A Series of Ligands Displaying a Remarkable Agonistic–Antagonistic Profile at the Adenosine A_1 Receptor

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Adenosine receptor agonists are usually variations of the natural ligand, adenosine. The ribose moiety in the ligand has previously been shown to be of great importance for the agonistic effects of the compound. In this paper, we present a series of nonadenosine ligands selective for the adenosine A_1 receptor with an extraordinary pharmacological profile. 2-Amino-4-benzo-[1,3]dioxol-5-yl-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (**70**, LUF 5853) shows full agonistic behavior comparable with the reference compound CPA, while also displaying comparable receptor binding affinity (K_i = 11 nM). In contrast, compound **58** (2-amino-4-(3-trifluoromethylphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile, LUF 5948) has a binding affinity of 14 nM and acts as an inverse agonist. Also present within this same series are compounds that show neutral antagonism of the adenosine A_1 receptor, for example compound **65** (2-amino-4-(4-difluoromethoxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile, LUF 5826).

Introduction

The adenosine A_1 receptor is the most extensively researched of the family of adenosine receptors, which also include the A_{2A}, A_{2B}, and A_3 receptors. Many different classes of ligands have been designed, synthesized, and tested at the A_1 receptor, and to date there are several recurring factors which almost define the affinity or efficacy of the compound. The antagonists can be generally split into two groups, those based on the xanthine core structure and those heterocycles which are nonxanthine compounds. This latter category encompasses all kinds of different mono-, bi-, tri-, and even quadricyclic mostly nitrogen-containing aromatic compounds.[1,2,3] On the contrary, until very recently (partial) agonists were all derivatives of the natural ligand itself, adenosine. Research had shown that, in general, manipulation of the ribose moiety results in a reduction of the intrinsic activity toward the adenosine A_1 receptor,[4–7] and thus accordingly all new nonadenosine ligands were assumed to be antagonists. The main exception to this is modification at the 5′-position, where replacement of the 5′-hydroxyl with 5′-*N*-carboxamides is particularly well tolerated, e.g., NECA and derivatives thereof, although these ligands tend not to be very selective.[3] Recently, based upon some data published in patent literature,[8,9] we showed that certain 2-amino-4-(substituted)-phenyl-6-(1*H*-imidazol-2-ylmethylsulfanyl)pyridine-3,5-dicarbonitriles had varying degrees of efficacy, ranging from full to partial agonists at the different adenosine receptors.[10] Most significantly, these compounds displayed a significant affinity and efficacy at the A_{2B} receptor that had not been reported previously. However, these ligands were not particularly selective at any of the adenosine receptors (being only slightly more favorable toward the A_1 receptor). In this paper we report on a novel series based upon this same template and examine their affinity and selectivity for the adenosine A_1 receptor. We also show their remarkable pharmacological profile in terms of their ability to block or activate the human A_1 receptor influencing the cAMP production in CHO cells stably expressing this receptor.

Results and Discussion

Chemistry. The compounds **55**–**72** were synthesized according to Scheme 1.[8,9] The aldehyde was reacted with malononitrile in a straightforward Knoevenagel condensation in the presence of a few drops of piperidine to give the intermediates **1**–**18** in moderate to good yields (30–93%). Pyridine formation occurred according to a preparation by Kambe et al.[11] to give the phenyl-protected sulfide in the 6-position of the ring. The functionalized malononitrile was refluxed with another equivalent of malononitrile and an equivalent of thiophenol in ethanol and triethylamine, resulting in compounds **19**–**36** (generally between 20 and 50% yields). To obtain the free thiol in the 6- position of the pyridine ring, 3.3 equiv of sodium sulfide in DMF at 80 °C for 2–3 h resulted in quantitative yields of compounds **37**–**54**. Throughout the synthesis, purification of the crude product was not performed, nor were the reactions optimized. The yields stated correspond to the crude material, and it was this crude substance that was used in subsequent reactions. The final step was the reaction of the free thiol with 2-bromoethanol in the presence of $NaHCO_3$ in DMF at room temperature to give compounds **55**–**72** in modest to good yields. The final products were purified by column chromatography, and subsequent recrystallization gave clean, fully characterized compounds.

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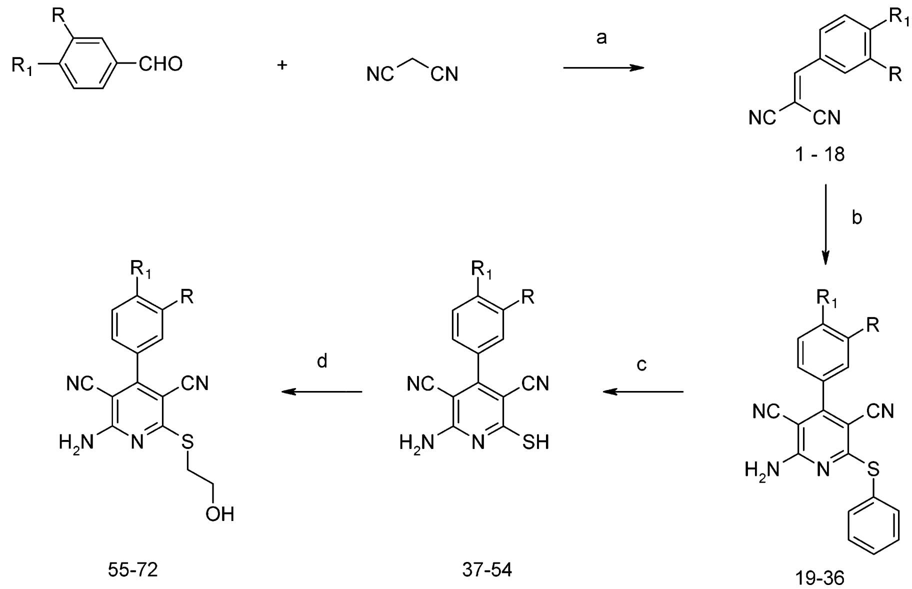
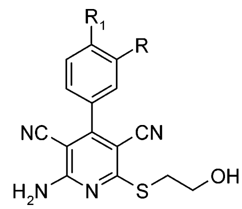
Scheme 1. Synthetic Route to 2-Amino-4-[(substituted)Phenyl]-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitriles **55−72**[a]



[a] (a) piperidine, EtOH, 1 h reflux; (b) malononitrile, thiophenol, triethylamine, EtOH, 4 h reflux; (c) (i) Na_2S, DMF, (ii) 1 M HCl; (d) 2-bromoethanol, $NaHCO_3$, DMF.

