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MEDICINAL CHEMISTRY RESEARCH

SYNTHESIS AND BIOLOGICAL EVALUATION OF ALLOSTERIC A_1-ADENOSINE RECEPTOR MODULATORS STRUCTURALLY RELATED TO (2-AMINO-4,5,6,7-TETRAHYDRO-BENZO[B]THIOPHEN-3-YL)-(4-CHLORO-PHENYL)-METHANONE, A POTENT COMPOUND USEFUL TO REDUCE NEUROPATHIC PAIN

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Abstract: New derivatives of (2-amino-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)-(4-chlorophenyl)-methanone (compound **1**), an allosteric enhancer of agonist binding to the A_1-adenosine receptor, have been synthesized and evaluated in an intact cell assay at different concentrations to determine which among them were potential allosteric enhancers of the action of adenosine to activate the human-A_1adenosine receptor. None of the synthesized compounds appear to be more potent than **1** at a concentration of 10 μM. Most of the compounds increase the cAMP content of CHO cells expressing the human A_1-adenosine receptor, indicating an antagonist activity. Only two of the evaluated compounds (**2** and **8**) appeared to be allosteric enhancers at high concentration (10 μM).

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Adenosine is an autocoid that modulates numerous functions in the cardiovascular and other organ systems. The actions of adenosine are mediated by at least four different cell surface P_1-purinoreceptor (P) subtypes classified as A_1, A_{2A}, A_{2B}, and A_3.[1] These receptor subtypes belong to the superfamily of G protein-coupled receptors and are widely distributed throughout the body.[2,3] Their indiscriminate activation may cause undesiderable side effects. For this reason, it would be desirable that drugs which target these receptors have some degree of organ selectivity.

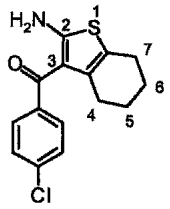
Extracellular adenosine, as a breakdown product of adenosine 5'-triphosphate (ATP), protects tissues from ischemic damage by lowering oxygen demand and increasing oxygen supply. The cytoprotective effect is thought to be due to the activation of the A_1-adenosine receptor. Agents that increase the activation of A_1-adenosine receptor in response to adenosine would be useful in conditions characterized by a localized oxygen deficit, such as angina, myocardial ischemia and stroke.[4]

A variety of adenosine-mediated effects occurs via the A_1 adenosine receptors, highly expressed in the central nervous system (CNS), and in other tissues such as kidney, lung, bladder and heart.[2,3] The widespread expression of A_1-adenosine receptors and the lack of sufficiently selective A_1-adenosine agonists have been a major impediment to the successful development of direct-acting adenosine receptor agonists to exploit the cytoprotective properties of adenosine. Thus, it would be of great therapeutic importance to have compounds that are able to enhance the activation of A_1-adenosine receptors by the endogenous ligand, adenosine, within specific target tissues. Such an opportunity of intervention is provided by the concept of allosteric modulation of G-protein-coupled receptors (GPCRs).[5]

Allosteric enhancers, upon binding, are believed to stabilize a conformation of the A_1-adenosine receptor that has a high affinity for agonists. This effect is manifested as a slowing of the rate of dissociation of agonist from the receptor.[6] In addition, an allosteric enhancer appears to stabilize an active conformation of the receptor even in the absence of an agonist. Thus, in cells with A_1-adenosine receptors that are active spontaneously in the absence of an agonist, such as the Chinese hamster ovary (CHO) cells used in this study, an allosteric enhancer may increase the number of receptors that are active at any given time, and thereby cause a change in cell function.[7,8] The currently available allosteric enhancers of agonist binding to the A_1-adenosine receptor have

several non-specific actions. These non-specific actions include antagonism of the A_1 adenosine receptor[7, 9] and inhibition of the activity of adenylyl cyclase.[8]

Bruns and co-workers reported that 2-amino-3-benzoylthiophene derivatives are capable both of enhancing the binding and activity of reference A_1 receptor agonists, such as N^6-cyclopentyladenosine (CPA), to the A_1-adenosine receptor and, usually at higher concentrations, acting as competitive antagonists at the same receptor.[9] Therefore, the concentration range where these compounds can enhance the effects of agonists is limited.[6] Among the compounds tested by Bruns, it was demonstrated that (2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-(4-chlorophenyl)-methanone (compound **1**) represents a specific and selective allosteric enhancer of agonist binding to the A_1 receptor, with the best ratio of enhancement to antagonistic action at this receptor.[10]



Compound **1**

Compound **1** is selective for adenosine A_1 receptors, having no effects on receptors of other classes or on other adenosine receptor subtypes. The same compound appears to be active for reducing or eliminating hyperexcited sensory nerve functions in several models of neuropathic pain.[10,11] Some examples of neuropathic pain are diabetic neuropathy, post-herpetic neuralgia, trigeminal neuralgia, pain associated with acquired immune deficiency syndrome (AIDS) infection, pain due to cancer treatment, traumatic injury and pain due to peripheral vascular disease.

