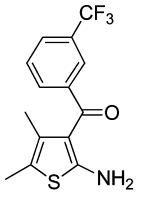
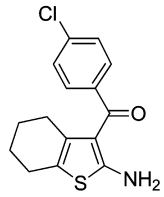
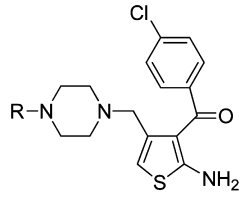
Chart 1. Chemical Structures of 2-Amino-3-aroylthiophene Derivatives 1, 2, 3a−e, and 4a-ao, Discovered as Allosteric Modulators for A_1 Adenosine Receptor



1, PD 81,723

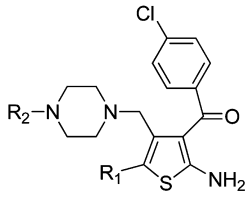


2, T 62

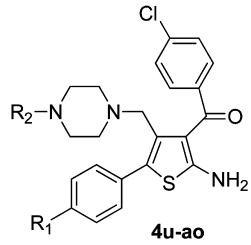


3a-e

3a; R=4-Cl-C_6H_4
3b; R=4-CF_3-C_6H_4
3c; R=3,4-F_2-C_6H_3
3d; R=3-Cl,4-F-C_6H_3,
3e; R=4-CF_3O-C_6H_4



4a-t



4u-ao

4a; R_1=CH_3, R_2=C_6H_5
4b; R_1=CH_3, R_2=$C_6H_5CH_2$
4c; R_1=CH_3, R_2=4-F-C_6H_4
4d; R_1=CH_3, R_2=4-Cl-C_6H_4
4e; R_1=CH_3, R_2=3-Cl-C_6H_4
4f; R_1=CH_3, R_2=3,4-Cl_2-C_6H_3
4g; R_1=CH_3, R_2=4-Br-C_6H_4
4h; R_1=CH_3, R_2=4-I-C_6H_4
4i; R_1=CH_3, R_2=4-CH_3-C_6H_4
4j; R_1=CH_3, R_2=4-CH_3O-C_6H_4
4k; R_1=CH_3, R_2=4-CN-C_6H_4
4l; R_1=CH_3, R_2=4-NO_2-C_6H_4
4m; R_1=CH_3, R_2=4-CF_3-C_6H_4
4n; R_1=CH_3, R_2=3-CF_3-C_6H_4
4o; R_1=CH_3, R_2=4-Cl,3-CF_3-C_6H_3
4p; R_1=C_2H_5, R_2=C_6H_5
4q; R_1=C_2H_5, R_2=4-F-C_6H_4
4r; R_1=C_2H_5, R_2=4-Cl-C_6H_4
4s; R_1=Br, R_2=C_6H_5
4t; R_1=Br, R_2=4-F-C_6H_4

4u; R_1=H, R_2=C_6H_5
4v; R_1=H, R_2=4-F-C_6H_4
4w; R_1=H, R_2=4-Cl-C_6H_4
4x; R_1=H, R_2=3,4-Cl_2-C_6H_3
4y; R_1=H, R_2=4-CF_3-C_6H_4
4z; R_1=H, R_2=3-CF_3-C_6H_4
4aa; R_1=H, R_2=4-Cl,3-CF_3-C_6H_3
4ab; R_1=H, R_2=2-CF_3-C_6H_4
4ac; R_1=H, R_2=4-CF_3O-C_6H_4
4ad; R_1=F, R_2=C_6H_5
4ae; R_1=F, R_2=4-F-C_6H_4
4af; R_1=Cl, R_2=C_6H_5
4ag; R_1=Cl, R_2=$C_6H_5CH_2$
4ah; R_1=Cl, R_2=4-F-C_6H_4
4ai; R_1=Cl, R_2=4-Cl-C_6H_4
4aj; R_1=Cl, R_2=3,4-Cl_2-C_6H_3
4ak; R_1=Cl, R_2=4-CF_3-C_6H_4
4al; R_1=Cl, R_2=3-CF_3-C_6H_4
4am; R_1=Cl, R_2=4-Cl,3-CF_3-C_6H_3
4an; R_1=Cl, R_2=2-CF_3-C_6H_4
4ao; R_1=Cl, R_2=4-CF_3O-C_6H_4

second wide lipophilic domain is able to interact with thiophene C-4 and C-5 substituents.[8,11] The presence of a carbonyl at C-3 and an adjacent primary amino group at C-2 of the thiophenes is an important feature of the A_1AR allosteric enhancer activity.[9] The benzoyl group was reported to be essential for activity, and hydrophobic substituents at either the meta or para position on the phenyl of the benzoyl moiety [such as CF_3, Cl, or $C(CH_3)_3$] are critical for high AE activity.[12] Replacing the aroyl with a naphthoyl group was well-tolerated.[12c,13]

In order to identify highly potent and selective AEs at the A_1AR, a wide range of 2-amino-3-aroylthiophene derivatives modified at the C-4 and C-5 positions of the thiophene ring have been reported.[12−14] Structure−activity relationship studies indicated that alkyl[12−14] and aryl[15] groups at the C-4 position favored AE activity. Only small alkyl groups were thought to be tolerated at the C-5 position, while bulky substitutents favored antagonistic properties.[16]

Among the synthesized compounds, derivative **2** [T-62, (2-amino-4,5,6,7-tetrahydro-benzo[*b*]thiophen-3-yl)(4-chlorophenyl)-methanone] was taken into phase II clinical trials by King Pharmaceuticals for the treatment of neuropathic pain associated with hyperalgesia and allodynia.[17]

In two previous articles,[18] we have reported the potent AE activity at the A_1AR of a series of 2-amino-3-(4-chlorobenzoyl)-4-[(4-arylpiperazin-1-yl)methyl]thiophene derivatives with general structure **3**. The enhancement was influenced by the number and position of electron-withdrawing or electron-releasing groups (EWGs or ERGs, respectively) on the phenyl tethered to the piperazine moiety. Among them, the 4-chloro (**3a**), 4-trifluoromethyl (**3b**), 3,4-difluoro (**3c**), 3-chloro-4-fluoro (**3d**), and 4-trifluoromethoxy (**3e**) derivatives were reported to be the most active compounds in binding (saturation and competition) and functional cAMP studies.

We hypothesized that the A_1AR contains an allosteric binding site able to accommodate the phenylpiperazine moiety, in which appropriate substituents on the phenyl ring influenced this interaction and contributed importantly to increase AE activity. On the basis of these findings, we continued the search for more active AEs for the A_1AR, by refining the structure−activity relationship around this class of compounds through the synthesis and biological evaluation of a new series of 2-amino-3-(4-chlorobenzoyl)-4-[(arylpiperazinyl)methyl]-thiophene derivatives with general structure **4**, characterized by the insertion of different groups at the 5-position. In detail, the substituents at the 5-position were selected from small alkyl

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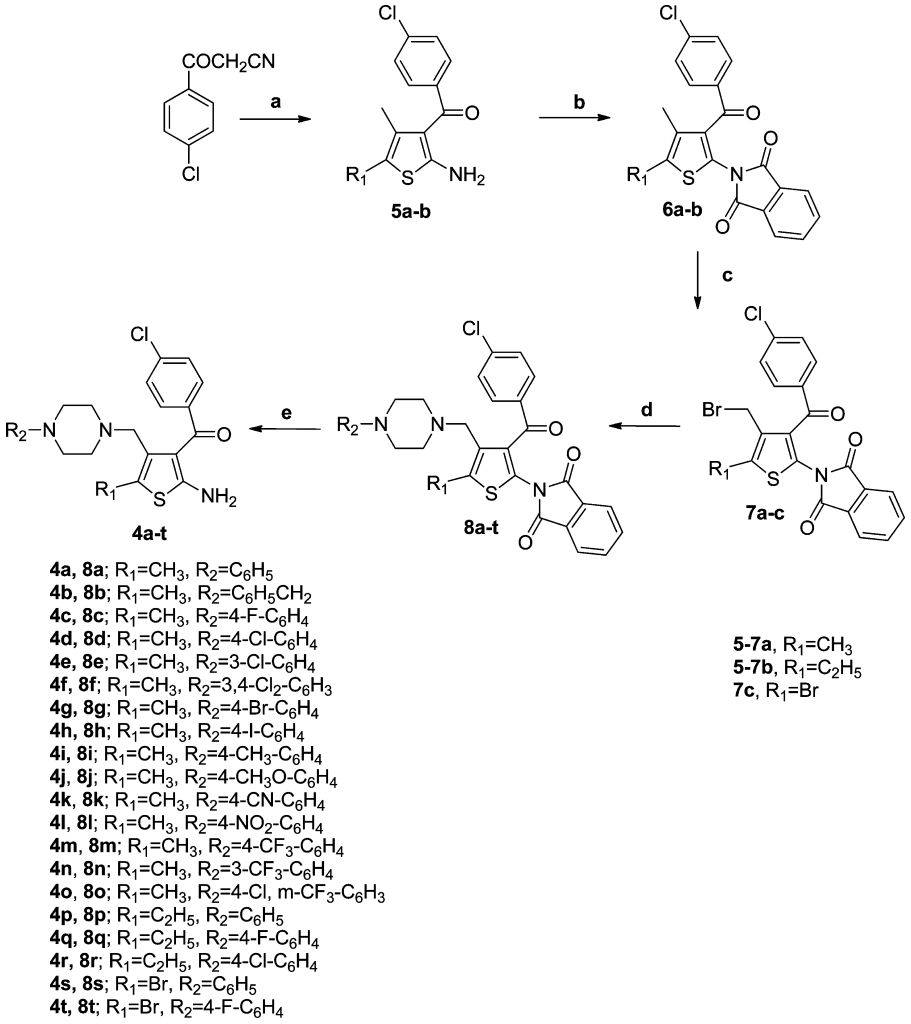
Scheme 1[a]



[a]Reagents: (a) 2-Butanone or 2-pentanone, S_8, TEA, EtOH, reflux, 2 h; (b) phthalic anhydride, AcOH, reflux; (c) NBS, CH_3CN, reflux; (d) *N*-arylpiperazine, TEA, CH_2Cl_2; (e) NH_2NH_2, EtOH.

groups such as methyl and ethyl for derivatives **4a**−**o** and **4p**−**r**, respectively, bromine for compounds **4s**−**t**, or an aryl moiety for the analogues **4u**−**ao**. For this latter class of compounds, we have also explored the effect on AE activity of electron-withdrawing fluorine (**4ad** and **4ae**) or chlorine (**4af**−**ao**) groups at the 4-position of the phenyl at the thiophene C-5 position. For the phenyl group linked to the piperazine moiety, a panel of substituents that had been reported to confer high AE activity in the previous series of thiophene 5-unsubstituted derivatives[18] with general structure **3** was chosen for this purpose.

By the preparation of this new series of 2-amino-3-(4-chlorobenzoyl)-4-[(arylpiperazinyl)methyl]-5-substituted-thiophene derivatives with general structure **4**, we are interested in investigating if modifications at the 5-position of this class of compounds are beneficial for interaction with the allosteric site of the A_1AR and if manipulation at this site could improve enhancer activity, separating this effect from the competitive antagonism at the same receptor subtype.

CHEMISTRY

Compounds **4a**−**r** were synthesized by the method depicted in Scheme 1. 4,5-Dimethyl thiophene derivative **5a**[12a] and its 4-methyl-5-ethylthiophene counterpart **5b** were directly obtained by the classical Gewald reaction[19] applied to butanone and 2-pentanone, respectively, and 3-(4-chlorophenyl)-3-oxopropanenitrile with elemental sulfur and triethylamine (TEA) as base in ethanol at reflux. Both of these compounds were transformed in excellent yields into the corresponding *N*-phthalimido derivatives **6a** and **6b** using phthalic anhydride in refluxing acetic acid. Regioselective monobromination at the C-4 methyl of **6a** and **6b** with *N*-bromosuccinimide proceeded in good yield to afford the 4-bromomethyl thiophene derivatives **7a** and **7b**, which were coupled with the appropriate *N*-arylpiperazines (2 equiv) in dichloromethane (DCM) to afford the derivatives **8a**−**o** and **8p**−**r**. Removal of the N-protected phthaloyl group was accomplished by the use of hydrazine in ethanol at reflux, to afford the final compounds **4a**−**o** and **4p**−**r.**

The 5-bromo derivatives **4s** and **4t** were prepared starting from the 4-bromomethyl-5-bromothiophene **7c**,[18a,b] which was condensed with 1-phenylpiperazine or 1-(4-fluorophenyl)-piperazine to yield **8s** and **8t**, respectively, and then subjected to treatment with ethanolic hydrazine.

The previously reported 5-bromothiophene derivative **9**[18a] served as the starting material in the sequence shown in Scheme 2 for the preparation of derivatives **4u**−**ao**. With our optimized

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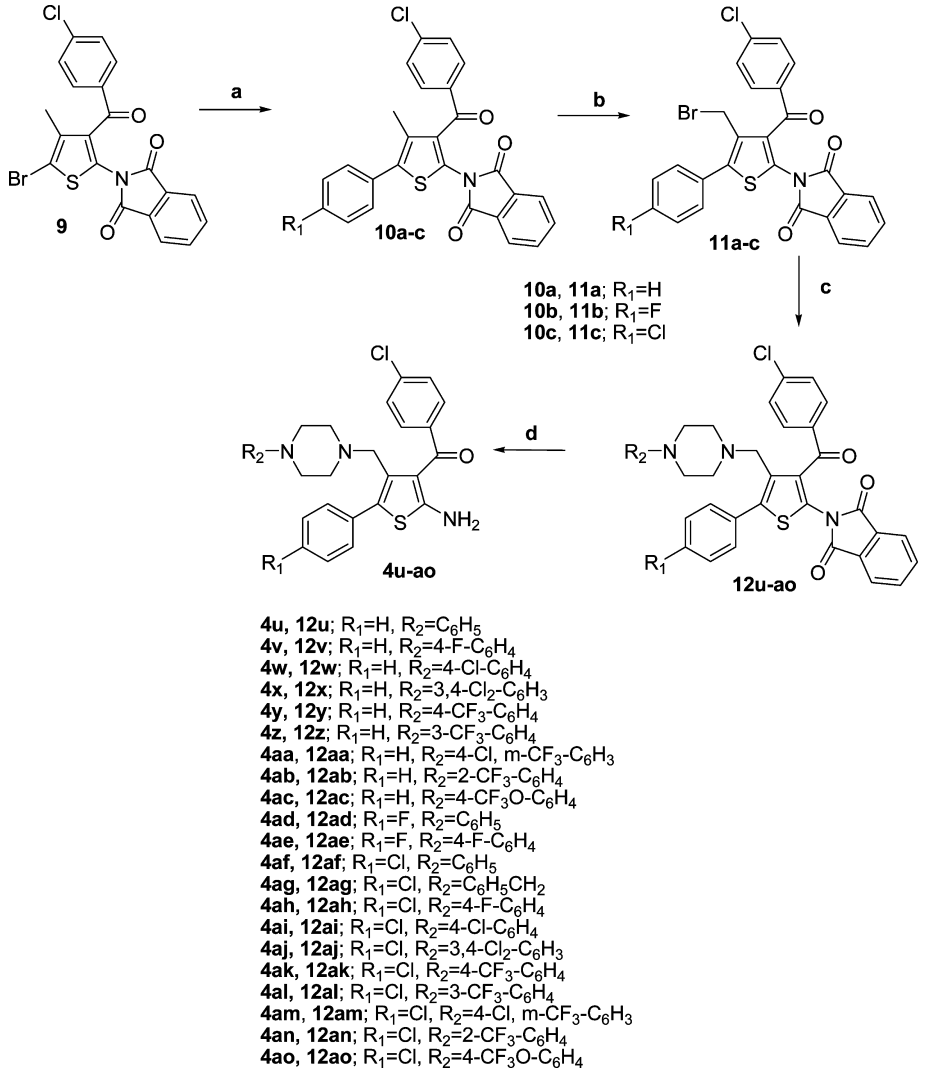
Scheme 2[a]



[a]Reagents: (a) $PdCl_2$(dppf), $ArB(OH)_2$, CsF, 1,4-dioxane, 65 °C; (b) NBS, 1,2-dichloroethane, reflux; (c) *N*-arylpiperazine, TEA, CH_2Cl_2; (d) NH_2NH_2, EtOH.

conditions in hand, intermediate **9** was subjected to palladium-catalyzed cross-coupling conditions in the presence of the appropriate arylboronic acid under heterogeneous conditions [$PdCl_2$(dppf), CsF] in 1,4-dioxane at 65 °C to furnish the 5-arylthiophene derivatives **10a–c** in good yields. These intermediates were brominated on the 4-methyl side chain with NBS in 1,2-dichloroethane to afford the 4-bromomethyl analogues **11a–c**, which were subsequently transformed into derivatives **12u–ao** by treatment with the appropriate *N*-arylpiperazines in DCM. The isolated compounds **12u–ao** were converted to the final compounds **4u–ao** by treatment with hydrazine in ethanol at reflux.

