2-Amino-6-furan-2-yl-4-substituted Nicotinonitriles as A_{2A} Adenosine Receptor Antagonists

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 A_{2A} adenosine receptor antagonists usually have bi- or tricyclic N aromatic systems with varying substitution patterns to achieve desired receptor affinity and selectivity. Using a pharmacophore model designed by overlap of nonxanthine type of previously known A_{2A} antagonists, we synthesized a new class of compounds having a 2-amino nicotinonitrile core moiety. From our data, we conclude that the presence of at least one furan group rather than phenyl is beneficial for high affinity on the A_{2A} adenosine receptor. Compounds **39** (LUF6050) and **44** (LUF6080) of the series had K_i values of 1.4 and 1.0 nM, respectively, with reasonable selectivity toward the other adenosine receptor subtypes, A_1 , A_{2B} , and A_3 . The high affinity of **44** was corroborated in a cAMP second messenger assay, yielding subnanomolar potency for this compound.

Introduction

Adenosine receptors have adenosine as their endogenous ligand and they belong to the class of G protein-coupled receptors (GPCRs). They occur as four different subtypes, A₁, A2A, A2B, and A3, all of which have been cloned successfully into artificial cell lines. All adenosine receptors are associated with the cAMP^a second messenger system. Activation of A₁ and A3 receptors mediates inhibition of adenylate cyclase, while A_{2A} and A_{2B} receptors, when activated, increase the intracellular cyclic AMP level. In contrast to the widespread distribution of A_1 and A_{2B} receptors in the brain, the A_{2A} receptor is highly expressed only in striatum, nucleus accumbens, olfactory tubercles, and globus pallidus pars externa in the rat and human brain.¹ The A_{2A} receptor is coexpressed with D_2 receptors in striatum. It is involved in the regulation of functional activity of D₂ dopamine receptors² as heterodimerization of A_{2A} and D₂ receptor subtypes inhibits D₂ receptor functions. It has been found that A_{2A} adenosine receptor antagonists improve motor function in animal models of basal ganglia disorders. Thus, such compounds are thought to serve as a therapeutic modality in Parkinson's disease.

Previously known A_{2A} antagonists can be generally classified as xanthine-like and nonxanthine like structures. KW-6002 (istradefylline) is a xanthine derivative having a styryl moiety on the 8 position and is already in phase III clinical trials for the treatment of Parkinson's disease³ (Figure 1). The 8-styryl xanthine moiety is associated with photochemical instability;⁴ however, also, xanthine-based structures have already been optimized and explored to a high extent. This led us to find new plausible A_{2A} antagonists from nonxanthine like structures. In this paper, we report the synthesis and SAR of a new class



istradefylline (KW 6002)

Figure 1. Istradefylline (KW-6002).

of A_{2A} antagonists from a pharmacophore model, which we constructed using previously reported A_{2A} nonxanthine antagonists.

Results and Discussion

Molecular Modeling. A molecular superimposition model was constructed using previously published antagonist ligands having high affinity and selectivity for the A_{2A} adenosine receptor. All these molecules were selected with the criteria of having diverse scaffolds based on the heterocyclic aromatic core of each molecule. The actual ligand taken from a specific scaffold class was the one that had a combination of high affinity and selectivity for the A_{2A} adenosine receptor. We also included in the overlap four prototypic nonxanthine A_{2A} adenosine receptor antagonists, viz. CGS15943 (in fact a nonselective compound), 8FB-PTP, SCH58261, and ZM241385.

The molecules shown in Figure 2 were drawn in the SPARTAN '04²¹ molecular modeling package, and each molecule was energy-minimized to yield the lowest energy conformer. Their electrostatic potentials were sampled over the entire Van der Waal's surface of each molecule and then electron density was mapped over it. As an example, SCH58261 is shown in Figure 3: shades of red and blue indicate relative electronegative and electropositive regions, respectively. After plotting the electrostatic potential over each individual molecule, we noticed that all of them share common features, i.e., an electron deficient region (blue) on the "top" of the molecule, accompanied by an electron rich region (red) on the "righthand" side of the molecule. The former represents the H-bond donating free amino group in most of the cases, while the latter usually constitutes the electron rich region of the oxygen atom in a furan ring.