Table 1. Affinities of the 2-Amino-4-[(substituted)phenyl]-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitriles (**55−72**) in Radioligand Binding Assays at the Human Adenosine Receptors and the Effect in cAMP Assays with the Human A_1 Adenosine Receptor



			K_i (nM) or % disp.[a]			
compound code	R	R_1	hA_1[b]	hA_{2A}[c]	hA_3[d]	% change in cAMP[e]
CPA	-	-	10 ± 1.3	790[22]	281 ± 56[23]	−100
DPCPX	-	-	6.1 ± 1.6	129[22]	1700 ± 170[13]	100
N0840	-	-	1081 ± 69	n.d.	15%[13]	72 ± 7
55	H	H	15 ± 4	23%	26%	−72 ± 6
56	F	H	40 ± 10	19%	8%	−102 ± 10
57	H	F	81 ± 5	4%	0%	−51 ± 10
58	CF_3	H	14 ± 2	22%	49%	85 ± 12
59	H	CF_3	150 ± 38	13%	0%	48 ± 6
60	CH_3	H	81 ± 17	29%	5%	−78 ± 6
61	H	CH_3	49 ± 9	20%	0%	−16 ± 6
62	OH	H	12 ± 3	25%	16%	−105 ± 22
63	H	OH	23 ± 3	37%	0%	−93 ± 12
64	OCF_2H	H	40 ± 20	8%	12%	6 ± 5
65	H	OCF_2H	34 ± 7	0%	23%	4 ± 6
66	OCF_3	H	30 ± 7	3%	11%	80 ± 13
67	H	OCF_3	156 ± 40	0%	10%	12 ± 12
68	OCH_3	H	4.3 ± 0.6	21%	18%	−58 ± 10
69	H	OCH_3	41 ± 8	8%	21%	−16 ± 19
70	$-OCH_2O-$		11 ± 2	33%	13%	−111 ± 16
71	OCH_3	OCH_3	25%	25%	0%	-
72	H	$N(CH_3)_2$	245 ± 100	0%	7%	−65 ± 23

[a] K_i ± SEM ($n = 3$), % displacement ($n = 2$). [b] Displacement of specific [^{3}H]DPCPX binding in CHO cells expressing human adenosine A_1 receptors or % displacement of specific binding at 1 μM concentrations. [c] Displacement of specific [^{3}H]ZM 241385 binding in CHO cells expressing human adenosine A_{2A} receptors or % displacement of specific binding at 1 μM concentrations. [d] Displacement of specific [^{125}I]AB-MECA binding in HEK 293 cells expressing human adenosine A_3 receptors or % displacement of specific binding at 1 μM concentrations. [e] % change of cAMP ± SEM ($n = 3$) compared to CPA (full agonist, −100%) and DPCPX (full inverse agonist 100%). cAMP generation was stimulated with 10 μM forskolin and compounds were tested at mostly 100 × their K_i value (see text).

Biology. All compounds were tested in radioligand binding assays to determine their affinities at the respective human adenosine receptors (Table 1). Compounds for which the affinities were determined (i.e., compounds which showed greater than 50% displacement of the radioligand at 1 μM) were tested in functional assays for their ability to influence the levels of cAMP in CHO cells expressing the human adenosine

A_1 receptor. The compounds were tested at concentrations of mostly $100 \times K_i$, and at least $20 \times K_i$, where the receptor sites should be almost fully occupied, and the reference ligands CPA, DPCPX, and N0840 were included in the assays. In $[^{35}S]GTP\gamma S$ binding assays on similar cell membranes from CHO cells these three particular ligands are respectively reported as a full agonist, an inverse agonist, and a neutral antagonist.[12,13] In cAMP assays on whole cells expressing the human A_1 receptor, CPA has been shown to be a full agonist and DPCPX as an inverse agonist.[12] To exclude the possibility of effects caused by endogeneous adenosine that may be present in the system, adenosine deaminase was added to the incubation medium.

Structure–Activity Relationships and Molecular Modeling. The results of the radioligand binding assays are presented in Table 1. The compounds are highly selective for the human A_1 adenosine receptor, with not one variation displaying more than 50% displacement of the radioligand at a concentration of 1 μM on the human A_{2A} or A_3 receptors. A selection of compounds was also tested on the human A_{2B} receptor. These also displayed either significantly less than or in the region of 50% displacement of the radioligand at a concentration of 1 μM.

Fluorine substitution caused many different effects on both the binding and efficacy of the compounds compared to the nonfluorinated analogues. Compounds **56** and **57**, the 3- and 4-fluorophenyl derivatives, possessed a K_i value of 40 and 81 nM, respectively, compared to the phenyl derivative **55**, which had an affinity of 15 nM. In general the highly electronegative fluorine atom was detrimental for binding affinity compared to its nonfluorinated analogue, but for two exceptions. One exception was compound **58** with a K_i value of 14 nM showing more than 5 times more affinity to the A_1 receptor than the 3-tolyl derivative **60** (K_i = 81 nM). Compound **59** abides by the generalization and possesses an affinity of 150 nM (cf., compound **61**, K_i = 49 nM).

The presence of the hydroxyl group (**62**, **63**) also increases the electronegativity of this region of the ligand and thus might also have been detrimental for binding affinity. However, the presence of the hydrogen atom probably induces hydrogen-bonding with the receptor, accounting for similarly high affinities (12 and 23 nM, respectively) to that of the nonsubstituted phenyl derivative (**55**, K_i = 15 nM). As mentioned earlier, the presence of the fluorine atom was generally detrimental for binding, and this was illustrated by the fluorine-substituted methoxyphenyl ligands (**64**, **66**, and **67**) which showed less affinity for the receptor than the analogous methoxyphenyl ligands (**68**, **69**). The other exception to the detrimental effect of fluorine was the 4-difluoromethoxyphenyl ligand **65**, which displayed comparable affinity to the 4-methoxyphenyl derivative **69** in the 30–40 nM range. With an affinity of 4 nM, the compound with the most encouraging potency at the A_1 receptor was the 3-methoxyphenyl ligand (**68**).

In general, substitution in the 3-position was more positive for binding than substitution in the 4-position of the phenyl ring. The only exceptions to this were the tolyl compounds (**60**, **61**) and the difluoromethoxyphenyl compounds (**64** and **65**), although these latter two compounds displayed very similar levels of potency. The spatial benefit of substitution in the 3- versus the 4-position is illustrated well by compounds **68**–**70**. The 3-methoxyphenyl derivative **68** displays almost 10-fold better affinity than the 4-methoxyphenyl ligand **69** at 4.3 nM and 41 nM, respectively. Compound **70** that can perhaps be seen as a compromise of these two moieties holds an affinity consistent with this theory at 11 nM. The 3,4-dimethoxyphenyl derivative **71**, a combination of compounds **68** and **69**, has a much-reduced affinity at the A_1 receptor, with a displacement of the radioligand of only 25% at a concentration of 1 μM. It is obvious that the two methoxy groups create too much steric bulk for good binding in the pocket to occur. The 4-dimethylaminophenyl derivative **72**, like the methoxy-analogues, possesses a methyl group connected to a hydrogen-bond acceptor. However, the existence of a second methyl substituent on this hydrogen-bond acceptor (causing steric hindrance, both in terms of optimal fit in the receptor and in perhaps preventing the occurrence of H-bonding) probably explains its relatively poor affinity at the A_1 adenosine receptor at 245 nM.

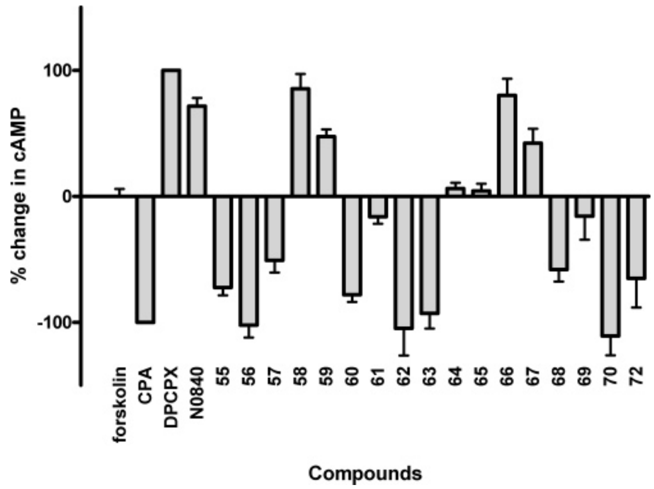


Figure 1. Effect of reference and synthesized ligands on forskolin-induced cAMP levels at hA_1 adenosine receptors compared to CPA (full agonist, 100% inhibition) and DPCPX (full inverse agonist, 100% stimulation).

