Synthesis and Biological Evaluation of a New Series of 2,3,5-Substituted [1,2,4]-Thiadiazoles as Modulators of Adenosine A_1 Receptors and Their Molecular Mechanism of Action

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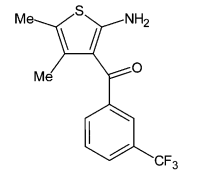
We synthesized two series (**7a**−**i** and **8a**−**i**) of 2,3,5-substituted [1,2,4]-thiadiazole analogues of SCH-202676 (**7a**, 2,3-diphenyl-5-*N*-methylimino-*2H*-[1,2,4]-thiadiazole) with emphasis on the *N*-imino substituent. Compounds **7a**−**g**,**i** and **8a**−**g** at a final concentration of 1 μM significantly inhibited [^{3}H]CCPA (2-chloro-N^6- cyclopentyladenosine) agonist binding to human A_1 adenosine receptors. At the same concentration, all compounds appeared to increase [^{3}H]DPCPX (1,3-dipropyl-8-cyclopentylxanthine) antagonist binding. Compound **7a** and LUF5855 (**7g**) were selected for further characterization and studied in both equilibrium and kinetic radioligand binding experiments. The results suggest a nonstoichiometric interaction with the receptor. Further bioanalytical procedures (HPLC and MS) provided proof for an unusual receptor interaction in which **7a** and **7g** upon incubation were transformed into their corresponding thioureas **5a** and **5g**. We suggest that the thiadiazoles are sulfhydryl modifying agents rather than allosteric modulators, as they appear to reversibly modify the sulfhydryl groups of cysteine residues in cell membrane preparations.

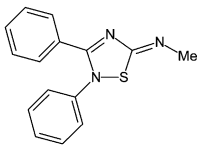
Introduction

Many G protein-coupled receptors (GPCRs) including adenosine receptors appear to have allosteric binding sites, occupation of which may modify the action of the endogenous ligand (i.e., hormone or neurotransmitter).[1,2] An allosteric modulator is thus best defined as a ligand that increases or decreases the action of an agonist or antagonist (the "orthosteric" ligand) by combining with a distinct (allosteric or allotopic) site on the receptor macromolecule.[3] Allosteric drugs are able to modulate receptor activity through conformational changes in the receptor protein that are transmitted from the allosteric to the orthosteric site. Allosteric modulators may have several advantages over orthosteric ligands as potential therapeutic agents, including a natural limitation of their effect (the endogenous ligand needs to be present) and tissue selectivity (the endogenous ligand concentrations may vary over different tissues).

In 1990, Bruns and co-workers reported that 2-amino-3-benzoylthiophene derivatives are capable of enhancing the binding and activity of reference A_1 receptor agonists, such as N^6-cyclopentyladenosine (CPA). However, the compounds were found to act as competitive antagonists at these receptors also.[4,5] PD81723 (Figure 1) became the reference allosteric modulator among them with the best ratio of enhancement to antagonistic action. Other derivatives of 2-amino-3-benzoylthiophene based on the structure of PD81723 have been synthesized. Among them, several compounds appeared more potent than PD81723.[6−10]



PD81723



SCH-202676, **7a**

Figure 1. Chemical structures of PD81723 and compound **7a**.

Recently, compound **7a** (Figure 1) was found to be a general allosteric modulator of both agonist and antagonist binding to GPCRs. The compound inhibited radioligand binding to a number of GPCRs, including the human μ-, δ-, and κ-opioid, α- and β-adrenergic, muscarinic M_1 and M_2, and dopaminergic D_1 and D_2 receptors.[11,12] In our previous work, a number of 2,3,5-substituted [1,2,4]-thiadiazole analogues of compound **7a**, with emphasis on substituents on both phenyl groups, were synthesized and tested as potential allosteric modulators of adenosine receptors.[13] They inhibited the binding of [^{3}H]CCPA (2-chloro-N^6- cyclopentyladenosine), a radiolabeled agonist for adenosine A_1 receptors, in a concentration-dependent manner (see also ref 14). Among them, 5-*N*-methylimino-2-(3-methylphenyl)-3-phenyl-2*H*-[1,2,4]-thiadiazole (**8a**) proved to be the most active with an EC_{50} value of 0.64 μM, 4 times more active than **7a**.

In the present study, we prepared two series of analogues based on **7a** and **8a**, in which the *N*-imino

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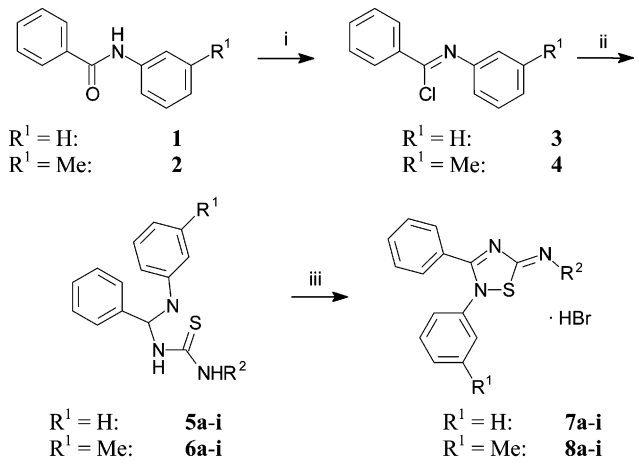
Scheme 1[a]



a: R^2 = Me; **b**: R^2 = Et; **c**: R^2 = Pr; **d**: R^2 = Bu; **e**: R^2 = 3-OH-Pr; **f**: R^2 = *i*Pr; **g**: R^2 = *c*Pentyl; **h**: R^2 = Ph; **i**: R^2 = Bn;

[a] Reagents: (i) $SOCl_2$; (ii) NaNCS, R^2NH_2; (iii) Br_2.

substituent (R^2 in Scheme 1) was now varied. Intrigued by the peculiar findings in both equilibrium and kinetic radioligand binding experiments, we examined their mechanism of action in more detail. We conclude that these compounds are best described as protein modifiers rather than allosteric modulators.

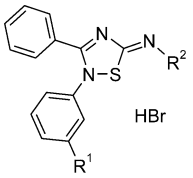
Results and Discussion

Chemistry. To prepare the compounds, we applied the following synthetic route.[15] Benzamides **1** and **2** were used as starting materials that were converted into benzimidoyl chlorides **3** and **4** (Scheme 1).[16] Substitution of chlorine by NCS followed by addition of an amine afforded thioureas **5a**−**i** and **6a**−**i** in moderate to good yields. Finally, an oxidation with bromine yielded the targeted 2,3,5-substituted [1,2,4]-thiadiazoles as their hydrobromide salts **7a**−**i** and **8a**−**i**.