All molecules were superimposed on each other by considering these two areas of electron density together with the aromatic

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^{*a*} Abbreviations: GPCR, G-protein-coupled receptor; cAMP, cyclic adenosine monophosphate; SAR, structure-activity relationship; CHO, chinese hamster ovary; HEK, human embryonic kidney; ADA, adenosine deaminase; AB-MECA, N⁶-(4-aminobenzyl)adenosine-5'-methyluronamide; CPA, N⁶-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxan-thine; NECA, 5'-*N*-ethylcarboxamidoadenosine; TLC, thin layer chromatography.



Figure 2. Set of molecules selected for their different scaffold structure. **a** CGS15943 K_i (hA_{2A}) = 0.4 nM,⁵ **b** 8FB-PTP, **c** SCH58261 K_i (hA_{2A}) = 0.6 nM,⁵ **d** ZM241385 K_i (hA_{2A}) = 0.8 nM,⁶ **f** K_i (rA_{2A}) = 0.1 nM,⁷ **g** K_i (hA_{2A}) = 4.3 nM,⁸ **h** K_i (hA_{2A}) = 0.5 nM,⁹ **i** K_i (hA_{2A}) = 1.1 nM,¹⁰ **j** K_i (hA_{2A}) = 0.2 nM,¹¹ **k** K_i (hA_{2A}) = 6.5 nM,¹² **l** K_i (hA_{2A}) = 20 nM,¹³ **m** K_i (hA_{2A}) = 1.2 nM,¹⁴ **n** K_i (hA_{2A}) = 0.12 nM,¹⁵ **o** K_i (hA_{2A}) = 4 nM,¹⁶ **p** K_i (hA_{2A}) = 1.1 nM,¹⁷ **q** K_i (hA_{2A}) = 6.6 nM,¹⁸ **r** K_i (hA_{2A}) = 1.4 nM,¹⁹ **s** K_i (hA_{2A}) = 0.1 nM.²⁰

nitrogen-containing ring system as a further basis of overlap (Figure 4a). Because molecules \mathbf{c} , \mathbf{d} , \mathbf{f} , \mathbf{h} , \mathbf{i} , \mathbf{k} , \mathbf{n} , \mathbf{s} , and \mathbf{o} (Figure 2) had bulky and flexible side chains, the orientation of these side chains, at the "left side" of the molecules, was very diverse (Figure 4a). To enhance the model for better overlap in this region, the single conformation of each of the molecules mentioned above compatible with the best overlap was chosen from the set of all possible conformers within a limit of 5 kcal/ mol above the lowest energy conformation (see Experimental Section for details). These selected conformations were overlaped with the other molecules used in the superimposition from Figure 4a to yield Figure 4b. A simplified version with only four molecules (\mathbf{e} , \mathbf{j} , \mathbf{m} , and \mathbf{r}) is shown in Figure 5 in which the electrostatic potential energy surface of \mathbf{m} was chosen to show the general pattern of superimposition.

Superimposition of these molecules led to a pharmacophore model shown in Figure 6. From the superimposition, it is clear that for a molecule to be an A_{2A} adenosine receptor antagonist, it requires a hydrogen bond donor (A) and electron rich regions (B and C), present on an *N*-containing aromatic ring (A and B) and a largely lipophilic L₁ group (C). These areas comply with the recently published 3D pharmacophore model for A_{2A} receptor antagonism by Wei et al.²² In addition to these regions, the *N*-containing ring also possesses an H-bond accepting region D. Furthermore, the lipophilic region L₂ seems to be desirable for good binding to the receptor. To this area an appropriately oriented heteroatom may be added (see also Figure 4b).