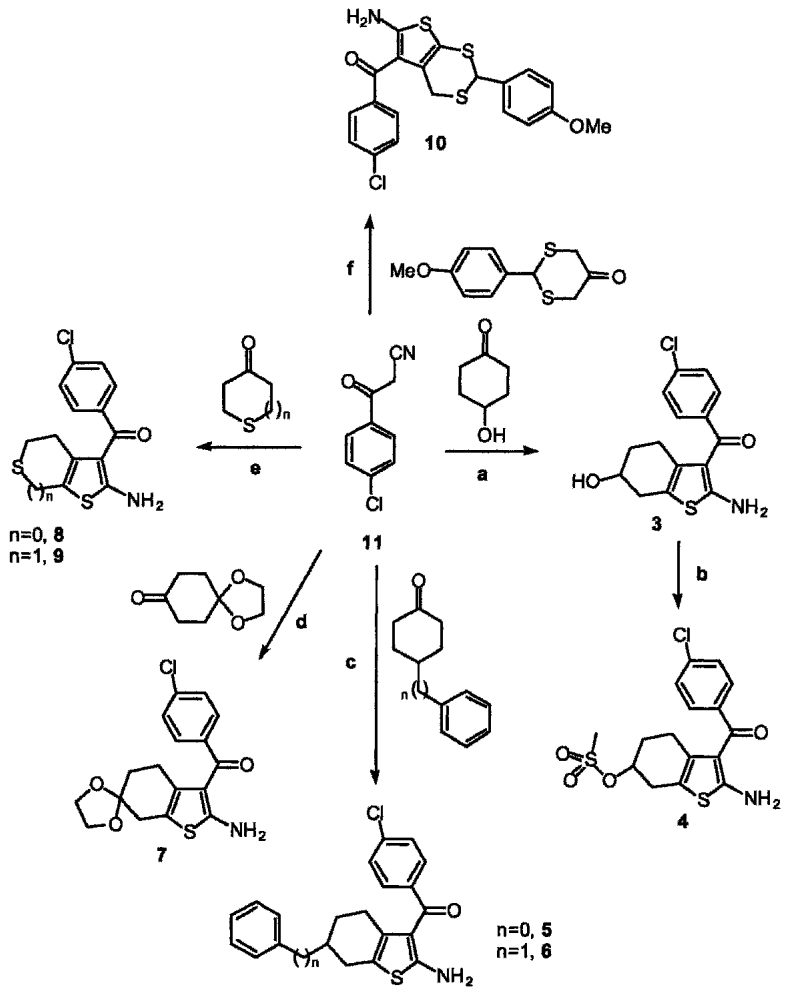
The purpose of our investigation was to synthesize and biologically evaluate a new series of derivatives of **1**, modified on the 4 and 5 position on the 2-amino-3-(4-chlorobenzoyl) moiety, and to establish the structural requirements and the structure-activity relationship for enhancement of

the action of the adenosine at the human A_1-adenosine receptor. In particular, we have examined different types of modifications of compound **1**. By the synthesis the benzo[b]thiophene derivative **2**, we have evaluated the effect due to aromatisation of the 4,5,6,7-tetrahydrobenzene ring fused with the thiophene. In a first series of derivatives (compounds **3-7**), the biological effect due to the insertion of different substituents in the 6-position of the 4,5,6,7-tetrahydrobenzo[b]thiophene moiety was studied. Finally, in a second series of derivatives (compounds **8-10**), we have evaluated the effect due to the presence of one or two sulphur atoms in the 6 or the 5 and 7 positions of the 4,5,6,7-tetrahydro-benzo[b]thiophene system.

Chemistry

The formation of 2-amino-3-(4-chlorobenzoyl)-thiophenes from the base-catalyzed condensation of carbonyl compounds and β-ketonitriles is achieved by the Gewald reaction.[16] This method was used for the synthesis of our new 2-amino-3-(4-chlorobenzoyl)thiophene derivatives **2-10** (Schemes 1-2), wherein the appropriate carbonyl compounds were reacted with the commercially available 4-chlorobenzoyl acetonitrile **11** and sulfur in ethanol in the presence of morpholine, according to the route shown in Scheme 1.

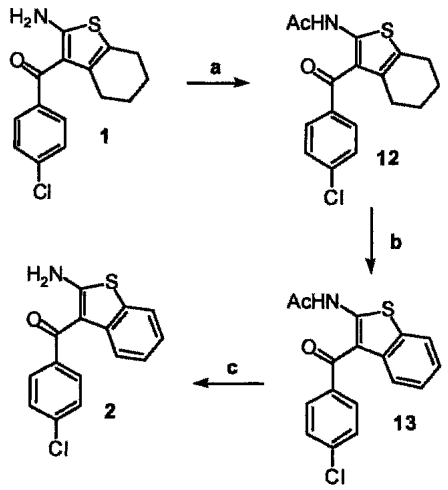
Scheme 1



Reagents. a: 4-hydroxycyclohexanone, S_8, morpholine, EtOH, 70¡C for 1h then 18 h at rt;
b: MsCl, DCM, TEA, 2h, r;t; c: 4-benzylcyclohexanone or 4-phenylcycloexanone, S_8, morpholine, EtOH, 70¡C for 1h then 18 h at rt;d: 1,4-cyclohexanedione monoethyleneacetal, S_8, morpholine, EtOH, 70¡C for 1h then 18 h at r.t.; e: 4,5-dihydro-3 (2H)-thiophenone or tetrahydro-4H-thiopyran-4-one, S_8, morpholine, EtOH, 70¡C for 1h then 18 h at rt; f: 2-(4-methoxy-phenyl)-[1,3]dithian-5-one, S_8, morpholine, EtOH, 70¡C for 1h then 18 h at rt.