The radioligand binding assays on the human A_1 adenosine receptor were performed with the inverse agonist ligand $[^3H]$DPCPX. This radioligand predominantly labels the low affinity state of the receptor, in contrast to the agonist (radio)ligands. Therefore, the K_i values obtained and given in Table 1 are a measure of the binding to low affinity receptors. For this reason, CPA exhibits a relatively large K_i value in this assay, which is probably also true for the other agonists in Table 1.

The behavior of the compounds in efficacy assays is displayed in Figure 1. The ligands were tested at concentrations of mostly $100 \times K_i$, and at least $20 \times K_i$, where the receptor sites should be almost fully occupied. The actual percentage inhibition or stimulation relative to the full agonist CPA (100% inhibition) and DPCPX (full inverse agonist, set at 100% stimulation) is given in Table 1. As the A_1 receptor is coupled to an inhibitory G protein, forskolin was used to induce the production of cAMP. N0840 (reported in $[^{35}S]GTP\gamma S$ assays to be a neutral antagonist)[12,13] was shown in this whole cell cAMP assay to have properties consistent with an inverse agonist, although on lower levels than DPCPX.

Almost full agonistic activity was found for four compounds (**56**, **62**, **63**, **70**), the 3-fluorophenyl, the hydroxyphenyl substitutents, and the benzo[1,3]dioxol-5-yl derivative. Five further compounds (**55**, **57**, **60**, **68**, **72**) display high levels of agonism, with efficacy ranging from 50 to 80%, with respect to full inhibition by CPA. Lower levels of partial agonism were demonstrated by compound **61** (the 4-tolyl derivative) and **69** (the 4-methoxyphenyl substitutent). The compounds with trifluoro derivatives (**58**, **59**, **66**, **67**) induce inverse agonism, resulting in an increase in cAMP levels. The two difluoromethoxyphenyl derivatives (**64** and **65**) show very little effect on the levels of cAMP even after stimulation by forskolin, indicating almost neutral antagonism. These compounds compare favorably with a previous exploration for neutral antagonists by De Ligt et al.[13] In that paper, an 8-substituted N^6-cyclopentyl-9-methyladenine, LUF 5674, possessed a binding affinity at the A_1 adenosine receptor of 75 nM while displaying neutral antagonism. In this study we have achieved neutral antagonism with K_i values in the region of 40 nM.

For each pair of 3- and 4-substituted analogues the 3-substituent shows more acute behavior than the 4-substituent. For example, the 3-fluorophenyl derivative **56** is a full agonist compared to partial agonism displayed by the 4-fluorophenyl derivative **57**, and the 3-CF_3 derivative **58** displays a higher inverse agonistic behavior than compound **59**, the 4-CF_3 analogue. Although binding affinity and efficacy are not necessarily correlated, these findings support the outcome of the binding studies. The results suggest that substitution in the 3-position fits the receptor pocket better than substitution in the 4-position to induce either activation of the receptor, or a stabilization to return the receptor to the ground state.

The mode of activation of these ligands at the adenosine A_1 receptor is far from clear. The long held theory of the necessity of the ribose group to activate the receptor proposed the existence of a ribose-specific binding domain in the receptor in which the activation 'trigger' resides. This current series of compounds, all without a ribose moiety, possess possible hydrogen-bonding groups (OH or NH_2) that may bind in the ribose-specific domain and activate the receptor. However, the potential to reside in this specific domain alone may not explain the observations recorded. All the compounds in the series consist of the same elements and only vary at the phenyl moiety, some distance away from the potential H-bonding hydroxyl and amino functions, and yet it is these substitutions that cause the great differences in receptor activation. A review by Visiers et al.[14] highlights the complexities involved with receptor activation/inactivation and summarizes our understanding of it. There are many proposed sites of importance in the activation of a G protein-coupled receptor (GPCR). The binding site itself for many endogenous ligands at rhodopsin-like receptors has been identified as being a spatially compact binding microdomain, most probably in transmembrane (TM) helices 3, 5, and 6, and there is evidence to suggest ligand

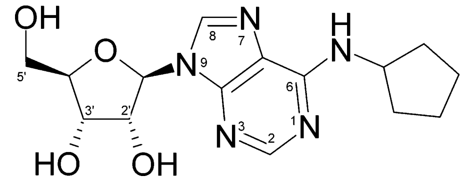


Figure 2. Structure of CPA and the numbering system of the molecule.

orientation can be crucial for efficacy. Almaula et al.[15] described how not only the binding affinity, but also the activation of the serotonin 5-HT_{2A} receptor was affected by small differences in the mode of interaction of the ligand with the receptor pocket. Following this, Ebersole et al.[16] investigated partial agonism of the human serotonin 5-HT_{2A} receptor. On the basis of mutagenesis studies and computational simulation, they found that ligand orientation affected the H-bonding at serine residues in TM3 and TM5 and caused a reduction in efficacy. In conclusion it was hypothesized that the same structurally specific mechanism holds for other GPCRs. Translating the proposals to the current findings, we speculate that the strongly electron-withdrawing trifluoromethyl and trifluoromethoxy groups cause such a change in electron density about the molecule that the manner of binding (with respect to the nonfluorinated ligands, e.g., **55**) at the receptor is distorted dramatically. This prevents not only activation of the receptor but induces the stabilization of an already active receptor to adopt an inactive form. In comparison, the difluoromethoxy derivatives (**64**, **65**) possess less electronegativity than the trifluoro species, distorting the manner of binding less and so resulting in neutral antagonists. In the same vein, the singularly substituted fluorine moiety (**55**, **56**) is probably not sufficiently electronegative and not large enough to induce a different binding mode; thus, agonism is conserved.

In an attempt to form a clearer basis to the potential mode of binding of the agonists, we looked at the electronic and spatial properties of these compounds in the molecular modeling package Spartan.[17] The agonists were modeled in comparison to the known agonist, CPA. For the clarity of the following discussion, CPA and its numbering system is shown in Figure 2.

Assuming that the conservation of the ribose-3′OH is necessary for activation of the agonist (as reported by Taylor et al.[18]), we propose two alternative models of superimposition depicted in Figures 3 and 4 (CPA and compound **68**). The first proposition suggests that the SCH_2CH_2OH region can be overlayed upon the 3′OH area. Examining the electronic configuration of the compounds, the electronegative regions of the H-bond accepting nitrile groups seem to correspond to the electronegative N1 and N7 of the purine ring. Spatially, the 3-substitution of the phenyl group in **68** also fits well with the cyclopentyl group of CPA, suggesting the advantage of 3-substitution above 4-substitution. The alternative model depicted in Figure 4 overlays the 2-amino group from the current series with the 3′OH of the ribose ring. The 3- and 5-nitrile groups of compound **68** now lie in the region of the 2′OH of the ribose group and the N7 of the purine ring, respectively. Again 3-substitution at the phenyl group confers overlap with the N^6-cyclopentyl domain of CPA, offering more optimal spatial compatibility than 4-substitution.