We decided to explore a monocyclic core ring structure in particular because very few A_{2A} adenosine receptor antagonists with such a scaffold have been reported (compound l in Figure



Figure 3. (I) SCH58261 in its lowest energy conformer as calculated with SPARTAN. (II) SCH58261 with electrostatic potential energy surface mapped as transparent cloud around stick model. (III) Molecular electrostatic potential energy surface of SCH58261.



Figure 4. (a) Superimposition of all the molecules in their lowest energy conformation. (b) Optimized superimposition of molecules after selecting a conformer with better overlap in L_2 lipophilic region for highly flexible molecules.

2 is one of the few examples). From the pharmacophore model in Figure 6, we learned that a monocyclic ring should have an aromatic character and a nitrogen atom at position B. At position D, we chose the nitrile function instead of an extra nitrogen atom in the ring to obtain a better distance separation between D and C. For exploring lipophilic region L₁, we tried various aromatic rings with substitutions that are discussed later in Table 1 and Table 2 Thus we came up with the nicotinonitrile template, having an amino group at position 2 as an H-bond donor. For the exploration of lipophilic region L₁, we included various aromatic rings that are discussed later in Table 2. It has been recently reported by Richardson et al.²³ that a furan group may



Figure 5. A simplified picture showing only four of the antagonists (**e**, **j**, **m**, **r**) superimposed on each other, also showing the electrostatic potential energy surface of **m**.



Figure 6. Simplified pharmacophore derived from superimposition of the investigated molecules. A represents a H-bond donating region, B, C and D represent electron-rich and H-bond accepting regions, respectively. L_1 and L_2 represent lipophilic regions. A molecule with a simple, monocyclic core and obeying the pharmacophore is shown below it.

carry a safety liability as it is prone to oxidative metabolism, hence we also searched for other rings at the L_1 lipophilic region.

Chemistry. The compounds were synthesized according to Scheme 1.²⁴ The aldehyde was reacted with malononitrile by adding a few drops of piperidine to give reaction intermediates 1-16 according to a Knoevenagel condensation.²⁵ In the case of heteroaromatic aldehydes, reactions were carried out at room temperature so as to avoid decomposition of the intermediate. In the next step, the malononitrile derivative and an aromatic acetyl ketone were fused with ammonium acetate in a microwave reactor at 120 °C for 1 h to form the nicotinonitriles 17-45.²⁶ The reaction was optimized for microwave conditions for this temperature and time to give maximum yield. The final product was purified by column chromatography and recrystallization.

Structure–Activity Relationships. The results of radioligand binding assays performed on the substituted nicotinonitriles 17–45 are shown in Table 1.

The unsubstituted phenyl derivative (**17**) had 11 nM affinity for the A_{2A} receptor but was not much selective against the A₁ receptor ($K_i = 28$ nM). It also showed 170 nM affinity for the A_{2B} receptor. The biphenyl derivative **18** was inactive at both

Table 1. Affinities of 2-Amino-4,6-substituted Nicotinonitriles in Radioligand Binding Assays of Human Adenosine Receptors