BIOLOGICAL RESULTS AND DISCUSSION

Functional Assays. Functional assays of allosteric enhancement activity were performed using Chinese hamster ovary (CHO) cells stably transfected to express the recombinant hA_1ARs. Activation of these receptors causes a detectable inhibition of adenylate cyclase activity and a reduction of cAMP content of CHO cells. Compounds with the potential to be allosteric enhancers of activation of hA_1ARs decrease the content of cAMP in CHO cells expressing hA_1ARs.

Allosteric enhancement was measured as the ability of the new synthesized compounds **4a–ao** and the reference derivative PD 81,723 to reduce the cAMP content of CHO:hA_1 cells in an in vitro assay on CHO:hA_1 cells. Two test paradigms were utilized: the test compound at a concentration of 10 μM alone and the test compound at 100-fold lower concentration (100 nM) in the presence of the selective hA_1AR agonist CCPA (1 pM). A decrease of cAMP content is indicated in Table 1 as a percentage inhibition of cAMP production from control (absence of test compound) or in the presence of the tested derivative. A similar reduction of cAMP content was observed with each of the tested compounds under both of the two test conditions.

Among the 41 synthesized derivatives, only three compounds (**4b**, **4k**, and **4l**) were less active than PD 81,723, and one compound (**4j**) was comparable to PD 81,723. All of the remaining compounds were considerably more active at 10 μM than PD 81,723 and decreased the percentage of cAMP production from 40% to 89% (Table 1).

Comparing compounds characterized by the presence of the same arylpiperazine moiety at the 4-position of the thiophene

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Table 1. Effect of the Novel Allosteric Enhancers 4a−ao and of PD 81,723 in cAMP Assay in hA_1CHO Cells

compd	% inhibn of cAMP production[a]	% inhibn of cAMP production + CCPA[b]
4a	59 ± 6	57 ± 6
4b	14 ± 1	19 ± 2
4c	50 ± 5	53 ± 5
4d	44 ± 4	40 ± 4
4e	58 ± 6	54 ± 6
4f	56 ± 5	51 ± 5
4g	49 ± 5	46 ± 5
4h	54 ± 5	45 ± 4
4i	61 ± 6	58 ± 6
4j	31 ± 3	30 ± 3
4k	11 ± 1	15 ± 2
4l	9 ± 1	13 ± 1
4m	60 ± 5	61 ± 6
4n	51 ± 5	52 ± 5
4o	52 ± 5	48 ± 5
4p	57 ± 6	50 ± 5
4q	63 ± 6	55 ± 6
4r	60 ± 6	53 ± 5
4s	48 ± 5	43 ± 4
4t	46 ± 5	41 ± 4
4u	79 ± 8	74 ± 7
4v	77 ± 8	71 ± 8
4w	68 ± 7	67 ± 7
4x	55 ± 5	56 ± 6
4y	72 ± 7	73 ± 7
4z	63 ± 7	60 ± 6
4aa	64 ± 6	65 ± 6
4ab	62 ± 6	66 ± 7
4ac	67 ± 8	69 ± 7
4ad	89 ± 9	81 ± 8
4ae	82 ± 9	77 ± 8
4af	75 ± 7	72 ± 7
4ag	65 ± 6	63 ± 6
4ah	71 ± 7	68 ± 7
4ai	85 ± 9	71 ± 7
4aj	69 ± 7	67 ± 7
4ak	83 ± 8	75 ± 8
4al	81 ± 8	78 ± 8
4am	76 ± 8	70 ± 7
4an	66 ± 7	62 ± 6
4ao	78 ± 8	72 ± 8
PD 81,723	19 ± 2	22 ± 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.

ring (**4s** vs **4a**, **4p**, **4u**, **4ad**, and **4af**; **4t** vs **4c**, **4q**, **4v**, **4ae**, and **4ah**), it is apparent that the 5-bromothiophene derivatives **4s** and **4t** were less active than the 5-methyl ones (**4a** and **4c**), which were less active than the 5-ethyl (**4p** and **4q**) and 5-aryl (**4u**, **4ad**, **4af**, **4v**, **4ae**, and **4ah**) counterparts as allosteric enhancers, although **4s** and **4t** were effective and more active than PD 81,723 at 10 μM. The greatest percentage reduction of cAMP production was achieved by compounds **4u−ao** that contained an aryl substituent at the C-5 position of the thiophene ring.

Within the series of 5-methylthiophene derivatives, **4a−o**, the introduction of a halogen at the 4-position of the phenyl moiety linked to the piperazine (**4c**, **4d**, **4g**, and **4h** for F, Cl, Br, and I, respectively) reduced the activity relative to the unsubstituted phenylpiperazine derivative **4a**, although there was no significant difference in activity between the four halogens. This may suggest that the adenosine A_1 receptor contains a large lipophilic binding domain around the arylpiperazine moiety able to accommodate these substituents. Moving the chlorine atom from the 4- to the 3-position, to furnish **4e**, resulted in activity comparable to that of **4a**. Interestingly, the 3,4-dichloro derivative (**4f**) was equally active with the 3-chloro derivative **4e**, but superior to the 4-chloro analogue **4d**. Replacement of the *N*-phenyl with a benzyl group (compound **4b**) was detrimental to the allosteric enhancement activity at 10 μM of concentration.

The *N*-(4-methylphenyl)piperazine derivative **4i** showed an inhibitory activity on cAMP production similar to the unsubstituted phenylpiperazine counterpart **4a**, suggesting that the presence of a lipophilic, electron-releasing moiety on the phenyl ring retained the activity and seemed to have no effect on the enhancement. In contrast, replacement of the 4-methyl group with a less lipophilic and more electron-releasing methoxy group (compound **4j**) significantly reduced the activity with respect to **4i**, even though it proved more active than PD 81,723. For derivative **4j**, the reduced enhancement activity may be attributed both to steric and electronic factors.

The introduction of a nitrile function (compound **4k**), which has a stronger electron-withdrawing effect but is less lipophilic than the halogens, led to a remarkable reduction in activity with respect to the halogen derivatives. A similar effect was observed by replacing the nitrile with the strongly electron-withdrawing but more hydrophilic nitro group (derivative **4l**). In contrast, derivative **4m**, which possesses a lipohilic and strongly electron-withdrawing trifluoromethyl moiety at the 4-position of the phenyl ring, showed an activity comparable to that of 4-methyl counterpart **4i**. Moving the trifluoromethyl from the 4-position to the 3-position (compound **4n**) led to a slight decrease in activity. Introduction of a chlorine in the 4-position, to furnish the 4-Cl, 3-CF_3 derivative **4o**, maintained activity relative to the 3-CF_3 derivative.

In the small series of 5-ethylthiophene derivatives **4p−r**, the unsubstituted phenylpiperazine derivative, **4p**, showed activity at least as high or equivalent to that of the 5-methyl counterpart, **4a**. The 4-fluoro- and 4-chlorophenylpiperazine (**4q** and **4r**, respectively) were more active than the corresponding 5-methyl analogues, **4c** and **4d**.

In the series of 5-phenylthiophene derivatives **4u−ac**, the phenyl and 4-fluorophenylpiperazine derivatives (**4u** and **4v**, respectively) were the most active compounds of the series. Replacement of fluorine with a chlorine (compound **4w**) caused a decrease of enhancement, which was more pronounced for the 3,4-dichloro analogue, **4x**. Among the isomeric trifluoromethylphenylpiperazine derivatives **4y**, **4z**, and **4ab**, the 4-CF_3 derivative **4y** was more active than the 3-CF_3 and 2-CF_3 counterparts (**4z** and **4ab**, respectively). Starting from **4z**, the insertion of chlorine at the 4-position, to furnish the 4-Cl, 3-CF_3 derivative **4aa**, maintained the same activity of **4z**. The replacement of the 4-trifluoromethyl group of **4y** with a less lipophilic and electron-releasing trifluoromethoxy moiety (compound **4ac**) slightly decreased the enhancement.

The effect of the substitution on the phenyl ring attached to the piperazine moiety, as well as on the phenyl at the thiophene C-5 position, to reduce the cAMP levels was also investigated

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Table 2. A_1AR Density Expressed as B_{max} Values Obtained by [^{3}H]CCPA Binding Assays in hA_1CHO Membranes in the Presence of 3a, 3b, 3e, 4a−ao, and Reference Compound PD 81,723 (10 μM) (A) and Modulation by the Novel Allosteric Enhancers (10 μM) on the CCPA Affinity (CCPA K_i shift) in [^{3}H]DPCPX Competition Binding Experiments (B)[a]

	A[b]		B[c]	
compd	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	685 ± 63	1.3 ± 0.1	9.6 ± 0.7	1.6 ± 0.1
3a	3626 ± 332	7.0 ± 0.6	2.7 ± 0.3	5.6 ± 0.5
3b	3989 ± 377	7.7 ± 0.7	2.4 ± 0.2	6.3 ± 0.5
3e	3502 ± 341	6.8 ± 0.6	2.9 ± 0.3	5.3 ± 0.5
4a	3584 ± 323	6.8 ± 0.6	2.4 ± 0.1	6.4 ± 0.5
4b	949 ± 85	1.8 ± 0.1	10.9 ± 0.9	1.4 ± 0.1
4c	3109 ± 302	5.9 ± 0.6	2.9 ± 0.1	5.2 ± 0.4
4d	2477 ± 238	4.7 ± 0.4	3.6 ± 0.3	4.2 ± 0.3
4e	3426 ± 287	6.5 ± 0.6	2.5 ± 0.2	6.1 ± 0.5
4f	3109 ± 273	5.9 ± 0.6	2.8 ± 0.2	5.4 ± 0.4
4g	2582 ± 227	4.9 ± 0.5	3.4 ± 0.3	4.5 ± 0.4
4h	2846 ± 273	5.4 ± 0.5	3.2 ± 0.3	4.8 ± 0.4
4i	3478 ± 326	6.6 ± 0.6	2.6 ± 0.2	5.9 ± 0.5
4j	1686 ± 154	3.2 ± 0.3	5.9 ± 0.5	2.6 ± 0.2
4k	738 ± 68	1.4 ± 0.1	12.8 ± 1.0	1.2 ± 0.1
4l	685 ± 66	1.3 ± 0.1	13.9 ± 1.1	1.1 ± 0.1
4m	4005 ± 384	7.6 ± 0.7	2.3 ± 0.1	6.8 ± 0.6
4n	3267 ± 292	6.2 ± 0.6	2.7 ± 0.2	5.7 ± 0.5
4o	2688 ± 234	5.1 ± 0.5	3.3 ± 0.3	4.6 ± 0.3
4p	2899 ± 222	5.5 ± 0.5	3.1 ± 0.2	4.9 ± 0.4
4q	3531 ± 332	6.7 ± 0.7	4.0 ± 0.3	3.8 ± 0.3
4r	3426 ± 311	6.5 ± 0.5	2.6 ± 0.2	6.0 ± 0.5
4s	2477 ± 213	4.7 ± 0.5	3.4 ± 0.3	4.5 ± 0.3
4t	2319 ± 211	4.4 ± 0.4	3.7 ± 0.3	4.1 ± 0.3
4u	6061 ± 575	11.5 ± 1.2	1.8 ± 0.1	8.3 ± 0.7
4v	5850 ± 527	11.1 ± 1.2	1.6 ± 0.1	9.4 ± 0.8
4w	5112 ± 485	9.7 ± 0.9	1.9 ± 0.1	7.9 ± 0.7
4x	3584 ± 321	6.8 ± 0.6	2.4 ± 0.2	3.0 ± 0.2
4y	5639 ± 516	10.7 ± 1.1	1.7 ± 0.1	8.9 ± 0.7
4z	3953 ± 329	7.5 ± 0.7	2.2 ± 0.2	7.1 ± 0.7
4aa	4480 ± 405	8.5 ± 0.8	1.9 ± 0.1	7.9 ± 0.7
4ab	4321 ± 412	8.2 ± 0.8	2.0 ± 0.2	7.6 ± 0.8
4ac	4954 ± 472	9.4 ± 0.9	1.8 ± 0.1	8.4 ± 0.8
4ad	7431 ± 653	14.1 ± 1.2	1.2 ± 0.1	13.3 ± 1.1
4ae	6166 ± 587	11.7 ± 1.1	1.6 ± 0.1	9.5 ± 0.8
4af	5375 ± 498	10.2 ± 1.0	1.6 ± 0.1	9.5 ± 0.8
4ag	4058 ± 384	7.7 ± 0.8	2.3 ± 0.1	6.8 ± 0.7
4ah	5007 ± 467	9.5 ± 0.9	1.8 ± 0.2	8.3 ± 0.6
4ai	6008 ± 512	11.4 ± 1.1	1.8 ± 0.1	8.7 ± 0.7
4aj	4374 ± 418	8.3 ± 0.8	2.0 ± 0.1	7.7 ± 0.8
4ak	5902 ± 498	11.2 ± 1.1	1.5 ± 0.1	10.5 ± 0.9
4al	6219 ± 519	11.8 ± 1.1	1.2 ± 0.1	13.0 ± 1.1
4am	5323 ± 503	10.1 ± 1.0	1.9 ± 0.1	8.0 ± 0.7
4an	4269 ± 375	8.1 ± 0.8	2.1 ± 0.1	7.2 ± 0.6
4ao	5639 ± 526	10.7 ± 1.0	1.7 ± 0.1	8.9 ± 0.7

[a]The values are expressed as the mean ± SEM, n = 3 independent experiments. [b]B_{max} (fmol/mg protein) and B_{max} shift obtained in [^{3}H]CCPA saturation binding experiments performed in the absence (B_{max} = 527 ±48 fmol/mg protein) or in the presence of 10 μM enhancers. [c]K_i values of CCPA in the presence of 10 μM test compounds and CCPA shift = K_i(CCPA)/K_i(CCPA + 10 μM enhancers), where the K_i of CCPA was 15.3 ± 1.5 nM.

(compounds **4ad−ao**). The presence of an electron-withdrawing fluorine or chlorine group at the 4-position of the phenyl at the thiophene C-5 position generally had a positive influence on the activity (see **4u** vs **4ad**, **4v** vs **4ae**, **4w** vs **4ai**, **4x** vs **4aj**, **4y** vs **4ak**, **4z** vs **4al**, **4aa** vs **4am**, **4ab** vs **4an**, **4ac** vs **4ao**). The 4-fluorophenyl derivative with an *N*-phenylpiperazine at the 4-position, **4ad,** was the most active compound of the entire series at the concentration tested, reducing the cAMP level by 82% at a concentration of 10 μM. We assume that the receptor environment around the thiophene 5-position binding site is lipophilic, and for this reason, substitution with a phenyl bearing an electron-withdrawing fluorine or chlorine group at this position is optimal.

Among the thiophene 5-(4-chlorophenyl) series of derivatives **4af−ao**, compounds bearing a 4-chlorophenyl (**4ai**), 4-trifluoromethylphenyl (**4ak**), and the isomeric 3-trifluoromethylphenyl (**4al**) moiety on the piperazine were the most active compounds of this series. As shown with the 5-methyl series, replacement of the *N*-phenyl (**4af**) with an *N*-benzyl group (**4ag**) was detrimental to the AE activity at a concentration of 10 μM. While **4af** and the 4-fluoro analogue (**4ah**) appeared to have similar activities, replacement of fluorine with chlorine (compound **4ai**) increased the reduction of cAMP production. By the synthesis of the 3,4-dichlorophenyl analogue **4aj**, it was confirmed that the addition of a second chlorine atom was detrimental for activity. The strong electron-withdrawing trifluoromethyl group at the 4-position and 3-position of the phenyl led to compounds **4ak** and **4al**, respectively, appearing to have similar activities, while the 2-CF_3 derivative **4an** has similar activity to the benzyl analogue **4ag**. As reported previously for derivative **4aa**, the 4-Cl, 3-CF_3 analogue **4am** displayed a similar pattern, being slightly less active than the 3-CF_3 counterpart. The replacement of the 4-CF_3 group with a 4-OCF_3 (compound **4ao**) maintained the level of cAMP reduction, which is comparable to that of the 4-Cl, 3-CF_3 derivative **4am**.