Biology. All compounds were initially characterized in equilibrium radioligand displacement studies on human adenosine A_1 receptors. Both a radiolabeled agonist ([^{3}H]CCPA) and a radiolabeled antagonist ([^{3}H]-DPCPX (1,3-dipropyl-8-cyclopentylxanthine)) were used. Subsequently, selected compounds were used for further characterization by, for example, studying their influence on association and dissociation kinetics of the adenosine A_1 receptor radioligands.

All final 2,3,5-substituted [1,2,4]-thiadiazoles **7a**−**i** and **8a**−**i** were tested on human adenosine A_1 receptors (Table 1). All compounds at a final concentration of 1 μM significantly inhibited [^{3}H]CCPA binding except **8h**. In particular, **7a**−**g** and **8a**,**b** appeared to be most potent with 16−36% specific binding of radioligand remaining. At the same concentration, all compounds appeared to increase [^{3}H]DPCPX binding. At a lower concentration, (0.1 μM) none of the compounds apparently affected [^{3}H]CCPA binding. Introduction of alkyl R^2 groups with an increasing number of carbon atoms, methyl, ethyl, propyl, *iso*propyl, butyl, or the introduction of a 3-hydroxypropyl group or a cyclic alkyl group, namely, *cyclo*pentyl, did not affect the results of binding experiments significantly. However, for compounds with aromatic phenyl or benzyl groups as R^2 substituents, a smaller decrease in the inhibition of [^{3}H]CCPA binding was observed for both series of compounds **7** (for **7h** 66% and for **7i** 43%) and **8** (for **8h** 89% and for **8i** 66% specific binding of radioligand remaining). Comparing the compounds bearing the same R^2 but a different R^1 substituent (compound series **7** and **8**), we concluded that the values were only marginally influenced by the R^1 (3-methyl) substituent.

Table 1. Modulation of [^{3}H]CCPA and [^{3}H]DPCPX Binding to Human A_1 Adenosine Receptors by 2,3,5-Substituted [1,2,4]-Thiadiazoles



			% specific binding of radioligand remaining		
			[^{3}H]DPCPX[a]	[^{3}H]CCPA[a]	
compound	R^1	R^2	10^{-6} M	10^{-6} M	10^{-7} M
7a	H	Me	122	16	97
7b	H	Et	134	20	99
7c	H	Pr	135	26	98
7d	H	Bu	126	27	101
7e	H	3-OH−Pr	127	27	106
7f	H	*i*Pr	126	23	97
7g	H	*c*Pentyl	123	25	100
7h	H	Ph	106	66	104
7i	H	Bn	124	43	101
8a	Me	Me	132	23	96
8b	Me	Et	125	24	100
8c	Me	Pr	126	29	97
8d	Me	Bu	114	36	99
8e	Me	3-OH−Pr	142	29	97
8f	Me	*i*Pr	121	29	100
8g	Me	*c*Pentyl	101	35	96
8h	Me	Ph	107	89	96
8i	Me	Bn	116	66	99

[a] Data are expressed as means from three independent experiments performed in duplicate; individual values varied less than 10% for [^{3}H]CCPA binding and less than 35% for [^{3}H]DPCPX binding. The results are given as percentage specific binding of radioligand remaining, where total control binding is 100% and nonspecific binding is 0%.

One compound (**7g**) was analyzed in more detail, together with **7a**, for reasons of comparison. Both inhibited [^{3}H]CCPA binding in a concentration-dependent manner. The EC_{50} value of **7g** was 503 ± 39 nM compared to the EC_{50} value of **7a** (2800 ± 200 nM). The displacement curve for **7g** was steep ($n_H = -2.7 \pm 0.1$), although less pronounced than for **7a** ($n_H = -6.5 \pm 2.8$), full effects being observed within 1−2 concentration log units for both. The aberrant pseudo-Hill coefficients, not compatible with a 1:1 stoichiometric interaction with the receptor, prompted us to examine the compounds' mechanism of action in more detail.

We first analyzed the behavior of **7a** and **7g** (at concentrations of 1 μM) on the kinetics of the interaction between the radiolabeled agonist [^{3}H]CCPA and the receptor. Both association and dissociation of [^{3}H]CCPA were affected by the two compounds in that the extent of association and dissociation was inhibited (Figures 2A and 2B). For compound **7a**, total radioligand association (i.e., the asymptotic level of binding) was only half of the control value (49% vs 93%), for **7g** it was slightly higher (71%). The dissociation process was similarly inhibited; for **7a**, the lowest value of the

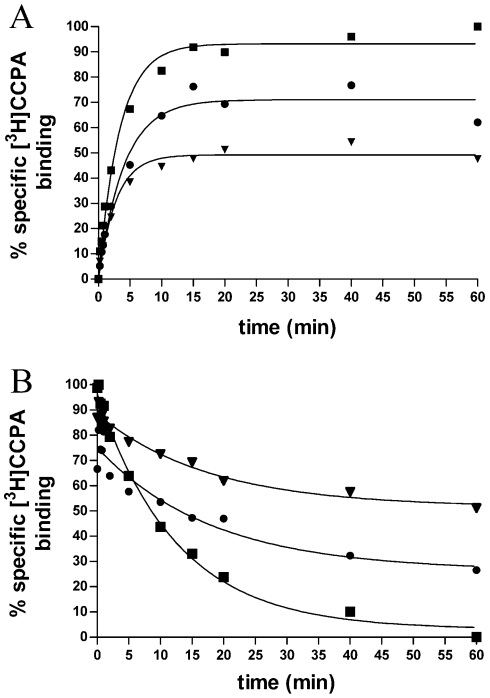


Figure 2. Association (A) and dissociation kinetics (B) of [^{3}H]-CCPA binding to and from human adenosine A_1 receptors. Data are from a representative experiment performed in duplicate. Radioligand binding is expressed as percent of specific binding. Concentrations of compounds **7a** (▼) and **7g** (●) were 1 μM. Control (■) was without compounds.