			K_i (nM) or % displacement ^a				
compound	R^1	\mathbb{R}^2	hA ₁ ^b	hA_{2A}^{c}	hA_{2B}^{d}	hA ₃ ^e	
17	Н	furan-2-yl	28 ± 7	11 ± 5	170 ± 39	28%	
18	4-phenyl	furan-2-yl	0%	0%	17%	4%	
19	4-Cl	furan-2-yl	48 ± 7	140 ± 35	33%	21%	
20	3,4-diCl	furan-2-yl	68 ± 23	190 ± 35	12%	14%	
21	4-CH ₃	furan-2-yl	47%	45%	15%	21%	
22	4-OCH ₃	furan-2-yl	34 ± 11	41 ± 6	33%	21%	
23	3,4-diOCH ₃	furan-2-yl	250 ± 99	92 ± 4	31%	20%	
24	3,4-OCH ₂ O-	furan-2-yl	11 ± 2	26 ± 2	24%	390 ± 110	
25	4-OH	furan-2-yl	54 ± 18	41 ± 15	370 ± 52	34%	
26	4-N(CH ₃) ₂	furan-2-yl	11%	3.8%	2%	17%	
27	$4-OCH(CH_3)_2$	furan-2-yl	19%	6%	8%	7%	
28	3-OCF ₃	furan-2-yl	270 ± 24	260 ± 13	29%	13%	
29	3-CH ₃	furan-2-yl	64 ± 18	140 ± 60	620 ± 220	18%	
30	Н	phenyl	14 ± 1	130 ± 10	26%	53%	
31	3,4-diOCH ₃	phenyl	66 ± 39	160 ± 10	13%	32%	
32	3,4-OCH ₂ O-	phenyl	7.0 ± 0.4	34%	0%	48%	
33	4-N(CH ₃) ₂	phenyl	12%	1%	4%	2%	
34	3-OCF ₃	phenyl	34 ± 2	58 ± 6	7%	28%	
35	3-CH ₃	phenyl	15 ± 1.5	130 ± 10	37%	33%	
36	4-OH	phenyl	61 ± 19	17%	18%	18%	
NECA		÷ •	7.8 ± 0.7		1270 ± 160	11 ± 1	
CPA			10 ± 1.3	1700 ± 300		280 ± 60	
DPCPX			6.1 ± 1.6	130 (n = 2)		1700 ± 170	

 ${}^{a}K_{i} \pm \text{SEM}$ (n = 3), % displacement (n = 2). b Displacement of specific [³H]DPCPX binding in CHO cell membranes expressing human adenosine A₁ receptors or % displacement of specific binding at 1 μ M concentrations. c Displacement of specific [³H]ZM241385 binding in HEK293 cell membranes expressing human adenosine A_{2A} receptors or % displacement of specific binding at 1 μ M concentrations. d Displacement of specific [³H]MRS1754 binding in CHO cell membranes expressing human adenosine A_{2B} receptors or % displacement of specific binding at 1 μ M concentrations. e Displacement of specific [³H]MRS1754 binding in CHO cell membranes expressing human adenosine A_{2B} receptors or % displacement of specific binding at 1 μ M concentrations. e Displacement of specific binding at 1 μ M concentrations.

Table 2. Affinities of 2-Amino-4,6-heteroaromatic-substituted Nicotinonitriles in Radioligand Binding Assays of Human Adenosine Receptors



			$K_{\rm i}$ (nM) or % displacement ^a			
compound	R^1	\mathbb{R}^2	hA ₁ ^b	$hA_{2A}{}^{c}$	hA_{2B}^{d}	hA3 ^e
37	furan-2-yl	phenyl	5.8 ± 1.5	19 ± 1	220 ± 40	29%
38	thiophen-2-yl	thiophen-2-yl	43 ± 6	32 ± 15	19%	31%
39	thiophen-2-yl	furan-2-yl	28 ± 6	1.4 ± 0.2	75 ± 15	51%
40	phenyl	thiophen-2-yl	12 ± 2	88 ± 25	41%	32%
41	phenyl	pyrrol-2-yl	28%	640 ± 370	35%	0%
42	furan-2-yl	thiophen-2-yl	29 ± 5	12 ± 1	380 ± 90	39%
43	thiophen-2-yl	phenyl	22 ± 10	54 ± 3	44%	11%
44	furan-2yl	furan-2-yl	12 ± 2	1.0 ± 0.1	34 ± 2	33%
45	5-Me-furan-2-yl	furan-2-yl	100 ± 30	15 ± 1	440 ± 110	24%

 ${}^{a}K_{i} \pm \text{SEM}$ (n = 3), % displacement (n = 2). b Displacement of specific [³H]DPCPX binding in CHO cell membranes expressing human adenosine A₁ receptors or % displacement of specific binding at 1 μ M concentrations. c Displacement of specific [³H]ZM241385 binding in HEK293 cell membranes expressing human adenosine A_{2A} receptors or % displacement of specific binding at 1 μ M concentrations. d Displacement of specific [³H]MRS1754 binding in CHO cell membranes expressing human adenosine A_{2B} receptors or % displacement of specific binding at 1 μ M concentrations. e Displacement of specific [¹²T]AB-MECA binding in HEK293 cell membranes expressing human adenosine A₃ receptors or % displacement of specific binding at 1 μ M concentrations.