Antagonistic Activity. A characteristic feature of allosteric enhancers at the A_1AR is the propensity to also cause antagonism at higher concentrations. The ability of compounds **4a−ao** 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_3ARs 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 A_3ARs.[20] None of the examined derivatives significantly inhibited the specific binding of the radioligands to A_1-, A_{2A}-, and A_3ARs, reaching a very low percentage inhibition of 12% or less at 10 μM concentration, suggesting that these novel enhancers are not able to bind to the orthosteric site. Substitution of the 5-position of the thiophene with small alkyl groups (methyl and ethyl), bromine or aryl moieties seemed to maintain or increase allosteric enhancement with a concomitant absence of antagonistic activity against A_1AR, as suggested from competition binding experiments.[22]

Effect of Enhancers on A_1AR Binding Parameters. Saturation and competition experiments of the selective

adenosine A_1 agonist [^{3}H]CCPA to A_1 receptors were performed to verify if the novel compounds **4a**−**ao** modified the agonist binding parameters. From these experiments, A_1 receptor affinity (K_D) and density (B_{max}) were evaluated in the presence and in the absence of the examined compounds (PD 81,723, **3a**, **3b**, **3e**, and **4a**−**4ao** at a concentration of 10 μM) and were used to calculate the fold increase of the affinity (K_D shift, column B) and density (B_{max} shift, column A) reported in Table 2. In [^{3}H]CCPA saturation binding experiments, the reference compound PD 81,723 induced a B_{max} shift to hA_1ARs of 1.3 fold. Under the same experimental conditions, with the exception of **4b**, **4k**, and **4l**, all of the new tested compounds proved to be superior to PD 81,723. From the receptor density calculated in the presence and in the absence of the novel enhancers, the derivatives **4u**, **4v**, **4y**, **4ad**, **4ae**, **4af**, **4ai**, **4ak**, **4am**, and **4ao** were the most active compounds, each causing a B_{max} shift of more than 10-fold. Interestingly, no differences were found in the affinity of [^{3}H]CCPA in the presence or in the absence of the tested compounds, suggesting that the enhancers were not able to modify the K_D values that ranged from 1.0 ± 0.1 to 1.2 ± 0.1 nM.

Table 2 also reports the derived apparent affinity (K_i) values for CCPA, based on a one-state model of analysis, in the absence and in the presence of tested enhancers. The enhancers were able to mediate a shift of the A_1 receptors toward the active state, as suggested by the increase of CCPA affinity, expressed as K_i values (Table 2). This table also shows 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 15.3 ± 1.5 nM as evaluated by a one-state model of analysis. 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 high affinity binding sites. In the presence of PD 81,723, the affinity of CCPA increased 1.6-fold. With the exception of derivatives **4b**, **4k**, and **4l**, the CCPA affinity data in the presence of the new tested derivatives **4a**−**ao** reveal that the displacement curves are shifted left, suggesting lower K_i values for CCPA. The largest affinity shift has been observed for compounds **4ad**, **4ak**, and **4al**, which decreased the apparent K_i values of CCPA approximately 13.3-, 10.5-, and 13.0-fold, respectively, resulting in these compounds being 2-fold more active than the 5-unsubstituted thiophene derivatives **3a**, **3b**, and **3e** (Table 2). Compounds **4u**, **4v**, **4y**, **4ac**, **4ae**, **4af**, **4ah**, **4ai**, **4am**, and **4ao** caused a similar shift of the apparent CCPA affinity, ranging from 8- to 9-fold, while derivatives **4a**, **4e**, **4m**, **4w**−**x**, **4aa**−**ab**, **4ag**, and **4an** afforded an increase ranging from 6- to 7-fold.

In Figure 1, we have shown the effect of the allosteric modulators PD 81,723, **4y**, **4ad**, and **4ai** at 10 μM concentration in [^{3}H]CCPA saturation binding experiments on A_1AR binding parameters such as affinity and density. The novel enhancers were able to significantly increase the A_1AR receptor density. In Figure 2A, representative binding curves for the displacement of [^{3}H]DPCPX by different concentrations of CCPA alone and in the presence of PD 81,723, **4y**, **4ad**, and **4ai** at 10 μM concentration were reported, showing the increase of the CCPA affinity in the presence of novel enhancers. Figure 2B,C reports the functional effect of the novel enhancers on cAMP experiments performed in hA_1CHO cells. In particular, Figure 2B shows the inhibitory effect of PD 81,723, **4y**, **4ad**, and **4ai** at 10 μM concentration on cAMP stimulated by 1 μM forskolin. The effect of the same compounds at 100 nM concentration in the presence of a low concentration of CCPA (1 pM) is depicted in Figure 2C. Interestingly, the novel enhancers were able to mediate a significant inhibition of the cAMP accumulation at lower concentration in the presence of the A_1AR agonist CCPA relative to the experiment in the absence of CCPA, confirming their specific role as A_1 allosteric enhancers.

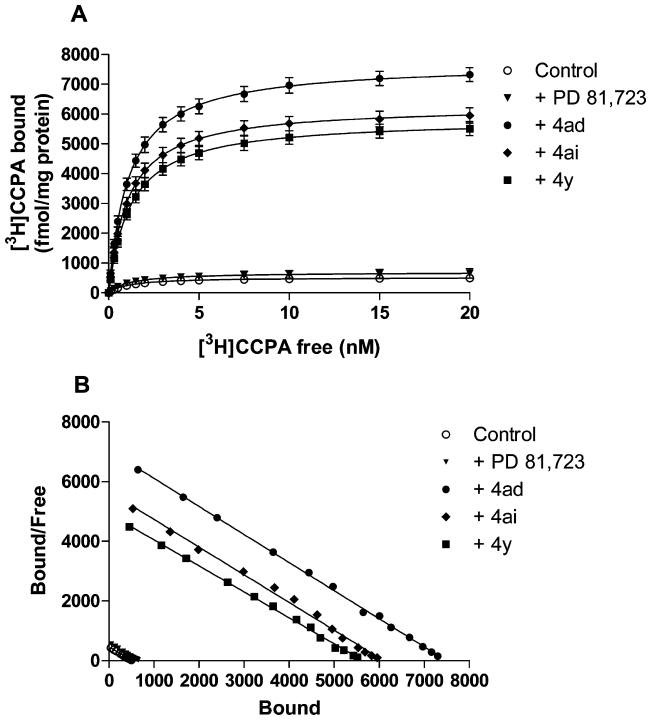

Figure 1. [^{3}H]CCPA saturation binding curves to human A_1 adenosine receptors (A). Under control conditions, the K_D value was 1.1 ± 0.1 nM and the B_{max} was 527 ± 48 fmol/mg protein. In the presence of novel enhancers (10 μM), K_D values were similar to those obtained in controls, and B_{max} values are 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 Section.

CONCLUSIONS

In this new series of compounds, **4a**−**ao**, we have maintained the 2-amino-3-(4-chlorobenzoyl)thiophene nucleus unchanged, which is thought to be essential for allosteric modulation at the A_1AR, while evaluating the effect on the allosteric activity of electron-releasing or electron-withdrawing groups on the phenyl of the arylpiperazine moiety at the C-4 position of thiophene ring. This modification was combined with the presence of methyl, ethyl, bromine, and aryl moieties at its C-5 position. The potential of these compounds to interact allosterically with the A_1AR was initially screened in an in vitro functional assay by evaluating their ability to reduce the cAMP production in CHO cells.

Comparing compounds characterized by the presence of same arylpiperazine moiety at the 4-position of the thiophene ring, it appeared that the 5-aryl substituted derivatives were more active than the methyl, ethyl, and bromine counterparts (i.e., **4u**, **4ad**, and **4af** vs **4a**, **4p**, and **4s**). While it was previously reported that bulky 5-alkyl substitution favored increased antagonistic properties, our series of 5-aryl derivatives **4u**−**ao** showed an absence of antagonistic activity against A_1AR. This presumably results from a favorable interaction of this portion of the molecule with the allosteric binding site of the A_1AR.

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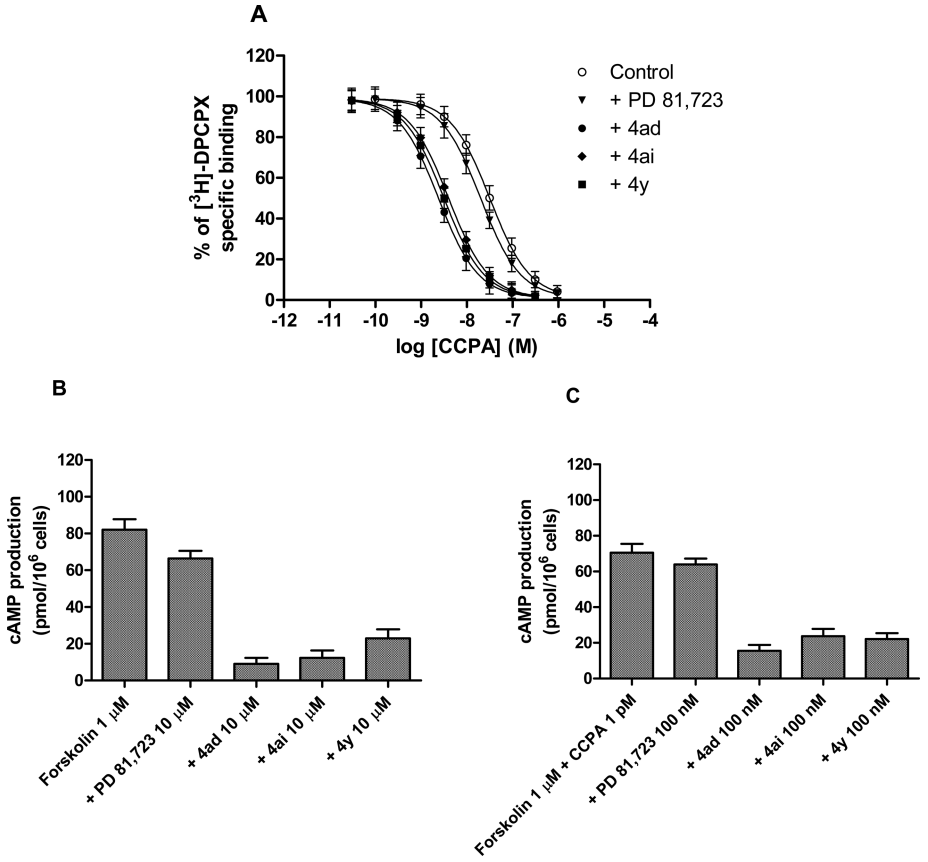

Figure 2. Inhibition curves of specific [^{3}H]DPCPX binding to human A_1ARs of CCPA in the absence and in the presence of novel enhancers (10 μM). Affinity values were calculated by using a one-state model of analysis. Values are the means and vertical lines are the SEM of three separate experiments, as described in the Experimental Section (A). Histograms relative to the cAMP inhibition, expressed in pmol/10^6 cells, mediated by novel allosteric enhancers (10 μM). The effect of the examined compounds (100 nM) was also studied in the presence of CCPA (1 pM) (B).

Although the series of 5-ethyl- and 5-bromothiophene derivatives **4p**−**r** and **4s,t**, respectively, were too small to draw many firm conclusions about the SAR, the biological data demonstrated that the insertion of a bromo or ethyl group at the C-5 position of the 2-amino-3-(4-chlorobenzoyl)-4-[(4-arylpiperazin-1-yl)methyl]thiophene nucleus maintained significant activity.

The therapeutic potential of selective AEs for the A_1AR is hampered by the fact that many of these molecules also possess antagonistic properties against this receptor subtype. None of the synthesized compounds (**4a**−**ao**) significantly inhibited antagonist binding at the hA_1AR, hA_2AR, or hA_3AR. Of the 41 newly synthesized molecules, only three compounds (**4b**, **4k**, and **4l**) were less active than PD 81,723. Those thiophene derivatives bearing a 5-aryl group, **4u**−**ao**, have substantially higher activity than PD 81,723. Among these, derivatives **4v**, **4ad**−**ae**, **4ak**, and **4al**, were the most active compounds in binding (saturation and displacement experiments) and functional cAMP assays. Saturation and competition experiments have also shown that the series of 5-aryl-substituted thiophene derivatives **4u**−**ao** were more active than the corresponding 5-unsubstituted analogues **3a**, **3b**, and **3e**. In competition binding experiments, the K_i values of CCPA in the presence of compounds **4ad**, **4ak**, and **4al** were decreased approximately 13.3-, 10.5-, and 13.0-fold, respectively, almost 2-fold more active at 10 μM than **3a**, **3b**, and **3e**.

EXPERIMENTAL SECTION

Chemistry. *Materials and Methods.* 3-(4-Chlorophenyl)-3-oxopropionitrile and all arylpiperazines were commercially available. ^{1}H NMR spectra were determined in $CDCl_3$ solutions and recorded with a Bruker AC-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. 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 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. 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) was distilled from calcium chloride and stored over molecular sieves (3 Å). Petroleum ether refers to the fractions boiling at 40−60 °C.

*Synthesis of (2-Amino-5-ethyl-4-methylthiophen-3-yl)(4-chlorophenyl)methanone (**5b**).* A stirred suspension of 3-(4-chlorophenyl)-3-oxopropionitrile (1.8 g, 10 mmol), sulfur (384 mg, 12 mmol), TEA (10 mmol, 1.4 mL), and pentan-2-one (861 mg, 1.1 mL, 10 mmol) in absolute EtOH (20 mL) was refluxed for 2 h. The solution was evaporated and the residue was dissolved in EtOAc (20 mL). The organic phase was subsequently washed with 1% (w/v) HCl

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(5 mL), a saturated solution of $NaHCO_3$ (5 mL), water (5 mL), and brine (5 mL); dried (Na_2SO_4); filtered; and concentrated. The residue was purified by column chromatography using a mixture of EtOAc:petroleum ether 1:9 as eluent, to give **5** as a yellow oil. Yield: 57%. ^{1}H NMR ($CDCl_3$): δ 1.18 (t, J = 7.6 Hz, 3H), 2.18 (s, 3H), 2.55 (q, J = 7.6 Hz, 2H), 5.77 (bs, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.46 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 279.8.

*General Procedure A for the Synthesis of Compounds **6a,b**.* To a solution of thiophene derivative **5a** or **5b** (4 mmol) in acetic acid (25 mL) was added phthalic anhydride (740 mg, 5 mmol) and the mixture was heated to reflux for 18 h. The solvent was evaporated in vacuo and the residue was dissolved in DCM (30 mL). The organic solution was washed with water (10 mL) and brine (10 mL), dried (Na_2SO_4), filtered, and concentrated. The crude product was stirred for 1 h in petroleum ether (20 mL) and then filtered to afford **6a**,**b**.

*2-[3-(4-Chlorobenzoyl)-4,5-dimethylthiophen-2-yl]isoindoline-1,3-dione (**6a**).* Following general procedure A, compound **6a** was isolated as a yellow powder. Yield: 92%. Mp: 154−156 °C. ^{1}H NMR ($CDCl_3$): δ 2.10 (s, 3H), 2.43 (s, 3H), 7.24 (d, J = 8.4 Hz, 2H), 7.64 (d, J = 8.4 Hz, 2H), 7.78 (m, 4H). MS (ESI): $[M]^+$ = 395.9.

*2-[3-(4-Chlorobenzoyl)-5-ethyl-4-methylthiophen-2-yl]-isoindoline-1,3-dione (**6b**).* Following general procedure A, compound **6b** was obtained as a white solid. Yield: 88%. Mp: 115−117 °C. ^{1}H NMR ($CDCl_3$): δ 1.34 (t, J = 7.6 Hz, 3H), 2.17 (s, 3H), 2.82 (q, J = 7.6 Hz, 2H), 7.26 (d, J = 8.8 Hz, 2H), 7.69 (d, J = 8.4 Hz, 2H), 7.77 (m, 2H), 7.79 (m, 2H). MS (ESI): $[M]^+$ = 409.9.