Compound **2** was synthesized by a three-step synthesis described in Scheme 2, starting from compound **1**. Acetylation of the amino group using a mixture of acetic anhydride (Ac_2O) and pyridine gave **12**. The subsequent dehydrogenation with palladium on activated charcoal (Pd/C) with heating furnished the benzo[b]thiophene derivative **13**, which was transformed by saponification into the desired product **2**.

Scheme 2



Reagents. a: Ac_2O, pyridine , rx; **b:** 10%Pd/C moinsted with a 50% of water, heating; **c:** KOH, EtOH, rx, 2h.

Results and Discussion

The assays of allosteric enhancement were performed using CHO cells stably transfected to express the recombinant human A_1-adenosine receptor. Activation of these receptors causes an inhibition of the activity of adenylyl cyclase and a reduction of cAMP content of CHO cells. Allosteric enhancement was measured as the ability of the compounds **1-10** at four different concentrations (0.01, 0.1, 1 and 10 μM) to reduce the cAMP content of CHO:hA_1 cells. The results are shown in Table 1. It is important to note that compounds **3-6** and **10** were synthesized and biologically evaluated in their racemic forms.

The effect of each tested compound on cAMP content was presented as a percentage of the value of cAMP content in the absence of drug (control, 100%). A decrease of cAMP content is indicated in Table 1 as a negative percentage change of cAMP content from control (absence of compound) in the presence of the tested compound. Compounds with the potential to be allosteric enhancers of activation of human A_1-adenosine receptors decrease the content of cAMP in CHO cells expressing human A_1-adenosine receptors. Receptors in an active conformation in CHO:hA_1 cells cause a detectable inhibition of adenylyl cyclase activity. Allosteric enhancers are thought to

stabilize the active conformation of the A_1-adenosine receptors, leading to a reduction in the cAMP content of the cells, whereas compounds that increased cAMP content are identified as A_1-adenosine receptor inverse agonists. Allosteric enhancers cannot be distinguished from agonists by use of this functional assay alone. Thus, this assay assessed the overall effect for each tested compound as enhancer or antagonist at the A_1-adenosine receptor.

Table 1. Percentage change in cAMP content of CHO cells in presence of compounds **1-10**

	Change in cAMP Content from Control (mean±SEM)			
	Concentration of compound			
Compound	0.01 μM	0.1 μM	1 μM	10 μM
1	-8±3	-23±3	-26±2	-55±2
2	-8±2	-5±6	-6±7	-29±3
3	-3±4	-13±4	-2±6	52±5
4	+11±4	-1±6	+15±6	+46±5
5	+20±0.4	-1±4	-11±6	-7±3
6	-11±7	-6±4	-1±4	+16±7
7	-17±8	-14±8	+3±8	+28±9
8	-1±4	+11±4	+1±3	-35±4
9	+15±3	-10±4	+16±5	-0.3±5
10	+4±5	-13±4	-17±4	-5±3

Two of the new tested compounds (derivatives **2** and **8**) appeared to be enhancers, whereas the remaining compounds appeared to be antagonists of the A_1-adenosine receptor. None of the tested compounds had a greater efficacy than **1**. Because most of the known allosteric enhancers are also A_1-adenosine receptor antagonists at some (usually high) concentration[9], the fact that none of the tested compounds had a greater efficacy than did compound **1** at 10 μM could be explained by a potential antagonist effect of the compounds at high concentrations.

The dehydrogenation of the compound **1** yields the corresponding benzo[b]thiophene derivative **2**, which is moderately active but less potent as an allosteric enhancer at higher concentrations (0.1-10 μM) with respect to **1**.

The introduction of a hydrophilic and electron-donating hydroxy group on the 6-position of the tetrahydrobenzo[b]thiophene moiety of compound **1** to furnish the compound **3** does not improve the allosteric enhancer activity at any of the tested concentrations. For this latter compound, the reduction in activity may be attributed to both steric and electronic factors. The same biological activity was observed for compound **4**, the methanesulfonyl ester (MsO) of **3**. The absence of allosteric enhancer activity observed for **4** could be due to the steric hindrance exerted by the MsO group. No increase of activity was observed with the introduction of bulky hydrophobic groups, such as phenyl and benzyl (compounds **5** and **6**, respectively) in the 6-position of the tetrahydrobenzo[b]thiophene ring of compound **1**. Also, the presence of a more hydrophilic [1,3] dioxolane moiety at this position was detrimental to the activity of this compound as an allosteric enhancer. In fact, the corresponding compound **7** did not lead to an increase of the allosteric enhancer activity at 1 and 10 μM. In contrast, compound **7** was more active than **1** at a lower concentration (0.01 μM).