 A_{2A} and A_1 receptors, indicating that the L_2 pocket cannot accommodate extended bulky lipophilic groups. Further systematic evaluation of substitutions as suggested from the Topliss scheme²⁷ provided detailed insight in the requirements for A_{2A} adenosine receptor affinity. Both chloro substitution at the para position (**19**) and 3,4-dichloro substitution (**20**) reduced affinity for the A_{2A} and A_1 receptors as compared to unsubstituted **17**. Further changing substitution to methyl at the para position on the phenyl ring (21) appeared even more unfavorable with respect to affinity for A_{2A} and A_1 receptors with a displacement of radioligand of only 45% and 47%, respectively, at a concentration of 1 μ M. Apparently, increased hydrophobicity and electron withdrawing character are detrimental for the affinity for the A_{2A} receptor. The introduction of a 4-methoxy



Thiophen-2-y

^a Reagents and conditions: (i) Piperidine, EtOH, 1 h reflux; (ii) NH₄OAc, toluene, 120 °C in microwave.

group on the phenyl ring (22) showed restored affinity for the A_{2A} receptor but without selectivity against the A_1 receptor (K_1) values of 41 nM and 34 nM, respectively). The 3,4-dimethoxy derivative 23 did show improvement in selectivity with respect to the A₁ receptor ($K_i = 250$ nM), however, its affinity for the A2A receptor was decreased to 92 nM. On the other hand, compound 24 with a 3,4-dioxymethylene substituent showed 11 nM affinity for A₁ and 26 nM affinity for the A_{2A} receptor, indicating the subtle receptor interactions at this part of the molecule.

Compound 25 also displayed nanomolar affinity but less selectivity (41 nM for the A_{2A} and 54 nM for the A_1 receptor). Substitution at the para position by dimethyl amine (26) or an isopropoxy group (27) showed a complete loss of affinity for A_{2A} and A₁ receptors, again indicating that bulky groups in the L₂ pocket are not favorable. Compounds 28 and 29, which have trifluoromethoxy and methyl groups at the meta position, respectively, showed 260 and 140 nM affinity for the A_{2A} adenosine receptor. However, these compounds were not selective as their affinity values for A₁ receptor were 270 nM (28) and 64 nM (29). Compounds 30-36 were synthesized to compare 2-amino-2-yl-6-furyl nicotinonitriles with 2-amino-2yl-6-phenyl nicotinonitriles. Compound 30 with two phenyl substituents showed 14 nM affinity for the A_1 and 130 nM affinity for the A_{2A} receptor. These data compare unfavorably with furan-substituted 17, suggesting that the presence of a furan moiety in the L_1 pocket is preferred for A_{2A} receptor affinity. This observation also holds true in case of the 3,4-dimethoxy substituent; the affinity of 23 changed from 92 to 160 nM after replacing the furan by a phenyl ring (31). In the case of the dioxymethylene ring, the change was even more prominent as compound 32 showed only 34% displacement of the A2A receptor radioligand. Substitution at the para position by dimethyl amine (33) in the case of the phenyl series, also caused a complete loss of affinity for A_{2A} and A₁ receptors due to the unfavorable interaction with the bulky groups in the L₂ pocket. Compound 34 with a trifluoromethoxy substituent at the meta position on the phenyl ring showed an affinity of 34 nM for A₁