*General Procedure B for the Synthesis of Compounds **10a−c**.* A stirred suspension of **9** (461 mg, 1 mmol) and the appropriate arylboronic acid (1.5 mmol) in 1,4-dioxane (10 mL containing 2−3 drops of water) was degassed under a stream of nitrogen over 10 min and then treated with $PdCl_2$(dppf) (82 mg, 0.1 mmol) and CsF (380 mg, 2.5 mmol). The reaction mixture was heated under nitrogen at 45 °C for 30 min and then at 75 °C for 5 h. The reaction mixture was cooled to ambient temperature, diluted with DCM (15 mL), filtered on a pad of Celite, and evaporated in vacuo. The residue was dissolved with DCM (20 mL), and the resultant solution was washed sequentially with 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.

*2-[3-(4-Chlorobenzoyl)-4-methyl-5-phenylthiophen-2-yl]-isoindoline-1,3-dione (**10a**).* Following general procedure B with phenylboronic acid, the residue was purified by chromatography on silica gel eluting with EtOAc:petroleum ether 2:8, to furnish **10a** as a white solid. Yield: 74%. Mp: 124−126 °C. ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H), 7.32 (d, J = 8.4 Hz, 2H), 7.46 (d, J = 8.4 Hz, 2H), 7.78 (m, 4H), 7.78 (m, 5H). MS (ESI): $[M]^+$ = 457.9.

*2-[3-(4-Chlorobenzoyl)-5-(4-fluorophenyl)-4-methylthiophen-2-yl]isoindoline-1,3-dione (**10b**).* Following general procedure B with 4-fluorophenylboronic acid, the residue was purified by chromatography on silica gel eluting with EtOAc:petroleum ether 2:8, to furnish **10b** as a yellow solid. Yield: 78%. Mp: 178−180 °C. ^{1}H NMR ($CDCl_3$): δ 2.18 (s, 3H), 7.14 (d, J = 6.4 Hz, 2H), 7.22 (d, J = 6.4 Hz, 2H), 7.46 (m, 2H), 7.72 (m, 6H). MS (ESI): $[M]^+$ = 475.8.

*2-[3-(4-Chlorobenzoyl)-5-(4-chlorophenyl)-4-methylthiophen-2-yl]isoindoline-1,3-dione (**10c**).* Following general procedure B with 4-chlorophenylboronic acid, the residue was purified by chromatography on silica gel eluting with EtOAc:petroleum ether 3:7, to furnish **10b** as a yellow solid. Yield: 72%. Mp: 194−196 °C. ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H), 7.27 (d, J = 8.4 Hz, 2H), 7.44 (d, J = 8.4 Hz, 2H), 7.72 (d, J = 8.4 Hz, 2H), 7.78 (m, 6H). MS (ESI): $[M]^+$ = 492.4.

*General Procedure C for the Synthesis of Compounds **7a,b**.* To a solution of thiophene derivative **6a** or **6b** (2 mmol) in acetonitrile (10 mL) was added *N*-bromosuccinimide (356 mg, 2 mmol), and the mixture was heated at reflux for 2 h. After this period, another portion of *N*-bromosuccinimide (356 mg, 2 mmol) was added and the reflux was continued for another 2 h and then the mixture was evaporated in vacuo. The residue was dissolved in DCM (15 mL) and the resultant solution was washed sequentially with water (5 mL) and brine (5 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated, and the residue was purified by flash chromatography on silica gel.

*2-[4-Bromomethyl-3-(4-chlorobenzoyl)-5-methylthiophen-2-yl]-isoindoline-1,3-dione (**7a**).* Following general procedure C, compound **7a** was purified by chromatography eluting with EtOAc:petroleum ether 2:8 to give a yellow solid. Yield: 53%. Mp: 173−175 °C. ^{1}H NMR ($CDCl_3$): δ 2.53 (s, 3H), 4.65 (s, 2H), 7.17 (d, J = 8.4 Hz, 2H), 7.63 (d, J = 8.4 Hz, 2H), 7.63 (m, 2H), 7.73 (m, 2H). MS (ESI): $[M]^+$ = 472.9, $[M + 2]^+$ = 474.8.

*2-[4-Bromomethyl-3-(4-chlorobenzoyl)-5-ethylthiophen-2-yl]-isoindoline-1,3-dione (**7b**).* Following general procedure C, compound **7b** was purified by chromatography eluting with EtOAc:petroleum ether 2:8 to give an orange oil. Yield: 37%. ^{1}H NMR ($CDCl_3$): δ 1.39 (t, J = 7.6 Hz, 3H), 2.96 (q, J = 7.6 Hz, 2H), 4.66 (s, 2H), 7.17 (d, J = 7.6 Hz, 2H), 7.64 (d, J = 7.6 Hz, 2H), 7.73 (m, 2H), 7.76 (m, 2H). MS (ESI): $[M]^+$ = 486.8, $[M + 2]^+$ = 488.8.

*General Procedure D for the Synthesis of Compounds **11a−c**.* To a solution of **10a**, **10b**, or **10c** (2 mmol) in 1,2-dichloroethane (10 mL) was added benzoyl peroxide (48 mg, 0.2 mmol) and *N*-bromosuccinimide (356 mg, 2 mmol), and the mixture was heated under reflux. After 2 h, additional *N*-bromosuccinimide (178 mg, 1 mmol) and benzoyl peroxide (24 mg, 0.1 mmol) were added, and heating at reflux was continued for 1 h further. The mixture was cooled to room temperature, diluted with DCM (10 mL), and washed with 5% $NaHCO_3$ solution (5 mL), water (5 mL), and brine (5 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated, to give a residue that was purified by column chromatography on silica gel.

*2-[4-Bromomethyl-3-(4-chlorobenzoyl)-5-phenylthiophen-2-yl]-isoindoline-1,3-dione (**11a**).* Following general procedure D, compound **11a** was purified by column chromatography eluting with EtOAc:petroleum ether 2:8 to give a light brown solid. Yield: 95%. Mp: 166−168 °C. ^{1}H NMR ($CDCl_3$): δ 4.73 (s, 2H), 7.21 (d, J = 8.6 Hz, 2H), 7.48 (d, J = 8.6 Hz, 2H), 7.52 (m, 1H), 7.68 (m, 8H). MS (ESI): $[M]^+$ = 534.8, $[M + 2]^+$ = 536.8.

*2-[4-Bromomethyl-3-(4-chlorobenzoyl)-5-(4-fluorophenyl)-thiophen-2-yl]isoindoline-1,3-dione (**11b**).* Following general procedure D, compound **11b** was purified by column chromatography eluting with EtOAc:petroleum ether 1.5:8.5 to give a yellow solid. Yield: 66%. Mp: 186−188 °C. ^{1}H NMR ($CDCl_3$): δ 4.67 (s, 2H), 7.16 (d, J = 6.6 Hz, 2H), 7.56 (d, J = 6.6 Hz, 2H), 7.72 (d, J = 6.4 Hz, 2H), 7.76 (m, 6H). MS (ESI): $[M]^+$ = 552.8, $[M + 2]^+$ = 554.8.

*2-[4-Bromomethyl-3-(4-chlorobenzoyl)-5-(4-chlorophenyl)-thiophen-2-yl]isoindoline-1,3-dione (**11c**).* Following general procedure D, compound **11c** was purified by column chromatography eluting with EtOAc:petroleum ether 1.5:8.5 to give a yellow solid. Yield: 88%. Mp: 178−180 °C. ^{1}H NMR ($CDCl_3$): δ 4.68 (s, 2H), 7.17 (d, J = 8.8 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H), 7.67 (d, J = 8.4 Hz, 2H), 7.76 (m, 4H). MS (ESI): $[M]^+$ = 569.1, $[M + 2]^+$ = 571.1.

*General Procedure E for the Synthesis of Compounds **8a−t**.* To a stirred solution of compound **7a−c** or **11a−c** (1 mmol) in dry DCM (5 mL) was added TEA (168 μL, 1.2 mmol). The mixture was cooled with a bath of ice/water, and then the appropriate arylpiperazine (2 equiv, 2 mmol) dissolved in DCM (1 mL) was slowly added. The mixture was then stirred at room temperature for 3 h, diluted with DCM (5 mL), and washed sequentially with water (5 mL) and brine (5 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated in vacuo to give a residue that was purified by column chromatography on silica gel.

*2-[3-(4-Chlorobenzoyl)-5-methyl-4-[(4-phenylpiperazin-1-yl)-methyl]thiophen-2-yl]isoindoline-1,3-dione (**8a**).* Following general procedure E using *N*-phenylpiperazine, compound **8a** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 92%. Mp: 103−105 °C. ^{1}H NMR ($CDCl_3$): δ 2.03 (t, J = 4.8 Hz, 4H), 2.24 (s, 3H), 2.88 (t, J = 4.8 Hz, 4H), 3.06 (s, 2H), 6.83 (d, J = 8.8 Hz, 2H), 6.90 (m, 3H), 7.20 (d, J = 8.4 Hz, 2H), 7.29 (d, J = 8.4 Hz, 2H), 7.39 (d, J = 6.8 Hz, 1H), 7.53 (t, J = 6.8 Hz, 2H), 7.82 (d, J = 6.8 Hz, 1H). MS (ESI): $[M]^+$ = 556.1.

*2-[4-[(4-Benzylpiperazin-1-yl)methyl]-3-(4-chlorobenzoyl)-5-methylthiophen-2-yl]isoindoline-1,3-dione (**8b**).* Following general procedure E using *N*-benzylpiperazine, compound **8b** was purified by chromatography eluting with EtOAc to give a yellow solid. Yield: 87%.

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Mp: 92−93 °C. ^{1}H NMR ($CDCl_3$): δ 1.84 (t, J = 5.2 Hz, 4H), 2.33 (s, 3H), 3.23 (t, J = 5.2 Hz, 4H), 3.39 (s, 2H), 3.49 (s, 2H), 7.28 (m, 5H), 7.37 (d, J = 8.8 Hz, 2H), 7.43 (t, J = 7.2 Hz, 2H), 7.53 (d, J = 7.2 Hz, 1H), 7.58 (d, J = 8.8 Hz, 2H), 7.82 (t, J = 7.2 Hz, 1H). MS (ESI): $[M]^+$ = 570.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-fluorophenyl)piperazin-1-yl)-methyl]-5-methylthiophen-2-yl}isoindoline-1,3-dione (**8c**).* Following general procedure E using *N*-(4-fluorophenyl)piperazine, compound **8c** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 92%. Mp: 103−105 °C. ^{1}H NMR ($CDCl_3$): δ 2.03 (t, J = 4.8 Hz, 4H), 2.24 (s, 3H), 2.88 (t, J = 4.8 Hz, 4H), 3.06 (s, 2H), 6.83 (d, J = 8.8 Hz, 2H), 6.90 (m, 3H), 7.20 (d, J = 8.4 Hz, 2H), 7.29 (d, J = 8.4 Hz, 2H), 7.39 (d, J = 6.8 Hz, 1H), 7.53 (t, J = 6.8 Hz, 2H), 7.82 (d, J = 6.8 Hz, 1H). MS (ESI): $[M]^+$ = 574.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-chlorophenyl)piperazin-1-yl)-methyl]-5-methylthiophen-2-yl}isoindoline-1,3-dione (**8d**).* Following general procedure E using *N*-(4-chlorophenyl)piperazine, compound **8d** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 89%. Mp: 135−136 °C. ^{1}H NMR ($CDCl_3$): δ 1.97 (t, J = 4.8 Hz, 4H), 2.32 (s, 3H), 2.84 (t, J = 5.2 Hz, 4H), 3.06 (m, 2H), 6.73 (d, J = 8.8 Hz, 2H), 6.82 (d, J = 9.2 Hz, 2H), 7.16 (d, J = 9.2 Hz, 2H), 7.24 (d, J = 8.8 Hz, 2H), 7.37 (d, J = 7.6 Hz, 1H, 1H), 7.60 (t, J = 7.6 Hz, 2H), 7.88 (d, J = 7.6 Hz, 1H). MS (ESI): $[M]^+$ = 590.5.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(3-chlorophenyl)piperazin-1-yl)-methyl]-5-methylthiophen-2-yl}isoindoline-1,3-dione (**8e**).* Following general procedure E using *N*-(3-chlorophenyl)piperazine, compound **8e** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 92%. Mp: 110−112 °C. ^{1}H NMR ($CDCl_3$): δ 1.96 (t, J = 5.4 Hz, 4H), 2.32 (s, 3H), 2.88 (t, J = 5.4 Hz, 4H), 3.06 (s, 2H), 6.66 (d, J = 7.8 Hz, 1H), 6.77 (d, J = 7.8 Hz, 1H), 6.84 (s, 1H), 7.12 (t, J = 7.8 Hz, 1H), 7.33 (d, J = 7.2 Hz, 1H), 7.40 (d, J = 8.4 Hz, 2H), 7.52 (t, J = 7.2 Hz, 2H), 7.59 (d, J = 8.4 Hz, 2H), 7.82 (d, J = 7.2 Hz, 1H). MS (ESI): $[M]^+$ = 590.5.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(3,4-dichlorophenyl)piperazin-1-yl)-methyl]-5-methylthiophen-2-yl}isoindoline-1,3-dione (**8f**).* Following general procedure E using *N*-(3,4-dichlorophenyl)piperazine, compound **8f** was purified by chromatography eluting with EtOAc:DCM 0.5:9.5 to give a yellow solid. Yield: 88%. Mp: 130−132 °C. ^{1}H NMR ($CDCl_3$): δ 1.96 (t, J = 4.8 Hz, 4H), 2.32 (s, 3H), 2.86 (t, J = 4.8 Hz, 4H), 3.07 (s, 2H), 6.62 (dd, J = 9.2 and 3.2 Hz, 1H), 6.94 (d, J = 2.8 Hz, 1H), 7.22 (d, J = 9.2 Hz, 1H), 7.37 (d, J = 7.2 Hz, 1H), 7.42 (d, J = 8.6 Hz, 2H), 7.53 (t, J = J = 7.2 Hz, 2H), 7.59 (d, J = 8.6 Hz, 2H), 7.82 (d, J = 7.2 Hz, 1H). MS (ESI): $[M]^+$ = 624.1.

*2-{4-[(4-(4-Bromophenyl)piperazin-1-yl)methyl]-3-(4-chlorobenzoyl)-5-methylthiophen-2-yl}isoindoline-1,3-dione (**8g**).* Following general procedure E using *N*-(4-bromophenyl)piperazine, compound **8g** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 94%. Mp: 135−137 °C. ^{1}H NMR ($CDCl_3$): δ 1.97 (t, J = 4.8 Hz, 4H), 2.32 (s, 3H), 2.85 (t, J = 4.8 Hz, 4H), 3.06 (m, 2H), 6.68 (d, J = 8.8 Hz, 2H), 6.82 (d, J = 9.2 Hz, 2H), 7.30 (d, J = 9.2 Hz, 2H), 7.32 (d, J = 8.8 Hz, 2H), 7.36 (d, J = 7.6 Hz, 1H, 1H), 7.58 (t, J = 7.6 Hz, 2H), 7.82 (d, J = 7.6 Hz, 1H). MS (ESI): $[M]^+$ = 633.0, $[M + 2]^+$ = 635.0.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-iodophenyl)piperazin-1-yl)-methyl]-5-methylthiophen-2-yl}isoindoline-1,3-dione (**8h**).* Following general procedure E using *N*-(4-iodophenyl)piperazine, compound **8h** was purified by chromatography eluting with EtOAc:DCM 0.25:9.75 to give a yellow solid. Yield: 94%. Mp: 133−135 °C. ^{1}H NMR ($CDCl_3$): δ 1.98 (t, J = 4.8 Hz, 4H), 2.32 (s, 3H), 2.87 (t, J = 4.8 Hz, 4H), 3.06 (m, 2H), 6.58 (d, J = 8.8 Hz, 2H), 6.66 (d, J = 9.2 Hz, 2H), 7.33 (d, J = 9.2 Hz, 2H), 7.38 (d, J = 8.8 Hz, 2H), 7.43 (d, J = 7.6 Hz, 1H, 1H), 7.56 (t, J = 7.6 Hz, 2H), 7.84 (d, J = 7.6 Hz, 1H). MS (ESI): $[M]^+$ = 681.1.