When the methylene in position six of the tetrahydrobenzo[b]thiophene ring of compound **1** was replaced by a sulphur atom to give **9**, we observed a loss of activity at all concentrations tested. Activity increased when, in this latter compound, the methylene unit between the sulphur and the 5-position of the thiophene ring was removed, to afford compound **8**. At 10 μM, compound **8** was more active than the homologue **9**, and only slightly less effective as an enhancer than **1**.
The insertion of two sulphur atoms at the 5 and 7-positions and a p-methoxyphenyl ring at the 6-position of compound **1**, furnished compound **10**, that demonstrated low activity as an enhancer at a concentration of 0.1 and 1 μM.

CHO cells expressing the human adenosine A_1 receptor (hCHO-A_1), and rat brain and human cortex membrane preparations containing native adenosine A_1 receptors were used to evaluate the effect of one selected compound, corresponding to derivative **5**, on the binding of the radiolabelled agonist [^{3}H]CCPA to A_1 receptor. An increase in the 1 nM [^{3}H]CCPA binding has been anticipated due to the presence of a putative allosteric enhancer.[6] Compound **5** at 10 μM increased the binding of [^{3}H]CCPA to hCHO-A_1, human brain and rat cortex membranes by 65, 29 and 33%, respectively. It should be mentioned, however, that the limited solubility of tested compound precluded the recording of a full concentration-effect curve.

To determine whether compound **5** enhance agonist binding to A_1 receptors, saturation binding experiments were performed using membranes prepared from $hCHOA_1$. The maximum specific binding (B_{MAX}) of the A_1 receptor agonist [^{3}H]CCPA increased up to 53% in the presence of **5** (10 μM).

In conclusion, we have identified two novel allosteric enhancers of agonist binding to adenosine A_1 receptors (compound **2** and **8**). None of the synthesized compounds proved superior to the reference enhancer **1**. Several of the tested compounds (**3**, **4**, **6** and **7**) increased the cAMP content of $CHO:hA_1$ cells at a concentration of 10 μM, and the action of these compounds is similar to that of the inverse agonist 8-cyclopentyl-1,3-dipropylxanthine (CPX). Thus, these compounds appear to stabilize the receptor in a conformation that reduces receptor coupling to G protein and adenylyl cyclase.

The substituent at position six of the tetrahydrobenzo[b]thiophene ring significantly influenced activity. In fact, in the series of derivatives **3-7**, the presence of both polar and non-polar substituents at this position was detrimental to the activity of these compounds as allosteric enhancers. These biological data support the importance of the lack of substituents on the 6-position of the tetrahydrobenzo[b]thiophene ring for the allosteric enhancer activity.

It should be noted however, that our assay of effects of putative enhancers on cAMP content of CHO cells expressing human A_1-adenosine receptors is not a specific assay of allosteric enhancement of agonist binding. Our assay does not measure directly the interaction between receptor activation and G protein activation, and our observations may be complicated by drug actions not related to enhancement, such as cell toxicity. However, the effect of a tested compound in the intact cell cAMP assay used in this study may be a more useful predictor of the effect of the compound *in vivo* than a binding assay that more specifically assesses allosteric enhancement. It is intended that these derivatives may be of value in better understanding adenosine A_1 receptor function.

EXPERIMENTAL SECTION

Abbreviations: acetic anhydride, Ac_2O; Chinese hamster ovary, CHO; chloroform, $CHCl_3$; cyclic adenosine-5'-monophosphate , cAMP; 8-cyclopentyl-1,3-dipropylxanthine, CPX; ethyl acetate,

EtOAc; N,N-dimethylformamide, DMF; N^6-cyclopentyladenosine, CPA; palladium on activated charcoal, Pd/C; potassium permanganate, $KMnO_4$; sodium sulphate, Na_2SO_4.