and 58 nM for A2A adenosine receptors. This represented improved affinity over its furan analogue (28), but no significant difference in selectivity was observed. The meta-methylsubstituted derivative (35) actually showed more selectivity toward A_1 as compared to A_{2A} adenosine receptors with a K_i value 15 nM for A1 and 130 nM for A2A receptors. Compound 36 with a para-substituted hydroxy group on the phenyl ring showed almost complete loss of affinity (17% displacement only at 1 μ M) for A_{2A} adenosine receptor. Comparing **36** with **25** is another confirmation that in case of nicotinonitriles the presence of a furan ring in the L₁ pocket is preferred over a phenyl ring with respect to A_{2A} adenosine receptor affinity. Concluding, the L₂ region can be substituted with various phenyl substituents. However, in most cases, the selectivity for the A2A receptor is decreased in favor of the A₁ receptor.

Table 2 shows the results of radioligand binding assays performed on heteroaromatic substituted nicotinonitriles (37 - 45).

We optimized the L_1 and L_2 regions of the pharmacophore by substituting the core molecule with various combinations of heteroaromatic ring systems. All compounds had affinities for A2A receptors in the low nanomolar range. Compounds 37 and 43, however, displayed even higher affinity toward A_1 than A_{2A} receptors with K_i values of 5.8 and 19 nM for 37 and 22 and 54 nM for 43. Compound 38 with two thiophene rings had 32 nM affinity for the $A_{2\text{A}}$ adenosine receptor but was not selective (43 nM on the A₁ receptor). Replacing one of the rings by furan as in 39 and 42 rendered these molecules more selective against the A₁ receptor. The K_i values of **39** and **42** for the A_{2A} adenosine receptor were 1.4 and 12 nM with 20- and 2.4-fold selectivity, respectively. Also, compound 39 showed 75 nM affinity for the A2B adenosine receptor. The only pyrrole derivative made in this series (41) displaced only 28% of the A_1 receptor radioligand, making it selective for A_{2A} , but its K_i value for this receptor subtype was modest (640 nM). These findings suggest that a five-membered heterocyclic ring is favored over a phenyl ring in the L₁ region, preferably with an H-bond accepting rather than donating heteroatom. Compound 44 with two furan rings yielded the highest affinity in this series for the A_{2A} receptor with a K_i value of 1.0 nM and 12-fold selectivity against the A₁ receptor. It also displayed 34-fold selectivity against the A_{2B} receptor (34 nM affinity). Recently, compound 44 has also been described as an inhibitor of mitogenactivated protein kinase-activated protein kinase-2.28 Compound 45 with a 5-methyl substituent on the furan ring in the L_2 pocket showed somewhat reduced affinity for the A_{2A} (15 nM) and A_1 adenosine receptor (100 nM). However, this substituted furan group is more metabolically stable and might thus be preferred over the simple furan moiety. Other less "reactive" heterocyclic moieties may also be explored, although the furan ring per se is a very common feature in A2A-selective adenosine receptor antagonists as found in many different research programs.

All compounds listed in both tables displayed little affinity for the human A₃ receptor. In most cases, there was less than 50% displacement of radioligand when compounds were tested at 1 μ M concentration.

Functional Assay at A2A Adenosine Receptors. For a functional evaluation, intact CHO cells expressing the human adenosine A2A receptor were used. The cells were stimulated with increasing concentrations of the prototypic A_{2A} receptor agonist CGS21680 (2-[4-(2-carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine). Further CGS21680 concentration–effect curves were recorded (n = 3) in the presence of two concentrations (10 and 100 nM) of 44 (Figure 7), causing



Figure 7. The effect of increasing concentrations of compound 44 (10 and 100 nM) on the cAMP production induced by increasing concentrations of CGS21680 in HEK 293 cells expressing the human A_{2A} adenosine receptor.

a rightward shift. These data were analyzed according to Gaddum and Schild, yielding a pA₂ value of 9.87 ± 0.36 , corresponding to an apparent affinity (K_B value) of 0.14 nM for **44**. This value is in fair agreement with the binding data (K_i value of 1 nM).