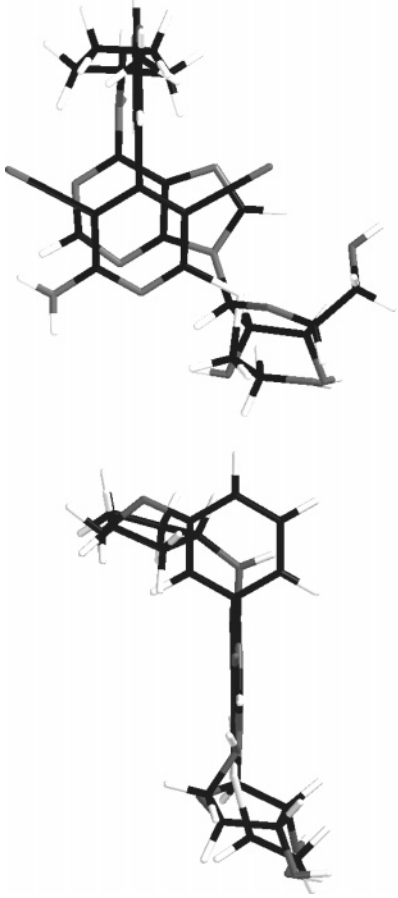


Figure 3. 2-Amino-4-(3-methoxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (**68**) superimposed upon CPA. The upper superimposition depicts the proposed overlap of electron density between the two nitrile groups and the purine ring. The lower picture is the superimposition shown from a different angle and displays the steric overlap of the N^6 substituent of CPA and the 3-methoxy substituent of the phenyl group. Also seen more clearly in this lower picture is the compatibility of the 3′OH group from the ribose ring and the hydroxyl moiety from compound **68**.

The rationale in this modeling procedure was to search for maximal overlap between the two structures. However, it should be kept in mind that the binding sites for the two compounds (CPA and **68**) may be (partially or even entirely) different, but converge in the subsequent activation of the receptor.

Conclusion

This paper describes a series of ligands displaying very different activity at the A_1 adenosine receptor. Several compounds are full agonists with affinity and efficacy comparable to the reference ligand CPA. There are also compounds in this series that display fully inverse agonistic behavior, almost on levels comparable to the reference ligand DPCPX. Most strikingly, neutral antagonists have also been discovered which retain high affinity and selectivity for the adenosine A_1 receptor. It is perhaps particularly noteworthy that these effects vary with the presence or absence of fluorine atoms distant from the phenyl ring. This suggests the substantial influence of hydrogen-bond acceptors in this part of the molecule upon the activation of the adenosine A_1 receptor.

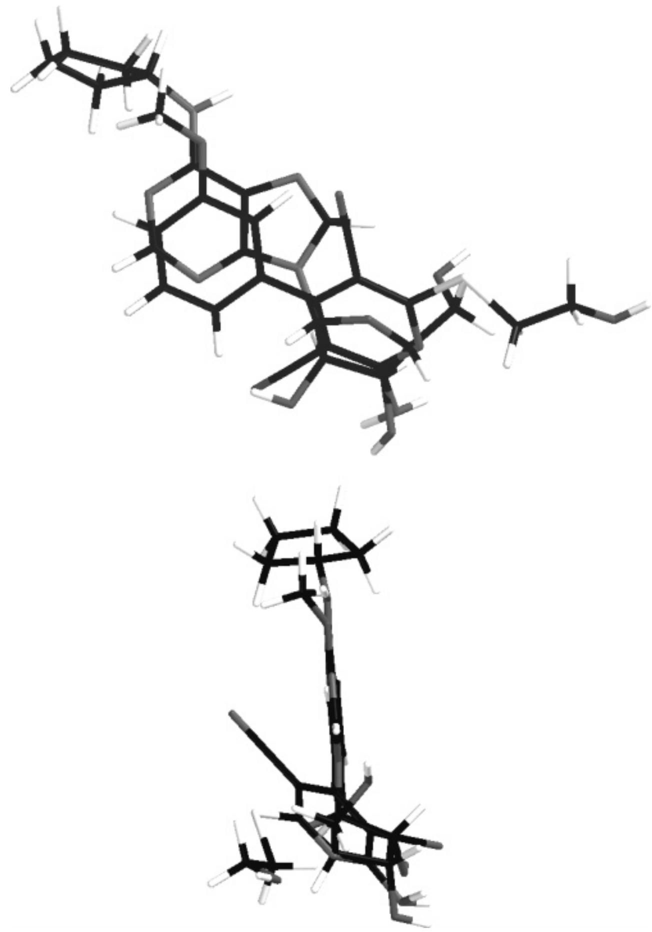


Figure 4. An alternative superimposition of compound **68** and CPA. Here the anchor point has been taken as the 2-amino group of **68** with the 3′OH of the ribose ring. The topmost picture suggests overlap of the ribose 2′OH group with the 3-nitrile moiety, and the purine N7 with the 5-nitrile group. The lower picture is the superimposition shown from a different angle and displays more clearly the overlap of the 2-amino group from **68** with the 3′OH of CPA.

Experimental Section

Materials and Methods. All reagents used were obtained from commercial sources and all solvents were of an analytical grade. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200 (^{1}H NMR, 200 MHz; ^{13}C NMR, 50.29 MHz) spectrometer with tetramethylsilane as an internal standard. Chemical shifts are reported in δ(ppm), and the following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. Melting points were determined on a Büchi melting point apparatus and are uncorrected. The value stated for each compound is the initial temperature at which the compound begins to melt. Elemental analyses were performed by the Leiden Institute of Chemistry and are within 0.4% of the theoretical values unless otherwise stated. Reactions were routinely monitored by TLC using Merck silica gel F_{254} plates.

General Procedure for Functionalized Malononitriles (1−18). To malononitrile (1.32 mL, 20.8 mmol) dissolved in EtOH (14 mL) was added an aldehyde (20 mmol) followed by 3 drops of piperidine. This reaction mixture was then refluxed for 1 h and then allowed to cool to room temperature, upon which a precipitate formed. The crude product was collected by filtration and used without any further purification.

Compounds **1**, **8**, **9**, **14**, **15** have been reported previously.[10] NMR values for compounds **2**, **3**, **7**, **17**, **18** were in accordance to literature data.[19,20]

2-(3-Trifluorobenzylidene)-malononitrile (4). Off white solid. ^{1}H NMR δ(DMSO): 8.67 (s, 1H, C*H*), 8.27−8.21 (m, 1H, phenyl-*H*), 8.09−8.05 (m, 1H, phenyl-*H*), 7.92−7.84 (m, 1H, phenyl-*H*).

2-(4-Trifluorobenzylidene)-malononitrile (5). Off white solid. ^{1}H NMR δ(DMSO): 8.69 (s, 1H, C*H*), 8.14−7.99 (m, 4H, phenyl-*H*).

2-(3-Methylbenzylidene)-malononitrile (6). Off white solid. ^{1}H NMR δ(DMSO): 8.51 (s, 1H, C*H*), 7.80−7.77 (m, 2H, phenyl-*H*), 7.54−7.51 (m, 2H, phenyl-*H*), 2.37 (s, 3H, CH_3).

2-(3-Difluoromethoxybenzylidene)-malononitrile (10). Off-white solid. ^{1}H NMR δ($CDCl_3$): 7.79−7.75 (m, 2H, phenyl-*H*), 7.66 (s, 1H, C*H*), 7.56 (m, 1H, phenyl-*H*), 7.43−7.39 (m, 1H, phenyl-*H*), 6.58 (t, 1H, J = 72.3 Hz, OCF_2H).

2-(4- Difluoromethoxybenzylidene)-malononitrile (11). Off-white solid. ^{1}H NMR δ($CDCl_3$): 7.96 (d, 2H, J = 8.76 Hz, phenyl-*H*), 7.75 (s, 1H, C*H*), 7.27 (d, 2H, J = 8.8 Hz, phenyl-*H*), 6.65 (t, 1H, J = 72.3 Hz, OCF_2H).