Materials and Methods

Cyclohexanone, 1,4-cyclohexanedione-*mono*-ethylene ketal, 4-phenyl cyclohexanone, tetrahydro-thiopyran-4-one and dihydrothiophen-3-one are commercially available and were purchased from Aldrich (Milan, Italy). The following carbonyl compounds were prepared as previously described: 4-benzylcyclohexanone[12], 2-(4-methoxy-phenyl)-[1,3]dithian-5-one[13], 4-hydroxy cyclohexanone[14] and 4-methanesulfonyloxy-cyclohexanone[15]. All commercially available compounds were used without further purification. Organic solutions were dried over anhydrous sodium sulphate (Na_2SO_4) (Aldrich, Milan, Italy). Dioxane was distilled from calcium hydride and dry N,N-dimethylformamide (DMF) was distilled from calcium chloride and stored over molecular sieves (3 Å). Dioxane and DMF were purchased from Aldrich (Milan, Italy). ^{1}H-NMR spectra were recorded on a Bruker AC 200 spectrometer (Rheinstetten, Germany). Chemical shifts (δ) are given in ppm upfield from tetramethylsilane (Fluka, Milan, Italy) as internal standard, and the spectra were recorded in appropriate deuterated solvents indicated in the procedure. All products reported showed ^{1}H-NMR spectra in agreement with the assigned structures. Melting points (mp) were determined on a Büchi B540 apparatus (Büchi, Zürich, Switzerland) and are uncorrected. Elemental analyses were conducted by the Microanalytical Laboratory of the Chemistry Department of the University of Ferrara using a Perkin-Elmer 240B CHN CHN analyser (Perkin-Elmer, Shelton, CT, USA). All reactions were performed under an inert atmosphere of dry nitrogen, unless otherwise described. Standard syringe techniques (glass/metal Luer, purchased from Aldrich, Milan, Italy) were applied for transferring dry solvents. Reaction progress and product mixtures were routinely monitored by TLC on silica gel (precoated F_{254} Macherey-Nagel plates, Macherey-Nagel, Düren, Germany) and visualized with aqueous potassium permanganate ($KMnO_4$). Flash chromatography was performed using 230-400 mesh silica gel (Merck, Darmstadt, Germany) and the solvent system indicated in the procedure. Reaction solvents, recrystallization and work-up solvents were used as received and were purchased from Carlo-Erba (Milan, Italy). In high-pressure hydrogenation experiments, a Parr (Parr Instrument, Moline, Illinois, USA) shaker in an autoclave was used.

N-[3-(4-chlorobenzoyl)-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl]-acetamide (12). Pyridine (10 drops) was added to a stirred solution of **1**[17] (1.46 g., 5 mmol) in Ac_2O (12 mL) at room temperature. The solution was refluxed for 2 h, poured in water, and extracted with ethyl acetate (EtOAc) (40 mL). The extract was washed successively with saturated aqueous sodium hydrogen

carbonate ($NaHCO_3$), water, and brine, dried over Na_2SO_4 and concentrated *in vacuo*. Recrystallization from petroleum ether afforded **12** as a yellow solid. Yield: 78%, m.p.= 213-215°C. ^{1}H-NMR ($CDCl_3$): δ 1.56 (m, 2 x H-5, 2H), 1.81 (m, 2 x H-6, 2H), 1.93 (m, 2 x H-4, 2H), 2.27 (s, CH_3, 3H), 2.68 (m, 2 x H-7, 2H), 7.42 (d, J=8.6 Hz, 2 x H aromatics, 2H), 7.50 (d, J=8.6 Hz, 2 x H aromatics, 2H), 11.3 (s, NH, 1H).

N-[3-(4-Chlorobenzoyl)-benzo[b]thiophen-2-yl]-acetamide (13). A mixture of **12** (601 mg, 1.8 mmol), 10% Pd-C (50% wet, 1.4 g.) and chloroform ($CHCl_3$) (20 mL) was stirred at room temperature for 10 min. and then the solvent was evaporated. The resulting powder was heated at 130°C for 20 h, cooled to room temperature, and extracted with EtOAc. The insoluble solids were removed by filtration, and the filtrate was concentrated *in vacuo*. Recrystallization from petroleum ether afforded **13** as a yellow solid. Yield:72%, m.p.=142-144°C. ^{1}H-NMR ($CDCl_3$) δ: 2.36 (s, CH_3, 3H), 6.95 (d, J=7.2 Hz, H-4, 1H), 7.14 (t, J=7.2 Hz, H-5, 1H), 7.19 (t, J=7.2 Hz, H-6, 1H), 7.43 (d, J=8.4 Hz, 2 x H aromatics, 2H), 7.58 (d, J=8.4 Hz, 2 x H aromatics, 2H), 7.73 (d, J=7.2 Hz, H-7, 1H), 11.9 (s, NH, 1H).

(2-Amino-benzo[b]thiophen-3-yl)-(4-chlorophenyl)-methanone (2). A mixture of **13** (153 mg, 0.464 mmol), 1N aqueous sodium hydroxide (0.5 mL, 0.5 mmol) and ethanol (10 mL) was refluxed for 5h, and then concentrated *in vacuo*. The residue was diluted with EtOAc, washed successively with water and brine, dried over Na_2SO_4, filtered, and evaporated *in vacuo*. The residue was purified by flash chromatography on silica gel using EtoAc-petroleum ether (2-8, v/v) as eluent. Recrystallization from petroleum ether furnished **2** as a yellow solid. Yield: 68%; mp 148-150°C. IR (KBr) cm^{-1} : 3296, 1578, 1465, 1418, 1238, 1014, 747. ^{1}H-NMR ($CDCl_3$) δ: 6.70 (d, J=9.0 Hz, H-4, 1H), 7.05 (m, H-5 and H-6, 2H), 7.27 (bs, NH_2, 2H), 7.41 (d, J=8.6 Hz, 2 x H aromatics, 2H), 7.48 (d, J=9.0 Hz, H-7, 1H), 7.54 (d, J=8.6 Hz, 2 x H aromatics, 2H). Elemental analysis for $C_{15}H_{10}ClNOS$; (calcd): C, 62,61; H, 3,50; Cl, 12,32; N, 4,87; (found): C, 62,33; H, 3,20; Cl, 12,08; N, 4,67.