Conclusion

In this paper, we presented a new series of 2-amino-6-furan-2-yl-4-substituted nicotinonitriles, designed and synthesized on the basis of a pharmacophore model derived through molecular modeling of previously known ligands. Comparing this series of compounds with a number of 2-amino-6-phenyl-2-yl-4substituted nicotinonitriles demonstrated the importance of the furan moiety to render these nicotinonitriles potent for the A2A adenosine receptor. Compound 44 is highly potent on this adenosine receptor subtype in both a radioligand binding and a second messenger assay. One possible advantage of these small molecules over some of the polycyclic antagonists resides in their more favorable physicochemical properties. With the Molecular Evoluator software,^{29,30} the following parameters for compound 44 were calculated: LogP 3.06; MW 251; hydrogen bond acceptors 5; hydrogen bond donors 2. These values comply with the often-used Lipinski Rule of Five.³¹

Experimental Section

Molecular Modeling. Molecular modeling was performed with the SPARTAN '04²¹ software package (Wave Function Inc.). Default values in the Merck Force field were used in molecular mechanics minimizations. Conjugate gradient energy minimization was continued until the rms energy derivative was less than 0.001 kcal·mol⁻¹ Å⁻¹. Conformers were generated using the systematic search method, and the lowest energy conformer was used for further calculations. The molecular electrostatic potential of these conformers was calculated with the semiempirical molecular orbital program AM1. The energy and molecular electrostatic potentials were sampled over the entire accessible surface of the molecules (equal to the van der Waals contact surface). In the figures, the most negative electrostatic potential is colored red and the most positive one is blue.

For molecules c, d, f, h, i, k, n, s, and o, all possible conformations were generated by calculating their conformation distribution at ground-state by molecular mechanics. All the conformations for each molecule were sorted on the basis of the energy parameter to which a cutoff value of 5 kcal/mol from the lowest energy was applied. From the remaining conformers, one was chosen that had a side chain orientation that was most compatible with rigid compound **j** in Figure 2.

Chemistry, Materials, and Methods. All reagents were obtained from commercial sources and all solvents were of analytical grade. ¹H and ¹³C NMR spectra were recorded on a Bruker AC 200 (¹H

NMR, 200 MHz; ¹³C NMR, 50.29 MHz) spectrometer with tetramethylsilane as an internal standard. Chemical shifts are reported in δ (ppm). Melting points were determined by Büchi melting point apparatus and are uncorrected. Elemental analyses were performed by Leiden Institute of Chemistry and are within 0.4% of theoretical values unless otherwise stated. Reactions were routinely monitored by TLC using Merck silica gel F₂₅₄ plates. Microwave reactions were performed on an Emrys Optimizer (Biotage AB). Wattage was automatically adjusted to maintain the desired temperature.

General Procedure for the Preparation of Malononitriles (1-16). To malononitrile (20 mmol) dissolved in EtOH (40 mL) was added an aromatic aldehyde (20 mmol) followed by 2 drops of piperidine. In the case of substituted benzaldehydes, the reaction mixture was refluxed for 1 h. In the case of heterocyclic aromatic aldehydes, the reaction was carried out for 1 h at room temperature. The precipitate was formed on cooling the reaction mixture to room temperature. The crude product was filtered, and it was fairly pure to carry out the further reactions.

General Procedure for the Preparation of Nicotinonitriles (17–45). To a solution of previously synthesized benzylidene malononitrile (3 mmol, 1 equiv) in toluene was added 2-acetyl furan (3 mmol, 1 equiv) and ammonium acetate (4.5 mmol, 1.5 equiv). The mixture was heated in a microwave at 120 °C for 1 h. The reaction mixture was purified by column chromatography using dichloromethane–methanol solvent system. Recrystallization from methanol or ethanol gave the corresponding nicotinonitrile in pure form. The yield of reaction varied from 40–70%.