Table 2. Association and Dissociation Kinetic Parameters of [^{3}H]CCPA Binding to and from Human Adenosine A_1 Receptors in the Presence or Absence of **7a** and **7g** (1 mM)[a]

	association		dissociation	
	$t_{1/2}$ (min)	k_{on} (nM^{-1} min^{-1})	$t_{1/2}$ (min)	k_{off} (min^{-1})
control	2.6 ± 0.3	0.27 ± 0.02	10.8 ± 2.8	0.067 ± 0.016
7a	2.0 ± 0.1	0.35 ± 0.01	12.4 ± 0.4	0.056 ± 0.001
7g	2.9 ± 0.8	0.25 ± 0.07	12.0 ± 0.6	0.058 ± 0.003

[a] The values are ±SEM (n = 3).

asymptote was 51%, for **7g** 26%, compared to the control value of 3% radioligand remaining after 60 min (Figure 2B). Interestingly, the association and dissociation kinetic parameters (i.e., $t_{1/2}$ and k values) did not change significantly compared to the control values (Table 2). This finding, in fact, discounts the compounds to be allosteric modulators, which by definition influence these parameters, for either the dissociation or the association process or both. Rather, it seems that in the association kinetic experiments the thiadiazoles mask the receptors' primary binding sites, preventing [^{3}H]-CCPA from binding. In a similar vein, in dissociation kinetic experiments the radioligand seems to be locked into the cavity of the binding site by the thiadiazoles, preventing complete dissociation. In our former publication, other substituted thiadiazoles were similarly tested, but at a concentration of 10 μM. Under these conditions, the association of [^{3}H]CCPA binding to human adenosine A_1 receptors was almost negligible (<5%) and could not be characterized numerically.[13]

To shed further light on these findings, we used a number of bioanalytical approaches. We first studied the stability of the compounds per se. It appeared that the ^{1}H NMR spectrum of compound **7a** in DMSO did not change either in the course of 1 month at room temperature or after incubation at 65 °C for 5 h. We then reasoned that the compounds might react with protein components as present during the radioligand binding experiments. In Figure 3, graph A is the HPLC chromatogram of compound **7a** after incubation with CHO cell membranes expressing hA_1 receptors in Tris buffer. In fact, using membranes of wild-type CHO cells (i.e., not expressing the receptor), we obtained almost identical results (data not shown). The chromatogram shows that in the presence of membranes a part of **7a** (t_r = 7.6 min) is transformed into its synthetic thiourea precursor **5a** (t_r = 6.8 min, also checked independently with **5a** itself). We subsequently carried out experiments in which compounds **7a** and **7g** were reacted with most of the 20 essential amino acids. Only after reaction of **7a** and **7g** with cysteine we found a significant change in the respective HPLC chromatograms. In the presence of cysteine, a full conversion of **7a** into **5a** was observed (Figure 3B). Next to its t_r value, the identity of **5a** was confirmed after isolation from the reaction mixture through its UV and ^{1}H NMR spectra. In a similar approach, we identified **5g** (now with HPLC-MS) as the resulting product of the interaction of **7g** with either cell membranes or cysteine.

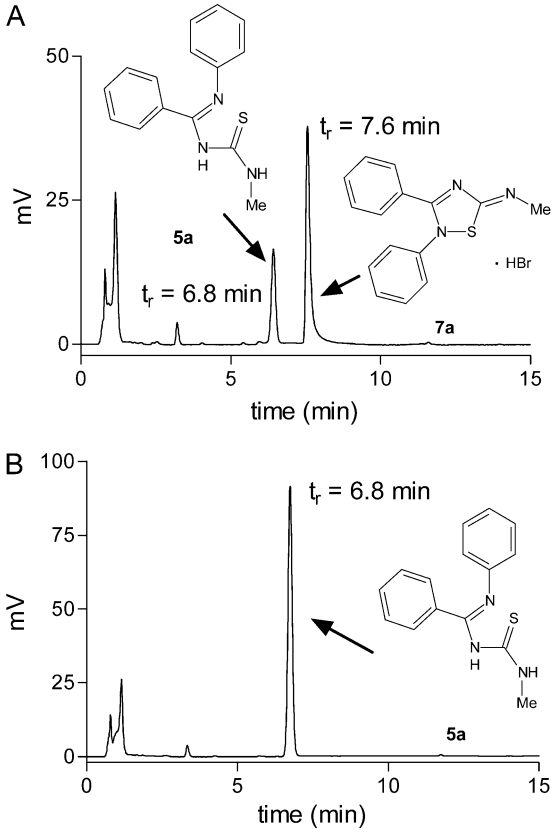
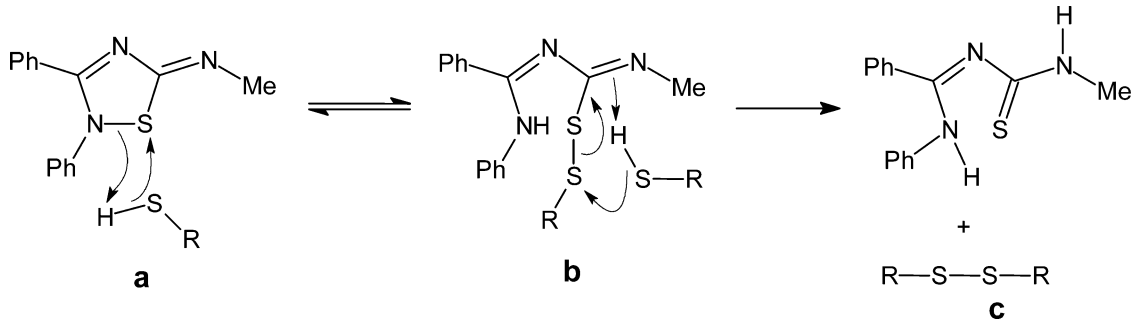


Figure 3. HPLC chromatograms (at λ = 270 nm). Graph A: product from the interaction of **7a** (10^{-5} M) with CHO cell membranes expressing human adenosine A_1 receptors. Graph B: product from the incubation of **7a** (10^{-5} M) with cysteine (10^{-3} M).

From graphs A and B in Figure 3, it appears that the amounts of **5a** formed are different. Complete conversion is obtained in the presence of cysteine alone (Figure 3B), whereas this is not the case in the presence of CHO cell membranes expressing hA_1 receptors. It can be calculated that with 20 μg of protein present and an average cysteine content in mammalian proteins of

Scheme 2



2.26%[17] the final cysteine concentration is approximately 10^{-5} M in the experimental setup that led to graph A. This is roughly equal to the thiadiazole concentration, whereas in graph B there is a 100-fold molar excess of cysteine compared to the thiadiazole.

We suggest that the mechanism of action is the following (Scheme 2). Compound **7a** (and analogues) binds to the receptor; however, it is reduced by cysteine residues (**a**), present either in the binding site or in any protein in the membrane preparation, into a covalent thiourea adduct (**b**). Formation of this adduct is not the end stage; it is either reverted to **7a** or transformed into the free thiourea **5a** in the presence of a reducing agent (e.g., another cysteine (R−SH, **c**)). In the latter case, two thiol groups are oxidized to form a disulfide bond. These disulfide bridges are supposed to be responsible for the behavior of **7a** and analogues in their capability of noncompetitively influencing radioligand binding. We anticipate the process to be reversible, since we[13] and Fawzi et al.[11] had demonstrated that the effect of **7a** on radioligand binding could be washed out.