General procedure for the synthesis of compounds 3-10. A mixture of 4-chlorobenzoyl acetonitrile (5 mmol), appropriate ketone (5 mmol), morpholine (0.44 mL, 5 mmol), and sulphur (164 mg, 5 mmol) was heated at 70° C for 1 h, then stirred at room temperature for 20 h. At the end of this period, the solvent was evaporated under reduced pressure and the residue diluted with ethyl acetate. After washing with water, the organic layer was dried (Na_2SO_4), filtered, then evaporated. The crude product was purified by flash column chromatography.

(2-Amino-6-hydroxy-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)-(4-chlorophenyl)-methanone (3). This product was purified by flash chromatography using a mixture of EtOAc-petroleum ether (6:4 v/v) as eluent. Pale yellow solid; Yield: 56%; m.p. 176-177 °C .IR (KBr) cm^{-1}: 3354, 3242, 3146, 2912, 1573, 1557, 1426, 1294, 1074, 773. ^{1}H-NMR ($CDCl_3$): δ 1.71 (m, 2 x H-4, 2H), 1.92 (m, 2 x H-5, 2H), 2.50 (dd, J=12.0 and 4.8 Hz, H-7a, 1H), 2.85 (dd, J=12.0 and 4.8 Hz, H-7b, 1H), 4.21 (bs, OH and H-6, 2H), 6.74 (bs, NH_2, 2H), 7.40 (m, H-aromatics, 4H). Elemental analysis for $C_{15}H_{14}ClNO_2S$; (calcd): C, 58,53; H, 4,58; Cl, 11,52; N, 4,55; (found): C, 58,21; H, 4,26; Cl, 11,23; N, 4,25.

[2-Amino-6-[(methanesulfonyl)oxy]-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl]-(4-chlorophenyl)-methanone (4). This product was purified by flash chromatography using a mixture of EtOAc-petroleum ether (3:7 v/v) as eluent. Yellow solid; Yield: 56%; m.p. 73-75 °C. IR (KBr) cm^{-1}: 3429, 2928, 1577, 1432, 1349, 1172, 944; ^{1}H-NMR ($CDCl_3$): δ 1.88 (t, J=6.2 Hz, 2 x H-4, 2H), 1.97 (t, J=4.8 Hz ,2 x H-5, 2H), 2.94 (dd, J=12.0 and 4.8 Hz, H-7a, 1H), 2.98 (dd, J=12.0 and 4.8 Hz, H-7b, 1H), 3.03 (s, CH_3, 3H), 5.07 (m, H-6, 1H), 6.74 (bs, NH_2, 2H), 7.41 (m, H-aromatics, 4H). Elemental analysis for $C_{16}H_{16}ClNO_4S_2$; (calcd): C, 49,80; H, 4,18; Cl, 9,19; N, 3,63; (found): C, 49,42; H, 3.92; Cl, 9,02; N, 3,42.

(2-Amino-6-phenyl-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-(4-chlorophenyl)-methanone (5). This product was purified by flash chromatography using a mixture of EtOAc-petroleum ether (2:8 v/v) as eluent. Orange solid; Yield: 76%; m.p. 102-104 °C. IR (KBr) cm^{-1} : 3296, 1573, 1427, 1266, 1089, 697. ^{1}H-NMR ($CDCl_3$): δ 1.56 (t, J=7.2 Hz, 2 x H-4, 2H), 1.56 (m, H-6, 1H), 1.89 (d, J=10.8 Hz ,2 x H-5, 2H), 2.72 (dd, J=12.0 and 4.8 Hz, H-7a, 1H), 3.12 (dd, J=12.0 and 4.8 Hz, H-7b, 1H), 7.22 (m, H phenyl and NH_2, 7H), 7.37 (d, J=8.6 Hz, 2 x H aromatics, 2H), 7.44 (d, J=8.6 Hz, 2 x H aromatics, 2H). Elemental analysis for $C_{21}H_{18}ClNOS$; (calcd): C, 68,56; H, 4,93; Cl, 9,64; N, 3,81; (found): C, 68,32; H, 4,64; Cl, 9,35; N, 3,68.

(2-Amino-6-benzyl-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)-(4-chlorophenyl)-methanone (6). This product was purified by flash chromatography using a mixture of EtOAc-petroleum ether (1:9 v/v) as eluent. Yellow solid; Yield: 56%; m.p. 60-62 °C. IR (KBr) cm^{-1}: 3435, 2922, 1576, 1430, 1088 and 700. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (m, H-6, 1H), 1.28 (t, J=7.4 Hz, 2 x H-5, 2H), 1.72 (m, H-4, 2H), 2.22 (dd, J=12.0 and 4.8 Hz, H-7a, 1H), 2.51 (dd, J=12.0 and 4.8 Hz, H-7b, 1H), 2.59 (d, J=7.0 Hz, CH_2Ph, 2H), 6.70 (bs, NH_2, 2H), 7.26 (m, H aromatics, 9H). Elemental analysis for $C_{22}H_{20}ClNOS$; (calcd): C, 69,19; H, 5,28; Cl, 9,28; N, 3,67; (found): C, 69,05; H, 5,02; Cl, 9,01; N, 3,42.