2-(3-Trifluoromethoxybenzylidene)-malononitrile (12). Off-white solid. ^{1}H NMR δ($CDCl_3$): 7.89−7.86 (m, 1H, phenyl-*H*), 7.80 (s, 1H, C*H*), 7.74 (m, 1H, phenyl-*H*), 7.62 (m, 1H, phenyl-*H*), 7.51−7.47 (m, 1H, phenyl-*H*).

2-(4-Trifluoromethoxybenzylidene)-malononitrile (13). Off-white solid. ^{1}H NMR δ($CDCl_3$): 7.99 (d, 2H, J = 8.8 Hz, phenyl-*H*), 7.78 (s, 1H, C*H*), 7.38 (d, 2H, J = 8.8 Hz, phenyl-*H*).

2-Benzo[1,3]dioxol-5-ylmethylenemalononitrile (16). Off white solid. ^{1}H NMR δ(DMSO): 8.39 (s, 1H, C*H*), 7.57−7.54 (m, 2H, phenyl-*H*), 7.24−7.19 (m, 1H, phenyl-*H*), 6.25 (s, 2H, −OCH_2O-).

General Procedure for 2-Amino-4-(substituted)phenyl-6-phenylsulfanylpyridine-3,5-dicarbonitriles (19−36). To a solution of the functionalized malononitrile (**1**−**18**) (10 mmol) in EtOH (10 mL), was added malononitrile (0.64 mL, 10 mmol), thiophenol (1.02 mL, 10 mmol), and triethylamine (50 μL), and the mixture heated at reflux for approximately 4 h. The reaction mixture was then allowed to cool to room temperature. The crude product precipitated upon cooling and was collected by filtration.

Compounds **19**, **26**, **27**, **32**, **33** have been reported previously.[10]

2-Amino-4-(3-fluorophenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (20). Yellow solid. ^{1}H NMR δ(DMSO): 7.90 (br s, 2H, NH_2), 7.71−7.40 (m, 9H, phenyl-*H*).

2-Amino-4-(4-fluorophenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (21). Yellow solid. ^{1}H NMR δ(DMSO): 7.64 (br s, 2H, NH_2), 7.62−7.59 (m, 4H, phenyl-*H*), 7.52−7.40 (m, 5H, phenyl-*H*).

2-Amino-4-(3-trifluoromethylphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (22). Yellow solid. ^{1}H NMR δ(DMSO): 7.85 (br s, 2H, NH_2), 7.83−7.70 (m, 4H, phenyl-*H*), 7.66−7.61 (m, 2H, phenyl-*H*), 7.56−7.51 (m, 3H, phenyl-*H*).

2-Amino-4-(4-trifluoromethylphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (23). Yellow solid. ^{1}H NMR δ(DMSO): 8.01−7.92 (m + br s, 3H, NH_2 + CF_3-phenyl-*H*), 7.83−7.78 (m, 2H, CF_3-phenyl-*H*), 7.64−7.60 (m, 2H, phenyl-*H*), 7.53−7.43 (m, 3H, phenyl-*H*).

2-Amino-4-(3-methylphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (24). Yellow solid. ^{1}H NMR δ(DMSO): 7.83 (br s, 2H, NH_2), 7.66−7.61 (m, 2H, phenyl-*H*), 7.56−7.49 (m, 3H, phenyl-*H*), 7.45−7.33 (m, 4H, tolyl-*H*), 2.41 (s, 3H, CH_3).

2-Amino-4-(4-methylphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (25). Yellow solid. ^{1}H NMR δ(DMSO): 7.60 (br s, 2H, NH_2), 7.55−7.52 (m, 2H, phenyl-*H*), 7.50−7.38 (m, 7H, phenyl-*H* + tolyl-*H*), 2.43 (s, 3H, CH_3).

2-Amino-4-(3-difluoromethoxyphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (28). Yellow solid. ^{1}H NMR δ(DMSO): 7.88 (br s, 2H, NH_2), 7.67−7.34 (m, 9H, phenyl-*H*), 6.95 (m, 1H, OCF_2H).

2-Amino-4-(4-difluoromethoxyphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (29). Yellow solid. ^{1}H NMR δ(DMSO): 7.85 (br s, 2H, NH_2), 7.69−7.38 (m, 9H, phenyl-*H*), 7.07 (m, 1H, OCF_2H).

2-Amino-4-(3-trifluoromethoxyphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (30). Yellow solid. ^{1}H NMR δ(DMSO): 7.89 (br s, 2H, NH_2), 7.75−7.51 (m, 9H, phenyl-*H*).

2-Amino-4-(4-trifluoromethoxyphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (31). Yellow solid. ^{1}H NMR δ(DMSO): 7.89 (br s, 2H, NH_2), 7.76 (d, 2H, J = 6.6 Hz, OCF_3-phenyl-*H*), 7.66 (m, 7H, OCF_3-phenyl-*H* + phenyl-*H*).

2-Amino-4-benzo[1,3]dioxol-5-yl-6-phenylsulfanylpyridine-3,5-dicarbonitrile (34). Yellow solid. ^{1}H NMR δ(DMSO): 7.80 (br s, 2H, NH_2), 7.64−7.60 (m, 2H, S-phenyl-*H*), 7.53−7.50 (m, 3H, S-phenyl-*H*), 7.20−7.04 (m, 3H, phenyl-*H*), 6.18 (s, 2H, −OCH_2O-).

2-Amino-4-(3,4-dimethoxyphenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (35). Yellow solid. ^{1}H NMR δ(DMSO): 7.77 (br s, 2H, NH_2), 7.65−7.50 (m, 7H, phenyl-*H*), 7.15 (d, 2H, J = 8.8 Hz, phenyl-*H*), 3.67 (s, 3H, CH_3), 3.63 (s, 3H, CH_3).

2-Amino-4-(4-(dimethylamino)phenyl)-6-phenylsulfanylpyridine-3,5-dicarbonitrile (36). Yellow solid. ^{1}H NMR δ(DMSO): 7.68 (br s, 2H, NH_2), 7.64−7.60 (m, 2H, phenyl-*H*), 7.54−7.48 (m, 2H, phenyl-*H*), 7.44 (d, 2H, J = 8.8 Hz, 4-NMe_2-phenyl-*H*), 6.86 (d, 2H, J = 8.8 Hz, 4-NMe_2-phenyl-*H*), 3.03 (s, 6H, 2 × CH_3).

General Procedure for 2-Amino-4-(substituted)-phenyl-6-mercaptopyridine-3,5-dicarbonitriles (37−54). The pyridine (**19**−**36**) (3 mmol) was dissolved in DMF (10 mL) and to this was added sodium sulfide (0.78 g, 10 mmol) and the mixture stirred at 80 °C for 2 h. Upon cooling to room temperature, 1 M HCl (20 mL) was added, resulting in the formation of a yellow precipitate. The crude product was collected by filtration.

Compounds **37**, **44**, **45**, **50**, **51** have been reported previously.[10] NMR values for compound **43** were in accordance to literature data.[11]

2-Amino-4-(3-fluorophenyl)-6-mercaptopyridine-3,5-dicarbonitrile (38). Yellow solid. ^{1}H NMR δ(DMSO): 7.65−7.59 (m, 1H, phenyl-*H*), 7.48−7.35 (m, 3H, phenyl-*H*).

2-Amino-4-(4-fluorophenyl)-6-mercaptopyridine-3,5-dicarbonitrile (39). Yellow solid. ^{1}H NMR δ(DMSO): 7.58−7.52 (m, 2H, phenyl-*H*), 7.44−7.39 (m, 2H, phenyl-*H*).