2-Amino-4-thiophen-2-yl-6-furan-2-yl-nicotinonitrile (39, LUF6050). mp: 180 °C. ¹H NMR δ (CDCl₃): 5.36 (s, 2H), 6.55–6.58 (m, 1H), 7.21 (s, 1H), 7.12–7.22 (m, 2H), 7.51–7.59 (m, 2H), 7.85–7.86 (d, 1H). Anal. (C₁₄H₉N₃OS) C, H, N.

2-Amino-4,6-difuran-2-yl-nicotinonitrile (44, LUF6080). mp: 199 °C. ¹H NMR δ (CDCl₃): 5.30 (s, 2H), 6.55 (m, 2H), 7.46 (d, 1H), 7.51 (s, 1H), 7.58 (d, 1H). Anal. (C₁₄H₉N₃O₂) C, H, N.

Biology

Binding Studies. [³H]DPCPX and [¹²⁵I]AB-MECA were purchased from Amersham Biosciences (NL). [³H]ZM241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3- α][1,3,5]triazin-5ylamino]ethyl)phenol) and [³H]MRS1754 (*N*-(4-cyanophenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1*H*-purin-8yl)-phenoxy]acetamide) were obtained from Tocris Cookson, Ltd. (UK). CHO cells expressing the human adenosine A₁ receptor were provided by Dr. Andrea Townsend-Nicholson, University College London, UK. Dr. S. Rees (GSK, Stevenage, UK) kindly provided CHO cells expressing the human A_{2B} receptor. HEK293 cells stably expressing the human adenosine A_{2A} and A₃ receptor were kind gifts from Dr. J. Wang (Biogen/ IDEC, Cambridge, MA) and Dr. K. N. Klotz (University of Würzburg, Germany), respectively.

All compounds were tested in radioligand binding assays to determine their affinities at human adenosine A_1 ([³H]DPCPX), A_{2A} ([³H]ZM241385), and A₃ ([¹²⁵I]AB-MECA) receptors as described previously in literature with the exception that nonspecific binding to the A2A receptor was determined in the presence of 10 µM CGS21680 instead of 100 µM CPA. For the adenosine A_{2B} receptor assay membranes containing 11 μ g of protein were incubated in a total volume of 100 μ L of 50 mM Tris/HCl, 0.1% CHAPS, ADA 0.8 IU/mL (pH 7.4) and [³H[MRS1754 (final concentration, 1.2 nM) for 1 h at 25 °C in a shaking water bath. Nonspecific binding was determined in the presence of 1 μ M NECA. The incubation was terminated by filtration over Whatman GF/C filters under reduced pressure with a Brandel harvester (Gaithersburg, MD). Filters were washed three times with ice cold 50 mM Tris/HCl pH 7.4, placed in vials and counted.

cAMP assay. HEK293 cells expressing the human A_{2A} adenosine receptor were grown as a monolayer on 6 cm culture plates. The cells were harvested and centrifuged two times for 5 min/1000 rpm. For cAMP production and determination, 7500 cells/well were used on 384-well plates. The cells were incubated for 45 min at room temperature with either CGS21680 alone or together with compound 44 in different concentrations. The assay medium also contained cilostamide (50 μ M), rolipram (50 μ M), and adenosine deaminase (0.8 IU/mL). Incubation was stopped with detection mix and antibody solution was added, these two steps according to the instructions of the supplier. The assay was performed with the Lance cAMP 384 kit from Perkin-Elmer based on the competition of the sample's cAMP with a europium-labeled cAMP tracer complex for binding sites on cAMP-specific antibodies labeled with Alexa Fluor dye.

Data Analysis. K_i values were calculated using a nonlinear regression curve fitting program (GraphPad Prism 4.0, GraphPad Software Inc., San Diego, CA). K_i values of radioligands were 1.6, 1.0, 1.3, and 5.0 nM for [³H]DPCPX, [³H]ZM 241385, [³H]MRS1754, and [¹²⁵I]AB-MECA, respectively.

Supporting Information Available: ¹H NMR data of compounds **1–16.** Physical data and elemental analysis of the target compounds **17–45**. This material is available free of charge via the Internet at http://pubs.acs.org.

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