*2-[3-(4-Chlorobenzoyl)-5-methyl-4-[(4-(4-methylphenyl)-piperazin-1-yl)methyl]thiophen-2-yl]isoindoline-1,3-dione (**8i**).* Following general procedure E using *N*-(4-methylphenyl)piperazine, compound **8i** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 83%. Mp: 110 °C. ^{1}H NMR ($CDCl_3$): δ 1.98 (t, J = 4.8 Hz, 4H), 2.25 (s, 3H), 2.32 (s, 3H), 2.83 (t, J = 4.8 Hz, 4H), 3.06 (s, 2H), 6.74 (d, J = 8.4 Hz, 2H), 6.82 (d, J = 8.4 Hz, 2H), 7.04 (d, J = 8.2 Hz, 2H), 7.07 (d, J = 8.2 Hz, 2H), 7.39 (d, J = 7.2 Hz, 1H), 7.49 (t, J = 7.2 Hz, 2H), 7.80 (d, J = 7.2 Hz, 1H). MS (ESI): $[M]^+$ = 570.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-methoxyphenyl)piperazin-1-yl)-methyl]-5-methylthiophen-2-yl}isoindoline-1,3-dione (**8j**).* Following general procedure E using *N*-(4-methoxyphenyl)piperazine, compound **8j** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 82%. Mp: 108−110 °C. ^{1}H NMR ($CDCl_3$): δ 1.98 (t, J = 4.8 Hz, 4H), 2.33 (s, 3H), 2.77 (t, J = 4.8 Hz, 4H), 3.06 (s, 2H), 3.77 (s, 3H), 6.85 (d, J = 8.8 Hz, 2H), 6.88 (d, J = 8.8 Hz, 2H), 7.38 (d, J = 7.2 Hz, 1H), 7.41 (d, J = 8.4 Hz, 1H), 7.52 (t, J = 7.2 Hz, 2H), 7.58 (m, 3H), 7.78 (d, J = 7.2 Hz, 1H). MS (ESI): $[M]^+$ = 586.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-cyanophenyl)piperazin-1-yl)-methyl]-5-methylthiophen-2-yl}isoindoline-1,3-dione (**8k**).* Following general procedure E using *N*-(4-cyanophenyl)piperazine, compound **8k** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 82%. Mp: 133−135 °C. ^{1}H NMR ($CDCl_3$): δ 2.03 (t, J = 4.8 Hz, 4H), 2.32 (s, 3H), 2.96 (t, J = 4.8 Hz, 4H), 3.08 (m, 2H), 6.75 (d, J = 9.2 Hz, 2H), 6.84 (d, J = 9.2 Hz, 2H), 7.42 (d, J = 7.6 Hz, 1H), 7.44 (d, J = 8.4 Hz, 2H), 7.63 (m, 4H), 7.82 (d, J = 7.6 Hz, 1H). MS (ESI): $[M]^+$ = 581.1.

*2-{3-(4-Chlorobenzoyl)-5-methyl-4-[(4-(4-nitrophenyl)piperazin-1-yl)methyl]thiophen-2-yl}isoindoline-1,3-dione (**8l**).* Following general procedure E using *N*-(4-nitrophenyl)piperazine, compound **8l** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 88%. Mp: 110−112 °C. ^{1}H NMR ($CDCl_3$): δ 1.99 (t, J = 5.4 Hz, 4H), 2.32 (s, 3H), 3.09 (s, 2H), 3.12 (t, J = 5.4 Hz, 4H), 6.71 (d, J = 9.6 Hz, 2H), 6.82 (d, J = 9.2 Hz, 2H), 7.33 (d, J = 7.6 Hz, 1H), 7.42 (d, J = 8.4 Hz, 2H), 7.53 (t, J = 7.6 Hz, 2H), 7.60 (d, J = 8.4 Hz, 2H), 8.82 (d, J = 7.6 Hz, 1H). MS (ESI): $[M]^+$ = 601.1.

*2-{3-(4-Chlorobenzoyl)-5-methyl-4-[(4-(4-(trifluoromethyl)-phenyl)piperazin-1-yl)methyl]thiophen-2-yl}isoindoline-1,3-dione (**8m**).* Following general procedure E using *N*-(4-(trifluoromethyl)-phenyl)piperazine, compound **8m** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow oil. Yield: 89%. ^{1}H NMR ($CDCl_3$): δ 1.98 (t, J = 4.8 Hz, 4H), 2.32 (s, 3H), 2.97 (t, J = 4.8 Hz, 4H), 3.07 (s, 2H), 6.81 (d, J = 8.8 Hz, 2H), 6.91 (d, J = 8.8 Hz, 2H), 7.42 (m, 3H), 7.48 (t, J = 6.8 Hz, 2H), 7.56 (d, J = 8.4 Hz, 2H), 7.86 (d, J = 6.8 Hz, 1H). MS (ESI): $[M]^+$ = 624.1.

*2-{3-(4-Chlorobenzoyl)-5-methyl-4-[(4-(3-(trifluoromethyl)-phenyl)piperazin-1-yl)methyl]thiophen-2-yl}isoindoline-1,3-dione (**8n**).* Following general procedure E using *N*-(3-(trifluoromethyl)-phenyl)piperazine, compound **8n** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 94%. Mp: 107−109 °C. ^{1}H NMR ($CDCl_3$): δ 1.99 (t, J = 4.8 Hz, 4H), 2.32 (s, 3H), 2.93 (t, J = 5.4 Hz, 4H), 3.08 (s, 2H), 6.92 (d, J = 7.8 Hz, 1H), 7.00 (s, 1H), 7.06 (m, 1H), 7.12 (d, J = 7.8 Hz, 1H), 7.36 (d, J = 8.4 Hz, 2H), 7.43 (d, J = 7.2 Hz, 1H), 7.52 (t, J = 7.2 Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H), 7.82 (d, J = 7.2 Hz, 1H). MS (ESI): $[M]^+$ = 624.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-chloro-3-(trifluoromethyl)-phenyl)piperazin-1-yl)methyl]-5-methylthiophen-2-yl}isoindoline-1,3-dione (**8o**).* Following general procedure E using *N*-(4-chloro-3-(trifluoromethyl)phenyl)piperazine, compound **8o** was purified by chromatography eluting with EtOAc:DCM 0.5:9.5. Yield: 92%. Mp: 118−120 °C. ^{1}H NMR ($CDCl_3$): δ 1.99 (t, J = 5.2 Hz, 4H), 2.32 (s, 3H), 2.90 (t, J = 5.2 Hz, 4H), 3.08 (s, 2H), 6.81 (dd, J = 8.8 and 3.0 Hz, 1H), 6.78 (d, J = 3.0 Hz, 1H), 7.28 (d, J = 8.8 Hz, 1H), 7.35 (d, J = 8.6 Hz, 2H), 7.39 (d, J = 7.4 Hz, 1H), 7.44 (d, J = 7.4 Hz, 1H), 7.53 (t, J = 7.4 Hz, 1H), 7.60 (d, J = 8.6 Hz, 2H), 7.79 (d, J = 7.4 Hz, 1H). MS (ESI): $[M]^+$ = 658.5.

*2-[3-(4-Chlorobenzoyl)-5-ethyl-4-[(4-phenyl-piperazin-1-yl)-methyl]thiophen-2-yl]-isoindoline-1,3-dione (**8p**).* Following general procedure E using *N*-phenylpiperazine, compound **8p** was purified by chromatography eluting with EtOAc:petroleum ether 1.5:8.5 to give an orange solid. Yield: 34%. Mp: 124−126 °C. ^{1}H NMR ($CDCl_3$): δ 1.34 (t, J = 7.6 Hz, 3H), 2.27 (m, 4H), 2.85 (m, 6H), 3.32 (s, 2H), 6.79 (d, J = 8.4 Hz, 2H), 7.21 (t, J = 7.6 Hz, 2H), 7.32 (d, J = 8.4 Hz, 2H), 7.71 (m, 5H), 7.81 (m, 2H). MS (ESI): $[M]^+$ = 570.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-fluorophenyl)piperazin-1-yl)-methyl]-5-ethylthiophen-2-yl}isoindoline-1,3-dione (**8q**).* Following general procedure E using *N*-(4-chlorophenyl)piperazine, compound **8q** was purified by chromatography eluting with EtOAc:petroleum ether 1.5:8.5 to give a white solid. Yield: 43%. Mp: 122−124 °C. ^{1}H NMR ($CDCl_3$): δ 1.20 (t, J = 7.6 Hz, 3H), 1.99 (m. 4H), 2.68 (q, J = 7.6 Hz, 2H), 3.02 (m, 4H), 3.22 (s, 2H), 6.84 (d, J = 8.4 Hz, 2H), 7.21 (d, J = 8.4 Hz, 2H), 7.38 (d, J = 8.4 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H), 7.72 (m, 2H), 7.84 (m, 2H). MS (ESI): $[M]^+$ = 588.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-chlorophenyl)piperazin-1-yl)-methyl]-5-ethylthiophen-2-yl}isoindoline-1,3-dione (**8r**).* Following general procedure E using *N*-(4-chlorophenyl)piperazine, compound **8r** was purified by chromatography eluting with EtOAc:petroleum ether 1.5:8.5 to give a white solid. Yield: 37%. Mp: 142−144 °C. ^{1}H NMR ($CDCl_3$): δ 1.19 (t, J = 7.6 Hz, 3H), 2.32 (m. 4H), 2.77 (q, J = 7.6 Hz, 2H), 3.02 (m, 4H), 3.32 (s, 2H), 6.82 (d, J = 8.4 Hz, 2H), 7.17 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 7.57 (d, J = 8.4 Hz, 2H), 7.71 (m, 2H), 7.81 (m, 2H). MS (ESI): $[M]^+$ = 604.5.

*2-[5-Bromo-3-(4-chlorobenzoyl)-4-[(4-phenylpiperazin-1-yl)-methyl]thiophen-2-yl]isoindoline-1,3-dione (**8s**).* Following general procedure E using *N*-phenylpiperazine, compound **8s** was purified by chromatography eluting with EtOAc:petroleum ether 4:6 to give a yellow solid. Yield: 86%. Mp: 191−193 °C. ^{1}H NMR ($CDCl_3$): δ 2.85 (t, J = 5.0 Hz, 2H), 3.14 (s, 2H), 3.34 (t, J = 5.0 Hz, 2H), 3.42 (t, J = 5.2 Hz, 2H), 3.94 (t, J = 5.2 Hz, 2H), 6.80−6.93 (m, 4H), 6.93−7.28 (m, 4H), 7.39 (d, J = 8.4 Hz, 2H), 7.58−7.62 (m, 2H), 7.82 (d, J = 6.8 Hz, 1H). MS (ESI): $[M]^+$ = 619.1, $[M + 2]^+$ = 621.1.

*2-{5-Bromo-3-(4-chlorobenzoyl)-4-[(4-(4-fluorophenyl)piperazin-1-yl)methyl]thiophen-2-yl}isoindoline-1,3-dione (**8t**).* Following general procedure E using *N*-(4-fluorophenyl)piperazine, compound **8t** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a white solid. Yield: 89%. Mp: 203−204 °C. ^{1}H NMR ($CDCl_3$): δ 2.29 (t, J = 4.8 Hz, 4H), 2.78 (t, J = 4.8 Hz, 4H), 3.38 (s, 2H), 6.72 (d, J = 8.6 Hz, 1H), 6.78 (d, J = 8.6 Hz, 1H), 6.86 (d, J = 8.6 Hz, 1H), 6.91 (d, J = 8.6 Hz, 1H), 7.22 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 7.672 (m, 2H),. 7.82 (m, 2H). MS (ESI): $[M]^+$ = 637.1, $[M + 2]^+$ = 639.1.

*2-[5-Phenyl-3-(4-chlorobenzoyl)-4-[(4-phenylpiperazin-1-yl)-methyl]thiophen-2-yl]isoindoline-1,3-dione (**12u**).* Following general procedure E using *N*-(4-phenyl)piperazine, compound **12u** was purified by chromatography eluting with EtOAc:petroleum ether 1.5:8.5 to give a yellow solid. Yield: 78%. Mp: 200−202 °C. ^{1}H NMR ($CDCl_3$): δ 2.19 (t, J = 5.0 Hz, 4H), 2.79 (t, J = 5.0 Hz, 4H), 3.41 (s, 2H), 6.78 (d, J = 8.8 Hz, 2H), 7.18 (7, J = 8.8 Hz, 2H), 7.37 (d, J = 6.8 Hz, 2H), 7.45 (m, 5H), 7.83 (m, 7H). MS (ESI): $[M]^+$ = 618.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-fluorophenyl)piperazin-1-yl)-methyl]-5-phenylthiophen-2-yl}isoindoline-1,3-dione (**12v**).* Following general procedure E using *N*-(4-fluorophenyl)piperazine, compound **12v** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 59%. Mp: 160−161 °C. ^{1}H NMR ($CDCl_3$): δ 2.18 (t, J = 4.8 Hz, 4H), 2.70 (t, J = 4.8 Hz, 4H), 3.41 (s, 2H), 6.71 (d, J = 8.6 and 4.4 Hz, 2H), 6.88 (t, J = 8.4 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 7.43 (m, 5H), 7.73 (m, 2H), 7.81 (d, J = 8.4 Hz, 2H), 7.86 (m, 2H). MS (ESI): $[M]^+$ = 636.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-chlorophenyl)piperazin-1-yl)-methyl]-5-phenylthiophen-2-yl}isoindoline-1,3-dione (**12w**).* Following general procedure E using *N*-(4-chlorophenyl)piperazine, compound **12w** was purified by chromatography eluting with EtOAc:petroleum ether 2:8 to give a yellow solid. Yield: 81%. Mp: 201−202 °C. ^{1}H NMR ($CDCl_3$): δ 2.17 (t, J = 4.8 Hz, 4H), 2.75 (t, J = 5.2 Hz, 4H), 3.42 (s, 2H), 6.64 (d, J = 9.0 Hz, 2H), 7.12 (d, J = 9.0 Hz, 2H), 7.33 (d, J = 9.0 Hz, 2H), 7.47 (m, 5H), 7.86 (m, 6H). MS (ESI): $[M]^+$ = 652.5.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(3,4-dichlorophenyl)piperazin-1-yl)-methyl]-5-phenylthiophen-2-yl}isoindoline-1,3-dione (**12x**).* Following general procedure E using *N*-(3,4-dichlorophenyl)piperazine, compound **12x** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a white solid. Yield: 81%. Mp: 153−155 °C. ^{1}H NMR ($CDCl_3$): δ 2.17 (t, J = 4.8 Hz, 4H), 2.76 (t, J = 4.8 Hz, 4H), 3.41 (s, 2H), 6.62 (dd, J = 8.8 and 2.8 Hz, 1H), 6.82 (d, J = 2.8 Hz, 1H), 7.20 (d, J = 8.8 Hz, 1H), 7.46 (d, J = 8.6 Hz, 2H), 7.72 (m, 7H), 7.82 (m, 4H). MS (ESI): $[M]^+$ = 687.1.

*2-{3-(4-Chlorobenzoyl)-5-phenyl-4-[(4-(4-(trifluoromethyl)-phenyl)piperazin-1-yl)methyl]thiophen-2-yl}isoindoline-1,3-dione (**12y**).* Following general procedure E, compound **12y** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 67%. Mp: 163−164 °C. ^{1}H NMR ($CDCl_3$): δ 2.20 (t, J = 4.8 Hz, 4H), 2.88 (t, J = 4.8 Hz, 4H), 3.42 (s, 2H), 6.75 (d, J = 8.4 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 7.38 (d, J = 8.4 Hz, 2H), 7.45 (m, 5H), 7.83 (m, 6H). MS (ESI): $[M]^+$ = 686.1.

*2-{3-(4-Chlorobenzoyl)-5-phenyl-4-[(4-(3-(trifluoromethyl)-phenyl)piperazin-1-yl)methyl]thiophen-2-yl}isoindoline-1,3-dione (**12z**).* Following general procedure E using *N*-(3-(trifluoromethyl)-phenyl)piperazine, compound **12y** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 81%. Mp: 118−120 °C. ^{1}H NMR ($CDCl_3$): δ 2.18 (t, J = 4.8 Hz, 4H), 2.84 (t, J = 4.8 Hz, 4H), 3.42 (s, 2H), 6.94 (m, 3H), 7.34 (d, J = 8.2 Hz, 2H), 7.46 (m, 4H), 7.83 (m, 8H). MS (ESI): $[M]^+$ = 686.1.