(2-Amino-6-spiro(1,4-dioxolan-2-yl)-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)-(4-chlorophenyl)-methanone (7). This product was purified by flash chromatography using a mixture of EtOAc-petroleum ether (1:4 v/v) as eluent. Pale yellow solid; Yield: 62%; m.p. 191-193 °C. IR (KBr) cm^{-1}: 3423, 3297, 1575, 1442, 1426, 1285, 1112, 1058, 949, 839, 678; ^{1}H-NMR ($CDCl_3$): δ 1.66 (t, J=6.4 Hz, 2 x H-5, 2H), 2.00 (t, J=6.4 Hz, 2 x H-4, 2H), 2.74 (s, 2 x H-7, 2H), 3.99 (s, OCH_2CH_2O, 4H), 6.77 (bs, NH_2, 2H), 7.41 (m, H aromatics, 4H). Elemental analysis for $C_{17}H_{16}ClNO_3S$; (calcd): C, 58,37; H, 4,61; Cl, 10,13; N, 4,00; (found): C, 58,03; H, 4,361; Cl, 9.87; N, 3.78.

(2-Amino-4,5-dihydrothieno[2,3-b]thiophen-3-yl)-(4-chlorophenyl)-methanone (8). This product was purified by flash chromatography using a mixture of ethyl acetate-petroleum ether (2:8 v/v) as eluent. Orange solid; Yield: 52%; m.p: 146-150 °C. IR (KBr) cm^{-1}: 3433, 1572, 1430, 1410, 1270 and 840; ^{1}H-NMR ($CDCl_3$): δ 2.38 (t, J=7.8 Hz, 2 x H-4, 2H), 3.51 (t, J=7.8 Hz, 2 x H-5, 2H), 6.89 (bs, NH_2, 2H), 7.42 (m, H-aromatics, 4H). Elemental analysis for $C_{13}H_{10}ClNOS_2$; (calcd): C, 52,78; H, 3,41; Cl, 11,98; N, 4,74; (found): C, 52,66; H, 3,21; Cl, 11,72; N, 4,57.

(2-Amino-4,7-dihydro-5*H*-thieno[2,3-c]thiopyran-3-yl)-(4-chlorophenyl)-methanone (9). This product was purified by flash chromatography using a mixture of ethyl acetate-petroleum ether (2:8 v/v) as eluent. Yellow solid; Yield: 66%; m.p. 142-146 °C. IR (KBr) cm^{-1}: 3422, 1582, 1440, 1405, 1255 and 830; ^{1}H NMR ($CDCl_3$): δ 2.18 (t, J=5.6 Hz , 2 x H-4, 2H), 2.60 (t, J=5.6 Hz, 2 x H-5, 2H), 3.63 (s, 2 x H-7, 2H), 6.62 (bs, NH_2, 2H), 7.39 (d, J=7.6 Hz, 2 x H-aromatics, 2H), 7.47 (d, J=7.6 Hz, 2 x H-aromatics, 2H). Elemental analysis for $C_{14}H_{12}ClNOS_2$; (calcd): C, 54,27; H, 3,90; Cl, 11,44; N, 4,52; (found): C, 54,03; H, 3,77; Cl, 11,23; N, 4,25.

[2-Amino-6-(4-methoxyphenyl)-4*H*-1,5,7-trithiainden-3-yl]-(4-chlorophenyl)-methanone (10). This product was purified by flash chromatography using a mixture of ethyl acetate-petroleum ether (1:1 v/v) as eluent. Yellow solid; Yield: 54%; m.p. 158-160 °C. IR (KBr) cm^{-1}: 3436, 3311, 1607, 1578, 1509, 1432, 1255, 1174, 1090, 1025, 838; ^{1}H-NMR ($CDCl_3$): δ 3.29 (d, J=6.8 Hz, 2 x H-4, 2H), 3.80 (s, OCH_3, 3H), 5.5 (s, H-6, 1H), 6.65 (bs, NH_2, 2H), 6.88 (t, J=8.6 Hz, 2 x H of p-chlorophenyl, 2H), 7.35 (d, J=8.6 Hz, 2 x H of p-chlorophenyl, 2H,), 7.40 (d, , J=8.4 Hz, 2 x H of p-methoxyphenyl 2H), 7.57 (d, , J=8.4 Hz, 2 x H of p-methoxyphenyl, 2H). Elemental analysis for $C_{20}H_{16}ClNO_2S_3$; (calcd): C, 55,35; H, 3,72; Cl, 8,17; N, 3,23; (found): C, 55,06; H, 3,48; Cl, 7.98; N, 3,02.