The stability and reactivity of other thiadiazoles have been studied by several authors. Pan et al. have demonstrated that thiadiazoles bearing aryl R^2 substituents shown to be active as melanocortin MC4 receptor agonists may undergo a thermal rearrangement to 2-amidinobenzothiazole in DMSO.[18,19] The same authors reduced 2-(4-methylphenyl)-3-phenyl-5-(4-methoxyphenylamino)-[1,2,4]-thiadiazolium bromide to its precursor imidoylthiourea by dithioerythritol. They suspected that this compound also reacts with the thiol group on cysteine residues or that it oxidizes two thiol groups to create a disulfide bond, causing irreversible binding to the receptor.[18] Marrano et al. found this type of compound to be irreversible inhibitors of guinea pig liver transglutaminase through a mechanism of action similar to that suggested by Pan and co-workers (i.e., irreversible inhibition by the formation of a disulfide bond upon opening of the thiadiazole ring).[20] Others found that 1,2,4-thiadiazoles are susceptible to nucleophilic attack at the sulfur atom followed by ring opening.[21] Moreover, 1,2,4-thiadiazole derivatives reacted with active site thiol residues of certain cysteine proteases to form covalent adducts.[22] A similar mechanism was suggested to explain how 1,2,4-thiadiazoles act as cathepsin B inhibitors. The compounds were proven to undergo ring opening with thiols but not with amines and/or alcohols. The C3 substituent can be tailored to tune the reactivity of the heterocycle, that is to increase or decrease the rate of ring opening by thiols.[23] Next to the current thiadiazoles, other and better-known sulfhydryl group modifying agents are *N*-ethylmaleimide (NEM) and phenylmercuric chloride (PMC). It is well established that treatment with 1 mM NEM shifts A_1 adenosine receptors from a high-affinity to a low-affinity state for agonists.[24] This effect has been ascribed to the alkylation of a sulfhydryl group of the inhibitory guanine nucleotide binding protein G_i. Phenylmercuric chloride (PMC) forms a covalent mercaptide with sulfhydryl groups, which can be reversed by treatment with dithiothreitol.[25−27] To this class of sulfhydryl modifiers may now be added the highly (re)active thiadiazoles, instead of classifying these compounds as allosteric modulators.

Conclusion

Thiadiazoles were synthesized with different *N*-imino substituents. Results of receptor−ligand binding experiments and stability studies by HPLC and HPLC-MS showed that the compounds are highly reactive sulfhydryl modifying agents rather than allosteric inhibitors. They appear to be reduced into their thiourea precursors by thiol groups of cysteine residues. This general feature explains their overall lack of selectivity.

Experimental Section

Chemicals. [^{3}H]DPCPX (128 Ci/mmol) was purchased from Amersham Pharmacia Biotech. [^{3}H]CCPA (54.9 Ci/mmol) was obtained from NEN. DPCPX was from Sigma-RBI. All other chemicals, including starting materials, were from standard sources and of the highest purity commercially available.

Instruments and Analysis. ^{1}H NMR spectra were measured at 200 MHz with a Bruker AC 200, Bruker AV 400, or Bruker DMX 600 spectrometer. ^{13}C NMR spectra were measured at 50, 100, or 150 MHz. Chemical shifts for ^{1}H and ^{13}C are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard; coupling constants are given in Hz. Melting points were determined with a Büchi capillary melting point apparatus and are uncorrected. Combustion analyses of new target compounds (series **7** and **8**) were performed by the analytical department of the Gorlaeus Laboratories, Leiden University (The Netherlands) and are within ±0.4% of theoretical values unless otherwise specified.

For HPLC analysis, samples were analyzed by means of a Gilson (Middleton, WI) solvent delivery system, equipped with a Gilson 234 sample injector (100 μL loop) and a Spectra focus (Thermo Separation Products, Breda, The Netherlands) diode array detector. The column was a 125 mm × 4.0 mm Prontosil Eurobond C18 (5.0 μm particles) from Bischoff (Leonberg, Germany). The effluent was monitored from 210 to 360 nm. For HPLC-MS analysis, the HPLC system was a Jasco low-pressure system, 900 series. The column was a 4.6 mm × 150 mm Alltima C18 with a flow of 1 mL/min. The MS system was an API 165 PE-SCJEX (Applied Biosystems), QI system.

Preparation of Benzamides and Benzimidoyl Chlorides. Compound **1** is commercially available, while compounds **2**−**4** were prepared according to known procedures.[16]

General Procedure for the Preparation of the Thioureas.[15]The appropriate benzimidoyl chloride was dissolved in acetone p.a. (1 mmol/3 mL). At −10 °C, a solution of NaNCS (1.1 equiv) in acetone (1 mmol/2 mL) was added dropwise. The mixture was stirred in the cooling bath until 0 °C was reached. The precipitate (NaCl) was removed by filtration over a glass filter with Hyflo. The filtrate was stirred on an ice bath, and the appropriate amine (1.1 equiv) was added. The reaction mixture was allowed to warm to room temperature (with stirring), then the solvent was removed by evaporation, and the residue was triturated with diethyl ether, collected on a glass filter, washed once more, and dried in vacuo to give the thiourea. This product was then used in the next step without further purification.

1-Methyl-3-(phenyl-phenyliminomethyl)thiourea (5a).[13] Scale: 20.9 mmol. Yield: 4.50 g (80%) of an off-white solid. ^{1}H NMR ($CDCl_3$): δ 3.26 (d, 3H, J = 4.4 Hz, NCH_3); 6.68 (d, 2H, J = 8.8 Hz, Ar); 6.98 (d, 1H, J = 9.5 Hz, Ar); 7.47 (m, 7H, Ar); 8.06 (s, 1H, NH); 11.84 (bs, 1H, NH).

1-Ethyl-3-(phenyl-phenyliminomethyl)thiourea (5b).[13] Scale: 9.2 mmol. Yield: 2.30 g (88%) of a pale yellow solid. ^{1}H NMR ($CDCl_3$): δ 1.33 (t, 3H, J = 7.3 Hz, CH_3); 3.76 (m, 2H, CH_2); 6.68 (d, 2H, J = 8.8 Hz, Ar); 6.97 (m, 1H, Ar); 7.24 (m, 7H, Ar); 8.00 (s, 1H, NH); 11.90 (bs, 1H, NH).

1-(Phenyl-phenyliminomethyl)-3-propyl-thiourea (5c). Scale: 7.0 mmol. Yield: 1.67 g (80%). ^{1}H NMR ($CDCl_3$): δ 1.02 (t, 3H, J = 7.30 Hz, CH_3); 1.69−1.79 (m, 2H, CH_2); 3.69 (q, 2H, J = 6.56 Hz, CH_2); 6.68 (d, 2H, J = 7.32 Hz, Ar); 6.92 (t, 1H, J = 7.30 Hz, Ar); 7.10−7.32 (m, 7H, Ar); 8.04 (s, 1H, NH); 12.00 (s, 1H, NH).