2-Amino-4-(3-trifluoromethylphenyl)-6-mercaptopyridine-3,5-dicarbonitrile (40). Yellow solid. ^{1}H NMR δ(DMSO): 7.91−7.78 (m, 4H, phenyl-*H*).

2-Amino-4-(4-trifluoromethylphenyl)-6-mercaptopyridine-3,5-dicarbonitrile (41). Yellow solid. ^{1}H NMR δ(DMSO): 7.96 (d, 2H, J = 8.0 Hz, phenyl-*H*), 7.76 (d, 2H, J = 8.0 Hz, phenyl-*H*).

2-Amino-4-(3-methylphenyl)-6-mercaptopyridine-3,5-dicarbonitrile (42). Yellow solid. ^{1}H NMR δ(DMSO): 7.57−7.30 (m, 4H, phenyl-*H*), 2.40 (s, 3H, CH_3).

2-Amino-4-(3-difluoromethoxyphenyl)-6-mercaptopyridine-3,5-dicarbonitrile (46). Yellow solid. ^{1}H NMR δ(MeOH): 7.46−7.33 (m, 4H, phenyl-*H*), 6.89 (t, 1H, J = 73.4 Hz, OCF_2H).

2-Amino-4-(4-difluoromethoxyphenyl)-6-mercaptopyridine-3,5-dicarbonitrile (47). Yellow solid. ^{1}H NMR δ(MeOD): 7.59 (m, 2H, phenyl-*H*), 7.30 (m, 2H, phenyl-*H*), 6.97 (t, 1H, J = 73.1 Hz, OCF_2H).

2-Amino-4-(3-trifluoromethoxyphenyl)-6-mercaptopyridine-3,5-dicarbonitrile (48). Yellow solid. ^{1}H NMR δ(MeOD): 7.67−7.62 (m, 1H, phenyl-*H*), 7.54−7.47 (m, 3H, phenyl-*H*).

2-Amino-4-(4-trifluoromethoxyphenyl)-6-mercaptopyridine-3,5-dicarbonitrile (49). Yellow solid. ^{1}H NMR δ(MeOD): 7.67−7.63 (m, 2H, phenyl-*H*), 7.48−7.44 (m, 2H, phenyl-*H*).

2-Amino-4-benzo[1,3]dioxol-5-yl-6-mercaptopyridine-3,5-dicarbonitrile (52). Yellow solid. ^{1}H NMR δ(DMSO): 7.13−7.00 (m, 3H, phenyl-*H*), 6.17 (s, 2H, OCH_2O).

2-Amino-4-(3,4-dimethoxyphenyl)-6-mercaptopyridine-3,5-dicarbonitrile (53). Yellow solid. ^{1}H NMR δ(DMSO): 7.17−7.13 (m, 3H, phenyl-*H*), 3.85 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3).

2-Amino-4-(4-(dimethylamino)phenyl)-6-mercaptopyridine-3,5-dicarbonitrile (54). Yellow solid. ^{1}H NMR δ(DMSO): 7.41 (d, 2H, J = 8.8 Hz, phenyl-H), 6.83 (d, 2H, J = 8.8 Hz, phenyl-H), 3.03 (s, 6H, 2 × CH_3).

General Procedure for 2-Amino-4-[(substituted)phenyl]-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitriles (55−72). The free thiol (**37**−**54**) (1 mmol) was stirred with 2-bromoethanol (84 μL, 1 mmol) and $NaHCO_3$ (0.34 g, 1 mmol) in DMF (2 mL) at room temperature for 2 h. Water (10 mL) was added to precipitate the crude product, which was collected by filtration. Purification by column chromatography on SiO_2, eluting with ethyl acetate and/or ethyl acetate−methanol mixtures, followed by recrystallization gave the desired products. The solvents used in the recrystallization process were CH_2Cl_2 and MeOH.

2-Amino-4-phenyl-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (55). White solid, 54%. mp: 211 °C. ^{1}H NMR δ(MeOD): 7.56−7.48 (m, 5H, phenyl-H), 3.80 (t, 2H, J = 6.6 Hz, OCH_2), 3.40 (t, 2H, J = 6.6 Hz, CH_2S). ^{13}C NMR δ(MeOD): 167.1, 159.6, 158.3, 134.0, 130.3, 128.7, 128.4, 115.4, 115.3, 96.1, 85.7, 59.5, 32.6. MS (ESI): 296.9. Anal. ($C_{15}H_{12}N_4OS$·0.3DMF) C, H, N, S.

2-Amino-4-(3-fluorophenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (56). White solid, 38%. mp: 189 °C. ^{1}H NMR δ(DMSO): 8.09 (br s, 2H, NH_2) 7.70−7.59 (m, 1H, phenyl-H), 7.52−7.37 (m, 3H, phenyl-H), 5.05 (t, 1H, J = 5.5 Hz, OH), 3.72−3.64 (m, 2H, OCH_2), 3.40−3.33 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.8, 159.5, 130.3, 130.2, 123.7, 117.1, 116.7, 115.2, 114.8, 114.3, 105.6, 83.3, 59.8, 31.5. MS (ESI): 314.8 Anal. ($C_{15}H_{11}FN_4OS$·0.3H_2O) C, H, N, S.

2-Amino-4-(4-fluorophenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (57). White solid, 54%. mp: 133 °C. ^{1}H NMR δ(DMSO): 8.06 (br s, 2H, NH_2) 7.68−7.60 (m, 2H, phenyl-H), 7.49−7.40 (m, 2H, phenyl-H), 5.04 (t, 1H, J = 5.5 Hz, OH), 3.73−3.64 (m, 2H, OCH_2), 3.45−3.33 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.0, 166.0, 160.0, 157.4, 131.1, 131.0, 130.4, 116.0, 115.6, 115.4, 115.2, 85.8, 83.9, 59.4, 32.6. MS (ESI): 315.0. Anal. ($C_{15}H_{11}FN_4OS$·0.3MeOH) C, H, N, S.

2-Amino-4-(3-trifluoromethylphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (58). White solid, 60%. mp: 164 °C. ^{1}H NMR δ(DMSO): 8.10 (br s, 2H, NH_2), 8.00−7.83 (m, 4H, phenyl-H), 5.05 (t, 1H, J = 5.5 Hz, OH), 3.75−3.66 (m, 2H, OCH_2), 3.42−3.36 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.2, 159.6, 156.8, 135.1, 132.7, 130.0, 129.8, 129.2, 127.0, 126.6, 125.6, 115.3, 115.1, 93.7, 85.9, 59.5, 32.7. MS (ESI): 364.9. Anal. ($C_{16}H_{11}F_3N_4OS$·0.07CH_2Cl_2) C, H, N, S.

2-Amino-4-(4-trifluoromethylphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (59). White Solid, 69%. mp: 158 °C. ^{1}H NMR δ(DMSO): 8.14 (br s, 2H, NH_2), 7.99 (d, 2H, J = 8.0 Hz, phenyl-H), 7.81 (d, 2H, J = 8.0 Hz, phenyl-H), 5.05 (t, 1H, J = 5.5 Hz, OH), 3.74−3.65 (m, 2H, OCH_2), 3.45−3.36 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 178.9, 167.2, 159.5, 157.0, 138.2, 130.8, 130.1, 129.6, 126.6, 125.7, 115.2, 115.0, 93.4, 85.7, 59.4, 32.7. MS (ESI): 364.6. Anal. ($C_{16}H_{11}F_3N_4OS$·0.2CH_2Cl_2) C, H, N, S.