*2-{3-(4-Chlorobenzoyl)-5-phenyl-4-[(4-(4-chloro-3-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-2-yl}-isoindoline-1,3-dione (**12aa**).* Following general procedure E using *N*-(4-chloro-3-(trifluoromethyl)phenyl)piperazine, compound **12aa** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a white solid. Yield: 78%. Mp: 228−230 °C. ^{1}H NMR ($CDCl_3$): δ 2.18 (t, J = 4.6 Hz, 4H), 2.80 (t, J = 4.6 Hz, 4H), 3.42 (s, 2H), 6.80 (dd, J = 8.8 and 2.8 Hz, 1H), 7.00 (d, J = 2.8 Hz, 1H), 7.24 (d, J = 8.8 Hz, 1H), 7.35 (d, J = 8.8 Hz, 2H), 7.46 (d, J = 8.8 Hz, 2H), 7.46 (m, 5H), 7.87 (m, 4H). MS (ESI): $[M]^+$ = 720.6.

*2-{3-(4-)Chlorobenzoyl)-5-phenyl-4-[(4-(2-(trifluoromethyl)-phenyl)piperazin-1-ylmethyl]thiophen-2-yl}isoindoline-1,3-dione (**12ab**).* Following general procedure E using *N*-(2-(trifluoromethyl)-phenyl)piperazine, compound **12ab** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a white solid. Yield: 89%. Mp: 201−202 °C. ^{1}H NMR ($CDCl_3$): δ 2.18 (t, J = 4.4 Hz, 4H), 2.46 (t, J = 4.4 Hz, 4H), 3.41 (s, 2H), 6.91 (d, J = 8.8 Hz, 1H), 7.12 (d, J = 8.8 Hz, 1H), 7.44 (m, 7H), 7.56 (d, J = 8.4 Hz, 2H), 7.76 (m, 6H). MS (ESI): $[M]^+$ = 686.1.

*2-{3-(4-Chlorobenzoyl)-5-phenyl-4-[(4-(4-(trifluoromethoxy)-phenyl)piperazin-1-yl)methyl]thiophen-2-yl}isoindoline-1,3-dione (**12ac**).* Following general procedure E using *N*-(4-(trifluoromethoxy)-phenyl)piperazine, compound **12ac** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow oil. Yield: 68%. ^{1}H NMR ($CDCl_3$): δ 1.88 (t, J = 4.8 Hz, 4H), 2.85 (t, J = 4.8 Hz, 4H), 3.12 (s, 2H), 6.82 (d, J = 9.0 Hz, 2H), 6.94 (d, J = 8.8 Hz, 2H), 7.34 (m, 3H), 7.38 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8.4 Hz, 2H), 7.68 (d, J = 8.2 Hz, 2H), 7.84 (m, 4H). MS (ESI): $[M]^+$ = 702.1.

*2-[5-(4-Fluorophenyl)-3-(4-chlorobenzoyl)-4-[(4-phenylpiperazin-1-yl)methyl]thiophen-2-yl]isoindoline-1,3-dione (**12ad**).* Following general procedure E using *N*-phenylpiperazine, compound **12ad** was purified by chromatography eluting with EtOAc:petroleum ether 2:8 to give a yellow solid. Yield: 98%. Mp: 188−190 °C. ^{1}H NMR ($CDCl_3$): δ 2.16 (t, J = 4.8 Hz, 4H), 2.78 (t, J = 4.8 Hz, 4H), 3.36 (s, 2H), 6.77 (t, J = 8.8 Hz, 2H), 7.13 (m, 4H), 7.35 (d, J = 8.6 Hz, 2H), 7.42 (m, 2H), 7.94 (m, 7H). MS (ESI): $[M]^+$ = 636.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-fluorophenyl)piperazin-1-yl)-methyl]-5-(4-fluorophenyl)thiophen-2-yl}isoindoline-1,3-dione (**12ae**).* Following general procedure E using *N*-(4-fluorophenyl)-piperazine, compound **12ae** was purified by chromatography eluting with EtOAc:petroleum ether 1.5:8.5 to give a yellow solid. Yield: 67%. Mp: 164−166 °C. ^{1}H NMR ($CDCl_3$): δ 2.18 (t, J = 4.8 Hz, 4H), 2.70 (t, J = 4.8 Hz, 4H), 3.37 (s, 2H), 6.68 (t, J = 8.6 Hz, 2H), 6.73 (t, J = 8.6 Hz, 2H), 7.16 (t, J = 8.6 Hz, 2H), 7.32 (d, J = 8.4 Hz, 2H), 7.37 (m, 4H), 7.78 (m, 4H). MS (ESI): $[M]^+$ = 654.1.

*2-[5-(4-Chlorophenyl)-3-(4-chlorobenzoyl)-4-[(4-phenylpiperazin-1-yl)methyl]thiophen-2-yl]isoindoline-1,3-dione (**12af**).* Following general procedure E using *N*-phenylpiperazine, compound **12af** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a white solid. Yield: 76%. Mp: 171−172 °C. ^{1}H NMR ($CDCl_3$): δ 2.22 (t, J = 5.0 Hz, 4H), 2.83 (t, J = 5.0 Hz, 4H), 3.38 (s, 2H), 6.74 (d, J = 8.6 Hz, 2H), 6.78 (d, J = 8.6 Hz, 2H), 7.22 (d, J = 8.6 Hz, 2H),

7.37 (d, J = 8.4 Hz, 2H), 7.43 (m, 5H), 7.75 (m, 4H). MS (ESI): $[M]^+$ = 652.6.

*2-[(4-(4-Benzylpiperazin-1-yl)methyl]-3-(4-chlorobenzoyl)-5-(4-chlorophenyl)thiophen-2-yl]isoindoline-1,3-dione (**12ag**).* Following general procedure E using *N*-benzylpiperazine, compound **12ag** was purified by chromatography eluting with EtOAc:petroleum ether 4:6 to give a yellow solid. Yield: 77%. Mp: 198−200 °C. ^{1}H NMR ($CDCl_3$): δ 2.12 (t, J = 5.2 Hz, 4H), 3.08 (t, J = 5.2 Hz, 4H), 3.44 (s, 2H), 3.89 (s, 2H), 7.28 (m, 5H), 7.34 (d, J = 8.8 Hz, 2H), 7.42 (d, J = 7.2 Hz, 2H), 7.46 (d, J = 7.2 Hz, 2H), 7.52 (d, J = 8.8 Hz, 2H), 7.84 (m, 4H). MS (ESI): $[M]^+$ = 666.6.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-fluorophenyl)piperazin-1-yl)-methyl]-5-(4-chlorophenyl)thiophen-2-yl}isoindoline-1,3-dione (**12ah**).* Following general procedure E using *N*-(4-fluorophenyl)-piperazine, compound **12ah** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a white solid. Yield: 68%. Mp: 168−170 °C. ^{1}H NMR ($CDCl_3$): δ 2.17 (t, J = 4.8 Hz, 4H), 2.70 (t, J = 4.8 Hz, 4H), 3.38 (s, 2H), 6.62 (d, J = 8.6 Hz, 1H), 6.64 (d, J = 8.6 Hz, 1H), 6.88 (t, J = 8.6 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 7.37 (d, J = 8.4 Hz, 2H), 7.44 (m, 2H), 7.83 (m, 6H). MS (ESI): $[M]^+$ = 670.6.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(4-chlorophenyl)piperazin-1-yl)-methyl]-5-(4-chlorophenyl)thiophen-2-yl}isoindoline-1,3-dione (**12ai**).* Following general procedure E using *N*-(4-chlorophenyl)-piperazine, compound **12ai** was purified by chromatography eluting with EtOAc:petroleum ether 2:8 to give a white solid. Yield: 78%. Mp: 144−145 °C. ^{1}H NMR ($CDCl_3$): δ 2.17 (t, J = 4.8 Hz, 4H), 2.74 (t, J = 5.2 Hz, 4H), 3.38 (s, 2H), 6.66 (d, J = 9.0 Hz, 2H), 7.12 (d, J = 9.0 Hz, 2H), 7.33 (m, 2H), 7.37 (d, J = 8.8 Hz, 2H), 7.42 (d, J = 7.6 Hz, 2H), 7.76 (m, 6H). MS (ESI): $[M]^+$ = 687.1.

*2-{3-(4-Chlorobenzoyl)-4-[(4-(3,4-dichlorophenyl)piperazin-1-yl)-methyl]-5-(4-chlorophenyl)thiophen-2-yl}isoindoline-1,3-dione (**12aj**).* Following general procedure E using *N*-(3,4-dichlorophenyl)-piperazine, compound **12aj** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a white solid. Yield: 81%. Mp: 146−147 °C. ^{1}H NMR ($CDCl_3$): δ 2.17 (t, J = 4.8 Hz, 4H), 2.82 (t, J = 4.8 Hz, 4H), 3.42 (s, 2H), 6.64 (dd, J = 9.2 and 3.2 Hz, 1H), 6.68 (d, J = 2.8 Hz, 1H), 7.84 (d, J = 9.2 Hz, 2H), 7.43 (m, 5H), 7.83 (m, 6H). MS (ESI): $[M]^+$ = 721.5.

*2-{3-(4-Chlorobenzoyl)-5-(4-chlorophenyl)-4-[(4-(4-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-2-yl}-isoindoline-1,3-dione (**12ak**).* Following general procedure E using *N*-(4-(trifluoromethyl)phenyl)piperazine, compound **12ak** was purified by chromatography eluting with EtOAc:petroleum ether 1.5:8.5 to give a yellow solid. Yield: 78%. Mp: 240−242 °C. ^{1}H NMR ($CDCl_3$): δ 2.17 (t, J = 4.8 Hz, 4H), 2.88 (t, J = 4.8 Hz, 4H), 3.36 (s, 2H), 6.77 (d, J = 8.8 Hz, 2H), 7.37 (d, J = 8.8 Hz, 2H), 7.42 (m, 4H), 7.43 (t, J = 6.8 Hz, 2H), 7.77 (m, 6H). MS (ESI): $[M]^+$ = 720.6.

*2-{3-(4-Chlorobenzoyl)-5-(4-chlorophenyl)-4-[(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]-thiophen-2-yl}-isoindoline-1,3-dione (**12al**).* Following general procedure E using *N*-(3-(trifluoromethyl)phenyl)piperazine, compound **12al** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow oil. Yield: 78%. ^{1}H NMR ($CDCl_3$): δ 2.17 (t, J = 4.8 Hz, 4H), 2.95 (t, J = 4.8 Hz, 4H), 3.39 (s, 2H), 6.82 (d, J = 8.8 Hz, 2H), 6.94 (d, J = 8.8 Hz, 2H), 7.39 (m, 3H), 7.44 (d, J = 6.8 Hz, 2H), 7.74 (m, 6H). MS (ESI): $[M]^+$ = 720.6.

*2-{3-(4-Chlorobenzoyl)-5-(4-chlorophenyl)-4-[(4-(4-chloro-3-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-2-yl}-isoindoline-1,3-dione (**12am**).* Following general procedure E using *N*-(4-chloro-3-(trifluoromethyl)phenyl)piperazine, compound **12am** was purified by chromatography eluting with EtOAc:petroleum ether 2:8 to give a yellow solid. Yield: 76%. Mp: 235−237 °C. ^{1}H NMR ($CDCl_3$): δ 2.17 (t, J = 4.8 Hz, 4H), 2.82 (t, J = 4.8 Hz, 4H), 3.039 (s, 2H), 6.82 (dd, J = 8.8 and 1.2 Hz, 1H), 6.92 (d, J = 1.2 Hz, 2H), 7.35 (m, 3H), 7.42 (t, J = 6.8 Hz, 2H), 7.44 (d, J = 8.4 Hz, 2H), 7.82 (m, 4H). MS (ESI): $[M]^+$ = 755.1.

*2-{3-(4-Chlorobenzoyl)-5-(4-chlorophenyl)-4-[(4-(2-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-2-yl}-isoindoline-1,3-dione (**12an**).* Following general procedure E using *N*-(2-(trifluoromethyl)phenyl)piperazine, compound **12an** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 78%. Mp: 208−210 °C. ^{1}H NMR ($CDCl_3$): δ 2.12 (t, J = 4.8 Hz, 4H), 2.98 (t, J = 4.8 Hz, 4H), 3.22 (s, 2H), 6.82 (d, J = 8.8 Hz, 2H), 6.88 (d, J = 8.8 Hz, 2H), 7.42 (m, 4H), 7.56 (d, J = 6.8 Hz, 2H), 7.70 (m, 6H). MS (ESI): $[M]^+$ = 720.6.

*2-{3-(4-Chlorobenzoyl)-5-(4-chlorophenyl)-4-[(4-(4-(trifluoromethoxy)phenyl)piperazin-1-yl)methyl]thiophen-2-yl}-isoindoline-1,3-dione (**12ao**).* Following general procedure E using *N*-(4-(trifluoromethoxy)phenyl)piperazine, compound **12ao** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a brown solid. Yield: 76%. Mp: 146−147 °C. ^{1}H NMR ($CDCl_3$): δ 2.08 (t, J = 4.8 Hz, 4H), 2.78 (t, J = 4.8 Hz, 4H), 3.42 (s, 2H), 6.76 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 8.8 Hz, 2H), 7.32 (d, J = 8.8 Hz, 2H), 7.43 (m, 4H), 7.76 (m, 6H). MS (ESI): $[M]^+$ = 736.6.

*General Procedure F for the Synthesis of Compounds **4a**−**ao**.* A stirred suspension of **8a**−**t** or **12u**−**ao** (0.5 mmol) and hydrazine monohydrate (58 μL, 1.2 mmol, 1.2 equiv) in absolute EtOH (10 mL) was refluxed for 1 h. The solvent was then evaporated, the residue suspended in DCM (10 mL), and the suspension filtered through Celite. The filtrate was concentrated in vacuo to obtain a residue that was purified by column chromatography.

*[2-Amino-5-methyl-4-[(4-phenylpiperazin-1-yl)methyl]thiophen-3-yl](4-chlorophenyl)methanone (**4a**).* Following general procedure F, compound **4a** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 96%. Mp: 78−80 °C. ^{1}H NMR ($CDCl_3$): δ 1.98 (t, J = 4.8 Hz, 4H), 2.22 (s, 3H), 2.91 (t, J = 4.8 Hz, 4H), 2.95 (s, 2H), 5.80 (bs, 2H), 6.83 (d, J = 8.4 Hz, 2H), 7.23 (t, J = 8.4 Hz, 3H), 7.35 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 426.0. Anal. ($C_{23}H_{24}ClN_3OS$): C, H, N.

*[2-Amino-4-[(4-benzylpiperazin-1-yl)methyl]-5-methylthiophen-3-yl](4-chlorophenyl)methanone (**4b**).* Following general procedure F, compound **4b** was purified by chromatography eluting with EtOAc to give a yellow solid. Yield: 73%. Mp: 48−50 °C.^{1}H NMR ($CDCl_3$): δ 1.84 (t, J = 5.2 Hz, 4H), 2.18 (s, 3H), 2.86 (t, J = 5.2 Hz, 4H), 3.40 (s, 2H), 3.49 (s, 2H), 5.79 (bs, 2H), 7.26 (m, 5H), 7.33 (d, J = 8.4 Hz, 2H), 7.51 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 440.0. Anal. ($C_{24}H_{26}ClN_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-fluorophenyl)piperazin-1-yl)methyl]-5-methylthiophen-3-yl}(4-chlorophenyl)methanone (**4c**).* Following general procedure F, compound **4c** was purified by chromatography eluting with EtOAc:DCM 0.5:9.5 to give a yellow solid. Yield: 55%. Mp: 70−72 °C. ^{1}H NMR ($CDCl_3$): δ 1.99 (t, J = 4.8 Hz, 4H), 2.23 (s, 3H), 2.83 (t, J = 4.8 Hz, 4H), 2.96 (s, 2H), 5.82 (bs, 2H), 6.76 (d, J = 8.6 Hz, 1H), 6.83 (d, J = 8.6 Hz, 1H), 6.94 (t, J = 8.6 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 444.1. Anal. ($C_{23}H_{23}ClFN_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-chlorophenyl)piperazin-1-yl)methyl]-5-methylthiophen-3-yl}(4-chlorophenyl)methanone (**4d**).* Following general procedure F, compound **4d** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 62%. Mp: 83−85 °C. ^{1}H NMR ($CDCl_3$): δ 1.98 (t, J = 4.8 Hz, 4H), 2.22 (s, 3H), 2.87 (t, J = 4.8 Hz, 4H), 2.95 (s, 2H), 5.80 (bs, 2H), 6.73 (d, J = 9.2 Hz, 2H), 7.16 (d, J = 9.2 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 460.5. Anal. ($C_{23}H_{23}Cl_2N_3OS$): C, H, N.