1-Butyl-3-(phenyl-phenyliminomethyl)thiourea (5d). Scale: 4.0 mmol. Yield: 0.64 g (52%). ^{1}H NMR ($CDCl_3$): δ 0.97 (t, 3H, J = 7.30 Hz, CH_3); 1.36−1.55 (m, 2H, CH_2); 1.60−1.78 (m, 2H, CH_2); 3.68−3.78 (m, 2H, CH_2); 6.67 (d, 2H, J = 8.04 Hz, Ar); 6.97 (t, 1H, J = 7.30 Hz, Ar); 7.11−7.36 (m, 7H, Ar); 7.99 (s, 1H, NH); 11.98 (s, 1H, NH).

1-(3-Hydroxypropyl)-3-(phenyl-phenyliminomethyl)-thiourea (5e). Scale: 7.0 mmol. Yield: 1.95 g (91%). ^{1}H NMR ($CDCl_3$): δ 1.83−1.93 (m, 2H, CH_2); 2.72 (s, 1H, OH); 3.75 (t, 2H, J = 7.76 Hz, CH_2); 3.87−3.96 (m, 2H, CH_2); 6.68 (d, 2H, J = 7.32 Hz, Ar); 6.97 (t, 1H, J = 7.30 Hz, Ar); 7.11−7.33 (m, 7H, Ar); 8.11 (s, 1H, NH); 12.06 (s, 1H, NH).

1-Isopropyl-3-(phenyl-phenyliminomethyl)thiourea (5f). Scale: 7.0 mmol. Yield: 1.90 g (91%). ^{1}H NMR ($CDCl_3$): δ 1.35 (d, 6H, J = 6.58 Hz CH_3); 4.47−4.64 (m, 1H, CH); 6.68 (d, 2H, J = 7.32 Hz, Ar); 6.97 (t, 1H, J = 7.30 Hz, Ar); 7.11−7.39 (m, 7H, Ar); 7.93 (s, 1H, NH); 11.90 (s, 1H, NH).

1-Cyclopentyl-3-(phenyl-phenyliminomethyl)thiourea (5g). Scale: 7.0 mmol. Yield: 1.96 g (87%). ^{1}H NMR ($CDCl_3$): δ 1.62−1.72 (m, 6H, CH_2); 2.04−2.22 (m, 2H, CH_2); 4.61−4.78 (m, 1H, CH); 6.66 (d, 2H, J = 7.30 Hz, Ar); 7.14 (t, 1H, J = 7.30 Hz, Ar); 7.10−7.36 (m, 7H, Ar); 7.94 (s, 1H, NH); 12.08 (s, 1H, NH).

1-Phenyl-3-(phenyl-phenyliminomethyl)thiourea (5h). Scale: 7.0 mmol. Yield: 1.98 g (85%). ^{1}H NMR ($CDCl_3$): δ 6.76 (d, 2H, J = 8.04 Hz, Ar); 7.01 (t, 1H, J = 7.30 Hz, Ar); 7.14−7.45 (m, 10H, Ar); 7.75 (d, 2H, J = 7.30 Hz, Ar); 8.16 (s, 1H, NH); 10.90 (s, 1H, NH).

1-Benzyl-3-(phenyl-phenyliminomethyl)thiourea (5i). Scale: 7.0 mmol. Yield: 2.28 g (95%). ^{1}H NMR ($CDCl_3$): δ 4.98 (d, 2H, J = 5.10 Hz, CH_2); 6.44 (d, 2H, J = 8.04 Hz, Ar); 6.95 (t, 1H, J = 7.30 Hz, Ar); 7.12 (t, 2H, J = 7.32 Hz, Ar); 7.27−7.40 (m, 10H, Ar); 8.10 (s, 1H, NH); 12.40 (s, 1H, NH).

1-Methyl-3-(phenyl-3-tolyliminomethyl)thiourea (6a).[13] Scale: 6.9 mmol. Yield: 1.08 g (56%). ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H, CH_3); 3.25 (d, 3H, J = 4.4 Hz, NCH_3); 6.43 (d, 1H, J = 8.0 Hz, Ar); 6.56 (s, 1H, Ar); 6.78 (d, 1H, J = 7.3 Hz, Ar); 7.00 (dd, 1H, J = 7.3, 8.0 Hz, Ar); 7.28 (m, 5H, Ar); 8.03 (s, 1H, NH); 11.83 (bs, 1H, NH).

1-Ethyl-3-(phenyl-3-tolyliminomethyl)thiourea (6b).[13] Scale: 7.0 mmol. Yield: 1.52 g (73%). ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 3H, J = 7.3 Hz, CH_3); 2.21 (s, 3H, CH_3); 3.75 (m, 2H, CH_2); 6.44 (d, 1H, J = 7.3 Hz, Ar); 6.56 (s, 1H, Ar); 6.78 (d, 1H, J = 7.3 Hz, Ar); 7.01 (dd, 1H, J = 7.3, 8.0 Hz, Ar); 7.32 (m, 5H, Ar); 7.96 (s, 1H, NH); 11.91 (bs, 1H, NH).

1-(Phenyl-3-tolyliminomethyl)-3-propyl-thiourea (6c). Scale: 5.0 mmol. Yield: 1.52 g (97%). ^{1}H NMR ($CDCl_3$): δ 1.02 (t, 3H, J = 7.30 Hz, CH_3); 1.69−1.79 (m, 2H, CH_2); 2.21 (s, 3H, CH_3); 3.69 (q, 2H, J = 7.30 Hz, CH_2); 6.43 (d, 1H, J = 8.80 Hz, Ar); 6.54 (s, 1H, Ar); 6.78 (d, 1H, J = 7.32 Hz, Ar); 7.00 (t, 1H, J = 7.32 Hz, Ar); 7.21−7.39 (m, 5H, Ar); 7.99 (s, 1H, NH); 12.01 (bs, 1H, NH).

1-Butyl-3-(phenyl-3-tolyliminomethyl)thiourea (6d). Scale: 4.0 mmol. Yield: 0.80 g (62%). ^{1}H NMR ($CDCl_3$): δ 0.97 (t, 3H, J = 7.30 Hz, CH_3); 1.37−1.55 (m, 2H, CH_2); 1.62−1.78 (m, 2H, CH_2); 2.21 (s, 3H, CH_3); 3.67−3.78 (m, 2H, CH_2); 6.43 (d, 1H, J = 7.30 Hz, Ar); 6.54 (s, 1H, Ar); 6.79 (d, 1H, J = 8.04 Hz, Ar); 7.01 (t, 1H, J = 7.30 Hz, Ar); 7.20−7.37 (m, 5H, Ar); 7.97 (bs, 1H, NH); 12.00 (s, 1H, NH).