2-Amino-4-(3-methylphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (60). White Solid, 74%. mp: 178 °C. ^{1}H NMR δ(DMSO): 8.02 (br s, 2H, NH_2), 7.47−7.30 (m, 4H, phenyl-H), 5.04 (t, 1H, J = 5.5 Hz, OH), 3.71−3.65 (m, 2H, OCH_2), 3.40−3.34 (m, 2H, CH_2S), 2.40 (s, 3H, CH_3). ^{13}C NMR δ(DMSO): 167.1, 159.6, 158.4, 138.1, 134.0, 131.0, 128.7, 125.5, 115.5, 115.3, 93.6, 85.9, 59.5, 32.7, 20.9. MS (ESI): 311.2. Anal. ($C_{16}H_{14}N_4OS$) C, H, N, S.

2-Amino-4-(4-methylphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (61). White Solid, 81%. mp: 206 °C. ^{1}H NMR δ(DMSO): 8.00 (br s, 2H, NH_2), 7.46−7.36 (m, 4H, phenyl-H), 5.03 (t, 1H, J = 5.5 Hz, OH), 3.73−3.64 (m, 2H, OCH_2), 3.39−3.33 (m, 2H, CH_2S), 2.41 (s, 3H, CH_3). ^{13}C NMR δ(DMSO): 167.0, 159.6, 158.3, 140.2, 131.1, 129.2, 128.4, 115.5, 115.3, 93.6, 85.7, 59.5, 32.6, 20.9. MS (ESI): 310.8. Anal. ($C_{16}H_{14}N_4OS$) C, H, N, S.

2-Amino-4-(3-hydroxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (62). White solid, 70%. mp: 227−229 °C dec ^{1}H NMR δ(DMSO): 8.01 (br s, 2H, NH_2) 7.41−7.33 (m, 1H, phenyl-H), 6.98−6.89 (m, 3H, phenyl-H), 5.05 (t, 1H, J = 5.5 Hz, OH), 3.73−3.64 (m, 2H, OCH_2), 3.42−3.33 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.1, 135.2, 130.0, 118.9, 117.3, 115.4, 115.3, 115.1, 93.6, 86.0, 59.5, 32.7. MS (ESI): 312.8. Anal. ($C_{15}H_{12}N_4O_2S$·0.6H_2O) C, H, N, S.

2-Amino-4-(4-hydroxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (63). White solid, 40%. mp: 224 °C dec ^{1}H NMR δ(DMSO): 7.95 (br s, 2H, NH_2) 7.39 (m, 2H, phenyl-H), 6.93 (m, 2H, phenyl-H), 5.04 (br s, 1H, OH), 3.70−3.64 (m, 2H, OCH_2), 3.32−3.19 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.1, 130.3, 124.3, 115.7, 115.4, 59.5, 32.7. MS (ESI): 312.7. Anal. ($C_{16}H_{14}N_4O_2S$·0.1MeOH), C, H, N, S.

2-Amino-4-(3-difluoromethoxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (64). White solid, 36%. mp: 140 °C. ^{1}H NMR δ(MeOD): 7.60−7.52 (m, 1H, phenyl-H), 7.38−7.31 (m, 3H, phenyl-H), 6.86 (t, 1H, 73.4 Hz, CF_2H), 3.79 (t, 2H, J = 6.6 Hz, OCH_2), 3.39 (t, 2H, J = 6.6 Hz, CH_2S). ^{13}C NMR δ(DMSO): 169.4, 161.4, 158.5, 152.8, 137.0, 131.7, 126.5, 122.0, 120.4, 117.6, 116.1, 116.0, 95.5, 87.2, 61.4, 33.5. MS (ESI): 363. Anal. ($C_{16}H_{12}F_2N_4O_2S$) C, H, N, S.

2-Amino-4-(4-difluoromethoxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (65). White solid, 44%. mp: 156 °C. ^{1}H NMR δ(DMSO): 8.04 (br s, 2H, NH_2) 7.65 (m, 2H, phenyl-H), 7.38 (m, 2H, phenyl-H), 7.43 (s, 1H, CF_2H), 5.04 (t, 1H, J = 5.5 Hz, OH), 3.73−3.64 (m, 2H, OCH_2), 3.38−3.33 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.1, 159.6, 157.4, 152.4, 130.7, 118.4, 115.5, 115.3, 93.4, 85.8, 59.5, 32.7. MS (ESI): 363.0. Anal. ($C_{16}H_{12}F_2N_4O_2S$), C, H, N, S.

2-Amino-4-(3-trifluoromethoxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (66). White solid, 69%. mp: 125 °C. ^{1}H NMR δ(DMSO): 8.08 (br s, 2H, NH_2) 7.73−7.59 (m, 4H, phenyl-H), 5.04 (t, 1H, J = 5.5 Hz, OH), 3.73−3.64 (m, 2H, OCH_2), 3.38−3.33 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.1, 159.5, 156.5, 148.2, 136.1, 131.0, 127.7, 123.3, 122.9, 121.5, 115.2, 115.0, 85.8, 59.5, 32.6. MS (ESI): 380.7. Anal. Calc. for $C_{16}H_{11}F_3N_4O_2S$ (C 50.53; H 2.92; N 14.73; S 8.43) found (C 50.46; H 2.39; N 15.01; S 8.16) %.

2-Amino-4-(4-trifluoromethoxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (67). White solid, 75%. mp: 144 °C. ^{1}H NMR δ(DMSO): 8.08 (br s, 2H, NH_2) 7.74−7.58 (m, 4H, phenyl-H), 5.04 (t, 1H, J = 5.5 Hz, OH), 3.73−3.64 (m, 2H, OCH_2), 3.37−3.33 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.2, 159.6, 157.1, 149.6, 133.2, 131.0, 121.2, 115.4, 115.2, 93.6, 85.8, 59.5, 32.7. MS (ESI): 380.8. Anal. ($C_{16}H_{11}F_3N_4O_2S$) C, H, N, S.

2-Amino-4-(3-methoxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (68). White solid, 63%. mp: 188 °C. ^{1}H NMR δ(DMSO): 8.01 (br s, 2H, NH_2) 7.53−7.45 (m, 1H, phenyl-H), 7.17−7.06 (m, 3H, phenyl-H), 5.06 (t, 1H, J = 5.5 Hz, OH), 3.82 (s, 3H, CH_3), 3.73−3.64 (m, 2H, OCH_2), 3.39−3.33 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.1, 159.7, 159.1, 158.1, 135.3, 130.1, 120.6, 115.9, 115.5, 115.3, 114.1, 93.7, 85.8, 59.5, 55.4, 32.7. MS (ESI): 326.9. Anal. ($C_{16}H_{14}N_4O_2S$·0.2MeOH), C, H, N, S.

2-Amino-4-(4-methoxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (69). White solid, 54%. mp: 188 °C. ^{1}H NMR δ(DMSO): 7.96 (br s, 2H, NH_2) 7.51 (d, 2H, J = 8.8 Hz, phenyl-H), 7.13 (d, 2H, J = 8.8 Hz, phenyl-H), 5.03 (t, 1H, J = 5.5 Hz, OH), 3.86 (s, 3H, CH_3), 3.73−3.64 (m, 2H, OCH_2), 3.37−3.33 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.1, 160.8, 159.8, 158.0, 130.2, 125.9, 115.7, 115.6, 114.1, 85.7, 59.7, 59.5, 55.4, 32.6. MS (ESI): 327.0. Anal. ($C_{16}H_{14}N_4O_2S$·0.2H_2O) C, H, N, S.