*{2-Amino-4-[(4-(3-chlorophenyl)piperazin-1-yl)methyl]-5-methylthiophen-3-yl}(4-chlorophenyl)methanone (**4e**).* Following general procedure F, compound **4e** was purified by chromatography eluting with EtOAc:DCM 0.75:9.25 to give a yellow solid. Yield: 56%. Mp: 58−60 °C. ^{1}H NMR ($CDCl_3$): δ 1.96 (t, J = 4.8 Hz, 4H), 2.22 (s, 3H), 2.92 (t, J = 4.8 Hz, 4H), 3.00 (s, 2H), 5.82 (bs, 2H), 6.68 (d, J = 7.6 Hz, 1H), 6.76 (d, J = 7.6 Hz, 1H), 6.78 (s, 1H), 7.12 (t, J = 8.4 Hz, 1H), 7.36 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 460.4. Anal. ($C_{23}H_{23}Cl_2N_3OS$): C, H, N.

*{2-Amino-4-[(4-(3,4-dichlorophenyl)piperazin-1-yl)methyl]-5-methylthiophen-3-yl}(4-chlorophenyl)methanone (**4f**).* Following general procedure F, compound **4f** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 51%. Mp: 63−65 °C. ^{1}H NMR ($CDCl_3$): δ 1.96 (t, J = 4.8 Hz, 4H), 2.22 (s, 3H), 2.88 (t, J = 4.8 Hz, 4H), 2.94 (s, 2H), 5.82 (bs, 2H), 6.63 (dd, J = 9.2 and 2.8 Hz, 1H), 6.86 (d, J = 2.8 Hz, 1H), 7.22 (d, J = 9.2 Hz, 1H), 7.36 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 494.9. Anal. ($C_{23}H_{22}Cl_3N_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-bromophenyl)piperazin-1-yl)methyl]-5-methylthiophen-3-yl}(4-chlorophenyl)methanone (**4g**).* Following general procedure F, compound **4g** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 72%. Mp: 73−75 °C. ^{1}H NMR ($CDCl_3$): δ 1.96 (t, J = 5.2 Hz, 4H), 2.22 (s, 3H), 2.87 (t, J = 5.2 Hz, 4H), 2.94 (s, 2H), 5.81 (bs, 2H), 6.68 (d, J = 9.2 Hz, 2H), 7.29 (d, J = 9.2 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 504.9, $[M + 2]^+$ = 506.9. Anal. ($C_{23}H_{23}BrClN_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-iodophenyl)piperazin-1-yl)methyl]-5-methylthiophen-3-yl}(4-chlorophenyl)methanone (**4h**).* Following general procedure F, compound **4g** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 75%. Mp: 72−73 °C. ^{1}H NMR ($CDCl_3$): δ 1.99 (t, J = 5.2 Hz, 4H), 2.04 (s, 3H), 2.89 (t, J = 5.2 Hz, 4H), 2.98 (s, 2H), 5.80 (bs, 2H), 6.58 (d, J = 8.8 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.47 (d, J = 8.8 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 551.9. Anal. ($C_{23}H_{23}ClIN_3OS$): C, H, N.

*[2-Amino-5-methyl-4-[(4-(4-methylphenyl)piperazin-1-yl)-methyl]thiophen-3-yl](4-chlorophenyl)methanone (**4i**).* Following general procedure F, compound **4i** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 55%. Mp: 77−79 °C. ^{1}H NMR ($CDCl_3$): δ 1.98 (t, J = 5.2 Hz, 4H), 2.22 (s, 3H), 2.25 (s, 3H), 2.85 (t, J = 5.2 Hz, 4H), 2.94 (s, 2H), 5.81 (bs, 2H), 6.75 (d, J = 8.8 Hz, 2H), 7.03 (d, J = 8.8 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 440.0. Anal. ($C_{24}H_{26}ClN_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-methoxyphenyl)piperazin-1-yl)methyl]-5-methylthiophen-3-yl}(4-chlorophenyl)methanone (**4j**).* Following general procedure F, compound **4j** was purified by chromatography eluting with EtOAc:DCM 1.5:8.5 to give a yellow solid. Yield: 67%. Mp: 63−65 °C. ^{1}H NMR ($CDCl_3$): δ 1.98 (t, J = 5.4 Hz, 4H), 2.21 (s, 3H), 2.80 (t, J = 5.4 Hz, 4H), 2.94 (s, 2H), 3.75 (s, 3H), 5.81 (bs, 2H), 6.81 (s, 4H), 7.34 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 456.1. Anal. ($C_{24}H_{26}ClN_3O_2S$): C, H, N.

*{2-Amino-4-[(4-(4-cyanophenyl)piperazin-1-yl)methyl]-5-methylthiophen-3-yl}(4-chlorophenyl)methanone (**4k**).* Following general procedure F, compound **4k** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 54%. Mp: 76−77 °C. ^{1}H NMR ($CDCl_3$): δ 1.96 (t, J = 4.8 Hz, 4H), 2.21 (s, 3H), 2.95 (s, 2H), 3.04 (t, J = 4.8 Hz, 4H), 5.84 (bs, 2H), 6.74 (d, J = 8.8 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.44 (d, J = 8.8 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 451.0. Anal. ($C_{24}H_{23}ClN_4OS$): C, H, N.

*{2-Amino-5-methyl-4-[(4-(4-nitrophenyl)piperazin-1-yl)methyl]-thiophen-3-yl}(4-chlorophenyl)methanone (**4l**).* Following general procedure F, compound **4l** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 53%. Mp: 85−87 °C. ^{1}H NMR ($CDCl_3$): δ 1.98 (t, J = 5.4 Hz, 4H), 2.331 (s, 3H), 3.08 (s, 2H), 3.11 (t, J = 5.4 Hz, 4H), 6.26 (bs, 2H), 6.70 (d, J = 8.8 Hz, 2H), 6.80 (d, J = 9.2 Hz, 2H), 8.05 (d, J = 9.2 Hz, 2H), 8.13 (d, J = 8.8 Hz, 2H). MS (ESI): $[M]^+$ = 471.0. Anal. ($C_{23}H_{23}ClN_4O_3S$): C, H, N.

*{2-Amino-5-methyl-4-[(4-(4-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4m**).* Following general procedure F, compound **4m** was purified by chromatography eluting with EtOAc:DCM 1:9 to give a yellow solid. Yield: 54%. Mp: 76−78 °C. ^{1}H NMR ($CDCl_3$): δ 2.04 (t, J = 4.8 Hz, 4H), 2.22 (s, 3H), 2.95 (s, 2H), 3.00 (t, J = 4.8 Hz, 4H), 5.82 (bs, 2H), 6.82 (d, J = 8.4 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.43 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 494.0. Anal. ($C_{24}H_{23}ClF_3N_3OS$): C, H, N.

*{2-Amino-5-methyl-4-[(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4n**).* Following general procedure F, compound **4n** was purified by chromatography eluting with EtOAc:DCM 0.5:9.5 to give a yellow solid. Yield: 68%. Mp: 60−61 °C. ^{1}H NMR ($CDCl_3$): δ 1.99 (t, J = 5.2 Hz, 4H), 2.22 (s, 3H), 2.96 (m, 6H), 5.81 (bs, 2H), 6.96 (d, J = 7.6 Hz, 1H), 7.00 (s, 1H), 7.03 (d, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 7.37 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 494.1. Anal. ($C_{24}H_{23}ClF_3N_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-chloro-3-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]-5-methylthiophen-3-yl}(4-chlorophenyl)methanone (**4o**).* Following general procedure F, compound **4o** was purified by chromatography eluting with EtOAc:DCM 0.5:9.5 to give a yellow solid. Yield: 54%. Mp: 133−135 °C. ^{1}H NMR ($CDCl_3$): δ 1.98 (t, J = 4.4 Hz, 4H), 2.22 (s, 3H), 2.93 (t, J = 4.4 Hz, 4H), 2.96 (s, 2H), 5.82 (bs, 2H), 6.84 (dd, J = 8.8 and 2.8 Hz, 1H), 7.07 (d, J = 2.8 Hz, 1H), 7.29 (d, J = 8.8 Hz, 1H), 7.37 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 528.5. Anal. ($C_{24}H_{22}Cl_2F_3N_3OS$): C, H, N.

*{2-Amino-5-ethyl-4-[(4-phenylpiperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4p**).* Following general procedure F, compound **4p** was purified by chromatography eluting with EtOAc:DCM 1.5:8.5 to give a yellow solid. Yield: 86%. Mp: 104−106 °C. ^{1}H NMR ($CDCl_3$): δ 1.22 (t, J = 7.6 Hz, 3H), 1.98 (m. 4H), 2.64 (q, J = 7.6 Hz, 2H), 2.92 (m, 4H), 2.96 (s, 2H), 5.78 (bs, 2H), 6.85 (m 3H), 7.23 (m, 2H), 7.37 (d, J = 8.4 Hz, 2H), 7.56 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 440.1. Anal. ($C_{24}H_{26}ClN_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-chlorophenyl)piperazin-1-yl)methyl]-5-ethylthiophen-3-yl}(4-fluorophenyl)methanone (**4q**).* Following general procedure F, compound **4q** was purified by chromatography eluting with EtOAc:petroleum ether 2:8 to give a yellow solid. Yield: 82%. Mp: 123−125 °C. ^{1}H NMR ($CDCl_3$): δ 1.21 (t, J = 7.6 Hz, 3H), 1.98 (m, 4H), 2.61 (q, J = 7.6 Hz, 2H), 2.84 (m, 4H), 2.96 (s, 2H), 5.77 (bs, 2H), 6.82 (m, 2H), 6.93 (d J = 9.0 Hz, 2H), 7.34 (d J = 8.6 Hz, 2H), 7.57 (d, J = 8.6 Hz, 2H). MS (ESI): $[M]^+$ = 458.0. Anal. ($C_{24}H_{25}ClFN_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-chlorophenyl)piperazin-1-yl)methyl]-5-ethylthiophen-3-yl}(4-chlorophenyl)methanone (**4r**).* Following general procedure F, compound **4r** was purified by chromatography eluting with EtOAc:petroleum ether 1.5:8.5 to give a yellow solid. Yield: 69%. Mp: 115−117 °C. ^{1}H NMR ($CDCl_3$): δ 1.23 (t, J = 7.6 Hz, 3H), 1.97 (m. 4H), 2.61 (q, J = 7.6 Hz, 2H), 2.88 (m, 4H), 2.96 (s, 2H), 5.77 (bs, 2H), 6.72 (d, J = 9.0 Hz, 2H), 7.19 (d, J = 9.0 Hz, 2H), 7.34 (d J = 8.2 Hz, 2H), 7.52 (d, J = 8.2 Hz, 2H). MS (ESI): $[M]^+$ = 474.4. Anal. ($C_{24}H_{25}Cl_2N_3OS$): C, H, N.

*{2-Amino-5-bromo-4-[(4-phenylpiperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4s**).* Following general procedure F, compound **4s** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 61%. Mp: 63−64 °C. ^{1}H NMR ($CDCl_3$): δ 2.04 (d, J = 4.8 Hz, 4H), 2.93 (d, J = 4.8 Hz, 4H), 3.00 (s, 2H), 6.07 (bs, 2H), 6.14 (s, 1H), 6.83 (m, 2H), 7.23 (m, 2H), 7.39 (d, J = 7.8 Hz, 2H), 7.53 (d, J = 7.8 Hz, 2H). MS (ESI): $[M]^+$ = 490.8, $[M + 2]^+$ = 492.9. Anal. ($C_{22}H_{21}BrClN_3OS$): C, H, N.

*{2-Amino-5-bromo-4-[(4-(4-fluorophenyl)piperazin-1-yl)methyl]-thiophen-3-yl}(4-chlorophenyl)methanone (**4t**).* Following general procedure F, compound **4t** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow oil. Yield: 52%. ^{1}H NMR ($CDCl_3$): δ 2.03 (t, J = 4.8 Hz, 4H), 2.87 (t, J = 4.8 Hz, 4H), 3.00 (s, 2H), 6.07 (bs, 2H), 6.14 (d, J = 8.6 Hz, 1H), 6.79 (t, J = 8.6 Hz, 1H), 6.88 (t, J = 8.6 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 508.8, $[M + 2]^+$ = 510.9. Anal. ($C_{22}H_{20}BrClFN_3OS$): C, H, N.

*{2-Amino-4-[(4-phenylpiperazin-1-yl)methyl]-5-phenylthiophen-3-yl}(4-chlorophenyl)methanone (**4u**).* Following general procedure F, compound **4u** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 46%. Mp: 74−76 °C. ^{1}H NMR ($CDCl_3$): δ 1.91 (t, J = 4.8 Hz, 4H), 2.87 (t, J = 4.8 Hz, 4H), 3.12 (s, 2H), 5.79 (bs, 2H), 6.78 (d, J = 8.4 Hz, 2H), 7.21 (7, J = 8.4 Hz, 2H), 7.32 (d, J = 7.2 Hz, 2H), 7.41 (m, 6H), 7.67 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 488.1. Anal. ($C_{28}H_{26}ClN_3OS$): C, H, N.

*{2-Amino-4-[4-[(4-fluorophenyl)piperazin-1-yl]methyl]-5-phenylthiophen-3-yl}(4-chlorophenyl)methanone (**4v**).* Following general procedure F, compound **4v** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 88%. Mp: 70−71 °C. ^{1}H NMR ($CDCl_3$): δ 1.91 (t, J = 4.8 Hz, 4H), 2.79 (t, J = 4.8 Hz, 4H), 3.12 (s, 2H), 5.82 (bs, 2H), 6.76 (dd, J = 8.8 and 4.8 Hz, 2H), 6.90 (t, J = 8.4 Hz, 2H), 7.34 (d, J = 7.2 Hz, 2H), 7.39 (m, 5H), 7.65 (d, J = 8.0 Hz, 2H). MS (ESI): $[M]^+$ = 506.1. Anal. ($C_{28}H_{25}FClN_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-chlorophenyl)piperazin-1-yl)methyl]-5-phenylthiophen-3-yl}(4-chlorophenyl)methanone (**4w**).* Following general procedure F, compound **4w** was purified by chromatography eluting

with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 67%. Mp: 79−80 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (t, J = 4.8 Hz, 4H), 2.02 (t, J = 4.8 Hz, 4H), 2.11 (s, 2H), 5.83 (bs, 2H), 6.71 (d, J = 9.2 Hz, 2H), 7.16 (d, J = 9.2 Hz, 2H), 7.34 (m, 7H), 7.64 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 522.5. Anal. ($C_{28}H_{25}Cl_2N_3OS$): C, H, N.

*{2-Amino-4-[(4-(3,4-dichlorophenyl)piperazin-1-yl)methyl]-5-phenylthiophen-3-yl}(4-chlorophenyl)methanone (**4x**).* Following general procedure F, compound **4x** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 62%. Mp: 70−71 °C. ^{1}H NMR ($CDCl_3$): δ 1.85 (t, J = 5.0 Hz, 4H), 2.88 (t, J = 5.0 Hz, 4H), 3.12 (s, 2H), 5.82 (bs, 2H), 6.58 (dd, J = 9.0 and 3.0 Hz, 1H), 6.81 (d, J = 3.0 Hz, 1H), 7.18 (d, J = 9.0 Hz, 1H), 7.32 (d, J = 8.4 Hz, 2H), 7.39 (m, 5H), 7.64 (d, J = 8.2 Hz, 2H). MS (ESI): $[M]^+$ = 556.9. Anal. ($C_{28}H_{24}Cl_3N_3OS$): C, H, N.

*{2-Amino-5-phenyl-4-[(4-(4-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4y**).* Following general procedure F, compound **4y** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 65%. Mp: 90−91 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (t, J = 4.8 Hz, 4H), 2.95 (t, J = 4.8 Hz, 4H), 3.12 (s, 2H), 5.83 (bs, 2H), 6.76 (d, J = 8.4 Hz, 2H), 7.39 (m, 9H), 7.68 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 556.1. Anal. ($C_{29}H_{25}ClF_3N_3OS$): C, H, N.