1-(3-Hydroxypropyl)-3-(phenyl-3-tolyliminomethyl)-thiourea (6e). Scale: 4.0 mmol. Yield: 1.01 g (71%). ^{1}H NMR ($CDCl_3$): δ 1.81−1.94 (m, 2H, CH_2); 2.21 (s, 3H, CH_3); 3.75 (t, 2H, J = 5.12 Hz, CH_2); 3.87−3.96 (q, 2H, J = 5.84 Hz, CH_2); 6.43 (d, 1H, J = 8.02 Hz, Ar); 6.55 (s, 1H, Ar); 6.79 (d, 1H, J = 8.04 Hz, Ar); 7.01 (t, 1H, J = 7.30 Hz, Ar); 7.20−7.38 (m, 5H, Ar); 8.08 (bs, 1H, NH); 12.10 (bs, 1H, NH).

1-Isopropyl-3-(phenyl-3-tolyliminomethyl)thiourea (6f). Scale: 4.0 mmol. Yield: 1.05 g (84%). ^{1}H NMR ($CDCl_3$): δ 1.34 (d, 6H, J = 6.58 Hz, $2CH_3$); 4.47−4.64 (m, 1H, CH); 6.43 (d, 1H, J = 8.02 Hz, Ar); 6.54 (s, 1H, Ar); 6.79 (d, 1H, J = 7.30 Hz, Ar); 7.01 (t, 1H, J = 7.30 Hz, Ar); 7.25−7.36 (m, 5H, Ar); 7.91 (bs, 1H, NH); 11.93 (bs, 1H, NH).

1-Cyclopentyl-3-(phenyl-3-tolyliminomethyl)thiourea (6g). Scale: 4.0 mmol. Yield: 1.15 g (85%). ^{1}H NMR ($CDCl_3$): δ 1.63−2.22 (m, 8H, $4CH_2$); 4.60−4.78 (m, 1H, CH); 6.44−6.52 (m, 2H, Ar); 6.79 (d, 1H, J = 4.2 Hz, Ar); 7.00 (t, 1H, J = 7.30 Hz, Ar); 7.24−7.32 (m, 5H, Ar); 7.93 (s, 1H, NH); 12.10 (bs, 1H, NH).

1-Phenyl-3-(phenyl-3-tolyliminomethyl)thiourea (6h). Scale: 5.0 mmol. Yield: 1.21 g (70%). ^{1}H NMR ($CDCl_3$): δ 2.23 (s, 3H, CH_3); 6.50 (d, 1H, J = 8.02 Hz, Ar); 6.63 (s, 1H, Ar); 6.82 (d, 1H, J = 7.30 Hz, Ar); 7.03 (t, 1H, J = 7.32 Hz, Ar); 7.12−7.45 (m, 8H, Ar); 7.74 (d, 2H, J = 8.04 Hz, Ar); 8.13 (bs, 1H, NH); 10.83 (bs, 1H, NH).

1-Benzyl-3-(phenyl-3-tolyliminomethyl)thiourea (6i). Scale: 6.0 mmol. Yield: 2.03 g (94%). ^{1}H NMR ($CDCl_3$): δ 2.18 (s, 3H, CH_3); 4.98 (d, 2H, J = 5.12 Hz, CH_2); 6.34−6.49 (m, 2H, Ar); 6.80 (d, 1H, J = 10.54 Hz, Ar); 6.94 (t, 1H, J = 7.30 Hz, Ar); 7.21−7.42 (m, 10H, Ar); 8.07 (s, 1H, NH); 12.43 (bs, 1H, NH).

General Procedure for the Preparation of [1,2,4]-Thiadiazoles.[15b] The thiourea was dissolved in a minimal amount of CH_2Cl_2 (usually 2−4 mL/mmol) and then diluted with twice the volume of ethyl acetate. At room temperature, a 0.5 M solution of bromine in ethyl acetate (2 equiv) was added dropwise. The thiadiazole hydrobromide precipitated. After addition of light petroleum ether, the mixture was left to stand at 5 °C overnight. Filtering, washing (2×) of the residue with petroleum ether, and drying in vacuo afforded the [1,2,4]-thiadiazole. The products were recrystallized from methanol.

2,3-Diphenyl-5-*N*-methylimino-*2H*-[1,2,4]-thiadiazole Hydrobromide (7a).[13] Scale: 16.3 mmol. Yield: 4.50 g (79%) as a white solid. Mp: 248−250 °C. ^{1}H NMR (MeOD-d_3): δ 3.37 (s, 3H, NCH_3); 7.49 (m, 10H, Ar). Anal. ($C_{15}H_{13}N_3S$·HBr) C, H, N.

2,3-Diphenyl-5-*N*-ethylimino-*2H*-[1,2,4]-thiadiazole Hydrobromide (7b).[13] Scale: 8.1 mmol. Yield: 2.41 g (82%) as a white solid. Mp: 240−242 °C. ^{1}H NMR (MeOD-d_3): δ 1.40 (t, 3H, J = 7.3 Hz, CH_3); 3.85 (q, 2H, J = 7.3 Hz, NCH_2); 7.48 (m, 10H, Ar). Anal. ($C_{16}H_{15}N_3S$·0.8 HBr·1.3 H_2O) C, H, N.

2,3-Diphenyl-5-*N*-propylimino-*2H*-[1,2,4]-thiadiazole Hydrobromide (7c). Scale: 5.0 mmol. Yield: 1.56 g (83%) as a white solid. Mp: 239−240 °C. ^{1}H NMR ($CDCl_3$): δ 1.07 (t, 3H, J = 7.30 Hz, CH_3); 1.70−1.91 (m, 2H, CH_2); 3.72 (q, 2H, J = 7.32 Hz, CH_2); 7.21−7.53 (m, 10H, Ar); 10.38 (bs, 1H, HBr). ^{1}H NMR ($CDCl_3$ + 1 drop of D_2O): δ 1.06 (t, 3H, J = 7.30 Hz,

CH_3); 1.71−1.89 (m, 2H, CH_2); 3.72 (t, 2H, J = 7.30 Hz, CH_2); 7.21−7.52 (m, 10H, Ar). Anal. ($C_{17}H_{17}N_3S$·HBr) C, H, N.