2-Amino-4-benzo[1,3]dioxol-5-yl-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (70). White solid, 42%. mp: 192 °C. ^{1}H NMR δ(DMSO): 7.99 (br s, 2H, NH_2) 7.16−7.01 (m, 3H, phenyl-H), 6.17 (s, 2H, −OCH_2O-), 5.02 (t, 1H, J = 5.5 Hz, OH), 3.67 (m, 2H, OCH_2), 3.38−3.32 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 197.7, 167.0, 159.7, 157.9, 148.9, 147.4, 127.4, 123.0, 115.6, 115.4, 108.9, 108.5, 101.8, 93.8, 59.5, 32.6. MS (ESI): 341.1. Anal. Calc. for $C_{16}H_{12}N_4O_3S$ (C 56.46; H 3.55; N 16.46; S 9.42) found (C 56.31; H 3.11; N 16.38; S 9.46) %.

2-Amino-4-(3,4-dimethoxyphenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (71). White solid, 35%. mp: 209 °C. ^{1}H NMR δ(DMSO): 7.96 (br s, 2H, NH_2) 7.19−7.13 (m, 3H, phenyl-H), 5.02 (m, 1H, OH), 3.86 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.69−3.66 (m, 2H, OCH_2), 3.32−3.19 (m, 2H, CH_2S). ^{13}C NMR δ(DMSO): 167.0, 159.7, 158.0, 150.4, 148.3, 126.0, 121.5, 115.7, 115.6, 112.3, 111.5, 92.7, 85.9, 59.5, 55.7, 55.6, 32.8. MS (ESI): 356.9. Anal. ($C_{17}H_{16}N_4O_3S$), C, H, N, S.

2-Amino-4-(4-(dimethylamino)phenyl)-6-(2-hydroxyethylsulfanyl)pyridine-3,5-dicarbonitrile (72). White solid, 43%. mp: 268 °C dec ^{1}H NMR δ(DMSO): 7.87 (br s, 2H, NH_2) 7.39 (d, 2H, J = 8.8 Hz, phenyl-H), 6.82 (d, 2H, J = 8.8 Hz, phenyl-H), 4.99 (t, 1H, J = 5.5 Hz, OH), 3.69−3.60 (m, 2H, OCH_2), 3.38−3.32 (m, 2H, CH_2S), 3.00 (s, 6H, 2 × CH_3). ^{13}C NMR δ(DMSO): 167.1, 160.0, 158.3, 151.5, 129.9, 120.0, 116.1, 116.0, 111.3, 93.2, 85.0, 59.6, 39.5, 32.6. MS (ESI): 339.8. Anal. ($C_{17}H_{17}N_5OS{\cdot}0.2CH_2Cl_2$) C, H, N, S.

Biology. Materials and Methods. [^{3}H]DPCPX and [^{125}I]-AB-MECA were purchased from Amersham Biosciences (NL). [^{3}H]ZM 241385 was obtained from Tocris Cookson, Ltd. (UK). CHO cells expressing the human adenosine A_1 receptor were provided by Dr. Andrea Townsend-Nicholson, University College London, UK. HEK 293 cells stably expressing the human adenosine A_{2A} and A_3 receptor were gifts from Dr. Wang (Biogen) and Dr. K.-N. Klotz (University of Würzburg, Germany), respectively. CHO cells expressing the human A_{2B} receptor were provided by Dr. Steve Rees (GlaxoSmithKline, UK).

All compounds made were tested in radioligand binding assays to determine their affinities at the human adenosine A_1, A_{2A}, and the A_3 receptors, whereas a selection of the compounds was tested at the human A_{2B} receptor (see text).[21] The human A_1 receptors were expressed in CHO cells, and [^{3}H]-DPCPX used as the radioligand. The A_{2A} and A_3 receptors were expressed in HEK 293 cells, and [^{3}H]ZM 241385 and [^{125}I]AB-MECA were used as the respective radioligands. At the A_{2B} receptor, radioligand displacement was determined on membranes from CHO cells stably transfected with human A_{2B} receptor, using [^{3}H]MRS1754 as the radioligand.

Compounds for which the affinities were determined (i.e., compounds which showed greater than 50% displacement of the radioligand at 1 μM) were tested in functional assays for their ability to influence the levels of cAMP in the test system. The compounds were tested at concentrations of mostly 100 × K_i, and at least 20 × K_i, where the receptor sites should be almost fully occupied. The behavior of the compounds was observed with reference to known adenosine receptor ligands: CPA (a full agonist), DPCPX (an inverse agonist), and N0840 (reported as a neutral antagonist).

CHO cells expressing the human adenosine A_1 receptor were grown overnight as a monolayer in 24-well tissue culture plates (400 μL/well; 2 × 10^5 cells/well). cAMP generation was performed in Dulbecco's Modified Eagles Medium (DMEM)/N-2-hydroxyethylpiperazin-N'-2-ethansulfonic acid (HEPES) buffer (0.60 g HEPES/50 mL DMEM pH 7.4). Each well was washed twice with HEPES/DMEM buffer (250 μL), and the following was added: adenosine deaminase (0.8 IU/mL), rolipram (50 μM), cilostamide (50 μM). This was then incubated for 30 min at 37 °C followed by the introduction of the compound of interest. After a further 10 min of incubation, forskolin was added (10 μM). After a subsequent 15 min, incubation was stopped by aspirating the assay medium and by adding 200 μL of ice-cold 0.1 M HCl. The amount of cAMP was determined by competition with [^{3}H]cAMP for protein kinase A (PKA). Briefly, the sample, approximately 1.8 nM [^{3}H]cAMP, and 100 μL of PKA solution were incubated on ice for 2.5 h. The incubations were stopped by rapid dilution with 2 mL of ice-cold Tris HCl buffer (pH 7.4), and bound radioactive material was then recovered by filtration through Whatman GF/C filters. Filters were additionally rinsed with 2 × 2 mL of Tris HCl buffer, and then the radioactivity counted in Packard Emulsifier Safe scintillation fluid (3.5 mL). All data reflect three independent experiments performed in duplicate.

Data Analysis. K_i values were calculated using a nonlinear regression curve-fitting program (GraphPad Prism 4, GraphPad Software Inc., San Diego, CA). K_D values of the radioligands were 1.6 nM, 1.0 nM, and 5.0 nM for [^{3}H]DPCPX, [^{3}H]ZM 241385, and [^{125}I]AB-MECA, respectively. The data from the functional assays were also analyzed with GraphPad Prism. Figure 1 and the results shown in Table 1 were generated by evaluating the data to relate to the known ligands CPA and DPCPX. The first step involved normalizing the raw data with respect to CPA and DPCPX. CPA was chosen as the baseline value as it has previously been shown to be a full agonist, and DPCPX as the highest value, assuming full inversely agonistic behavior. After normalization, the Y-axis was shifted to present the data as being above or below that of the forskolin-generated levels of cAMP. Finally, the data were again normalized to assume 100% inhibition of cAMP by the agonist CPA and 100% full inverse agonism by DPCPX.

Molecular Modeling. Molecular modeling work was performed with the SPARTAN molecular modeling package Spartan Pro 1.0.2. Default values in the Merck Force Field were used in Molecular Mechanics minimizations. The molecular electrostatic potential was calculated using the semiempirical molecular orbital program AM1. The electrostatic potential was sampled over the entire accessible surface of the molecules (roughly to a van der Waals contact surface).

Supporting Information Available: A table of the elemental analyses data. This material is available free of charge via the Internet at http://pubs.acs.org.

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