Cyclic AMP accumulation in CHO cells. Chinese hamster ovary cells expressing human recombinant A_1-adenosine receptors (CHO:hA_1 cells) at a density of approximately 8000 fmol/mg protein were prepared as previously described[18] and aliquots of these cells at low passage numbers were frozen and stored in liquid nitrogen. Upon the arrival of a group of compounds for testing in the laboratory, an aliquot of cells was removed from liquid nitrogen storage and grown in Ham's F-12 culture medium with 10% fetal bovine serum (Flow, Irvine, UK) and 0.5 mg/ml of antibiotic G-418 (Calbiochem, Darmstadt, Germany).[19] Cells were passaged thrice weekly. For experiments, aliquots of cells were placed into 12-well culture plates with culture medium, serum, and antibiotic for 48 hours, by which time the cells had grown to a confluent monolayer.

To begin an experiment, growth medium was removed from the culture plates and cells were washed once with Hanks' buffered saline solution. The wash solution was then removed and replaced with fresh Hanks' solution containing forskolin (1 μM), rolipram (20 μM), CPA (0.01 nM), adenosine deaminase (2 U/mL), and the allosteric enhancer to be tested. Forkolin, rolipram, CPA and adenosine deaminase were purchased from Sigma RBI (Milan, Italy). Forskolin was used to stimulate the activity of adenylyl cyclase, rolipram to inhibit cyclic adenosine-5'-monophosphate (cAMP) phosphodiesterase, adenosine deaminase to degrade endogenous adenosine, and CPA to cause a small increase of the number of activated adenosine receptors.

After 6 min of incubation at 36°C in the presence of drugs, the incubation solution was removed and hydrochloric acid (final concentration, 50 mM) was added to cells to terminate drug action. The content of cAMP in acidified extracts of cells was determined by radioimmunoassay as previously described.[18] Because the magnitude of the effects of allosteric enhancers on CHO:hA_1 cells changed subtly with passage number and differed slightly among different aliquots of cells, the action of tested compounds **1-10** were assayed in each experiment.

Allosteric enhancement was measured as the action of a test compound at different concentrations (0.01, 0.1, 1 and 10 μM) to reduce the cAMP content of CHO:hA_1 cells in the presence of 0.05-0.1 nM CPA. CPA (0.05-0.1 nM) alone causes a slight reduction of cAMP content of cells by activation of A_1-adenosine receptors. Allosteric enhancement of the action of CPA causes a further reduction of the cAMP content of CHO:hA_1 cells. Because the spontaneous activity of adenosine receptors in CHO:hA_1 cells causes an inhibition of adenylyl cyclase activity even in the absence of an agonist[19], antagonists of adenosine receptors increase cAMP content of cells. Therefore, compounds that

increased cAMP content of cells in this study were provisionally identified as A_1-adenosine receptor antagonists.

Cell line propagation. The expression of the human A_1 receptors in CHO cells has been previously described.[20] The CHO-A_1 cell clone was grown aderently 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) 0.2mg/ml at 37°C in 5% CO_2/95% air. Cells were subcultured (1:10) weekly.

Membrane preparation from CHO-A1 cells. For membrane preparation the culture medium was removed. The cells were washed with PBS and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with Polytron and the homogenate was spun for 10 min at 1,000 x g. The supernatant was then centrifuged for 30 min at 100,000 x g. The membrane pellet was resuspended in 50 mM Tris HCl buffer pH 7.4 and incubated with 2UI/ml of ADA for 30 min at 37°C. Then the suspension was stored at -80°C. The protein concentration was determined according to a Bio-Rad method[21] with bovine albumin as a standard reference.

Membrane preparation from rat cortex and human brain. Human cerebral cortex was obtained from the University of Ferrara, Medicina Legale Section with approval of human tissue use protocol. Rat cerebral cortex was harvested from adult male Wistar rats. All tissue culture reagents were obtained from Sigma.Cerebral cortical tissue from each species was homogenized using a Polytron (setting 6, 20s) in 20 volumes of ice-cold 50 mM Tris-HCl, pH 7.4. This crude membrane homogenate was then centrifuged at 48,000 x g for 15 min at 4°C. The resulting pellet was resuspended in buffer containing 2 IU/ml ADA to 20 mg/ml original tissue weight and incubated at 37°C for 30 min to remove endogenous adenosine. This membrane homogenate was recentrifuged at 48,000 x g for 15 min at 4°C. The resulting membrane pellet was resuspended and recentrifuged at 48,000 x g for 15 min at 4°C. The final membrane pellets were stored at –80°C until the time of assay.

^{3}[H]CCPA competition binding experiments. Binding of 1 nM [^{3}H]CCPA (2-chloro-N^6-cyclopentyladenosine) to A_1 receptors in hCHO-A_1, rat and human brain membranes in the absence and presence of increasing concentrations of tested compound was carried out in triplicate at 25°C for 90 min in 50 mM Tris-HCl, pH 7.4. Non specific binding was defined as binding in the presence of 10 μM R-PIA. [^{3}H]CCPA (specific activity, 55 Ci/mmol) were obtained from NEN Research Products (Boston, MA).

^{3}[H]CCPA saturation binding experiments. Saturation binding experiments of [^{3}H]CCPA (0.05 to 10 nM) to A_1 receptors expressed in hCHO-A_1 were performed in triplicate at 25°C for 90 min in 50 mM Tris-HCl, pH 7.4, in the absence and presence of tested compound.

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