2,3-Diphenyl-5-*N*-butylimino-*2H*-[1,2,4]-thiadiazole Hydrobromide (7d). Scale: 1.8 mmol. Yield: 0.37 g (54%) as a white solid. Mp: 206−207 °C. ^{1}H NMR ($CDCl_3$): δ 0.96 (t, 3H, J = 7.30 Hz, CH_3); 1.43−1.62 (m, 2H, CH_2); 1.73−1.87 (m, 2H, CH_2); 3.81 (q, 2H, J = 6.58 Hz, CH_2); 7.27−7.56 (m, 10H, Ar); 9.69 (bs, 1H, HBr). Anal. ($C_{18}H_{19}N_3S$·HBr) C, H, N.

2,3-Diphenyl-5-*N*-(3-hydroxypropyl)imino-*2H*-[1,2,4]-thiadiazole Hydrobromide (7e). Scale: 5.4 mmol. Yield: 1.75 g (83%) as a white solid. Mp: 165−167 °C. ^{1}H NMR ($CDCl_3$): δ 1.96−2.05 (m, 2H, CH_2); 3.80−3.99 (m, 4H, $2CH_2$); 7.25−7.54 (m, 10H, Ar); 10.41 (bs, 1H, HBr). Anal. ($C_{17}H_{17}N_3OS$·HBr·0.3 H_2O) C, H, N.

2,3-Diphenyl-5-*N*-isopropylimino-*2H*-[1,2,4]-thiadiazole Hydrobromide (7f). Scale: 5.7 mmol. Yield: 1.69 g (79%) as a white solid. Mp: 250−251 °C. ^{1}H NMR ($CDCl_3$): δ 1.42 (d, 6H, J = 6.58 Hz, CH_3); 4.39−4.56 (m, 1H, CH); 7.21−7.51 (m, 10H, Ar); 10.32 (bs, 1H, HBr). Anal. ($C_{17}H_{17}N_3S$·HBr·0.3 H_2O) C, H, N.

2,3-Diphenyl-5-*N*-cyclopentylimino-*2H*-[1,2,4]-thiadiazole Hydrobromide (7g). Scale: 5.4 mmol. Yield: 1.65 g (76%) as a white solid. Mp: 242−243 °C. ^{1}H NMR ($CDCl_3$): δ 1.68−2.15 (m, 8H, $4CH_2$); 4.58−4.76 (m, 1H, CH); 7.27−7.56 (m, 10H, Ar); 9.53 (bs, 1H, HBr). Anal. ($C_{19}H_{19}N_3S$·HBr) C, H, N.

2,3-Diphenyl-5-*N*-phenylimino-*2H*-[1,2,4]-thiadiazole Hydrobromide (7h). Scale: 5.1 mmol. Yield: 0.95 g (45%) as a light yellow solid. Mp: 227−228 °C. ^{1}H NMR ($CDCl_3$): δ 7.23−7.60 (m, 13H, Ar); 7.88 (d, 2H, J = 8.02 Hz); 12.2 (bs, 1H, HBr). Anal. ($C_{20}H_{15}N_3S$·HBr·0.3 H_2O) C, H, N.

2,3-Diphenyl-5-*N*-benzylimino-*2H*-[1,2,4]-thiadiazole Hydrobromide (7i). Scale: 5.8 mmol. Yield: 2.21 g (90%) as a light yellow solid. Mp: 226−227 °C. ^{1}H NMR ($CDCl_3$): δ 4.97 (d, 2H, J = 5.84 Hz, CH_2); 7.27−7.54 (m, 15H, Ar); 10.4 (bs, 1H, HBr). ^{1}H NMR ($CDCl_3$ + 1 drop of D_2O): δ 4.97 (s, 2H, CH_2); 7.27−7.88 (m, 15H, Ar). Anal. ($C_{21}H_{17}N_3S$·HBr) C, H, N.

5-*N*-Methylimino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]-thiadiazole Hydrobromide (8a).[13] Scale: 3.9 mmol. Yield: 1.25 g (89%) as a white solid. Mp: 237−238 °C. ^{1}H NMR ($CDCl_3$): δ 2.37 (s, 3H, CH_3); 3.35 (d, 3H, J = 5.1 Hz, NCH_3); 7.00 (m, 1H, Ar); 7.12 (s, 1H, Ar); 7.34 (m, 4H, Ar); 7.53 (m, 3H, Ar); 10.21 (bs, 1H, HBr). Anal. ($C_{16}H_{15}N_3S$·0.8 HBr·1.2 H_2O) C, H, N.

5-*N*-Ethylimino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]-thiadiazole Hydrobromide (8b).[13] Scale: 5.1 mmol. Yield: 1.45 g (76%) as a white solid. Mp: 226−227 °C. ^{1}H NMR ($CDCl_3$): δ 1.43 (t, 3H, J = 7.3 Hz, CH_3); 2.37 (s, 3H, CH_3); 3.81 (m, 2H, NCH_2); 6.98 (m, 1H, Ar); 7.13 (s, 1H, Ar); 7.33 (m, 4H, Ar); 7.53 (m, 3H, Ar); 10.33 (bs, 1H, HBr). Anal. ($C_{17}H_{17}N_3S$·HBr·0.3 H_2O) C, H, N.

5-*N*-Propylimino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]-thiadiazole Hydrobromide (8c). Scale: 4.0 mmol. Yield: 1.13 g (72%) as a white solid. Mp: 214−215 °C. ^{1}H NMR ($CDCl_3$): δ 0.86 (m, 3H, CH_3); 1.70−1.86 (m, 2H, CH_2); 2.40 (s, 3H, CH_3); 3.70−3.90 (m, 2H, CH_2); 7.08−7.54 (m, 9H, Ar); 9.57 (bs, 1H, HBr). Anal. ($C_{18}H_{19}N_3S$·HBr) C, H, N.

5-*N*-Butylimino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]-thiadiazole Hydrobromide (8d). Scale: 2.5 mmol. Yield: 0.63 g (64%) as a white solid. Mp: 170−171 °C. ^{1}H NMR ($CDCl_3$): δ 0.99 (t, 3H, J = 7.30 Hz, CH_3); 1.42−1.82 (m, 4H, $2CH_2$); 2.36 (s, 3H, CH_3); 3.69−3.83 (m, 2H, CH_2); 6.99−7.56 (m, 9H, Ar); 10.32 (bs, 1H, HBr). Anal. ($C_{19}H_{21}N_3S$·HBr) C, H, N.

5-*N*-(3-Hydroxypropyl)imino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]-thiadiazole Hydrobromide (8e). Scale: 2.0 mmol. Yield: 0.95 g (96%) as a white solid. Mp: 180−181 °C. ^{1}H NMR ($CDCl_3$): δ 2.02 (s, 1H, OH); 2.36 (s, 3H, CH_3); 3.65−3.94 (m, 6H, $3CH_2$); 7.01−7.52 (m, 9H, Ar); 10.36 (bs, 1H, HBr). Anal. ($C_{18}H_{19}N_3S$·HBr) C, H, N.