*{2-Amino-5-phenyl-4-[(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4z**).* Following general procedure F, compound **4z** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 67%. Mp: 77−78 °C. ^{1}H NMR ($CDCl_3$): δ 1.92 (t, J = 4.8 Hz, 4H), 2.92 (t, J = 4.8 Hz, 4H), 3.12 (s, 2H), 5.82 (bs, 2H), 6.82 (d, J = 8.4 Hz, 2H), 6.97 (d, J = 8.4 Hz, 2H), 7.36 (m, 7H), 7.63 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 556.1. Anal. ($C_{29}H_{25}ClF_3N_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-chloro-3-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]-5-phenylthiophen-3-yl}(4-chlorophenyl)methanone (**4aa**).* Following general procedure F, compound **4aa** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 63%. Mp: 80−82 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (t, J = 4.8 Hz, 4H), 2.87 (t, J = 4.8 Hz, 4H), 3.12 (s, 2H), 5.82 (bs, 2H), 6.84 (d, J = 8.4 and 2.4 Hz, 1H), 7.02 (d, J = 2.4 Hz, 1H), 7.36 (m, 6H), 7.43 (d, J = 8.2 Hz, 2H), 7.65 (d, J = 8.2 Hz, 2H). MS (ESI): $[M]^+$ = 590.5. Anal. ($C_{29}H_{24}Cl_2F_3N_3OS$): C, H, N.

*{2-Amino-5-phenyl-4-[(4-(2-(trifluoromethyl)phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4ab**).* Following general procedure F, compound **4ab** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 67%. Mp: 80−81 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (t, J = 4.8 Hz, 4H), 2.61 (t, J = 4.8 Hz, 4H), 3.12 (s, 2H), 5.75 (bs, 2H), 6.84 (d, J = 8.4 Hz, 1H), 7.34 (d, J = 8.4 Hz, 2H), 7.46 (m, 8H), 7.70 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 556.2. Anal. ($C_{29}H_{25}ClF_3N_3OS$): C, H, N.

*{2-Amino-5-phenyl-4-[(4-(4-(trifluoromethoxy)phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4ac**).* Following general procedure F, compound **4ac** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 64%. Mp: 68−69 °C. ^{1}H NMR ($CDCl_3$): δ 1.87 (t, J = 4.8 Hz, 4H), 2.84 (t, J = 4.8 Hz, 4H), 3.11 (s, 2H), 5.82 (bs, 2H), 6.75 (d, J = 9.0 Hz, 2H), 7.04 (d, J = 8.8 Hz, 2H), 7.33 (m, 3H), 7.38 (d, J = 8.4 Hz, 2H), 7.43 (d, J = 8.4 Hz, 2H), 7.64 (d, J = 8.2 Hz, 2H). MS (ESI): $[M]^+$ = 572.1. Anal. ($C_{29}H_{25}ClF_3N_3O_2S$): C, H, N.

*{2-Amino-4-[(4-phenylpiperazin-1-yl)methyl]-5-(4-fluorophenyl)-thiophen-3-yl}(4-chlorophenyl)methanone (**4ad**).* Following general procedure F, compound **4ad** was purified by chromatography eluting with EtOAc:petroleum ether 2:8 to give a yellow solid. Yield: 53%. Mp: 113−114 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (t, J = 4.8 Hz, 4H), 2.87 (t, J = 4.8 Hz, 4H), 3.05 (s, 2H), 5.79 (bs, 2H), 6.80 (d, J = 8.4 Hz, 2H), 7.07 (t, J = 8.4 Hz, 2H), 7.21 (t, J = 8.4 Hz, 2H), 7.22 (m, 3H), 7.28 (d, J = 8.6 Hz, 2H), 7.62 (d, J = 8.6 Hz, 2H). MS (ESI): $[M]^+$ = 506.1. Anal. ($C_{28}H_{25}ClFN_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-fluorophenyl)piperazin-1-yl)methyl]-5-(4-fluorophenyl)thiophen-3-yl}(4-chlorophenyl)methanone (**4ae**).* Following general procedure F, compound **4ae** was purified by chromatography eluting with EtOAc:petroleum ether 2:8 to give a yellow solid. Yield: 86%. Mp: 105−107 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (t, J = 4.8 Hz, 4H), 2.78 (t, J = 4.8 Hz, 4H), 3.06 (s, 2H), 5.82 (bs, 2H), 6.74 (dd, J = 8.8 and 4.8 Hz, 2H), 6.93 (t, J = 8.8 Hz, 2H), 7.08 (d, J = 8.8 Hz, 2H), 7.24 (m, 2H), 7.42 (d, J = 8.4 Hz, 2H), 7.62 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 524.1. Anal. ($C_{28}H_{24}ClF_2N_3OS$): C, H, N.

*[2-Amino-5-(4-chlorophenyl)-4-[(4-phenylpiperazin-1-yl)methyl]-thiophen-3-yl](4-chlorophenyl)methanone (**4af**).* Following general procedure F, compound **4af** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 74%. Mp: 90−91 °C. ^{1}H NMR ($CDCl_3$): δ 1.88 (t, J = 5.0 Hz, 4H), 2.87 (t, J = 5.0 Hz, 4H), 3.08 (s, 2H), 5.86 (bs, 2H), 6.79 (d, J = 8.4 Hz, 2H), 6.82 (d, J = 8.4 Hz, 2H), 7.23 (d, J = 8.4 Hz, 2H), 7.27 (m, 3H), 7.37 (d, J = 8.4 Hz, 2H), 7.62 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 522.5. Anal. ($C_{28}H_{25}Cl_2N_3OS$): C, H, N.

*[2-Amino-4-[(4-benzylpiperazin-1-yl)methyl]-5-(4-chlorophenyl)-thiophen-3-yl]-(4-chlorophenyl)methanone (**4ag**).* Following general procedure F, compound **4ag** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow oil. Yield: 68%. ^{1}H NMR ($CDCl_3$): δ 1.82 (t, J = 5.2 Hz, 4H), 2.42 (t, J = 5.2 Hz, 4H), 3.06 (s, 2H), 3.54 (s, 2H), 5.78 (bs, 2H), 7.17 (d, J = 8.6 Hz, 2H), 7.26 (m, 5H), 7.32 (d, J = 8.4 Hz, 2H), 7.43 (d, J = 8.4 Hz, 2H), 7.63 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 536.5. Anal. ($C_{29}H_{27}Cl_2N_3OS$): C, H, N.

*{2-Amino-5-(4-chlorophenyl)-4-[(4-(4-fluorophenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4ah**).* Following general procedure F, compound **4ah** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 63%. Mp: 90−91 °C. ^{1}H NMR ($CDCl_3$): δ 1.85 (t, J = 4.8 Hz, 4H), 2.78 (t, J = 4.8 Hz, 4H), 3.08 (s, 2H), 5.82 (bs, 2H), 6.74 (d, J = 8.6 Hz, 1H), 6.78 (d, J = 8.6 Hz, 1H), 6.90 (t, J = 8.6 Hz, 2H), 7.26 (m, 2H), 7.34 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8.4 Hz, 2H), 7.67 (d, J = 8.6 Hz, 2H). MS (ESI): $[M]^+$ = 540.5. Anal. ($C_{28}H_{24}Cl_2FN_3OS$): C, H, N.

*{2-Amino-4-[(4-(4-chlorophenyl)piperazin-1-yl)methyl]-5-(4-chlorophenyl)thiophen-3-yl}(4-chlorophenyl)methanone (**4ai**).* Following general procedure F, compound **4ai** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 74%. Mp: 77−78 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (t, J = 4.8 Hz, 4H), 2.81 (t, J = 4.8 Hz, 4H), 3.09 (s, 2H), 5.80 (bs, 2H), 6.70 (d, J = 9.0 Hz, 2H), 7.14 (d, J = 9.0 Hz, 2H), 7.22 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8.4 Hz, 2H), 7.65 (d, J = 8.6 Hz, 2H). MS (ESI): $[M]^+$ = 556.8. Anal. ($C_{28}H_{24}Cl_3N_3OS$): C, H, N.

*{2-Amino-5-(4-chlorophenyl)- 4-[(4-(3,4-dichlorophenyl)-piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4aj**).* Following general procedure F, compound **4aj** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 64%. Mp: 108−110 °C. ^{1}H NMR ($CDCl_3$): δ 2.17 (t, J = 4.8 Hz, 4H), 2.82 (t, J = 4.8 Hz, 4H), 3.22 (s, 2H), 5.83 (bs, 2H), 6.62 (dd, J = 9.2 and 2.8 Hz, 2H), 6.82 (d, J = 2.8 Hz, 1H), 7.11 (d, J = 8.8 Hz, 2H), 7.34 (d, J = 8.8 Hz, 2H), 7.44 (d, J = 7.4 Hz, 2H), 7.68 (d, J = 7.4 Hz, 2H). MS (ESI): $[M]^+$ = 591.4. Anal. ($C_{28}H_{23}Cl_4N_3OS$): C, H, N.

*{2-Amino-5-(4-chlorophenyl)-4-[(4-(4-(trifluoromethyl)phenyl)-piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4ak**).* Following general procedure F, compound **4ak** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 72%. Mp: 75−77 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (t, J = 4.8 Hz, 4H), 2.94 (t, J = 4.8 Hz, 4H), 3.09 (s, 2H), 5.82 (bs, 2H), 6.78 (d, J = 8.8 Hz, 2H), 7.24 (d, J = 8.4 Hz, 2H), 7.28 (m, 2H), 7.34 (d, J = 8.4 Hz, 2H), 7.68 (d, J = 8.4 Hz, 2H), 7.44 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 556.1. Anal. ($C_{29}H_{25}ClF_3N_3OS$): C, H, N.

*{2-Amino-5-(4-chlorophenyl)-4-[(4-(3-(trifluoromethyl)phenyl)-piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4al**).* Following general procedure F, compound **4al** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 62%. Mp: 94−96 °C. ^{1}H NMR ($CDCl_3$): δ 2.02 (t, J = 4.8 Hz, 4H), 2.92 (t, J = 4.8 Hz, 4H), 3.04 (s, 2H), 5.82 (bs, 2H), 6.84 (d, J = 8.4 Hz, 2H), 7.02 (d, J = 8.4 Hz, 2H), 7.44 (m, 6H), 7.63 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 556.1. Anal. ($C_{29}H_{25}ClF_3N_3OS$): C, H, N.

*{2-Amino-5-(4-chlorophenyl)-4-[(4-(4-chloro-3-(trifluoromethyl)-phenyl)piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)-methanone (**4am**).* Following general procedure F, compound **4am**

was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid, yield: 63%. Mp: 170−172 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (t, J = 4.8 Hz, 4H), 2.89 (t, J = 4.8 Hz, 4H), 3.09 (s, 2H), 5.84 (bs, 2H), 6.82 (dd, J = 8.4 and 1.2 Hz, 2H), 7.00 (d, J = 1.2 Hz, 2H), 7.27 (m, 1H), 7.34 (d, J = 8.4 Hz, 2H), 7.44 (d, J = 8.4 Hz, 2H), 7.63 (d, J = 8.4 Hz, 2H). MS (ESI): $[M]^+$ = 624.9. Anal. ($C_{29}H_{23}Cl_3F_3N_3OS$): C, H, N.

*{2-Amino-5-(4-chlorophenyl)-4-[(4-(2-(trifluoromethyl)phenyl)-piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4an**).* Following general procedure F, compound **4an** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow oil. Yield: 67%. ^{1}H NMR ($CDCl_3$): δ 1.88 (t, J = 4.8 Hz, 4H), 2.62 (t, J = 4.8 Hz, 4H), 3.08 (s, 2H), 5.77 (bs, 2H), 7.22 (m, 4H), 7.34 (d, J = 8.8 Hz, 2H), 7.44 (d, J = 8.8 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H), 7.73 (d, J = 8.2 Hz, 2H). MS (ESI): $[M]^+$ = 556.0. Anal. ($C_{29}H_{25}ClF_3N_3OS$): C, H, N.

*{2-Amino-5-(4-chlorophenyl)-4-[(4-(4-(trifluoromethoxy)phenyl)-piperazin-1-yl)methyl]thiophen-3-yl}(4-chlorophenyl)methanone (**4ao**).* Following general procedure F, compound **4ao** was purified by chromatography eluting with EtOAc:petroleum ether 3:7 to give a yellow solid. Yield: 64%. Mp: 90−91 °C. ^{1}H NMR ($CDCl_3$): δ 1.89 (t, J = 4.8 Hz, 4H), 2.85 (t, J = 4.8 Hz, 4H), 3.09 (s, 2H), 5.80 (bs, 2H), 6.75 (d, J = 9.4 Hz, 2H), 7.03 (d, J = 8.4 Hz, 2H), 7.22 (m, 2H), 7.34 (d, J = 8.6 Hz, 2H), 7.42 (d, J = 8.6 Hz, 2H), 7.67 (d, J = 8.2 Hz, 2H). MS (ESI): $[M]^+$ = 606.5. Anal. ($C_{29}H_{24}Cl_2F_3N_3O_2S$): C, H, N.

Biology Experiments. *Materials.* [^{3}H]DPCPX ([^{3}H]-1,3-dipropyl-8-cyclopentylxanthine; specific activity, 120 Ci/mmol) and [^{3}H]CCPA ([^{3}H]-2-chloro-N6-cyclopentyladenosine; specific activity, 55 Ci/mmol) were obtained from Perkin-Elmer Research Products (Boston, MA); [^{3}H]ZM 241385 ([^{3}H](4-(2-[7-amino-2-(2-furyl)-[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 Amersham International (Buckinghamshire, UK). DPCPX (1,3-dipropyl-8-cyclopentylxanthine), R-PIA ((*R*)-*N*^{6}-(L-2-phenylisopropyl)adenosine), and CPA (*N*^{6}-cyclopentyladenosine) were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade and obtained from commercial sources.

Cell Membrane Preparation. 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*g*, and the supernatant was then centrifuged for 30 min at 100 000*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; and 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.[21]

Binding Experiments in hA_1CHO Membranes. *[^{3}H]CCPA Binding Experiments.* Saturation binding experiments of [^{3}H]CCPA (0.05−20 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.[18a] Binding experiments were carried out in triplicate in a final volume of 250 μL containing diluted membranes, 1 nM [^{3}H]CCPA, 50 mM Tris-HCl (pH 7.4), and the tested compounds at 10 μM concentration for 90 min at 25 °C.[18a] Nonspecific binding was defined as binding in the presence of 1 μM R-PIA.

[^{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.[22] Nonspecific binding was defined as binding in the presence of 1 μM DPCPX.

Assay of the Antagonist Activity versus A_1-, A_{2A}- and A_3ARs. A_1-, A_{2A}-, and A_3AR 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.[22−24] 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 MgCl2 (pH 7.4) at 4 °C for 60 min, and in 50 mM Tris HCl, 10 mM MgCl2, 1 mM EDTA (pH 7.4) at 4 °C for 120 min to study A_1-, A_{2A}-, and A_3ARs, respectively. Nonspecific 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 a Packard Tri Carb 2810 TR scintillation counter (Perkin-Elmer).

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 effect of allosteric enhancers were studied at 10 μM concentration that was added to the mixture for a further 10 min. The effect of 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 2000*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.[24] 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 2000*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).

Data Analysis. Saturation and competition binding experiments were analyzed with the program LIGAND, which performed a weighted, nonlinear, least-squares curve fitting.[25] 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.[26] All experimental data are expressed as mean ± standard error of the mean (SEM.) of three or four independent experiments performed in duplicate.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

GPCRs, G protein-coupled receptors; [^{3}H]DPCPX, [^{3}H]1,3-dipropyl-8-cyclopentylxanthine; [^{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; [^{3}H]CCPA, [^{3}H]-2-chloro-N^6-cyclopentyladenosine; [^{3}H]ZM 241385, [^{3}H](4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]-triazin-5-ylamino]ethyl)phenol); CPA, *N*^{6}-cyclopentyladenosine; CHO, Chinese hamster ovary; cAMP, adenosine 3′,5′-cyclic monophosphate; AEs, allosteric enhancers; NBS, *N*-bromosuccinimide; $PdCl_2$(DPPF), [1,1′-bis(diphenylphosphino)-ferrocene]dichloropalladium(II) complex with dichloromethane; CsF, cesium fluoride; EWG, electron-withdrawing group; ERG, electron-releasing group; CNS, central nervous system.

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(20) 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 (ref 9) 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 (ref 12) reported an inhibition of [^{3}H]DPCPX binding to human A_1 receptors by PD 81,723 of only 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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