5-*N*-Isopropylimino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]-thiadiazole Hydrobromide (8f). Scale: 2.5 mmol. Yield: 1.25 g (80%) as a white solid. Mp: 243−245 °C. ^{1}H NMR ($CDCl_3$): δ 1.47 (d, 6H, J = 6.58 Hz, $2CH_3$); 2.43 (s, 3H, CH_3); 4.47−4.62 (m, 1H, CH); 7.04−7.57 (m, 9H, Ar); 9.64 (bs, 1H, HBr). Anal. ($C_{18}H_{19}N_3S$·HBr·0.3 H_2O) C, H, N.

5-*N*-Cyclopentylimino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]-thiadiazole Hydrobromide (8g). Scale: 4.0 mmol. Yield: 1.05 g (63%) as a white solid. Mp: 238−239 °C. ^{1}H NMR ($CDCl_3$): δ 1.70−2.15 (m, 8H, CH_2); 2.38 (s, 3H, CH_3); 4.52−4.71 (m, 1H, CH); 7.02 (s, 1H, Ar); 7.16 (s, 1H, Ar); 7.27−7.40 (m, 5H, Ar); 7.54 (t, 2H, J = 8.04 Hz, Ar); 9.91 (bs, 1H, HBr). Anal. ($C_{20}H_{21}N_3S$·HBr) C, H, N.

5-*N*-Phenylimino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]-thiadiazole Hydrobromide (8h). Scale: 4.0 mmol. Yield: 1.25 g (74%) as a white solid. Mp: 219−220 °C. ^{1}H NMR ($CDCl_3$): δ 2.40 (s, 3H, CH_3); 7.04−7.63 (m, 12H, Ar); 7.90 (d, 2H, J = 8.04 Hz, Ar); 12.40 (bs, 1H, HBr). Anal. ($C_{21}H_{17}N_3S$·HBr·0.7 H_2O) C, H, N.

5-*N*-Benzylimino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]-thiadiazole Hydrobromide (8i). Scale: 4.0 mmol. Yield: 0.98 g (56%), as an off-white solid. Mp: 206−207 °C. ^{1}H NMR ($CDCl_3$): δ 2.38 (s, 3H, CH_3); 4.97 (d, 2H, J = 3.64 Hz, CH_2); 7.05−7.57 (m, 14H, Ar); 10.13 (bs, 1H, HBr). Anal. ($C_{22}H_{19}N_3S$·HBr·0.1 H_2O) C, H, N.

Radioligand Binding Assays. For displacement experiments, membranes of CHO cells expressing recombinant human adenosine A_1 receptors (20 μg of protein for [^{3}H]CCPA and 10 μg of protein for [^{3}H]DPCPX) were incubated at 25 °C for 60 min with ∼1.0 nM of [^{3}H]CCPA or ∼1.6 nM of [^{3}H]DPCPX and a fixed concentration or increasing concentrations of the compounds in a final volume of 0.4 mL Tris-HCl buffer (50 mM, pH = 7.4 at 25 °C). Nonspecific binding was measured in the presence of 10 μM DPCPX. Binding reactions were terminated by dilution with ice-cold 50 mM Tris HCl buffer. Samples were then filtered through Whatman GF/B glass-fiber filters using a Brandell cell harvester or a Millipore manifold. Filters were washed three times with 2−3 mL of the same buffer. Bound radioactivity was measured in a liquid scintillation counter (LKB Wallac) after the addition of 3.5 mL of scintillation liquid (Emulsifier-Safe, Packard).

In kinetic studies, the association of the radiolabeled agonist [^{3}H]CCPA (1 nM) was started by addition of the membranes (30 μg) to the radioligand, in the presence or absence of allosteric modulators. To study dissociation of [^{3}H]CCPA, membranes were preincubated with [^{3}H]CCPA (1 nM) at 25 °C for 60 min. Dissociation of [^{3}H]CCPA was then initiated by the addition of 100 μM 8-cyclopentyltheophylline (CPT) in the presence or absence of allosteric modulators. Samples were handled as mentioned before.

Statistical Analysis. Binding parameters were estimated by GraphPAD Prism software (GraphPAD, San Diego, CA). Data are expressed as mean ± SEM for the number of experiments indicated.

HPLC Analysis. As eluent, a gradient between (A) acetonitrile 10%, 10 mM acetic acid, 5 mM sodium dodecyl sulfate in water and (B) acetonitrile 90%, 10 mM acetic acid, 5 mM sodium dodecyl sulfate in water with a flow of 0.6 mL/min was used. The gradient was 0 min/40% B, 1 min/40% B, 15 min/100% B, 18 min/100% B, 18.2 min/40% B, and 20 min/40% B. Samples were injected dissolved in Tris or eluent A. Compounds were identified on the basis of their retention times (for **5a** t_r = 6.8 min, for **7a** t_r = 7.6 min) and typical UV spectra (from 210 to 360 nm). To convert compounds **7a** and **7g** into their precursors, the compound (10^{-5} M) in Tris buffer was incubated with cysteine (10^{-3} M) at room temperature for 10 min.

Reaction of compound **7a** with CHO cell membranes expressing hA_1 receptors was investigated under the same conditions as in radioligand binding experiments. To isolate the product formed from the reaction of **7a** and cysteine, a 20 mL solution in Tris was made, containing 10^{-4} M **7a** and 10^{-3} M cysteine. After 10 min of incubation at room temperature, the solution was extracted twice with CH_2Cl_2 (2 mL). The combined organic phases were dried over Na_2SO_4 and evaporated under reduced pressure.

The same samples were analyzed with an HPLC-MS system also. As eluent, a gradient between (A) water, (B) acetonitrile, and (C) 1% TFA in water with a flow of 1.0 mL/min was used. The gradient was 0 min/30% B, 2 min/30% B, 17 min/90% B, 22 min/90% B, and 30 min/30% B. C was 10% throughout the gradient. Compounds were identified on the basis of their retention times and their masses. For **7g** t_r = 11.0 min, MS = 322.0; for **5g** t_r = 15.7 min, MS = 324.0; for the product obtained from the reaction of **7g** and cysteine and for the product obtained from the interaction of **7g** with CHO cell membranes expressing hA_1 receptors t_r and MS values were identical with the values obtained for compound **5g**.

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Supporting Information Available: ^{13}C NMR spectra for **5a**−**i**, **6a**−**i**, **7a**−**i**, and **8a**−**i** and elemental analyses for **7c**−**i** and **8c**−**i**. This information is available free of charge via the Internet at http://pubs.acs.org.

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